THE UNIVERSITY OF MANITOBA

STRUCTURE - FUNCTION RELATIONS IN THE CALCIUM-DEPENDENT PROTEIN MODULATOR OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a Ph.D. thesis entitled: "STRUCTURE - FUNCTION RELATIONS IN THE CALCIUM-DEPENDENT PROTEIN MODULATOR OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE"

submitted by Michael P. Walsh,

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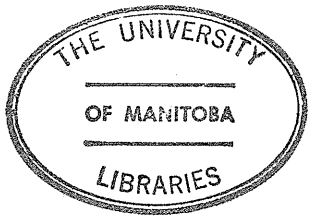
STRUCTURE - FUNCTION RELATIONS IN THE CALCIUM-DEPENDENT PROTEIN MODULATOR OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

BY

MICHAEL WALSH

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY



To Mary, Katy and Emma

"An experiment is never a failure solely because it fails to achieve predicted results. An experiment is a failure only when it also fails adequately to test the hypothesis in question, when the data it produces don't prove anything one way or another".

Robert M. Pirsig in "Zen and the Art of Motorcycle Maintenance", (1974), William Morrow and Company, Inc., New York, N.Y. 10016.

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ABSTRACT

Three aspects relating to the structure and function of the Ca^{2+}-dependent protein modulator of cyclic nucleotide phosphodiesterase were investigated: (1) the mode of interaction between the modulator and phosphodiesterase, (2) the conformational changes induced in the modulator by the binding of Ca^{2+}, and (3) the structural homology between the modulator and troponin C, the calcium-binding subunit of the muscle troponin complex.

Chemical modification of selected functional groups of the protein modulator indicated that the site for interaction with phosphodiesterase is located between the second and third calcium-binding regions and is on the surface of the molecule. The integrity of both methionine and lysine residues is essential for the expression of phosphodiesterase-stimulating activity. It is proposed that interaction with phosphodiesterase occurs via an initial site recognition involving Lys 75 and Lys 77, and possibly other neighbouring charged side chains, followed by formation of a strong binding interaction through hydrophobic interactions involving Met 71, 72 and 76. Chemical modification of histidine, tyrosine and arginine residues, on the other hand, did not affect the stimulation of phosphodiesterase - none of these residues are located in the vicinity of the proposed phosphodiesterase binding domain. Similar studies indicated that the site for interaction with phosphodiesterase is distinct from the site for interaction with troponin I.

A combination of circular dichroism, chemical modification and controlled enzymatic digestion of the protein modulator in the presence and absence of Ca^{2+} provided insight into the conformational changes

occurring as the modulator binds Ca^{2+}. Removal of Ca^{2+} results in a substantial loss of secondary structure: the helical content decreases from 49% to 40%, whereupon the molecule becomes much more susceptible to tryptic digestion and urea denaturation. On a more refined level, His 107, for example, becomes more accessible as the modulator binds Ca^{2+}, as revealed by kinetic analysis of carbethoxylation of this residue. Similar studies revealed altered reactivity of the other functional groups of the modulator upon binding of Ca^{2+}.

The protein modulator and troponin C exhibit approximately 80% sequence homology, taking conservative replacements into account (Vanaman et al, 1977). Circular dichroism studies of the protein modulator indicated the overall secondary structure of the molecule to be very similar to that of troponin C. Furthermore, controlled tryptic digestion of the modulator with structural and functional characterization of the resultant fragments revealed that cleavage occurred at positions homologous to those previously observed (Drabikowski et al, 1977a) in troponin C. The two calcium-binding proteins, therefore, exhibit similar tertiary structures. In addition, they undergo similar conformational changes upon binding of Ca^{2+} ions. The structural differences between the modulator and troponin C are small, but, in functional terms, these subtle differences have profound effects.

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ABBREVIATIONS

CD	Circular dichroism
DEAE	Diethylaminoethyl
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDC	1-ethyl-3-dimethylaminopropylcarbodiimide
EDTA	Ethylenediamine-N,N'-tetraacetic acid
EGTA	Ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid
MBA	N,N'-methylenebisacrylamide
NCS	N-chlorosuccinimide
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PDE	Cyclic nucleotide phosphodiesterase (EC 3.1.4.17)
PIPES	Piperazine-N,N'-bis(2-ethane sulfonic acid)
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
TPCK	N-tosyl-L-phenylalanylchloromethyl ketone
TN-C	Troponin C
TN-I	Troponin I
TN-T	Troponin T

MATERIALS

5'-nucleotidase was obtained from Sigma Chemical Co. (St. Louis, Mo.) and TPCK-trypsin from Worthington Biochemicals (Freehold, N.J.).

Troponin I (bovine cardiac) was a generous gift from Dr. C.M. Kay at the University of Alberta.

Cyclic 3',5' -adenosine monophosphate (cAMP), N-chlorosuccinimide, diethylpyrocarbonate, 1-ethyl-3-dimethylaminopropylcarbodiimide, glycine methyl ester, imidazole, iodoacetic acid and iodoacetamide, mercaptoacetic acid, 3-nitrotyrosine, PIPES, and SDS were all obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cyanogen bromide, 1,2-cyclohexanedione, and dithioerythritol were products of Pierce Chemical Co. (Rockford, Ill.).

Acrylamide, chloramine-T, EGTA, MBA, 2-mercaptoethanol, potassium isocyanate, and TEMED were obtained from Eastman Organic Chemicals (Rochester, N.Y.).

Ammonium persulfate, EDTA, and hydrogen peroxide were obtained from Fisher Scientific Co. (New Jersey), benzyl bromide and formic acid from Aldrich Chemical Co, (Milwaukee, Wis.), O-methylisourea from National Biochemicals (Cleveland, Ohio), homocitrulline from Cyclo Chemical Corp. (Los Angeles, Calif.), tetranitromethane from Schwarz/Mann (Orangeburg, N.Y.) and phenol from BDH Ltd. (Poole, England)

$^{45}CaCl_2$ was purchased from The Radiochemical Centre (Amersham, England) and 1-^{14}C-iodoacetic acid from New England Nuclear (Boston, Mass.).

All other chemicals were reagent grade or better.

INTRODUCTION

Considerable evidence has been provided in recent years that a ubiquitous Ca^{2+}-binding protein, originally discovered as an activator of cyclic nuclectide phosphodiesterase, may serve as the central regulatory element coupling Ca^{2+} to the regulation of cyclic nucleotide metabolism and non-muscle contractile and secretory processes. This protein modulator exhibits many Ca^{2+}-dependent regulatory activities, including : (1) activation of a specific cyclic nucleotide phosphodiesterase, (2) activation of a specific adenylate cyclase, (3) troponin C-like activity with a reconstituted Ca^{2+}-sensitive actomyosin ATPase system, (4) activation of erythrocyte membrane (Ca^{2+}-Mg^{2+}) ATPase, (5) stimulation of erythrocyte membrane Ca^{2+} transport, and (6) activation of skeletal and smooth muscle myosin light chain kinases. Furthermore, an unidentified protein, which appears to be an additional modulator-regulated enzyme, has been isolated from brain and shown to interact with the modulator in a Ca^{2+}-dependent manner. It is conceivable, and intuitively appealing, that all proteins observed to exhibit Ca^{2+}-dependent interaction with the protein modulator possess a common modulator binding domain.

The modulator protein exhibits none of the above-mentioned activities in the absence of Ca^{2+} ions. The protein binds 4 Ca^{2+} ions per mole and the binding of Ca^{2+} is accompanied by a substantial conformational change in the modulator whereupon the protein assumes an active form. The Ca^{2+}-binding properties of the modulator indicate that it functions as a physiological regulator alternating between active and inactive forms with fluctuations in the intracellular

Ca^{2+} concentration. This Ca^{2+}-mediated conformational change may thus serve a crucial role in the regulation of numerous Ca^{2+}-dependent physiological processes.

The protein modulator is also interesting from an evolutionary standpoint. Comparison of amino acid sequences indicated that the modulator is a member of a family of homologous Ca^{2+}-binding proteins which includes parvalbumins, myosin light chains, and troponin C. Within the family, the modulator and troponin C exhibit the greatest sequence homology (approximately 80%). This and other observations suggest that the tertiary structures of these two Ca^{2+}-binding proteins are very similar and that they have evolved from a common ancestor. Sequence data and studies of functional properties indicate that the modulator protein may have been one of the earliest Ca^{2+}-binding proteins to emerge with a defined function and may, indeed, be a precursor of troponin C.

I. Purposes of this study

(1) Identification of the amino acid residues of the protein modulator which are essential for its activation of PDE. The modulator may contain a single binding domain which is involved in its association with each of the modulator binding proteins noted above; hence, a detailed study of the mechanism of interaction of the modulator with PDE may provide a basis for understanding of all modulator-mediated, physiological regulatory mechanisms.

(2) Investigation of the conformational changes which occur in the protein modulator as it binds Ca^{2+}.

(3) Further study of the structural homology between the protein

modulator and troponin C.

II. Strategy

The strategy employed in approaching these problems involved the use of two widely used and proven methods of protein chemistry, namely, chemical modification of specific functional groups in the protein, and controlled enzymatic digestion of the native protein followed by structural and functional characterization of the resultant fragments.

Chemical modification represents the easiest and most direct means for the identification of amino acid residues of a protein which are involved in functional expression, e.g., residues at catalytic and binding sites of a protein. While this method often provides very conclusive evidence, the inherent drawbacks must be considered. Thus, e.g., while a loss of enzymatic activity may be observed upon treatment of an enzyme with a particular reagent, this is not necessarily due to the modification of active-site residues. Changes in activity may be due to structural changes, often the result of nonspecificity of the reagent, harsh reaction conditions, or extensive modification. The possibility of such conformational effects can be minimized by the use of mild reaction conditions with specific modification. It is always desirable to assess the likelihood of observed activity losses being due to conformational effects, as shown in this study : chemically modified, inactive derivatives were examined by circular dichroic spectrophotometry and their structures compared with that of the native protein. If chemical modification has no effect on the properties of interest, it is reasonable to assume that the modified residues are not

essential to the activity under investigation.

Controlled enzymatic digestion with isolation and characterization of the fragments obtained provides a very useful tool for identifying regions of the molecule which are required for the expression of functional activity. This approach has also proven very informative as regards the conformational changes occurring in both troponin C and the modulator protein upon binding of Ca^{2+}, and also as regards the structural homology between these two proteins.

LITERATURE REVIEW

I. Introduction

Cyclic nucleotides play important roles in the regulation of many biological functions (Robinson et al, 1968; Jost and Rickenberg, 1971; Wells and Hardman, 1977) and, consequently, their intracellular concentrations are very precisely controlled. The only established mechanism for the disposal of cyclic nucleotides in mammalian cells is their hydrolysis catalyzed by the enzyme cyclic nucleotide phosphodiesterase. This enzyme was first demonstrated by Sutherland and Rall (1958) and was subsequently partially purified from bovine heart by Butcher and Sutherland (1962) and from rabbit brain by Drummond and Perrott-Yee (1961). Several mechanisms exist whereby the activity of this enzyme is regulated, including allosteric modulation (Goren and Rosen, 1971; Beavo et al, 1971; Cheung, 1967), isoenzymes (Thompson and Appleman, 1971), enzyme localization (Cheung, 1967), and protein-protein interaction (Cheung, 1971; Goren and Rosen, 1971; Kakiuchi and Yamazaki, 1970).

Virtually all mammalian tissues contain multiple forms of cyclic nucleotide phosphodiesterase which differ in molecular weight and catalytic and regulatory properties (Appleman et al, 1973) and may therefore play different physiological roles in the cells. At least one form of the enzyme requires a calcium-dependent protein modulator for maximum activity.

II. Discovery of protein modulator

The protein modulator of bovine brain cyclic nucleotide

phosphodiesterase (EC 3.1.4.17) was discovered independently by Cheung (1970) in bovine brain and by Kakiuchi et al, (1970) in rat brain during attempts to purify the phosphodiesterase. It had earlier been reported (Cheung, 1969) that phosphodiesterase activity was gradually lost during the purification of the enzyme from beef brain and that the partially purified phosphodiesterase was activated upon incubation with snake venom. It was apparent that the venom contained a stimulatory factor(s) which activated the purified but not the crude enzyme. This venom factor was found to be non-dialyzable, insensitive to ribonuclease and deoxyribonuclease, and moderately susceptible to trypsin attack. It was heat and pH-sensitive, but part of the activity survived boiling at pH 1.2 for several minutes. Subsequently, Cheung (1970, 1971) reported that the crude bovine brain phosphodiesterase contained an activator which was removed from the enzyme during purification; this phenomenon accounted for the relative inactivity of the purified enzyme. Cheung demonstrated that the activator, isolated free of phosphodiesterase activity, effectively reconstituted the activity of the purified enzyme. Cheung (1971) also isolated an activator and a relatively inactive PDE from human brain, porcine brain, rat brain and bovine heart. An activator from one tissue effectively cross-activated a purified PDE from another tissue, indicating a lack of tissue specificity.

Independently, Kakiuchi and Yamazaki (1970) and Kakiuchi et al, (1971) resolved the phosphodiesterase activity of the high speed supernatant of rat brain extract into two peaks of phosphodiesterase activity by gel filtration on Sepharose 6B. Peak I was referred to as the "Ca^{2+}-independent and Mg^{2+}-dependent" phosphodiesterase; peak II represented the "Ca^{2+}-plus Mg^{2+}-dependent" PDE. The latter activity

required a heat-stable, non-dialyzable factor (PDE activating factor, PAF) present in brain extract. PAF stimulated the activity of peak II only when the Ca^{2+} concentration was above a threshold value (about 2 μM with 0.4-1 μM substrate) and the stimulatory effect of Ca^{2+} was dependent upon the presence of the modulator (PAF) (Kakiuchi et al, 1973). The modulator was shown to be protein in nature since its activity was destroyed by trypsin or Nagarse, but not by treatment with lecithinase C or pancreatic ribonuclease.

Goren and Rosen (1971) separated a bovine heart PDE and its endogenous activator by DEAE-cellulose column chromatography. The activator was non-dialyzable and relatively heat-stable, retaining 67% activity when heated at 90^{o} for 5 minutes. Kinetic analysis revealed that the activator decreased the K_m of PDE for cAMP approximately 8-fold, but did not influence the V_{max}.

The protein modulator of PDE isolated from various tissues and species has subsequently been studied by numerous investigators and their major findings will be discussed in the remainder of this review.

III. Occurrence

The Ca^{2+}-dependent protein modulator has been purified to apparent homogeneity from bovine heart (Teo et al, 1973) bovine brain (Lin et al , 1974; Watterson et al, 1976), porcine brain (Teshima and Kakiuchi, 1974; Klee, 1977a), rat testis (Beale et al, 1977), porcine and bovine adrenal medulla (Egrie and Siegel, 1975), blood platelets (Muszbek et al, 1977), chicken gizzard (Dabrowska et al, 1977a), earthworm (Waisman et al, 1978) and the electroplax of Electrophorus electricus (Childers and Siegel, 1975). Purified Ca^{2+}-binding phosphoproteins

from porcine brain (Wolff and Siegel, 1972) and beef adrenal medulla (Brooks and Siegel, 1973) were later shown to be the protein modulator (Wolff and Brostrom, 1974); the phosphate found in these proteins was probably due to contamination. Kuo and Coffee (1976a) purified and characterized a troponin C-like protein from bovine adrenal medulla. This protein shares several physical and chemical properties with the protein modulator and it was recently demonstrated that it is identical to the bovine brain Ca^{2+}-dependent protein modulator (Dabrowska et al, 1977a).

The presence of the modulator protein has been indicated in several other tissues and species, e.g., rat anterior pituitary (Azhar and Menon, 1977), bovine posterior pituitary (Russell and Thorn, 1977), and porcine coronary arteries (Wells et al, 1975). The modulator has also been identified in various cell cultures, e.g., in cultures of C-6 glial tumor cells (Brostrom and Wolff, 1974), in cultured human lymphoblastic leukemia and retinoblastoma, and in Brown-Pierce (rabbit) carcinoma (Liu et al, 1977), and in cultured normal fibroblasts and fibroblasts transformed by Rous sarcoma virus (Vanaman and Watterson, 1976).

Several instances were reported of the isolation of troponin C-like proteins from various tissues. At least in some cases, it is possible that the so-called "troponin C" referred to is actually Ca^{2+}-dependent protein modulator. For example, Fine et al, (1975) isolated a TN-C-like protein from chick embryo brain which exhibited a Ca^{2+}-dependent change in mobility on alkaline urea-PAGE and formed a urea-stable complex with troponin I in the presence, but not absence, of Ca^{2+}; these are characteristics of TN-C which are shared by the

protein modulator. Furthermore, when it was substituted for rabbit skeletal muscle TN-C in an actomyosin ATPase assay system containing tropomyosin, TN-I and TN-T, the brain protein restored Ca^{2+} sensitivity to the muscle actomyosin ATPase, presumably by interacting with TN-I, thereby relieving the inhibition induced by the tropomyosin-TN-I-TN-T complex, when Ca^{2+} was present. More brain protein than muscle TN-C was required to relieve the inhibitory effect, however, and the resulting Ca^{2+} sensitivity was lower than when muscle TN-C was used. It has since been demonstrated (Amphlett et al, 1976; Dedman et al, 1977a) that brain modulator protein can substitute for TN-C in a reconstituted Ca^{2+}-sensitive actomyosin ATPase system. Furthermore, Fine et al, (1975) observed that the TN-C-like protein has a molecular weight slightly less than that of muscle TN-C; the brain modulator also has a molecular weight (17,000) slightly less than that of muscle TN-C (18,000). These observations suggest the likelihood that this chick embryo TN-C-like protein is, in fact, the Ca^{2+}-dependent modulator protein.

Drabikowski et al, (1977c) analyzed several tissues for the presence of modulator protein and/or TN-C: rabbit skeletal muscle, bovine cardiac muscle, smooth muscle (rabbit uterus and chicken gizzard), bovine adrenal medulla, bovine brain and bovine platelets. Each tissue was homogenized and centrifuged at 100,000 x g prior to fractionation of both supernatant and pellet on Sephadex DEAE A-50. The acidic proteins were collected and subjected to preparative urea gel electrophoresis. The band revealing a common property of both TN-C and modulator, i.e., the Ca^{2+}-dependent change in mobility on urea-PAGE, was isolated and analyzed. Three properties were used to distinguish between TN-C and the modulator protein: (1) the difference in mobility

on SDS-PAGE (15% polyacrylamide) due to different molecular weights; (2) the difference in mobility of the complex with troponin I in urea gel in the presence of Ca^{2+}; and (3) the ability of the modulator, but not of TN-C, to stimulate PDE activity. TN-C was found only in the 100,000 x g pellet of skeletal and cardiac muscle. Modulator, on the other hand, was found in the cytosol of all tissues examined. The results suggest that the TN-C-like protein isolated by Fine et al, (1975) is indeed modulator. Furthermore, other TN-C-like proteins recently found in smooth muscle (Head et al, 1977), adrenal medulla (Kuo and Coffee, 1976a) and platelets (McGowan et al, 1976) are, in fact, identical to the Ca^{2+}-dependent protein modulator (Drabikowski et al, 1977c).

IV. The modulator in transformed cells

The concentration of the Ca^{2+}-dependent protein modulator is increased in chick embryo fibroblasts when they are transformed by Rous sarcoma virus (RSV). The modulator comprises 1.32% of the soluble protein in homogenates of fibroblasts infected and transformed by RSV. In comparison, the modulator comprises only 0.30% of the soluble protein in homogenates of normal fibroblasts from confluent cultures and 0.36% of the soluble protein in homogenates of fibroblasts infected with a transformation-defective mutant of RSV (Watterson et al, 1976a; Vanaman and Watterson, 1976). Modulator levels in normal fibroblasts at subconfluent cell densities, i.e., rapidly growing normal fibroblasts, are 0.42-0.76% of the homogenate soluble protein, i.e., between that found in confluent normal fibroblasts and that in fibroblasts transformed by RSV. These observations suggest that the levels of the modulator protein are elevated under conditions in which chick embryo fibroblasts are undergoing rapid growth and have decreased cAMP levels.

Lynch et al, (1975) studied the effects of agents that elevate intracellular levels of cAMP, such as dibutyryl cAMP, on PDE activity in normal and virally transformed cells. They found that such treatment led to increased PDE activity in normal mouse fibroblasts (3T3 cells) and mouse fibroblasts transformed by Simian virus 40 (3T3-SV40 cells). These observed increases in PDE activity were presumably due to increased protein synthesis; stimulation appeared to be at the level of transcription. Further studies revealed that the increased PDE activities were due to the stimulation of the synthesis of PDE, but not the modulator protein, by dibutyryl cAMP. The PDE and modulator thus appear to be controlled by separate genes.

V. Mechanism of activation of PDE

Using preparations of modulator-deficient PDE, Teo and Wang (1973) demonstrated that activation of PDE by Ca^{2+} and by the protein modulator are mutually dependent, i.e., the enzyme is not activated by Ca^{2+} in the absence of the protein modulator nor is the enzyme activated by the protein modulator at free Ca^{2+} ion concentrations much below 1 μM. On the other hand, at saturating levels of the modulator, PDE is activated more than 10-fold by Ca^{2+}, the concentration of Ca^{2+} at half-maximal activation being 2.3 μM. At least two mechanisms could account for the mutual dependence of Ca^{2+} and the protein modulator for PDE activation. Firstly, activation could be the result of binding of both activators (Ca^{2+} and the protein modulator) to the enzyme, or, secondly, the protein modulator must bind Ca^{2+} in order to activate the enzyme. Teo and Wang (1973) suggested that the second mechanism is operative in the PDE activation on the basis of equilibrium binding studies with $^{45}Ca^{2+}$ which indicated specific interaction between the protein modulator

and Ca^{2+}; furthermore, no significant binding of Ca^{2+} by the modulator-dependent PDE was observed in gel filtration studies. Liu et al, (1974) independently demonstrated Ca^{2+} binding to the protein modulator: the concentration of Ca^{2+} needed to give half-maximum activation of PDE in the presence of saturating amounts of the modulator was 4 μM. Ca^{2+} alone did not activate PDE.

Teshima and Kakiuchi (1974) observed by gel filtration that an active PDE-modulator complex (molecular weight ~200,000) was formed in the presence of Ca^{2+}. Addition of excess EGTA resulted in dissociation of this enzyme-modulator complex into relatively inactive PDE (molecular weight ~150,000) and modulator (~28,000). Comparable observations were made by Lin et al, (1975) suggesting that formation of the enzyme-modulator complex is dependent on Ca^{2+}. The effect of Ca^{2+} on the formation of this PDE-modulator complex was shown to be instantaneous (Lin et al, 1975). Similar observations were also reported by Wickson et al, (1975).

Based on these results, a mechanism for the activation of cyclic nucleotide PDE by the Ca^{2+}-dependent protein modulator was proposed by several groups (Teshima and Kakiuchi, 1974; Liu et al, 1974; Wang et al, 1975). According to this model, the activation of PDE is a stepwise process initiated by the binding of Ca^{2+} to the protein modulator. Upon Ca^{2+} binding the modulator is converted from an inactive to an active conformation. The active Ca^{2+}-modulator complex then associates with the PDE to form a ternary complex simultaneously inducing a conformational change in the PDE through this protein-protein interaction. The overall result is an enhancement of the PDE activity some 6- to 10-fold. The mechanism of activation may be represented schematically as follows:

$$Ca^{2+} + \text{Modulator} \rightleftharpoons \left[Ca^{2+} - \text{Modulator}\right]$$

$$\left[Ca^{2+} - \text{Modulator}\right] \rightleftharpoons \left[Ca^{2+} - \text{Modulator}^*\right]$$

$$\left[Ca^{2+} - \text{Modulator}^*\right] + PDE_{inactive} \rightleftharpoons \left[Ca^{2+} - \text{Modulator}^* - PDE\right]$$

$$\left[Ca^{2+} - \text{Modulator}^* - PDE\right] \rightleftharpoons \left[Ca^{2+} - \text{Modulator}^* - PDE^*\right]$$

where * denotes a different conformation.

The individual steps in the mechanism of activation will be considered in more detail in the following sections.

VI. Calcium binding properties

As mentioned in the previous section Teo and Wang (1973) showed, by equilibrium binding studies, that $^{45}Ca^{2+}$ binds to the protein modulator. A Scatchard plot of the data exhibited two linear regions suggesting the presence of two sets of calcium binding sites on the protein with different affinities: one high affinity site and 2 or 3 low affinity sites per protein molecule. The dissociation constants for Ca^{2+} bound at the high and low affinity sites were calculated to be 3×10^{-6} M and 12×10^{-6} M, respectively.

Several investigators have since studied the Ca^{2+} binding properties of the protein modulator. While there is no doubt that the protein modulator is a Ca^{2+} binding protein, discrepancies are apparent in the literature regarding the number of classes of Ca^{2+} binding sites on the protein and their respective affinities and capacities. Liu et al, (1974) reported that atomic absorption spectrophotometry revealed the presence of one mole of Ca^{2+} per mole of modulator. However, a Scatchard plot of data obtained from equilibrium Ca^{2+} binding studies revealed 4 Ca^{2+} binding sites per mole with dissociation constants

ranging from 4×10^{-6} M to 18×10^{-6} M (Liu et al, 1974; Lin et al, 1974). More recently (Watterson et al, 1976) Scatchard plots of $^{45}Ca^{2+}$-binding data obtained using the method of equilibrium dialysis indicated two sets of Ca^{2+} binding sites: high affinity binding ($K_d = 1 \times 10^{-6}$ M) of 2 moles of Ca^{2+} per mole of modulator protein, and low affinity binding ($K_d = 8.6 \times 10^{-4}$ M)of 2 moles of Ca^{2+} per mole of modulator.

Klee (1977a) independently observed two classes of Ca^{2+} binding sites - two high affinity sites ($K_d = 4 \times 10^{-6}$ M) and two low affinity sites ($K_d = 1.2 \times 10^{-5}$ M) - by equilibrium dialysis.

Wolff et al, (1977) pointed out that in each of these studies, the determination of bound Ca^{2+} involved the measurement of differences of distribution of radioactively labeled $^{45}Ca^{2+}$ across a membrane combined with a measurement of the specific activity of added Ca^{2+}. Furthermore, it is unclear in these studies whether the buffers and modulator employed were free of divalent cations in the form of either unlabeled Ca^{2+}, which would reduce the specific activity of the Ca^{2+} in the systems, or in the form of Mg^{2+} or Mn^{2+} which would competitively inhibit Ca^{2+} binding. Wolff et al, (1977) bypassed these technical difficulties in their studies of Ca^{2+} binding to the modulator by the technique of equilibrium dialysis since their experiments were based upon direct measurement in each sample of the concentration of spectral grade divalent cations by atomic absorption spectrophotometry and were not conducted with either chelators or $^{45}Ca^{2+}$. In this way they established the existence of two classes of Ca^{2+} binding sites: a high affinity class with a capacity of 3 moles of Ca^{2+} per mole of modulator and a dissociation constant of 2×10^{-7} M, and a lower affinity class with a capacity of 1 mole of Ca^{2+} per mole of modulator and a dissociation

constant of 10^{-6} M. More recently, Dedman et al (1977b) demonstrated, by the technique of equilibrium dialysis, that rat testis modulator protein contains 4 equivalent Ca^{2+}-binding sites with $K_d = 2.4 \times 10^{-6}$ M.

VII. Stoichiometry

The stoichiometry of the interaction between PDE and the modulator has not been established. However, estimates have been made on the basis of molecular weights for the free enzyme and the enzyme-modulator complex determined by gel filtration. Thus, Teshima and Kakiuchi (1974) and Lin et al, (1975) obtained values of 150,000 daltons for the free enzyme and 200,000 daltons for the enzyme-modulator complex. These values are inconclusive due to the fact that crude enzyme preparations were used in the determinations and the modulator itself exhibits anomalous behaviour on gel filtration, probably due to aggregation phenomena. More recently, utilizing an 80% pure preparation of the bovine heart modulator-dependent PDE, Ho et al, (1977) estimated a molecular weight of 155,000 for the free enzyme and 230,000 for the enzyme-modulator complex by gel filtration on a calibrated Sephadex G-200 column.

Dedman et al, (1977b) studied the relationship of Ca^{2+} binding by rat testis modulator protein to the stimulation of PDE activity. They observed, by circular dichroic spectrophotometry and tyrosine fluorescence studies, that Ca^{2+} produces marked conformational changes in the modulator. While there is no apparent cooperativity in the binding of Ca^{2+} to the modulator, there appears to be positive cooperativity for the interaction of the Ca^{2+}-modulator complex with PDE. The Hill coefficient of PDE activation by increasing amounts of modulator was calculated to be 2.0, implying the interaction of multiple Ca^{2+}-modulator complexes per phosphodiesterase. Thus, the binding of Ca^{2+}-modulator

to the first site may greatly enhance the binding of other Ca^{2+}-modulator molecules to the enzyme. Dedman et al, (1977b) proposed the following model for the Ca^{2+}-and modulator-mediated regulation of PDE activity:

$$Ca^{2+} + \text{Modulator}_{inactive}$$

$$\Big\Vert$$

$$\left[Ca^{2+} - \text{Modulator}^*\right]_{active}$$

$$\Big\Vert \text{ PDE}$$

$$\left[Ca^{2+} - \text{Modulator}^*\right]\text{-PDE}$$

$$\Big\Vert \; n\left[Ca^{2+} - \text{Modulator}^*\right]_{active}$$

$$\left[Ca^{2+} - \text{Modulator}^*\right]_{n+1}\text{-PDE}$$

(activated state)

where * denotes a different conformation.

VIII. Conformational change accompanying Ca^{2+} binding

An essential phase in the proposed mechanism of activation of PDE by the Ca^{2+}-dependent protein modulator is a conformational change in the modulator as it binds Ca^{2+}. This results in the formation within the modulator of a specific binding domain which is involved in the interaction between the modulator and PDE. Considerable evidence has now accumulated which substantiates the occurrence of such a conformational change. Upon binding Ca^{2+}, the modulator undergoes changes in several of its physical properties: the ultraviolet absorption spectrum shows a small decrease; the tyrosine fluorescence emission at 315 nm exhibits a 30% enhancement in intensity; Ca^{2+} stabilizes the modulator against inactivation (Wang et al,1975); Ca^{2+} strengthens the protein modulator against tryptic or chymotryptic inactivation (Ho et al,

1975; Liu and Cheung, 1976).

Optical rotatory dispersion measurements by Liu and Cheung (1976) showed that the Ca^{2+}-free modulator exhibited a negative minimum at 231 nm and a reduced mean residue rotation ($[m']_{231\ nm}$) of -5700 deg cm^2 $dmole^{-1}$ corresponding to a helical content of 39%. The binding of Ca^{2+} by the modulator is accompanied by a decrease in the value of $[m']_{231\ nm}$ to -7500 deg cm^2 $dmole^{-1}$ corresponding to a helical content of 57%. Liu and Cheung (1976) also studied the change in $[m']_{231\ nm}$ as a function of Ca^{2+} concentration and observed a half-maximum decrease of $[m']_{231\ nm}$ at 8 μM Ca^{2+}; this is comparable to the Ca^{2+} concentration giving half-maximal activity of the modulator. Thus, binding of Ca^{2+} to the modulator increases its helicity and the more helical conformation is the active form of the protein.

Kuo and Coffee (1976a) purified and characterized a troponin C-like protein from bovine adrenal medulla (adrenal medullary calcium-binding protein, AM-CBP) which has now been shown to be identical to the Ca^{2+}-dependent protein modulator of phosphodiesterase (Dabrowska et al, 1977a). Subsequently, Kuo and Coffee (1976b) studied the conformational change occurring in this protein as it binds Ca^{2+}. The far-ultraviolet circular dichroism (CD) spectrum of the native AM-CBP exhibited characteristic helical ellipticity bands at 222 and 207 nm. The helical content estimated from these data was between 40 and 45%. Removal of Ca^{2+} was accompanied by a change in ellipticity corresponding to a decrease in helical content to 20%. The near-UV CD spectrum of AM-CBP exhibited negative dichroic bands at 262 and 268 nm characteristic of phenylalanine. These bands were found to be relatively insensitive to removal of Ca^{2+} indicating no significant change in the asymmetry of the microenvironment

of these phenylalanine residues upon removal of Ca^{2+}.

Kuo and Coffee (1976b) also observed a Ca^{2+}-dependent conformational change in AM-CBP by sedimentation velocity studies: the sedimentation coefficient of the native protein in the presence of Ca^{2+} was 1.89S, while in the absence of Ca^{2+} it was 1.50S. From the elution properties of AM-CBP on Sephadex G-100, the Stokes radius was observed to be 19.8Å in the presence of Ca^{2+} and 21.9Å in the absence of Ca^{2+}. The reduced helical content, the decreased sedimentation coefficient, and the increased Stokes radius observed upon removal of Ca^{2+} from the AM-CBP indicated that removal of Ca^{2+} results in a transformation from a compact, symmetrical structure to one that is less ordered and more asymmetrical.

Klee (1977a) conducted similar studies of the Ca^{2+}-induced conformational change in porcine brain modulator protein. Circular dichroism studies indicated that Ca^{2+} induces an increase of 5 - 8% in α-helix content with a concomitant decrease in amount of random coil; in the absence of Ca^{2+} the protein contains 30 - 35% α-helix, 50% random coil and 15 - 20% β-pleated sheet. Spectrophotometric titration indicated that the two tyrosine residues have pK's of 10.4 and 11.9 and are, therefore, in different environments. One tyrosine residue (pK' = 11.9) is apparently buried within the hydrophobic interior of the protein and is not significantly affected by the conformational changes accompanying Ca^{2+} binding. The other tyrosine residue (pK' = 10.4) is partially exposed in the absence of Ca^{2+}; upon binding of Ca^{2+}, the pK' of this residue shifts to 10.1 indicating that increased exposure of this tyrosine residue to solvent occurs as part of the conformational change accompanying Ca^{2+} binding.

The far-UV CD spectrum of bovine brain protein modulator is characterized by negative maxima at 207 and 222 nm (Wolff et al, 1977). In the absence of divalent cations the mean residue ellipticity at 208 nm, $[\theta]_{208\ nm}$, was -12,000 deg cm^2 $dmole^{-1}$, indicative of approximately 28% helical content. Addition of saturating amounts of Ca^{2+} resulted in a decrease in $[\theta]_{208\ nm}$ to -16,200 deg cm^2 $dmole^{-1}$ indicating an increase in helical content to 42%. The near-UV CD spectrum in the absence of Ca^{2+} displayed a positive maximum at 266 nm, and negative maxima at 269 and 262 nm characteristic of phenylalanine were also observed. Addition of Ca^{2+} resulted in marked changes in the near-UV CD spectrum, notably the appearance of a negative maximum at 282 nm. The difference CD spectrum obtained with and without bound Ca^{2+} displayed maxima at 280, 269, 262 and 258 nm, revealing contributions from both tyrosine and phenylalanine; hence changes in the asymmetry of the microenvironment of both tyrosine and phenylalanine residues accompanied the binding of Ca^{2+} to the modulator.

Rat testis protein modulator has also been shown to undergo marked conformational changes upon Ca^{2+} binding (Dedman et al, 1976b). The Ca^{2+}-induced changes in conformation were monitored by circular dichroism and tyrosine fluorescence studies. Ca^{2+} was found to increase the α-helical content of the modulator from 45% at 10^{-8} M Ca^{2+} to 54% at 10^{-5} M Ca^{2+}. Over the same Ca^{2+} concentration range, the tyrosine fluorescence of the modulator increased 2.5-fold.

IX. Modulator-induced conformational change in PDE

Also inherent in the mechanism of activation of PDE by the Ca^{2+}-dependent protein modulator is the occurrence of a conformational change in the PDE upon binding of the Ca^{2+}-modulator complex. In order

to see whether or not such a conformational change takes place, Wang et al, (1975) examined the effects of Ca^{2+} and the protein modulator on the thermal stability of the enzyme. The PDE was found to be relatively stable at 55°C and pH 7.5 either in the free state or when either Ca^{2+} or the protein modulator was present. In the presence of both Ca^{2+} and the protein modulator, however, the enzyme rapidly lost its activity. The inactivation rate for the free enzyme was about seven times lower than that for the complexed enzyme. These observations suggest that the PDE does indeed undergo a conformational change upon association with the Ca^{2+}-modulator complex.

X. Physical properties

Several groups of investigators have conducted thorough physical and chemical characterization of the modulator protein derived from different sources. The different modulators exhibit very similar physical parameters.

A. Molecular weight and isoelectric point

Tables I and II summarize the major physical properties of the protein modulator derived from various sources. Considerable discrepancies are apparent in the reported values of molecular weight of the protein modulator; the values obtained depend more on the method of determination used than on the source of the protein. It is apparent that the modulator is a monomeric protein of molecular weight approximately 17,000 regardless of source. The protein evidently exhibits anomalous behaviour on gel filtration, since molecular weights obtained by this technique are always too high. Protein modulator from all sources is a very acidic protein as evidenced by the fact that all exhibit an isoelectric point around 4.

TABLE I. Molecular weight determinations of the protein modulator.

Tissue	Molecular weight Value	Obtained by	Reference
Bovine brain	15,000	SDS-PAGE	Lin et al (1974)
	15,000	sucrose density gradient centrifugation	
	15,000	sedimentation equilibrium	
	14,500	sedimentation velocity and diffusion	
	18,920	amino acid analysis	
	31,000	gel filtration	
	17,800	sedimentation equilibrium	Watterson et al (1976)
	18,000	SDS-PAGE	
	16,723	amino acid sequence	Vanaman et al (1977)
Bovine heart	27,000	gel filtration	Teo et al (1973)
	19,200	sedimentation diffusion	
Bovine adrenal medulla	11,900	equilibrium ultracentrifugation	Brooke and Siegel (1972)
	35,000	gel filtration	
	16,000	SDS-PAGE	Kuo and Coffee (1976a)
	16,000	sedimentation equilibrium	
Porcine brain	11,500	equilibrium ultracentrifugation	Wolff and Siegel (1972)
	34,500 } 46,000 }	gel filtration	
Rat testis	16,900	amino acid composition	Beale et al (1977)
Earthworm	15,700-18,200	analytical ultracentrifugation	Waisman et al (1978)
	18,000	SDS-PAGE	

TABLE II. Other physical properties of the protein modulator

Tissue	Sedimentation coefficient ($S_{20,w}$)	Diffusion constant ($D_{20,w}$) $cm^2\ s^{-1}$	Partial specific volume ($\bar{v}$) $ml\ g^{-1}$	Isoelectric point (pI)	Reference
Bovine brain	1.85 S	1.09×10^{-6}	0.72	4.3	Lin et al, (1974)
Bovine heart	2.0 S	9.0×10^{-7}	0.71	-	Teo et al, (1973)
Bovine adrenal medulla	1.89 S	-	0.72	4.27	Kuo and Coffee (1976a)
Rat testis	1.9 S	-	-	3.85	Beale et al, (1977)
Earthworm	1.95 S	9.25×10^{-7}	0.72	4.0	Waisman et al, (1978)

B. Ultraviolet absorption spectrum

The ultraviolet absorption spectra of modulators derived from various sources are indistinguishable from each other, but quite different from those of globular proteins in general. Instead of having an absorption maximum at 280 nm, the spectra exhibit considerable fine structure in the region of 250 - 280 nm with absorption peaks at 253, 259, 265, 268 and 276 nm (Wang et al, 1975; Stevens et al, 1976; Watterson et al, 1976; Liu and Cheung, 1976; Kuo and Coffee, 1976a; Klee, 1977a; Dabrowska et al, 1977a). This unusual optical property is due to the absence of tryptophan and a high phenylalanine: tyrosine ratio (8:2).

XI. Chemical structure

A. Amino acid composition

Comparison of the amino acid compositions of the protein modulators isolated from bovine heart (Wang et al, 1975; Stevens et al, 1976), bovine brain (Lin et al, 1974; Waterson et al, 1976), porcine brain (Wolff and Siegel, 1972; Klee, 1977a), bovine adrenal medulla (Kuo and Coffee, 1976a), rat testis (Beale et al, 1977) electroplax of Electrophorus electricus (Childers and Siegel, 1975), and earthworm (Waisman et al, 1978) indicates that modulators isolated from different sources exhibit a large degree of similarity. Appendix I shows a comparison of the amino acid compositions of bovine heart and bovine brain modulators; these compositions are virtually identical.

The protein modulator has a high content of acidic residues with a relatively low basic content, consistent with the observed isoelectric point of about 4.0. As expected from the UV absorption spectra, tryptophan is absent and the modulator has a high ratio of

phenylalanine to tyrosine. Cysteine is also absent.

B. Amino terminal analysis

Conflicting reports concerning the results of amino terminal analysis of the protein modulator have appeared in the literature. Thus, Lin et al, (1974) identified valine as the amino terminal residue of the bovine brain modulator using the dansyl chloride procedure. Kuo and Coffee (1976a) demonstrated, also by the dansyl chloride procedure, amino terminal isoleucine in the adrenal medulla modulator. On the other hand, Watterson et al, (1976) working with bovine brain modulator and Stevens et al, (1976) working with the bovine heart protein failed to detect an amino terminal amino acid using a Beckman automatic sequencer, and concluded that the amino terminus of the modulator is blocked. In determining the amino acid sequence of the bovine brain protein modulator, Vanaman et al, (1977) isolated a peptide containing a blocked amino terminus and identified this peptide as coming from the amino terminal. They noted that the amino terminus of the modulator is acetylated, as is also true of the homologous proteins, troponin C, the DTNB light chain of myosin, myosin alkali light chains, and parvalbumin. Rat testis modulator was also reported to have an acetylated amino terminus, the amino terminal sequence being Ac-Ala-Asp-Glu (Dedman et al, 1977b).

C. Amide determination

Liu and Cheung (1976) determined the number of acidic amino acids in the hydrolyzate of bovine brain modulator protein derived from glutamine and asparagine by hydrolyzing a sample of the pure protein in 2N HCl at 100° in vacuo and measured the amount of NH_3 in the resultant hydrolyzate using an amino acid analyzer. They concluded that out of a

total of 54 acidic amino acids found in the acid hydrolyzate, 16 of them derived from glutamine and asparagine, and 38 from glutamic and aspartic acids. Independently, Walsh and Stevens, (1977) determined the total free carboxyl group content, i.e., glutamic plus aspartic acids, of the bovine heart protein modulator by measurement of the increase in glycine methyl ester in the presence of a water-soluble carbodiimide. They concluded that out of a total of 55 acidic residues in the acid hydrolyzate, 40 derived from glutamic and aspartic acids, and 15 from glutamine and asparagine.

D. Amino acid sequence

The amino acid sequence of the bovine brain modulator protein was determined by Vanaman et al, (1977) and is shown in Appendix II aligned with the sequences reported previously for bovine cardiac muscle troponin C and rabbit skeletal muscle troponin C. The modulator protein sequence was deduced from analyses of peptides derived from the intact protein by a number of different procedures. Tryptic peptides encompassing the entire sequence of the protein were obtained following digestion of the performic acid oxidized protein or of the unmodified protein in the presence of EGTA. The amino acid sequences of these peptides were determined by automated or manual Edman degradation of the intact tryptic peptides and, where necessary, of thermolysin, chymotrypsin, or cyanogen bromide sub-fragments of individual peptides. The order of the individual tryptic peptides was unequivocally established by studies of cyanogen bromide peptides derived from the intact protein and of peptides isolated following trypsin cleavage of the citraconylated protein. Sequence assignments and overlaps were further confirmed by studies of chymotryptic peptides prepared from the performic acid

oxidized protein.

Vanaman et al, (1977) noted that the Asp-Lys-Asp sequences at residues 19-21 and 93-95 are relatively resistant to trypsin cleavage. Trypsin also did not cleave at the trimethyllysine residue (position 115). On the other hand, tryptic cleavage was observed to occur between the methionine residues at positions 71 and 72 and following the Met-Met sequence at positions 144 and 145.

E. Presence of an unusual amino acid

Watterson et al, (1976) originally reported the presence in the acid hydrolyzate of the bovine brain modulator protein of an unidentified ninhydrin-positive basic compound not found in muscle TN-C. Vanaman and Watterson (1976) subsequently showed the presence of this compound in hydrolyzates of brain modulators from all species tested, namely bovine, porcine, rabbit, rat and chicken to the extent of one mole per mole of modulator. This material was subsequently identified (Vanaman et al, 1977) as ϵ-N-trimethyllysine; it occurs at position 115 in the primary sequence of the bovine brain modulator.

Jackson et al (1977) independently demonstrated the presence of an unidentified component in the acid hydrolyzate of purified rat testis modulator; amino acid analysis showed one residue of this component per mole of protein; it eluted on the analyzer between histidine and lysine. The elution time for this component corresponded to that of synthetic ϵ-N-trimethyllysine (TML). Natural abundance ^{13}C Fourier transform NMR spectra of rat testis modulator displayed a narrow resonance at 53.8 ppm which is characteristic of the carbon resonance of a trimethylammonium group. A single tryptic peptide of the modulator was isolated which contained the residue of TML; digestion of this peptide with thermolysin

yielded a peptide with the sequence Leu-Gly-Glu-TML. This is identical to the corresponding region (residues 112-115) of the bovine brain protein (Vanaman et al, 1977).

The presence of this unusual residue in all modulator proteins examined, but its absence from TN-C, represents the most obvious significant difference between these proteins, and it may account for those functions which are specific to the modulator.

XII. Application of the modulator protein in enzyme purifications

A. Ion-exchange chromatography

As was indicated earlier, most mammalian tissues contain multiple forms of the enzyme cyclic nucleotide phosphodiesterase. For example, bovine heart is known to contain two cytoplasmic phosphodiesterases (Ho et al, 1976). One of these forms is Ca^{2+}-independent while the other requires both Ca^{2+} and the protein modulator for maximum activity. Considerable difficulty has been encountered in all attempts to purify PDE to homogeneity. Ho et al, (1977) have succeeded in obtaining a preparation of the bovine heart Ca^{2+}-and modulator-dependent PDE which is approximately 80% pure as judged by analytical disc gel electrophoresis by making use of the reversible interaction between the Ca^{2+}-dependent modulator and this form of PDE. The purification procedure exploits the significantly different affinity of the free enzyme and the enzyme-modulator complex for DEAE-cellulose. The procedure involves ammonium sulfate fractionation, three chromatographic steps on DEAE-cellulose, and gel filtration on Sephadex G-200, with a 5,000-fold purification over the crude extract.

The first DEAE-cellulose employs EGTA-containing buffers so

that the modulator-dependent enzyme is present in the modulator-free form. In this state, the modulator-dependent PDE is loosely bound to the column and well separated from the Ca^{2+}-independent form of the enzyme which is more tightly bound. The second DEAE-cellulose column also employs EGTA-containing buffers and serves to maximize the separation of the two forms of the enzyme. The third DEAE-cellulose column then employs Ca^{2+}-containing buffers so that the enzyme is in the PDE-modulator complex state. This complex binds much more tightly to the column than does the modulator-free form of the enzyme so that, since the majority of contaminating proteins must bind only loosely to this column, considerable purification is achieved in this step; indeed, this is the most effective step in the purification, providing an enrichment of over 75-fold.

B. Affinity chromatography

The fact that the protein modulator, in the presence of Ca^{2+}, exhibits strong interaction with the Ca^{2+}-and modulator-dependent PDE led several investigators to attempt the preparation of an affinity column in which the modulator is linked to the matrix in such a way that its biological activity is retained, with a view to achieving rapid and effective purification of PDE. Watterson and Vanaman (1976) succeeded in coupling the modulator protein to Sepharose 4B under conditions which allowed retention of PDE-stimulating activity. They used this conjugate to demonstrate directly the Ca^{2+}-dependent formation of a reversible modulator-PDE complex and to purify the modulator-dependent PDE by affinity chromatography. The resultant PDE preparation was still heterogeneous by the criterion of analytical disc gel electrophoresis, however, giving rise to 3 major bands of molecular weights 60,000,

40,000, and 18,000 daltons, as well as a number of minor bands.

Independently, Klee (1977b) also attempted to purify the modulator-dependent PDE by affinity chromatography on modulator-Sepharose. A 20- to 30-fold purification was achieved and the enzyme so prepared was stimulated 15- to 20-fold by the Ca^{2+}-dependent modulator. The preparation again was not homogeneous but further purification could be achieved by gel filtration on Sephadex G-200. It was especially interesting that a lower molecular weight component which could be resolved by SDS-PAGE into two polypeptides of 65,000 and 15,000 daltons eluted from the affinity column after the PDE activity. This component was shown to inhibit the activation of the purified PDE by limiting concentrations of modulator protein; no such inhibition was observed in the presence of a large excess of modulator or in the presence of EGTA. It is now apparent that this component is identical to a modulator binding protein characterized by Wang and Desai (1976, 1977), and discussed below in XV F.

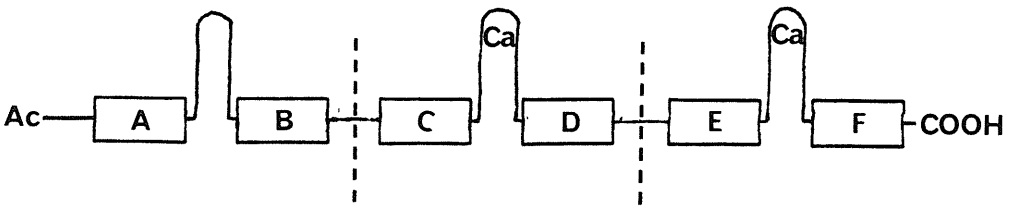
Recently, Sakai et al, (1977) demonstrated the presence of three forms of PDE in a rat brain homogenate supernatant by DEAE-cellulose column chromatography. Only one form of the enzyme was stimulated by Ca^{2+} and the protein modulator; this form of the enzyme could be bound to an affinity column which was prepared by coupling purified modulator protein to cyanogen bromide-activated Sepharose.

It is interesting to note that Wallace and Harary (1975) achieved partial purification of rat heart modulator protein by a procedure involving an affinity chromatographic step utilizing PDE bound to Sepharose 4B.

XIII. A family of homologous calcium-binding proteins produced by gene replication

A. Parvalbumins

Parvalbumins are small (molecular weight approximately 12,000), acidic, Ca^{2+}-binding proteins found in the skeletal muscle of vertebrates. While the physiological function(s) of these proteins remains a subject of speculation, their structure has been thoroughly characterized. Complete amino acid sequences of parvalbumins from pike (Frankenne et al, 1973), hake (Capony et al, 1973), carp (Coffee and Bradshaw, 1973), frog (Capony et al, 1975), and rabbit (Enfield et al, 1975; Capony et al, 1976) and partial sequences of several others (Demaille et al, 1974) have been reported. The three-dimensional structure of a carp parvalbumin has been determined at 1.85 Å resolution by X-ray diffraction (Kretsinger et al, 1971; Kretsinger and Nockolds, 1973). The molecule contains two Ca^{2+}-binding sites, each of which is confined to an 11-residue segment along the polypeptide chain. The structure of the complete molecule consists of six α-helices, denoted A through F, which are represented by the boxes in the following schematic representation of the molecule:



One Ca^{2+} ion is coordinated in the loop joining helix C to helix D; the second Ca^{2+} ion is coordinated in the EF loop between the E and F helices. This conformation of a helix, calcium-binding loop and helix is referred to as an "EF hand". The entire molecule can be divided into 3 segments, each 33-37 residues long which contain two helical regions each, as indicated by the dashed lines in the scheme above. A considerable degree of sequence and structural homology is apparent between the 2 Ca^{2+}-binding regions, which led Kretsinger (1972) and McLachlan (1972) to suggest that the parvalbumin molecule arose from gene duplication and fusion with subsequent divergent evolution. In addition to the 2 Ca^{2+}-binding regions, the parvalbumin molecule contains 2 helices at its N-terminal end and the packing of the six helices produces a hydrophobic core. On the basis of amino acid sequence data it is most probable that the tertiary structures of all other parvalbumins resemble very closely that of the carp muscle protein.

B. Troponin C

Troponin is a complex of three proteins (Greaser and Gergely, 1973) found in the thin filaments of skeletal muscle of a wide variety of species (Lehman et al, 1972). This protein complex, in combination with tropomyosin, regulates muscle contraction by conferring Ca^{2+} sensitivity on the interaction of actin and myosin (Weber and Murray, 1973). The components of the troponin complex are: troponin I (TN-I; 20,900 daltons) which inhibits the actin-myosin interaction; troponin C (TN-C; 18,000 daltons) which binds calcium and relieves the inhibition caused by TN-I; and troponin T (TN-T; 30,500 daltons) which attaches the subunit complex to tropomyosin.

The amino acid sequence determination of rabbit skeletal muscle

TN-C (Collins et al, 1973; 1977) bore out predictions (Kretsinger, 1972; Benzonana et al,1972) that this calcium-binding protein is similar to parvalbumins in primary structure. Collins et al, (1973) observed that the TN-C molecule can be divided into 4 segments, each 36-38 residues in length, which are similar in sequence to each other and to the two Ca^{2+}-binding regions of parvalbumins. From comparison of the amino acid sequences and knowledge of the three-dimensional structure of parvalbumin, they located 4 apparent Ca^{2+}-binding sites in TN-C. On this basis, they proposed that the Ca^{2+}-binding regions of TN-C have three-dimensional structures similar to those of parvalbumins, and that the two proteins are homologous, each having evolved by replication of a small (about 35 residues) precursor with a single calcium-binding site. Thus, rabbit skeletal muscle TN-C appears to contain 4 EF hand regions; the 4 calcium-binding octahedra (each Ca^{2+} ion is bound by 6 coordinating ligands) are easily recognised within the primary structure of the molecule.

Subsequently, Kretsinger and Barry (1975) predicted the tertiary structure of rabbit skeletal muscle TN-C on the basis of 3 assumptions: (I) TN-C contains 4 regions homologous to the Ca^{2+}-binding EF hand of parvalbumin; (2) the 4 EF hands are arranged in 2 pairs corresponding to Ca^{2+}-binding sites I and II, and III and IV, respectively; and (3) the regions of TN-C which are not in the 4 EF hands connect the hands within each pair, I to II and III to IV, and connect the pairs, region II to region III. The predicted model has a well-defined hydrophobic core made from side chains of all 8 helical regions and of the 4 Ca^{2+}-binding loops. The Ca^{2+} ions within pairs are separated by 11 Å, while the pairs of Ca^{2+} ions are separated from one another by over 30 Å.

The Ca^{2+} ion in binding site I is suggested to be accessible to solvent and most readily replaced by a lanthanide ion.

C. Myosin light chains

Myosin is composed of two heavy chains and four light chains. In rabbit white skeletal muscle there exist two structurally distinct classes of light chains, most commonly known as alkali light chains (ALC) and DTNB light chains (DLC). ALCs are believed to have an essential function in the ATPase activity of myosin, but the function of DLCs remains unclear. Some evidence suggests that Ca^{2+} exerts a direct effect on rabbit skeletal muscle myosin, and that DLCs may somehow be involved in regulation of actin-myosin interaction, but there has, as yet, been no direct demonstration of myosin-linked Ca^{2+} control. DLCs bind one Ca^{2+} ion per mole, but Mg^{2+} competes strongly, so that significant amounts of Ca^{2+} may not be bound in physiological conditions. ALCs do not bind Ca^{2+} at all under physiological conditions.

The amino acid sequences of rabbit skeletal muscle DLC (Collins, 1976) and ALC (Frank and Weeds, 1974) have been determined and comparison with the known sequences of Ca^{2+}-binding parvalbumins and TN-C indicates that the myosin light chains are homologous both to each other and to the parvalbumins and TN-C. Again 4 homologous regions can be recognised within both DLC and ALC; however, the internal sequence repeats in both of these proteins are very weak. The single Ca^{2+}-binding site of DLC is probably located in region I, i.e., the amino-terminal EF hand region. The pattern of hydrophobic residues predicted to form the internal core of TN-C is highly conserved in both DLC and ALC, suggesting that all these proteins have similar three-dimensional structures.

In relation to the evolutionary aspects of these homologous Ca^{2+}-binding proteins, it has been suggested (e.g. Collins 1976) that the ancestral protein from which these homologous proteins were derived contained a single calcium binding site with an EF hand structure; this protein underwent 2 successive gene duplications and fusions to produce a protein 4 times the length of the ancestral protein and containing 4 Ca^{2+}-binding sites. This protein is then the common ancestor of TN-C, ALC, DLC and parvalbumin. Of these four proteins, TN-C appears to be most closely related to this common ancestor since it has the strongest internal sequence repeats. Parvalbumin apparently evolved incomplete copying of the gene for the TN-C-like precursor, causing deletion of region I and loss of Ca^{2+} binding in region II. Since internal sequence repeats in the myosin light chains are weak, DLC and ALC may have evolved from TN-C (or a precursor very similar to TN-C). This divergent evolution has involved structural changes which lead to distortion of the Ca^{2+}-binding sites and consequent reduction in the affinity for Ca^{2+} observed in both DLC and ALC.

D. Ca^{2+}-dependent protein modulator

It was originally observed by Wang et al, (1975) and Stevens et al, (1976) that the mechanism of Ca^{2+} activation of cyclic nucleotide phosphodiesterase closely resembles the mechanism of Ca^{2+} regulation of skeletal muscle actomyosin ATPase: both systems are modulated by a Ca^{2+}-binding protein, namely the modulator protein and TN-C, respectively. The observation suggested the possibility that TN-C and the modulator may be structurally related, a possibility which has been substantiated by the extensive studies of several groups of investigators.

Stevens et al, (1976) showed that the protein modulator resembles

rabbit skeletal muscle TN-C in amino acid composition, molecular weight, isoelectric point, and ultraviolet absorption spectrum. On the other hand, the tryptic peptide maps of the modulator and TN-C bear little similarity to each other. Most of the major peptides were eluted from the tryptic peptide map of the modulator and subjected to amino acid analysis; of the peptides examined, only one was found to have an amino acid composition identical to that of a peptide from TN-C located in a comparable position on the map. This fact would appear to indicate little or no similarity between the two Ca^{2+}-binding proteins. However, closely related homologous proteins do not necessarily give very similar tryptic peptide maps.

Watterson et al, (1976) independently compared the physical and chemical properties of the bovine brain modulator and TN-C. They observed both proteins to have blocked N-termini, similar and characteristic ultraviolet absorption spectra, similar Ca^{2+} binding properties, very similar amino acid compositions, and they co-migrate on SDS-polyacrylamide gels. They also reported that the primary structures of selected tryptic peptides isolated from the modulator protein are similar or identical to regions of the primary sequences of rabbit skeletal muscle and bovine cardiac muscle TN-C. Dedman et al, (1977b) compared the physicochemical properties of rat testis modulator protein and rabbit skeletal muscle TN-C and similarly concluded that the two proteins are very similar and probably homologous, having evolved from a common ancestor.

Determination of the amino acid sequence of the protein modulator from bovine brain (Vanaman et al, 1977) established unequivocally that the primary structure of the modulator is homologous to that of both

rabbit skeletal muscle and bovine cardiac muscle TN-Cs (see Appendix II). Indeed, the sequence homology between the modulator and TN-Cs is considerably greater than that between TN-C and myosin light chains and parvalbumins, indicating that the modulator and TN-C form a family of structurally and functionally related proteins distinct from the more distantly related myosin light chains and parvalbumins. By aligning the sequence of the modulator so that residue 1 corresponds to residue 9 in cardiac TN-C and residue 8 in skeletal muscle TN-C, maximum homology to both TN-Cs is maintained throughout the entire linear sequence by introducing only two small gaps in the modulator protein sequence. The majority of the modulator sequence is either identical to or functionally very similar to that of the muscle TN-Cs. The total number of identical plus functionally conserved residues shared by the modulator and either TN-C is 114 out of 148 positions compared, only slightly lower than the total number (122) shared between the two TN-Cs.

As mentioned previously, the amino acid sequence of TN-C can be divided into 4 homologous domains, each of which contains a potential Ca^{2+}-binding site. The amino acid sequence of the modulator also possesses internal homology. All 4 domains are related in sequence, but the level of homology is greatest when the first domain (residues 8-40) is aligned with the third domain (residues 81-113) and the second domain (residues 44-76) is aligned with the fourth (residues 117-148). The level of internal homology in the protein modulator appears to be greater thanthat observed within the TN-Cs.

On the basis of the sequence homology between the modulator protein and TN-C, the predicted three-dimensional structure of TN-C (Kretsinger and Barry, 1975) can be applied, with minor alterations,

to the modulator. It is evident from the primary structure that all 4 domains within the modulator contain potential Ca^{2+}-binding sites, having calcium-liganding residues in the appropriate positions. The regions of sequence adjacent to the Ca^{2+}-binding loops contain the appropriate hydrophobic residues necessary for the helices thought to be essential for the formation of functional Ca^{2+}-binding loops.

The proposal that the three-dimensional structures of modulator protein and TN-C are very similar was substantiated recently by comparison of the peptide fragments obtained by controlled tryptic digestion of the protein modulator (Walsh et al, 1977) with those obtained in similar fashion from TN-C (Drabikowski et al, 1977a): the tryptic cleavages in the modulator protein, both in the presence and absence of Ca^{2+} were observed to occur in positions homologous to those in TN-C.

Since the protein modulator can substitute for TN-C in a reconstituted Ca^{2+}-sensitive actomyosin ATPase system, and because of its ubiquitous distribution, it appears that the protein modulator may have been an evolutionary precursor of TN-C. Indeed the modulator may be very similar to the common ancestral protein, alluded to earlier, which gave rise to TN-C, myosin light chains and parvalbumins by divergent evolution.

XIV. Evolutionary studies

On the basis of the structural homology between the Ca^{2+}-binding proteins, troponin C, parvalbumin and the light chains of myosin, Kretsinger (1975, 1976) has proposed that all intracellular Ca^{2+}-modulated proteins and enzymes have evolved from a common ancestral protein. The determination of the amino acid sequence of the Ca^{2+}-

dependent modulator protein (Vanaman et al, 1977) unequivocally demonstrated that this protein is also a member of this class of homologous Ca^{2+}-binding proteins. Indeed, the modulator may have been one of the earliest Ca^{2+}-binding proteins to emerge with a defined function.

Animal species representative of the major phyla were shown to contain activating factors similar to the modulator protein (Waisman et al, 1975). These factors were found to be heat stable, non-dialyzable and susceptible to proteolytic digestion. All activated a preparation of bovine heart PDE to a comparable extent and in a Ca^{2+}-dependent and reversible fashion. Subsequently, Waisman et al, (1978) confirmed that the activating factors from earthworm, sea anemone, lobster and starfish are indeed proteins which exhibit comparable electrophoretic mobility to the bovine brain modulator on analytical disc gel electrophoresis in 15% polyacrylamide gels. Furthermore, purification and characterization of the earthworm modulator revealed it to share many physical and chemical properties with the bovine modulator protein. Thus, both proteins have molecular weights around 17,000-18,000, isoelectric points of approximately 4.0, similar and characteristic ultraviolet absorption spectra, and similar amino acid compositions. Both proteins bind Ca^{2+} with high affinity; however, earthworm modulator binds 2 moles of Ca^{2+} per mole of protein with equal affinity ($K_d = 6 \times 10^{-6}$ M), whereas, the bovine modulator binds 4 moles of Ca^{2+} per mole with differing affinity, as seen earlier.

Similarly, Childers and Siegel (1975) purified and characterized the modulator from the electroplax of Electrophorus electricus and showed it to be physically and chemically indistinguishable from modulators isolated from mammalian brain, adrenal medulla and testis.

In conclusion, the modulator appears to have a ubiquitous distribution throughout the animal kingdom and its structure has been strongly conserved throughout evolution suggesting that it has some basic function(s) in animal cells.

XV. Other activities of the Ca^{2+}-dependent protein modulator

A. Are the modulator and TN-C functionally interchangeable?

1. Substitution of modulator for troponin C

Since the protein modulator and troponin C are so remarkably similar in structure it seemed reasonable to propose that they may be functionally interchangeable. Amphlett et al, (1976) demonstrated that the brain modulator protein possesses a number of the biological properties of skeletal muscle TN-C: in the presence of TN-I and tropomyosin from skeletal muscle it is as effective in restoring Ca^{2+} sensitivity to desensitized actomyosin as is the whole skeletal muscle troponin complex and tropomyosin; furthermore, the protein modulator forms Ca^{2+}-dependent complexes with TN-T and TN-I, as evidenced by electrophoresis in 10% polyacrylamide gels, the modulator -TN-I complex being stable to high urea concentration.

Subsequently, Dedman et al, (1977a) showed that rat testis protein modulator forms a complex with rabbit skeletal muscle TN-I and TN-T; this hybrid complex could regulate rabbit skeletal muscle actomyosin ATPase activity, albeit less effectively than native troponin.

It is particularly interesting that the modulator can replace both TN-C and TN-T in a reconstituted Ca^{2+}-sensitive actomyosin ATPase system (Amphlett et al, 1976); the reason for this apparent anomaly remains unknown.

2. Effect of TN-C on PDE activity

Various reports (Wang et al, 1975; Klee, 1977b) have indicated that TN-C does not activate PDE. For example, Klee (1977b) showed that TN-C does not activate PDE up to 10^{-5} M, while a modulator concentration of 8×10^{-9} M gave half-maximal stimulation of the enzyme. Furthermore, when TN-C was mixed with different amounts of modulator no inhibition of PDE activity was observed even at subsaturating amounts of modulator. Hence, despite its similarity to the modulator, TN-C is unable to activate PDE or to displace the modulator from the enzyme.

On the other hand Dedman et al, (1977a) claimed that rabbit skeletal muscle TN-C can substitute for the modulator in the stimulation of PDE; however, the affinity of TN-C for PDE is 600-fold lower than that of the modulator. They demonstrated that both rabbit skeletal muscle TN-C and rat testis protein modulator require micromolar concentrations of free Ca^{2+} to induce PDE activation: TN-C requires 1.9×10^{-6} M and modulator 1.2×10^{-6} M free Ca^{2+} for half-maximal stimulation of PDE. In view of the close similarity in physical and chemical properties of TN-C and modulator, it is conceivable that the preparation of rabbit muscle TN-C is contaminated with a minute amount of modulator and this trace contaminant accounts for the observed stimulation of PDE. Potter et al, (1977) subsequently claimed similar stimulation of PDE by parvalbumin.

B. Activation of brain adenylate cyclase

Brostrom et al, (1975) obtained an activating factor of adenylate cyclase from detergent-dispersed preparations of porcine cerebral cortex by ECTEOLA-cellulose column chromatography. Polyacrylamide gel electrophoresis and enzyme activation studies identified this factor as

the Ca^{2+}-dependent protein modulator of PDE. The modulator confers a Ca^{2+}-dependent activation upon the adenylate cyclase, and this activation is reversed by the subsequent addition of EGTA in excess of the free Ca^{2+}.

Subsequently, during studies of adenylate cyclase activity in C-6 glioma (glial tumor) cells, Brostrom et al, (1976) found that, on separating homogenates of these tumor cells into particulate and supernatant fractions by centrifugation at 27,000 x g for 20 minutes, nearly all the adenylate cyclase activity was found in the particulate fraction. This adenylate cyclase activity was stimulated approximately 40% by the addition of untreated supernatant fraction, by boiled or dialyzed supernatant fraction, and by brain Ca^{2+}-dependent protein modulator. The adenylate cyclase activator found in the supernatant fraction was subsequently identified as the Ca^{2+}-dependent protein modulator in PAGE studies. Brostrom et al, (1976) prepared dispersed adenylate cyclase by treating the particulate fraction with the nonionic detergent, Lubrol PX, and found that such treatment resulted in large losses of activity. This enzyme preparation could be activated several-fold by the addition of homogeneous Ca^{2+}-dependent modulator at low Ca^{2+} concentrations.

Recently, Brostrom et al, (1977), while studying particulate preparations of rat cerebral cortex, found that the adenylate cyclase activity was comprised of two contributing components, only one of which requires the Ca^{2+}-dependent protein modulator for activity. It appears that adenylate cyclase, like PDE, may exist in multiple forms which are subject to different types of regulation.

Independently, Cheung et al, (1975a) separated an activator from bovine or rat brain adenylate cyclase solubilized by Lubrol PX by an

anionic exchange resin column. This dissociation of the activator from adenylate cyclase rendered the enzyme less active; addition of exogenous activator restored full enzyme activity. Stimulation of adenylate cyclase by the activator is a Ca^{2+}-dependent process, the effect being immediate and reversible. The purified Ca^{2+}-dependent protein modulator of PDE from bovine brain was also observed to stimulate this dispersed adenylate cyclase preparation. Subsequently, Lynch et al, (1976a,b) separated a Lubrol PX - solubilized rat brain adenylate cyclase from its activator by Sephadex G-200 column chromatography in the presence of EGTA; in the presence of Ca^{2+}, however, some of the activator eluted with the enzyme. These observations suggested the occurrence of a Ca^{2+}-dependent complex formation between adenylate cyclase and the activator. The mechanism of stimulation of adenylate cyclase by the Ca^{2+}-dependent protein modulator is apparently analogous to that of PDE:

$$Ca^{2+} + \text{Modulator}_{\text{inactive}} \rightleftharpoons \left[Ca^{2+}\text{-Modulator*}\right]_{\text{active}}$$

$$\left[Ca^{2+}\text{ - Modulator*}\right]_{\text{active}} + \text{Enzyme}_{\text{inactive}} \rightleftharpoons \left[\text{Enzyme*- } Ca^{2+}\text{ - Modulator*}\right]_{\text{active}}$$

where * denotes a different conformation.

Kinetic studies revealed that the activator increased the V_{max} several-fold but did not affect the K_m for ATP. Stimulation required Ca^{2+}, with half-maximum effect at 15 μM. The activator conferred thermal stability to adenylate cyclase indicating that the enzyme assumes a more stable conformation as it complexes with the activator.

Lynch et al, (1977) subsequently showed that particulate rat brain adenylate cyclase, like the detergent-solublized enzyme, required

the Ca^{2+}-dependent modulator for maximum activity. They prepared modulator-deficient adenylate cyclase by extracting a brain particulate fraction with a hypertonic sodium chloride solution containing EGTA. The modulator increased the V_{max} of this adenylate cyclase preparation without affecting its apparent K_m for ATP. The adenylate cyclase was found to be more stable against thermal inactivation in the presence of Ca^{2+} and the protein modulator than in the free form indicating that the modulator probably induces a conformational change in the enzyme.

C. Activation of $(Ca^{2+}-Mg^{2+})$ATPase

A soluble activator of human erythrocyte membrane $(Ca^{2+}-Mg^{2+})$ ATPase was originally reported by Bond and Clough (1973) and partially purified by Luthra et al, (1976). This activator was subsequently purified 475-fold from human erythrocyte ghosts by Jarret and Penniston (1977). SDS gel electrophoresis of this partially purified activator showed predominance of a protein of molecular weight about 18,000. This protein exhibited properties similar to those of the Ca^{2+}-dependent protein modulator of PDE. Comparison of the ATPase activator with bovine brain protein modulator of PDE showed that both stimulated the $(Ca^{2+}-Mg^{2+})$ ATPase, both stimulated PDE, and they co-electrophoresed.

Independently, Gopinath and Vincenzi (1977) observed similarities between the human erythrocyte cytoplasmic activator of $(Ca^{2+}-Mg^{2+})$ ATPase and various Ca^{2+}-binding proteins such as parvalbumins, troponin C and the protein modulator of PDE. They tested the ability of these three proteins to stimulate human erythrocyte membrane $(Ca^{2+}-Mg^{2+})$ ATPase. They observed that high concentrations of parvalbumin and troponin C produced a modest increase in $(Ca^{2+}-Mg^{2+})$ ATPase activity, whereas the protein modulator of PDE at relatively low concentration activated

(Ca^{2+}-Mg^{2+}) ATPase to the same extent (370%) as a partially purified erythrocyte cytoplasmic activator preparation. Both activators increase the V_{max} and the apparent affinity of (Ca^{2+}-Mg^{2+}) ATPase for Ca^{2+}. The observations of these two groups of investigators suggest that the protein modulator of PDE and the soluble activator of erythrocyte membrane (Ca^{2+}-Mg^{2+}) ATPase may be similar, or even identical, proteins.

This possibility has been enhanced very recently by the report of Luthra et al, (1977) who purified this activator of human erythrocyte membrane (Ca^{2+}-Mg^{2+}) ATPase to apparent homogeneity from a membrane-free hemolysate by a combination of carboxymethyl-Sephadex chromatography and preparative polyacrylamide gel electrophoresis. The protein was found to have a highly negative charge and migrated as a single, fast moving band on analytical polyacrylamide gel electrophoresis. Preliminary observations on the amino acid composition of the purified activator revealed a high content of glutamic acid (17%) and aspartic acid (14%) and relatively low levels of lysine (4%) and arginine (5%); these values correspond very closely to those of the modulator. The molecular weight, as determined by SDS gel electrophoresis, was approximately 16,000.

D. Stimulation of Ca^{2+} transport

During studies of Ca^{2+} transport using inside-out vesicles of human erythrocyte membranes, Macintyre and Green (1977) observed that addition of a portion of a membrane-free hemolysate from lysed red cells to the transport assay system consisting of the inside-out vesicles, Mg^{2+}, ATP and $^{45}Ca^{2+}$, resulted in a 50% increase in $^{45}Ca^{2+}$ transport from the extravesicular space into the vesicle lumen relative to controls. They indicated that this activation is probably due to

the presence of a protein in the hemolysate shown by other workers to activate the Mg^{2+}- and Ca^{2+}-dependent ATPase of erythrocyte membranes. This protein, as we have seen, is probably identical to the Ca^{2+}-dependent protein modulator. Hence, it appears likely that the modulator protein may also play a role in erythrocyte Ca^{2+} transport processes.

E. Activation of myosin light chain kinase

The DTNB - light chain of white skeletal muscle myosin has been shown to be phosphorylatable by myosin light chain kinase prepared from crude myosin (Perrie et al, 1973; Pires et al, 1974). In order to investigate this phosphorylation in more detail, Yazawa and Yagi (1977) recently isolated myosin light chain kinase from rabbit skeletal muscle. This kinase was separated into two components of approximate molecular weights 100,000 and 20,000 by DEAE-cellulose chromatography; the 20,000 dalton component eluted between 0.35 and 0.45 M NaCl. It was shown that both components and Ca^{2+} ions were essential for the myosin light chain kinase activity. An ultraviolet absorption difference spectrum of the 20,000 dalton component was observed upon addition of Ca^{2+}, indicating the protein to be a Ca^{2+}-binding protein. The shape of the difference spectrum was observed to be similar to that of the Ca^{2+}-induced difference spectra of troponin C and the DTNB - light chain of myosin, both from rabbit skeletal muscle. However, distinct differences between the 3 spectra were noted, indicating that the 20,000 dalton subunit of myosin light chain kinase differs from troponin C and DTNB - light chain of myosin. It is quite possible that this subunit is identical to the protein modulator.

This possibility was enhanced by recent observations reported by Dabrowska et al, (1977a) during their studies of the Ca^{2+}-dependent

regulation of smooth muscle actomyosin which involves a myosin light chain kinase. This kinase is composed of two subunits of approximate molecular weights 105,000 and 17,000 (Dabrowska et al, 1977b). They characterized the 17,000 dalton subunit and concluded that it is identical to the Ca^{2+}-dependent protein modulator (Dabrowska et al, 1977a). This conclusion was based on several instances in which the 17,000 dalton subunit and the protein modulator were shown to exhibit identical behaviour or properties. Thus, in the presence of a constant amount of 105,000 dalton kinase component, the 17,000 dalton subunit (17K) and protein modulator from both brain and adrenal medulla induced identical activation of gizzard actomyosin Mg^{2+}-ATPase. Activation in each case required Ca^{2+}, and troponin C did not activate the enzyme. Similarly, 17K, brain and adrenal medulla modulators were equally effective in promoting phosphorylation of gizzard myosin, again in a Ca^{2+}-dependent process. Also, 17K and adrenal medulla modulator were equally effective in activating PDE; neither protein activated the enzyme in the absence of Ca^{2+}.

Addition of the 105,000 dalton kinase component (105K) to the PDE assay system in the presence of 17K resulted in an inhibition of PDE and 105K. Association between 17K and 105K was also demonstrated by gel filtration on Sepharose 6B: 17K and 105K eluted as a complex in the presence of Ca^{2+} and at low ionic strength, whereas, in the absence of Ca^{2+} or at high ionic strength, the two proteins were dissociated and eluted separately.

17 K and protein modulator were also shown to share a number of physical and chemical properties. The amino acid composition of 17K is very similar to the reported compositions of adrenal medulla, heart and

brain modulators. Most notable is the absence of tryptophan and the presence of one residue per mole of the unusual amino acid trimethyllysine. The absorption spectrum of 17K is very similar to that reported for modulator from various sources. 17K, adrenal medulla and brain modulators exhibited identical electrophoretic mobility in urea-polyacrylamide gels, while troponin C had a higher mobility and could be resolved from 17K upon electrophoresis of a mixture of the two proteins. 17K was also shown to form a urea-stable complex with troponin I. Tyrosine fluorescence data indicated the occurrence of a Ca^{2+}-dependent conformational change in 17K. Clearly 17K is identical to the modulator protein and distinct from troponin C in both functional and physical properties.

The significance and possible physiological role of the protein modulator in the regulation of smooth muscle myosin light chain kinase will be discussed in a later section (XVI B).

F. Modulator binding protein

Wang and Desai (1976) discovered a PDE inhibitory factor in bovine brain extracts as a result of the observation that higher concentrations of the protein modulator were required for activation of bovine brain PDE than for the heart enzyme. This inhibitor was separated from PDE in brain extracts by gel filtration on Sephadex G-200. Its effect on the PDE was found to be reversible; both PDE and protein modulator activities could be completely recovered from the inhibited reaction. The inhibitor was shown to be protein in nature since it was susceptible to digestion with trypsin or chymotrypsin, but not ribonuclease, deoxyribonuclease or amylase.

The inhibitor appears to act specifically on the modulator-dependent PDE since it has no effect on the Ca^{2+}-and modulator-independent

form of the enzyme or the basal activity of the Ca^{2+}-activatable enzyme; it counteracts the activation of PDE induced by the protein modulator. The Ca^{2+}-dependent form of PDE can be desensitized of its activation by Ca^{2+} by treatment with trypsin; such treatment also abolishes the inhibition by the inhibitory factor.

Gel filtration analysis on Sephadex G-200 revealed that the inhibitory protein and the Ca^{2+}-dependent modulator protein can associate in the presence of Ca^{2+}, whereas the inhibitory protein and the Ca^{2+}-activatable PDE do not interact (Wang and Desai, 1977). These observations suggested that the inhibitory protein inhibits the Ca^{2+}-dependent PDE by forming a complex with the protein modulator, thereby effectively removing the protein modulator so that it can no longer bind to and activate PDE. The molecular weights of the free inhibitor and the complex of inhibitor and protein modulator were estimated by gel filtration on a calibrated Sephadex G-200 column to be 95,000 and 160,000, respectively.

The inhibitory protein may be either another regulator of PDE or another modulator-regulated enzyme. In view of the observed association between the inhibitor and the protein modulator, the latter possibility appears more likely, i.e., the inhibitor is probably an enzyme (or protein) whose activity, like that of PDE and adenylate cyclase, is regulated by the Ca^{2+}-dependent protein modulator. The inhibitor was tested for various enzyme activities, including ATPase, GTPase, adenylate cyclase and 5'-nucleotidase, but found to exhibit none of these activities (Wang and Desai, 1977). Consequently, they have designated the inhibitory protein "modulator binding protein" until its identity is established.

The modulator binding protein is apparently distinct from a protein inhibitor of PDE discovered by Dumler and Etingof (1976) in bovine retinal tissue and purified to electrophoretic homogeneity by DEAE-cellulose column chromatography. This inhibitor differs from the modulator binding protein, which is heat-labile, in that it is stable to boiling at pH 1.5 and has a molecular weight, as estimated by gel filtration on a calibrated column of Sephadex G-75, of about 38,000.

XVI. Physiological Roles

A. Regulation of cyclic nucleotide metabolism

While the Ca^{2+}-dependent modulator protein can regulate PDE activity *in vitro*, this does not necessarily imply that this is a physiological regulatory mechanism. In an attempt to determine whether or not the modulator does regulate PDE activity *in vivo*, Smoake *et al*, (1974) investigated the distribution and developmental changes of the enzyme and the modulator in mammalian tissues and cells.

1. Tissue distribution

Smoake *et al*, (1974) examined eight rat tissues and found that the ratio of the activity of the modulator to that of the PDE varied greatly from tissue to tissue. For example, PDE and modulator activities were both high in brain, whereas testicular enzyme activity was low but modulator activity high. A similar tissue distribution was subsequently observed in the rabbit (Cheung *et al*, 1975b).

Egrie and Siegel (1975) isolated the Ca^{2+}-dependent modulator from bovine adrenal medulla and investigated its possible role in this tissue by characterizing adrenal medullary PDE. They observed that neither crude nor partially purified adrenal medullary PDE was inhibited

by EGTA or stimulated by Ca^{2+} and the modulator, whereas similar brain enzyme preparations displayed sensitivity to these agents. In contrast, Uzunov et al, (1976a) identified a PDE in rat and beef adrenal medulla which exhibited a high K_m for cAMP and was stimulated by Ca^{2+} and the protein modulator. The ratio of modulator to modulator-dependent PDE is high which probably accounts, at least in part, for the failure of Egrie and Siegel (1975) to demonstrate the presence of a modulator-dependent form of the enzyme in bovine adrenal medulla.

2. Developmental changes

Changes in the activities of PDE and the modulator during development were examined in rat brain, testis, thymus and liver (Smoake et al, 1974). In brain, the activity of PDE increased 25-fold while that of the modulator doubled in the period from 8 days before birth to adulthood. Testicular PDE activity decreased from birth to adulthood while modulator activity increased 4-fold during the same period. In thymus, PDE activity decreased from birth to 30 days and then increased slightly to the adult level. In contrast, liver PDE activity increased from 5 days before birth to 20 days of age and then decreased to the original level by adulthood. In both these tissues (liver and thymus) the level of modulator remained essentially constant throughout. This apparent lack of correlation between the activities of PDE and its modulator in a given tissue during development may indicate separate genetic regulation of the two proteins. On the other hand, it may be a result of either cellular heterogeneity or the existence of multiple forms of PDE, some of which are modulator-independent.

To investigate the possibility that the lack of correlation of activities may be due to cellular heterogeneity, Smoake et al, (1974)

separated whole blood into its constituent cell populations and examined the distribution of the activities of PDE and the modulator in each cell population. The erythrocytes contained 29% of the whole blood PDE and 87% of the modulator, while the platelets contained 66% of the PDE and 10% of the modulator. The leukocytes had trace levels of PDE activity but no modulator activity. The plasma had neither. Clearly, the ratio of modulator to enzyme activity varied greatly in the different cell populations. Most tissues consist of several cell types and their ratios may vary from one tissue to another. Furthermore, each cell type might have its own ratio of PDE to modulator. This could account for the apparent lack of parallel distribution of the activities of the two proteins in different tissues and during development of the same tissue.

Independently, Tanigawa et al, (1976) investigated the changes in hepatic Ca^{2+}/modulator-dependent and independent PDE activities during the development of the chick embryo. Nearly all the PDE found in the liver during the early embryonic stage (eighth day) was the modulator-dependent form of the enzyme; however, this form of the enzyme decreased proportionately with embryonic development. In the adult liver, little or no dependent enzyme activity could be observed. The intracellular cAMP level may thus be regulated by these two forms of PDE in compliance with the development of the chick.

3. Subcellular distribution

Smoake et al, (1974) examined the subcellular distribution of PDE and the protein modulator in human platelets. They found that approximately 70% of the activities of PDE and the modulator were present in the soluble fraction. They also examined isolated rat liver

parenchymal cells and found that the activities of the two proteins were localized in the soluble (35% of PDE, 44% of modulator), nuclear (27% of PDE, 16% of modulator), and microsomal (11.5% of PDE, 12% of modulator) fractions. The mitochondrial fraction had traces of PDE activity (3%) and little or no modulator activity. The fact that the subcellular distribution of the two proteins was in parallel in both platelets and liver parenchymal cells indicated possible in vivo regulation of PDE activity by the modulator. This conclusion was enhanced by the subsequent observation (Cheung et al, 1975b) that the subcellular distribution of the two activities also appears parallel in bovine brain cortex, a tissue far more complex than isolated hepatocytes or human platelets.

4. Possible mechanisms of regulation

The fact that the protein modulator stimulates the activities of both adenylate cyclase (which catalyzes the formation of cAMP) and cyclic nucleotide phosphodiesterase (which catalyzes its degradation) appears paradoxical. The physiological mechanism of regulation of cyclic nucleotide metabolism by the protein modulator remains speculative. One popular theory, that of Lynch et al, (1976a, 1976b), is based on the sequential activation of the two enzymes which causes a transient elevation of cAMP in many tissues in response to appropriate stimuli. These stimuli induce an influx of Ca^{2+} through the plasma membrane or a release of membrane-bound Ca^{2+}; since the low K_m, membrane-bound PDE is not affected by the modulator, this Ca^{2+} flux may activate the plasma membrane associated adenylate cyclase leading to an increase of intracellular cAMP. Ca^{2+} subsequently arriving at the cytosol then activates the soluble PDE, thus returning

the elevated level of cAMP to its prestimulated level. Several indirect lines of evidence support this mechanism. Kinetic studies with a highly purified modulator-deficient rat brain PDE (Uzunov et al, 1976b) revealed that the modulator increases the affinity of the PDE for cAMP 4-fold without changing the V_{max}. The V_{max} of the modulator-dependent PDE is several-fold higher than that of the other molecular forms of PDE, including those forms that have low K_m for cAMP. This PDE, with K_m = 350 μM, is highly unlikely to hydrolyze the concentration of cAMP normally present in the cell (10^{-6} M). When the PDE is saturated with modulator the affinity of the enzyme for cAMP is enhanced and the high hydrolytic capacity of this form of the enzyme could make it very suitable for rapid catabolism of cAMP when the tissue content is increased following adenylate cyclase stimulation by hormones and neurotransmitters. Hence, the high K_m, modulator-dependent PDE may be operative when the concentrations of cAMP are high and most probably protects the cell from exceptionally high accumulation of the second messenger.

Another school of thought (Kakiuchi et al, 1973; Brostrom et al, 1975) proposes, also on the basis of kinetic evidence, that the modulator-dependent phosphodiesterase is in reality a cGMP rather than a cAMP hydrolyzing enzyme. The enzyme, while having a high K_m for cAMP has a low K_m (5-9 μM) for cGMP. Thus, calcium influx in response to a stimulus results in the formation of a Ca^{2+}-modulator complex which simultaneously stimulates adenylate cyclase, causing an increase in intracellular cAMP, and modulator-dependent PDE, causing a decrease in cGMP.

Considerable evidence has been provided which indicates that elevations of intracellular cAMP induce a release of membrane-bound

modulator from particulate fractions. Uzunov et al, (1976b) observed that doses of carbamylcholine or reserpine that increase the cAMP content of rat adrenal medulla also increase the activity of the modulator in the supernatant fraction; this is presumably due to the release of modulator from a membrane-bound state. The increase in cAMP content precedes that of the modulator. The modulator release, which is Ca^{2+} independent, appears to be associated with a cAMP-dependent phosphorylation of its binding site in the membrane: a purified cAMP-dependent protein kinase, in the presence of ATP and cAMP, stimulated 3-fold the release of modulator from the particulate fraction of rat brain and adrenal medulla (Gnegy et al, 1976a, 1976b). The substrate for this phosphorylation could be either a membrane protein that binds modulator or modulator itself. However, when rats were injected intraventricularly with $\left[\gamma\text{-}^{32}P\right]$ ATP, no ^{32}P was found in the modulator; furthermore, the modulator could not be phosphorylated by protein kinase in vitro. Hence, the substrate for phosphorylation is likely a membrane protein that binds modulator. Thus an increase in cAMP may trigger a feedback mechanism which leads to an activation of the PDE and ultimately, to a decrease in the elevated cAMP concentration. These conclusions were substantiated by Gnegy et al, (1977) who studied the location and the protein kinase-elicited release of modulator from its binding sites in subcellular fractions of rat brain homogenate. In the subcellular particulate fractions, the concentration of modulator is highest in the microsomal fraction, followed by the mitochondrial and nuclear fractions. Modulator was released from membranes by cAMP-dependent phosphorylation of membrane protein, release occurring mainly from the mitochondrial subfractions containing synaptic membranes and

synaptic vesicles. These observations infer that, in vivo, high concentrations of cAMP stimulate a specific, or specifically located, cAMP-dependent protein kinase which, when activated by cAMP, phosphorylates membrane protein. As a result of this process, the efflux of modulator from physiological binding sites is promoted. The modulator released into the cytosol interacts with the high K_m PDE in the presence of Ca^{2+} and lowers its K_m, thereby facilitating the hydrolysis rate of cAMP.

B. Regulation of smooth muscle contraction

It has long been known that actin-myosin interaction in smooth muscle, as in skeletal muscle, is regulated by the intracellular Ca^{2+} concentration. Most investigators now agree that this Ca^{2+}-mediated regulation in smooth muscle is due to the concerted action of a protein kinase and a phosphatase acting on myosin. Myosin in the phosphorylated state can interact with actin, hydrolyze ATP and thus generate tension; removal of the phosphate groups by a phosphatase eliminates the actin activation of myosin Mg^{2+}-ATPase activity, with the net result that relaxation follows. At the contractile protein level, it appears that either the myosin light chain kinase or the phosphatase must be subject to Ca^{2+} regulation. This was borne out by the studies of Dabrowska et al, (1977a,b) who isolated and characterized chicken gizzard myosin light chain kinase. This enzyme was shown to consist of two subunits of molecular weights 105,000 and 17,000. The 17,000 dalton component was shown to be identical to the Ca^{2+}-dependent protein modulator. Thus, a coherent molecular mechanism for the Ca^{2+} regulation of smooth muscle contraction emerged in which the protein modulator plays a central role. In the resting state (essentially Ca^{2+}-free) the modulator

(17,000 dalton kinase component) is in the inactive conformation and dissociated from the 105,000 subunit; hence, the kinase is inactive. Contraction is initiated by an increase in intracellular Ca^{2+} concentration which is detected by the modulator. The resultant binding of Ca^{2+} to the modulator converts it to the active conformation in which state it can bind to the 105,000 dalton kinase component, thereby activating the myosin light chain kinase activity. The activated kinase phosphorylates the 20,000 dalton light chain of myosin (to the extent of one mole of phosphate per light chain) and thereby allows the activation by actin of the Mg^{2+}-ATPase activity. As long as Ca^{2+} is present, cyclic actin-myosin interactions proceed with the concurrent hydrolysis of ATP and development of tension or shortening. In the absence of Ca^{2+}, i.e., when the muscle relaxes, the kinase dissociates into its subunits and is thereby inactivated; the phosphatase removes the phosphate groups from the myosin thereby preventing further actin-activation of myosin Mg^{2+}-ATPase activity. The contractile apparatus is thus turned off.

It is now well established that a wide variety of non-muscle eucaryotic cells contain actin and myosin. The regulation of actin-myosin interactions by the modulator protein by a mechanism similar to that postulated to occur in smooth muscle may thus be a general phenomenon.

C. Mediation of Ca^{2+}-regulated processes in general

In view of the structural and functional similarities of the protein modulator and troponin C, it appears very likely that the modulator functions *in vivo* in the Ca^{2+}-mediated regulation of non-fast muscle contractile systems and, therefore, is involved in the regulation

of such processes as smooth muscle contraction, endo- and exocytosis, inter- and intracellular movement, etc. Vanaman et al, (1977) suggested, on the basis of the troponin C-like activity, the Ca^{2+}-dependent regulation of cyclic nucleotide levels, and calcium sequestration in smooth muscle and non-muscle tissues, exhibited by the modulator protein, that the modulator may be a central regulator for the complete cycle of stimulus, response and relaxation in animal cells. For the modulator to regulate so many biological activities in a concerted manner it is reasonable to assume that all modulator-regulated enzymes share a common modulator binding domain. Experimental observations discussed earlier support this possibility.

XVII. Other effectors of modulator-dependent PDE

A. Lipids

Phosphatidyl inositol (PI) and lysophosphatidyl choline (LPC) have been identified as activators of partially purified brain modulator-dependent PDE (Wolff and Brostrom, 1976). In the absence of Ca^{2+} and the protein modulator, microgram quantities of either of these phospholipids produced a linear, immediate and reversible activation of the enzyme. Fatty acids were also found to activate this form of PDE to varying degrees, with oleic acid being the most effective. Other phospholipids, such as phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and lysophosphatidyl ethanolamine, were shown to be ineffective as activators. Of a variety of nonionic, cationic and anionic detergents tested, only SDS activated the PDE; a modest degree of activation was produced over a narrow concentration range, with enzyme denaturation occurring at higher concentrations.

The interaction of the PDE with the phospholipid activators was

compared to its interaction with the Ca^{2+}-modulator complex. Both Ca^{2+}-modulator and LPC decreased the thermal stability of the enzyme to a similar extent. Pichard (1976) also showed that LPC or modulator decreased the thermal stability and the activation energy of the modulator-dependent PDE from 17.9 to 11.9 Kcal/mole. The apparent K_m of the LPC-dependent PDE activity was $\sim 30\ \mu M$ with cGMP as substrate and 1 mM with cAMP as substrate. With increasing LPC concentrations, the apparent K_m for each nucleotide remained unchanged while the V_{max} increased. Pichard and Cheung (1977) independently showed that LPC increased the V_{max} of brain cytoplasmic PDE without affecting its apparent K_m for cAMP.

Lipid activators and Ca^{2+}-modulator compete for activation of the PDE and appear to act at a common site on the PDE since all activations appear to be competitively inhibited by fluphenazine, a pharmacological agent known to act as a potent inhibitor of the modulator-dependent PDE. Studies of the structural requirements for enzyme activation emphasized the importance of the hydrophobic portion of the lipid activator, since LPC was a potent activator of the PDE, while L-α-phosphatidyl choline, L-α-glycerophosphate and phosphoryl choline were inactive.

Independently, Pichard (1976) demonstrated that LPC mimics the stimulation of PDE by the Ca^{2+}-dependent modulator. Half-maximal stimulation of the PDE was achieved at 30 μM LPC and the maximal extent of stimulation was comparable to that induced by the modulator. Stimulation by LPC was independent of Ca^{2+}; the effect was immediate and reversible, and was abolished by 0.01% Lubrol-PX (a nonionic detergent), which has no effect on the basal activity of PDE or the stimulation by

the modulator. Stimulation of PDE activity by a mixture of LPC and Ca^{2+}-dependent modulator protein was found to be less than that obtained with LPC or modulator alone, suggesting that the action of one interferes with that of the other. Clearly, the action of LPC appears similar to that of the protein modulator so that an understanding of the mode of action of LPC may shed light on the molecular events associated with the regulation of PDE activity. PDE was also observed to be stimulated by oleic acid, cardiolipin and phosphatidyl inositol, but in each case the degree of stimulation achieved was less than that induced by LPC (Pichard and Cheung, 1977).

B. Pharmacological agents

Weiss et al, (1974) studied the effects of various inhibitors of PDE on the multiple forms of the rat brain enzyme. Trifluoperazine was found to be a very potent inhibitor of the modulator-dependent PDE but only weakly inhibits the other forms of the enzyme, including the basal activity of the modulator-dependent form. The inhibition of the modulator-dependent enzyme induced by low concentrations of trifluoperazine could be overcome by increasing concentrations of the modulator, suggesting that the drug is competitively blocking the modulator activation. Fluphenazine was shown to be a competitive inhibitor of both Ca^{2+}-modulator and LPC activation of the enzyme, (Wolff and Brostrom, 1976) which lends further support to the suggestion that these two activators act at a common site on the PDE. However, it has since been shown (Levin and Weiss, 1977) that this class of drugs in fact bind to the protein modulator.

C. Others

Stimulation of the modulator-dependent PDE by the protein

modulator depends on the presence of free Ca^{2+} and Mg^{2+}, since the modulator requires Ca^{2+} for activity and PDE is Mg^{2+}-dependent. Consequently, any agents showing preferential chelation of Ca^{2+} rather than Mg^{2+} would be expected to inhibit the activated enzyme activity; such agents include EGTA, citrate and ATP (Kakiuchi and Yamazaki, 1970, 1970a).

Imidazole and ammonium sulfate have been shown to potentiate specifically the activated state of the modulator-dependent PDE (Ho et al, 1976). These agents cause an increase in maximal levels of the enzyme activation by the modulator protein.

GENERAL EXPERIMENTAL PROCEDURES

I. Purification of bovine brain protein modulator

The bovine brain protein modulator was isolated and purified by a modification of the procedure described by Teo et al, (1973) for the purification of the bovine heart modulator. The modifications were necessary to remove protein contaminants, notably the S100 protein (Watterson et al, 1976), not found in bovine heart. The purification was monitored by electrophoresis on 15% polyacrylamide slab gels (Fig. 1). The following steps were performed at 4° C unless indicated otherwise.

Homogenization

1 Kg of beef brain (stored frozen at -20° C) was thawed, chopped into small pieces and homogenized in 2.5 volumes of 0.1 M Tris, 2 mM EDTA, pH 7.5 in a Waring blender at high speed for 10 min. The homogenate was centrifuged at 11,000 g for 45 min. The homogenate supernatant is represented in slot 1 of Fig. 1.

Ammonium sulfate fractionation

The homogenate supernatant was adjusted to pH 8.8 with 5 N NaOH and brought to 40% saturation with ammonium sulfate, stirred well and left for 30 min. The mixture was then centrifuged at 11,000 g for 30 min. This step of the purification successfully separates the modulator-dependent phosphodiesterase, which is found in the pellet, from the modulator, located in the supernatant. Slot 2 (Fig. 1) represents this supernatant fraction.

Heat treatment

To the supernatant from the previous step was added 100 ml of 1 M magnesium acetate and additional ammonium sulfate to give a final

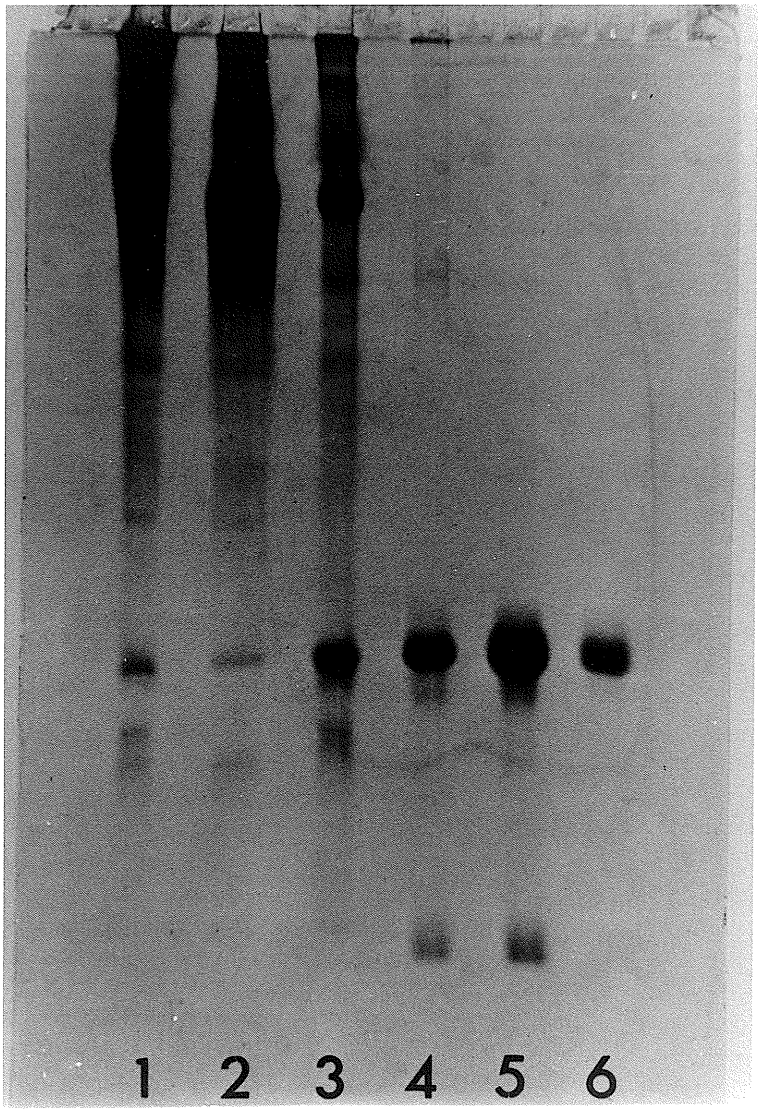


Figure 1

Purification of the bovine brain protein modulator monitored by electrophoresis in 15% polyacrylamide slab gels by the method of Davis (1964).
1: homogenate supernatant; 2: 40% ammonium sulfate fractionation supernatant; 3: heat treatment supernatant; 4: first DEAE-cellulose pool; 5: Sephadex G-100 pool; 6: second DEAE-cellulose pool.

60% saturation with respect to ammonium sulfate. The pH was adjusted to 4.2 and the resulting mixture left in the cold overnight. The mixture was then centrifuged at 11,000 g for 30 min. The supernatant was discarded and the pellet was suspended in 300 ml of 0.08 M Tris, 1 mM magnesium acetate, pH 8.0. The pH was adjusted to 8.0 with 5 N NaOH. Calcium chloride (45 mM) was added to a final concentration of 20 μM. The slurry was immersed in a boiling water bath with constant stirring until a precipitate formed, heating was continued for a further minute, the sample cooled immediately on ice and centrifuged at 23,500 g for 20 min. The supernatant was dialyzed against 0.02 M imidazole, 1 mM magnesium acetate, pH 6.5; this fraction is represented in slot 3 (Fig. 1).

First DEAE-cellulose column chromatography

The dialyzed supernatant was applied to a DEAE-cellulose column (2.5 x 90 cm) previously equilibrated with Buffer A (0.02 M imidazole, 1 mM magnesium acetate, 0.2 M NaCl, 20 μM $CaCl_2$, pH 6.5). Excess protein was washed off with Buffer A and elution achieved with a linear salt gradient consisting of one litre each of Buffer A and Buffer B (0.02 M imidazole, 1 mM magnesium acetate, 0.65 M NaCl, 20 μM $CaCl_2$, pH 6.5). The flow rate was 68 ml/h and 6.5 ml fractions were collected. The elution profile is shown in Fig. 2. Selected fractions were subjected to assay of modulator activity (Fig. 2) and electrophoresis on 15% polyacrylamide slab gels (Fig. 3), and the appropriate fractions enriched in modulator were pooled, dialyzed against water and lyophilized. This fraction is represented in slot 4 (Fig. 1).

Sephadex G-100 gel filtration

The lyophilized material was dissolved in 5 ml of 0.02 M Tris, 1 mM imidazole, 1 mM magnesium acetate, 0.01 mM $CaCl_2$, 0.5 M NaCl, pH 7.5

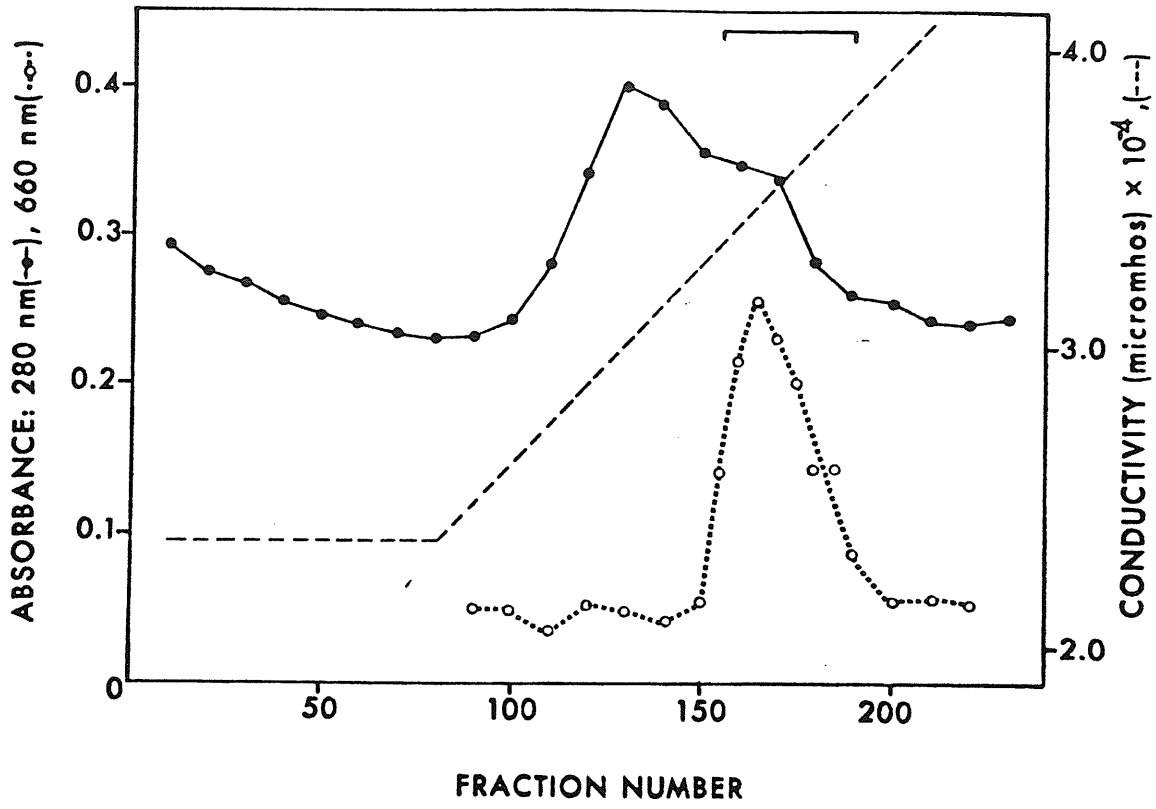


Figure 2 Purification of bovine brain modulator: first DEAE-cellulose column chromatography. The separation shown was performed and monitored as described in the text. 6.5 ml fractions were collected and those fractions indicated by the bar were pooled. Protein was monitored by measurement of the absorbance at 280 nm (●—●) and selected fractions were assayed for protein modulator activity as described in "General Experimental Procedures" IV; modulator activity is shown as absorbance at 660 nm (○···○).

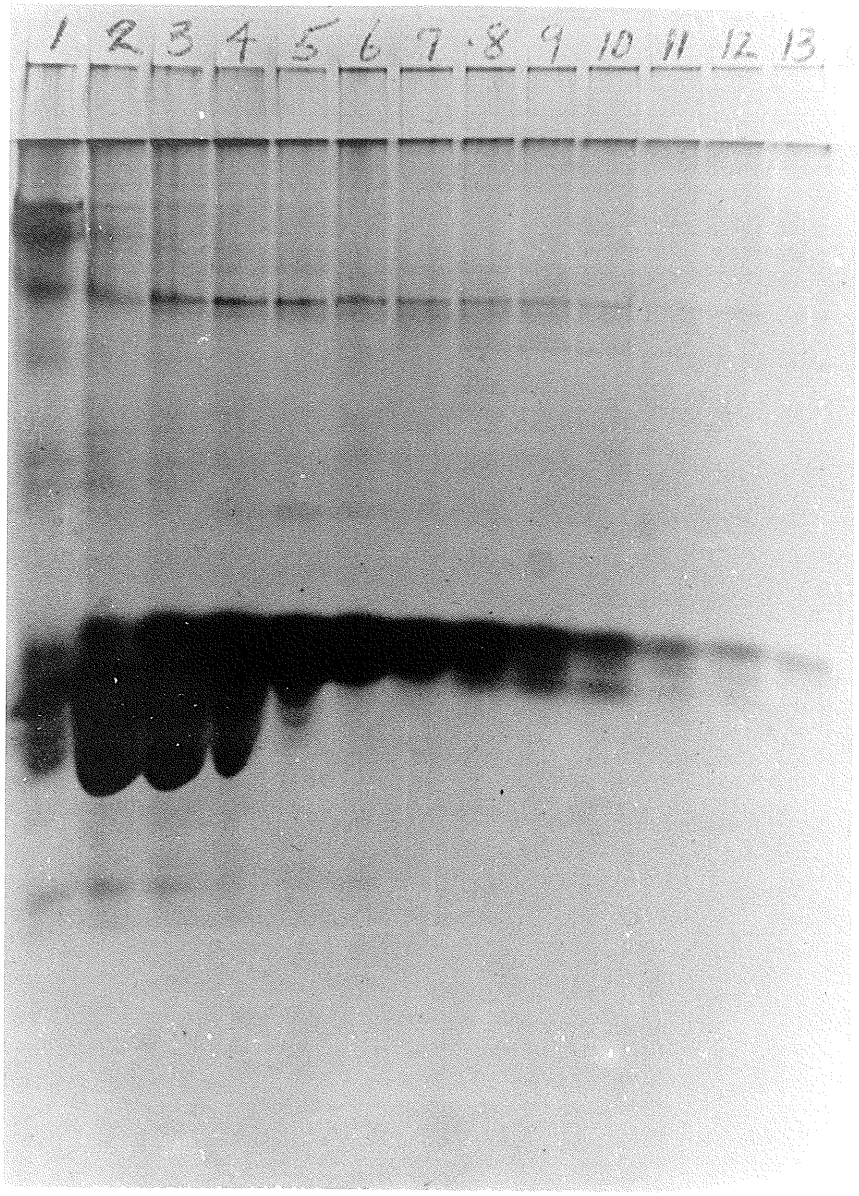


Figure 3

Purification of bovine brain modulator: first DEAE-cellulose column chromatography. Monitoring of selected fractions by electrophoresis on 15% polyacrylamide slab gel. Reading from left to right (slot 1-13), the fractions represented on the gel are 140, 150, 155, 160, 165, 170, 175, 180, 185, 190, 200, 210, 220.

and applied to a Sephadex G-100 column (2.5 x 90 cm) equilibrated with the same buffer and 3.2 ml fractions collected at a rate of 26 ml/h (Fig. 4). The fractions were monitored as described above (Fig. 5) and the appropriate fractions were pooled, dialyzed against water and lyophilized. This fraction is represented in slot 5 (Fig. 1).

Second DEAE-cellulose column chromatography

The lyophilized material was dissolved in 10 ml of Buffer A, applied to a DEAE-cellulose column (2.5 x 90 cm) and elution achieved as described above for the first DEAE-cellulose column. The elution profile is illustrated in Fig. 6. The fractions were monitored as before (Fig. 7), the appropriate fractions pooled, dialyzed against water and lyophilized. The resultant purified modulator is shown in slot 6 (Fig. 1).

A summary of the purification of the bovine brain modulator is shown in Table III. The average yield of purified modulator protein by this method is 50-70 mg per Kg of bovine brain.

Criteria of homogeneity

The purity of the final product was assessed by gel electrophoresis (Fig. 8) and electrofocusing (Fig. 9). One major band and several minor, slower migrating bands are seen by nondenaturing discontinuous electrophoresis (gel A, Fig. 8) in agreement with the observations of Watterson et al, (1976). On the other hand, a single major band is detected by discontinuous electrophoresis in the presence of 6 M urea (gel B) and by SDS-PAGE in the presence of 6 M urea (gel C). The apparent contaminants seen in gel A are, therefore, probably aggregates of the modulator protein. Examination of the purified modulator by isoelectric focusing (Fig. 9) revealed the presence of a

TABLE III. Purification of protein modulator from 1 Kg of bovine brain.

	Fraction	Volume (ml)	Protein (mg)[a]	Total units[b]	Specific activity (units/mg protein)	Purification factor	Yield (%)
I	Homogenate supernatant	1440	33,984	19.2×10^6	565	1	100
II	40% ammonium sulfate supernatant	1440	21,456	19.9×10^6	926	1.64	103
III	Heat treatment supernatant	440	1,478	17.6×10^6	11,940	21.1	92
IV	First DEAE-cellulose pool	276	199	13.8×10^6	69,350	122.7	72
V	Sephadex G-100 pool	71	148	13.5×10^6	91,220	161.4	70
VI	Second DEAE-cellulose pool	405	72	10.1×10^6	140,820	249.2	53

[a] Determined by the method of Lowry et al, (1951)

[b] One unit of modulator activity is defined as the amount giving 50% maximal activation of a standard amount (0.4-0.5 unit) of modulator-deficient phosphodiesterase (Teo et al, 1973).

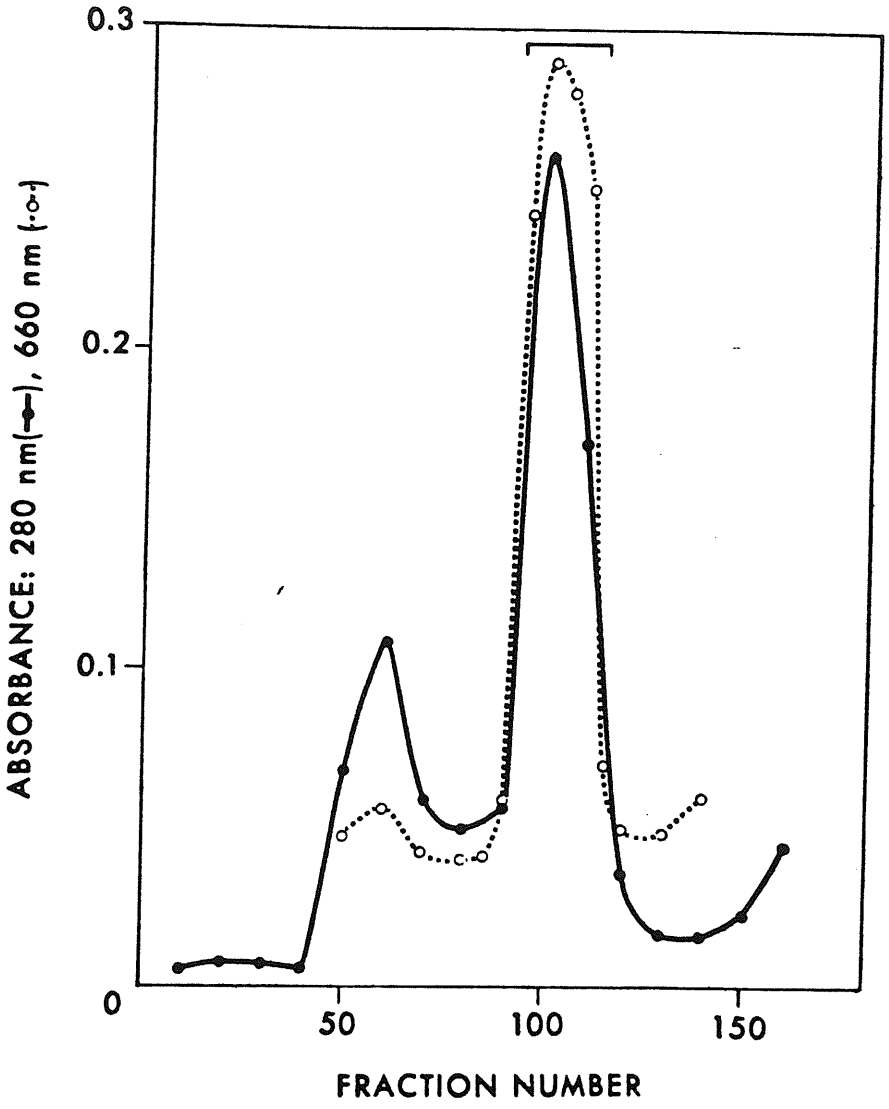


Figure 4

Purification of bovine brain modulator: gel filtration on Sephadex G-100. The separation shown was performed and monitored as described in the text. 3.2 ml fractions were collected and those fractions indicated by the bar were pooled.

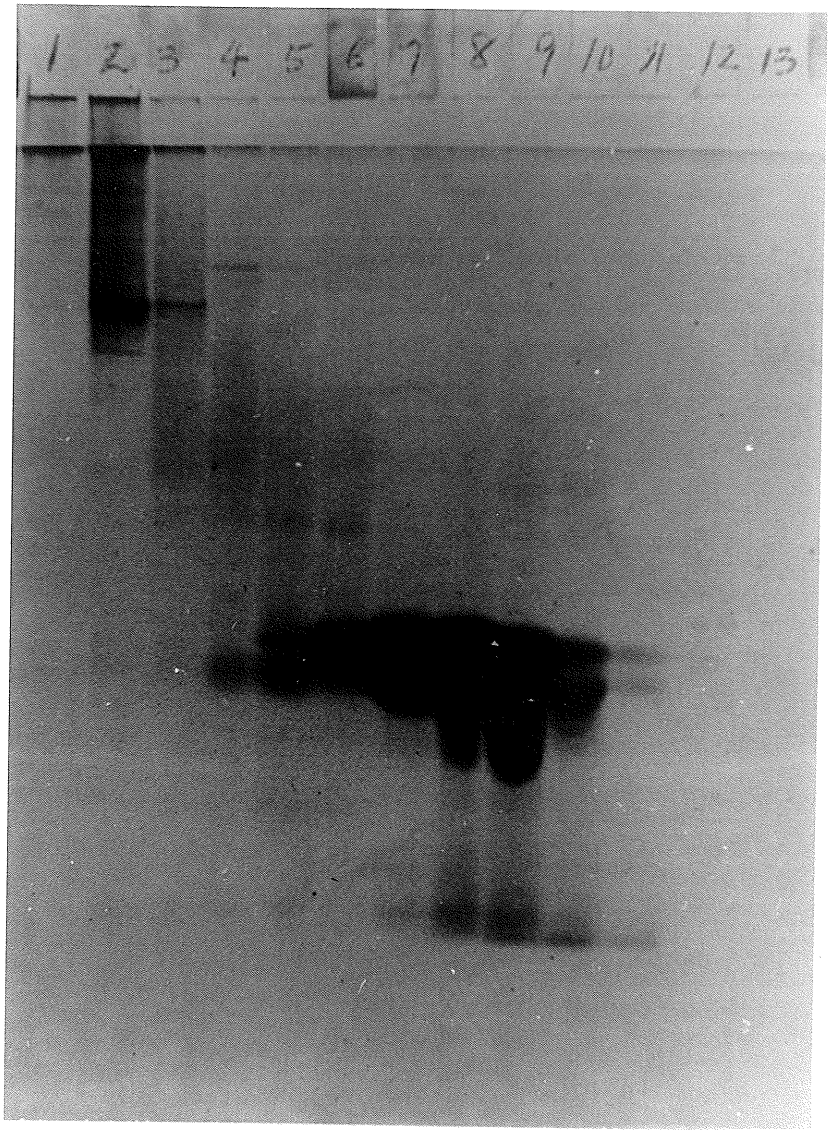


Figure 5

Purification of bovine brain modulator: gel filtration on Sephadex G-100. Monitoring of selected fractions by electrophoresis on 15% polyacrylamide slab gel. Reading from left to right (slot 1-13), the fractions represented on the gel are 50, 60, 70, 80, 90, 95, 100, 105, 110, 115, 120, 130, 140.

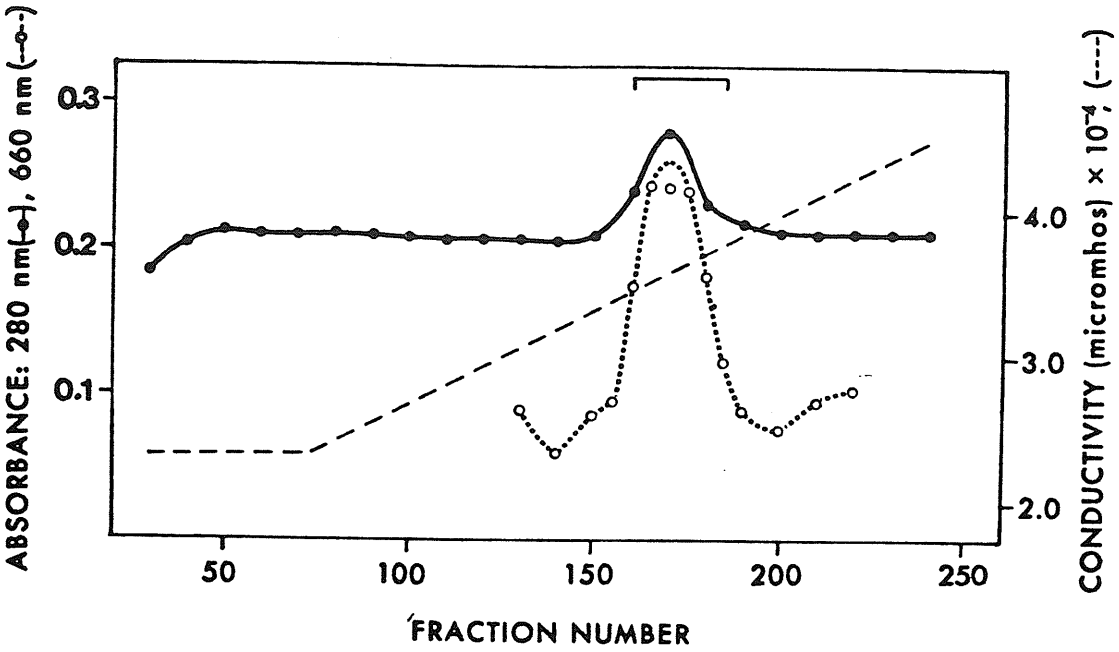


Figure 6

Purification of bovine brain modulator: second DEAE-cellulose column chromatography. The separation shown was performed and monitored as described in the text. 6.5 ml fractions were collected and those fractions indicated by the bar were pooled.

Figure 7

Purification of bovine brain modulator: second DEAE-cellulose column chromatography. Monitoring of selected fractions by electrophoresis on 15% polyacrylamide slab gel. Reading from left to right (slot 1-13) the fractions represented on the gel are 130, 140, 150, 155, 160, 165, 170, 175, 180, 185, 190, 200, 210.

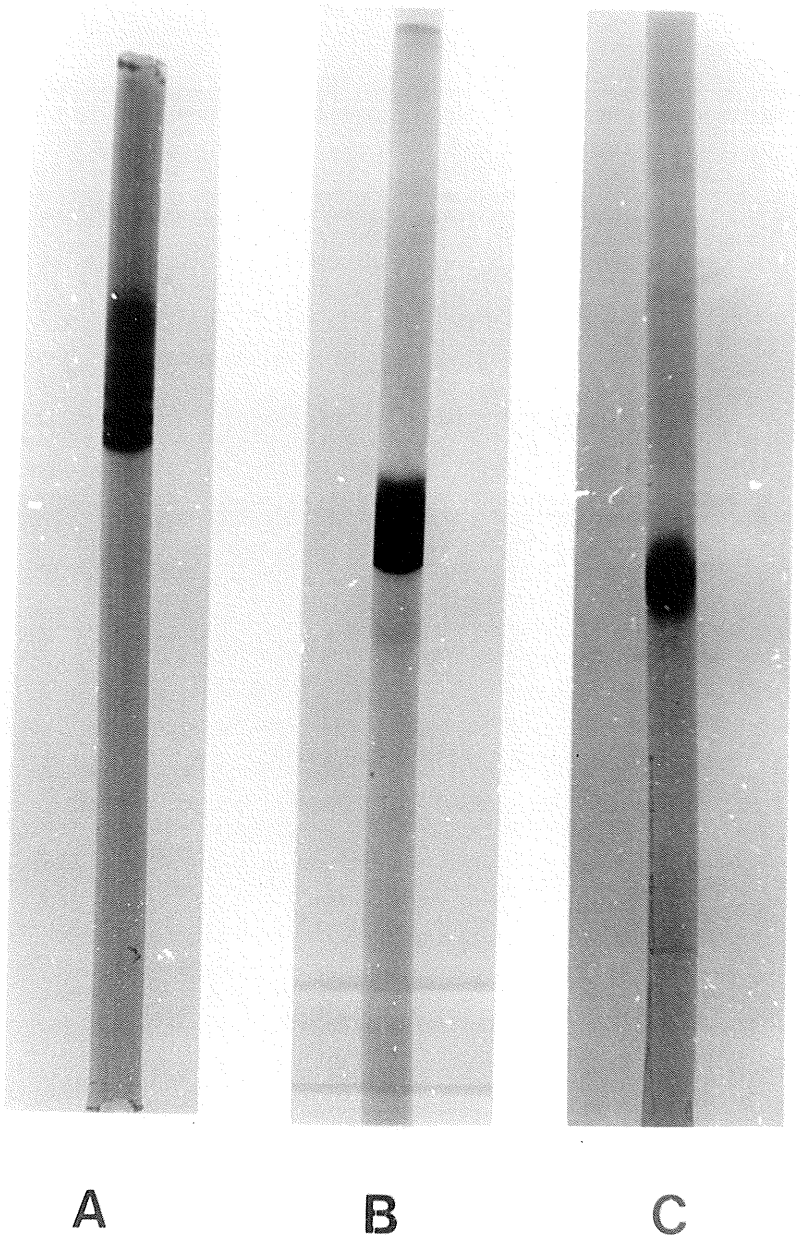


Figure 8

Criteria of purity of bovine brain modulator protein. Gel A: 15% discontinuous electrophoresis, 50 µg of protein; Gel B: 15% discontinuous electrophoresis in the presence of 6 M urea, 50 µg of protein; Gel C: 15% discontinuous electrophoresis in the presence of 0.1% SDS and 6 M urea, 50 µg of protein. Gels were run, stained and destained as described in **V.**

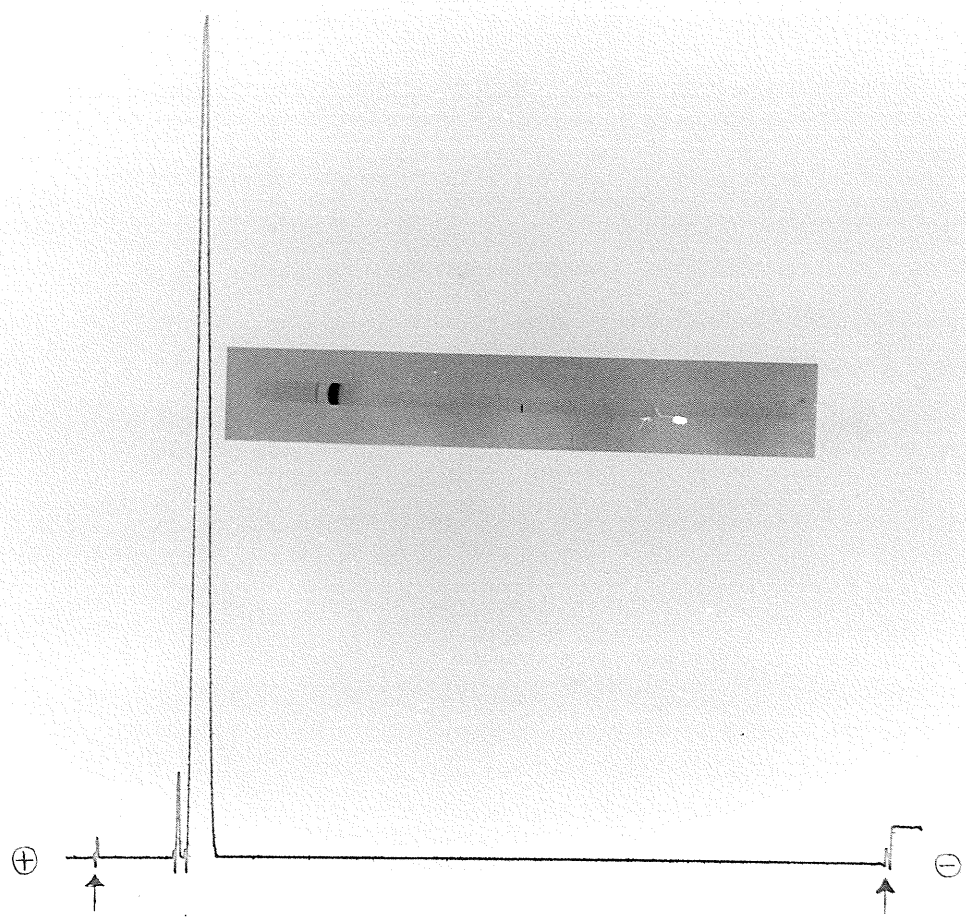


Figure 9

Criterion of purity of bovine brain modulator protein: gel isoelectric focusing, 50 μg of protein. The gel was run, stained and destained as described in "General Experimental Procedures" VD and is shown in the inset (the anode is at the left end of the gel). The gel was also scanned at 620 nm: the ends of the gel correspond to the arrows, and the anode is at the left end as shown.

single major band with trace amount of contamination by a more acidic protein. The gel was also scanned at 620 nm (Fig. 9); clearly the contaminant represents a very small percentage of the total protein. The isoelectric point of the modulator protein was determined to be 4.1, in excellent agreement with the observations of others.

Amino acid composition

Table IV shows the results of a typical amino acid analysis of the bovine brain protein modulator; the composition calculated from the amino acid sequence (Vanaman et al, 1977) is shown for comparison.

II. Preparation of modulator-deficient phosphodiesterase

Modulator-deficient phosphodiesterase (PDE) was prepared from bovine heart as described by Ho et al, (1976) which was a modification of the method of Teo et al, (1973). All steps of the preparation were performed at 4° C unless noted otherwise.

Homogenization

The fat was dissected from fresh beef heart (about 1.5 Kg) which was then chopped into half-inch cubes and stored frozen at -20° C. When needed, the frozen heart muscle was thawed, minced and homogenized with 2.5 litres of 0.1 M Tris, 2 mM EDTA, pH 7.5 in a Waring blender at top speed for about 10 min. The homogenate was centrifuged at 11,000 g for 30 min.

Ammonium sulfate fractionation

The pH of the homogenate supernatant was adjusted to 8.8 with 5 N NaOH and the solution was brought to 60% saturation with ammonium sulfate, stirred well and left for 30 min. The mixture was then centrifuged at 14,000 g for 30 min. The pellet (which contains PDE and some modulator protein) was retained, and the supernatant (which contains

TABLE IV. Amino acid composition of bovine brain protein modulator.

Amino Acid	Amino acid composition	
	By analysis[a]	By sequence[b]
Lysine	8.4	8[c]
Histidine	0.84	1
Arginine	5.72	6
Aspartic acid	22.00	23
Threonine	10.28	12
Serine	3.47	4
Glutamic acid	31.09	27
Proline	1.94	2
Glycine	11.30	11
Alanine	11.59	11
Half-cystine	0.0	0
Valine	6.85	7
Methionine	8.06	9
Isoleucine	7.46	8
Leucine	9.14	9
Tyrosine	1.89	2
Phenylalanine	7.93	8
Tryptophan	0.0	0

[a] Calculated using a molecular weight of 16,723 daltons (Vanaman et al, 1977).

[b] From Vanaman et al, (1977).

[c] Includes one residue of trimethyllysine per mole of modulator.

mostly modulator protein) was discarded. The pH 8.8 pellet was suspended in a minimum volume of 0.02 M Tris, 1 mM imidazole, 1 mM magnesium acetate, pH 7.0, and dialyzed against 0.02 M Tris, 1 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.01 mM $CaCl_2$, pH 7.0.

Ultracentrifugation

The dialyzed solution was centrifuged at 160,000 g in a Beckman Model L5-65 ultracentrifuge for 1 h. The pellet was discarded and the supernatant filtered with the aid of a Büchner funnel.

First DEAE-cellulose column chromatography

The filtered supernatant was applied to a DEAE-cellulose column (2.5 x 90) previously equilibrated with Buffer A (0.02 M Tris, 1 mM imidazole, 1 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.01 mM $CaCl_2$, 0.09 M NaCl, pH 7.0). Excess protein was washed off with Buffer A and elution achieved with a linear salt gradient consisting of one litre each of Buffer A and Buffer B (0.02 M Tris, 1 mM imidazole, 1 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.01 mM $CaCl_2$, 0.4 M NaCl, pH 7.0). The flow rate was 47.3 ml/h and 6.5 ml fractions were collected. The elution profile is shown in Fig. 10. Selected fractions were subjected to assay of PDE activity in the presence and absence of EGTA, conductivity measurement, and measurement of absorbance at 280 nm. PDE I (modulator-dependent PDE) is identified as the peak of activity which is inhibited by EGTA; PDE II is unaffected by EGTA and represents modulator-independent PDE. The fractions corresponding to PDE I (modulator-dependent PDE) were pooled and dialyzed against Buffer C (0.02 M Tris, 1 mM imidazole, 1 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.1 mM EGTA, 0.05 M NaCl, pH 7.0).

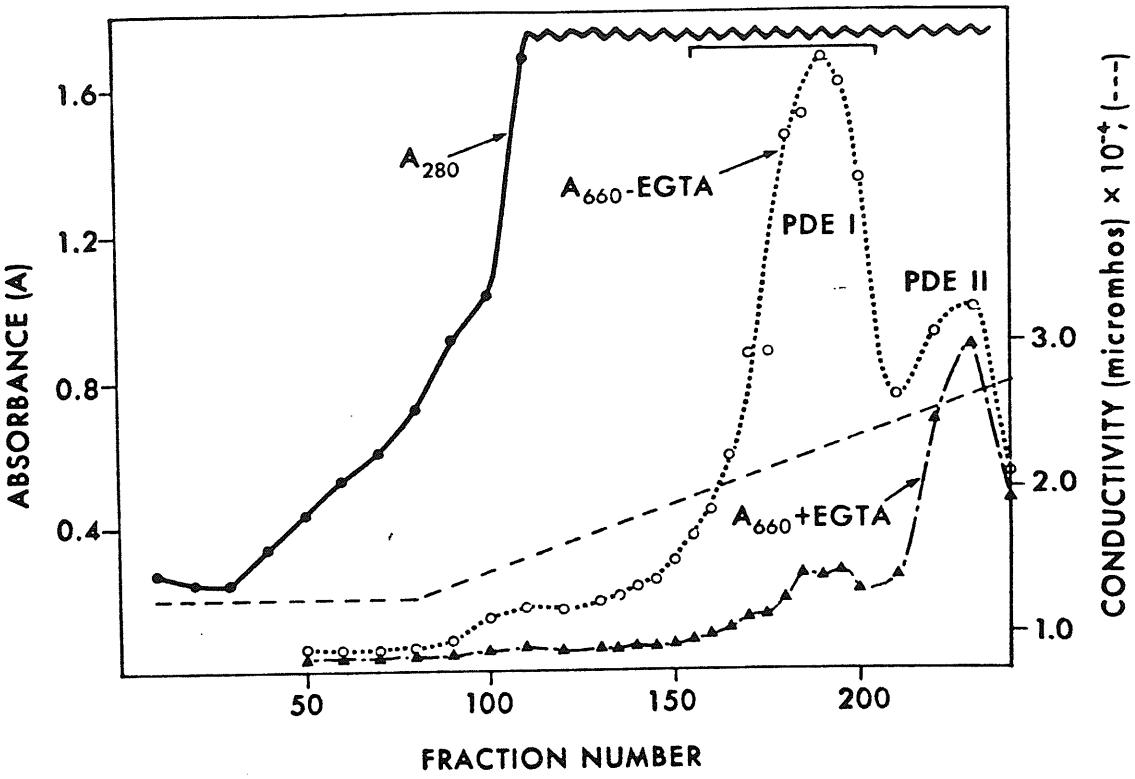


Figure 10

Preparation of bovine heart phosphodiesterase: first DEAE-cellulose column chromatography. The separation shown was performed and monitored as described in the text. 6.5 ml fractions were collected and those fractions indicated by the bar were pooled.

Second DEAE-cellulose column chromatography

The dialyzed material was applied to a DEAE-cellulose column (2.5 x 90 cm) previously equilibrated with Buffer C and eluted with a linear salt gradient consisting of 500 ml each of Buffer C and Buffer D (0.02 M Tris, 1 mM imidazole, 1 mM magnesium acetate, 10 mM 2-mercaptoethanol, 0.1 mM EGTA, 0.4 M NaCl, pH 7.0). The flow rate was 47.3 ml/h and 3.6 ml fractions were collected. The elution profile is shown in Fig. 11. The fractions indicated by the bar were pooled and stored frozen in 1 ml aliquots for subsequent use.

III. Assay of cyclic nucleotide phosphodiesterase

PDE activity was measured by the method of Butcher and Sutherland (1962) as modified by Wang et al. (1972). The assay involved the conversion of 5'-AMP, the product of the cAMP phosphodiesterase reaction, to adenosine and inorganic phosphate by 5'-nucleotidase. The reaction mixture, in a volume of 0.9 ml, contained, in addition to PDE and the protein modulator (sufficient to cause maximal activation of the enzyme), 1.2 mM cAMP and 0.25 unit of 5'-nucleotidase in 40 mM Tris, 40 mM imidazole, 3 mM magnesium acetate, 0.11 mM $CaCl_2$, pH 7.5. The reaction mixture was incubated at 30° C for 30 min, at which time reaction was terminated by the addition of 0.1 ml of 55% (w/v) trichloroacetic acid. The resulting mixture was centrifuged at 3,000 r.p.m. in a Sorvall GLC-2 general laboratory centrifuge for 10 min. To the supernatant (0.5 ml) was added 0.5 ml of 0.55% (w/v) ammonium molybdate in 1.1 N sulfuric acid, followed by 0.05 ml of reducing agent (1.15 M sodium bisulfite, 0.05 M sodium sulfite, 0.01 M 1-amino-2-naphthol-4-sulfonic acid). Colour development was allowed to proceed for at least 10 min and the absorbance

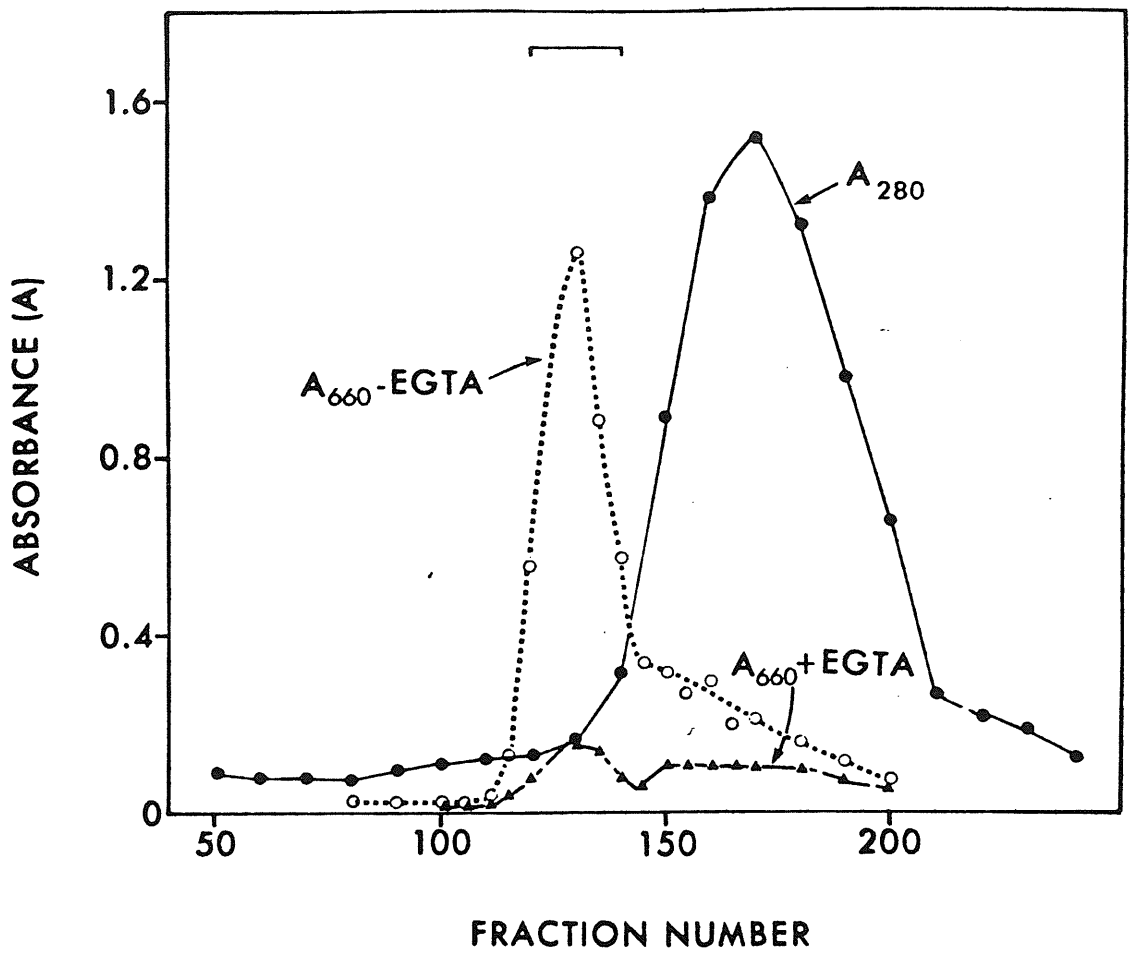


Figure 11

Preparation of bovine heart phosphodiesterase: second DEAE-cellulose column chromatography. The separation shown was performed and monitored as described in the text. 3.6 ml fractions were collected and those fractions indicated by the bar were pooled.

at 660 nm measured against distilled water in a Beckman Model 25 Spectrophotometer. A typical standard curve relating amount of PDE to $A_{660\ nm}$ is illustrated in Fig. 12. An optimum amount of protein modulator was always used in the assay. The curve does not plateau since further addition of enzyme increases the activity by the basal level, i.e., that seen in the absence of Ca^{2+} and the protein modulator.

One unit of PDE activity is equivalent to the amount of enzyme which, when maximally activated by the protein modulator and Ca^{2+}, hydrolyzed 1 μmole of cAMP per min at 30^{o} under standard conditions.

IV. Assay of protein modulator activity

Modulator activity was assayed by measuring the extent of stimulation of a fixed amount of modulator-deficient PDE (0.4-0.5 unit) under standard conditions. The reaction mixture, in a volume of 0.9 ml, contained in addition to the modulator and a standard amount of PDE (prepared as described in "General Experimental Procedures"), 1.2 mM cAMP and 0.25 unit of 5'-nucleotidase in 40 mM Tris, 40 mM imidazole, 3 mM magnesium acetate, 0.11 mM $CaCl_2$, pH 7.5. The reaction mixture was incubated at 30^{o} C for 30 min, at which time reaction was terminated by the addition of 0.1 ml of 55% (w/v) trichloroacetic acid. The resulting mixture, if cloudy, was centrifuged at 3,000 r.p.m. for 10 min in a Sorvall GLC-2 general laboratory centrifuge. To the supernatant (0.5 ml) was added 0.5 ml of 0.55% (w/v) ammonium molybdate in 1.1 N sulfuric acid, followed by 0.05 ml of reducing agent (1.15 M sodium bisulfite, 0.05 M sodium sulfite, 0.01 M 1-amino-2-naphthol-4-sulfonic acid). Colour development was allowed to proceed for at least 10 min and the absorbance at 660 nm was measured against distilled water in a Beckman Model 25 Spectrophotometer.

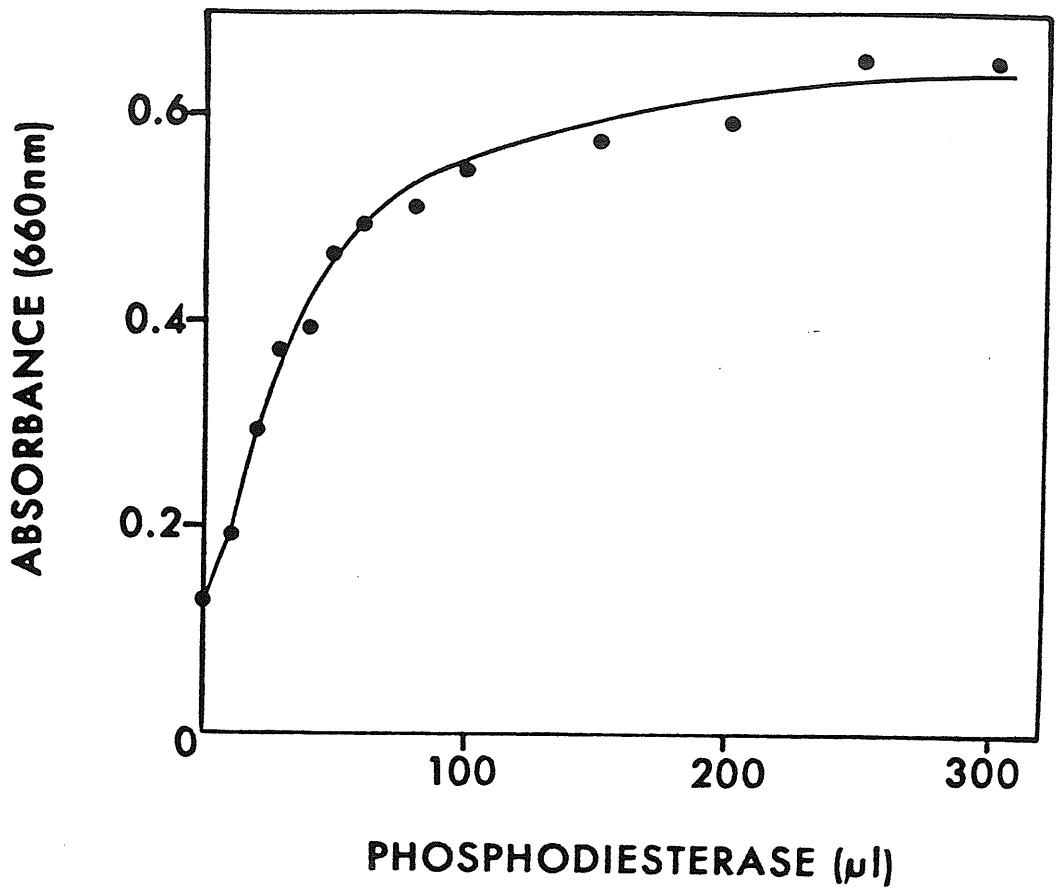


Figure 12

Standard curve of phosphodiesterase activity. Varying amounts of PDE, prepared as described in "General Experimental Procedures" II, were assayed as described in "General Experimental Procedures" III. Enzyme activity is represented as absorbance at 660 nm (see "General Experimental Procedures" III).

Several concentrations of each modulator sample were always assayed following an initial range-finding assay in order to construct a standard curve relating stimulation of PDE activity to amount of modulator (see Fig. 13, for example); this enabled accurate determination of the amount of modulator required to induce half-maximal stimulation of the standard amount of PDE. In the absence of added modulator, PDE possesses low catalytic activity. With increasing modulator concentration, the catalytic activity of the enzyme is increased in a nonlinear manner. With saturating concentration of the modulator, the enzyme is stimulated 6- to 10-fold. One unit of modulator is defined as the amount required to give half-maximal stimulation of the standard amount of PDE.

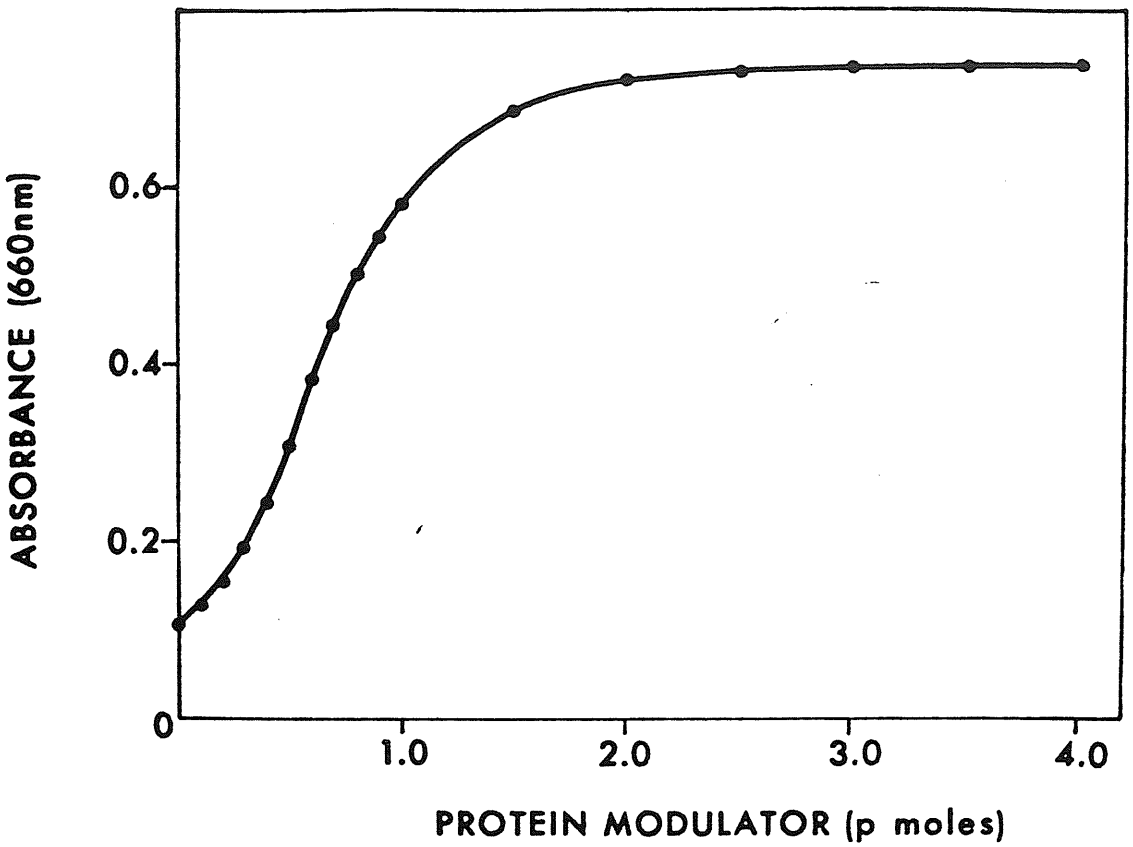


Figure 13

Standard curve of protein modulator activity. Varying amounts of purified protein modulator were assayed for their capacity to stimulate a fixed amount of modulator-deficient PDE as described in "General Experimental Procedures" IV. Protein modulator activity is represented as absorbance at 660 nm (see "General Experimental Procedures" IV).

V. Electrophoretic procedures

A. 15% PAGE

Gel electrophoresis was performed in 15% acrylamide slab or tube gels employing the discontinuous buffer system of Davis (1964). The following stock solutions were used:

Solution	Composition
A	22.2 g acrylamide 0.6 g MBA H_2O → 100 ml
B	0.6 M Tris-HCl, pH 8.9
C	0.6 M Tris-phosphate, pH 6.9
D (separating gel buffer)	40 g sucrose 40 ml solution B H_2O → 100 ml
E (sample buffer)	70 g sucrose 20 ml solution 2.5 mg bromophenol blue H_2O → 100 ml
F (Stock running buffer)	144 g glycine 30 g Tris H_2O → 1000 ml
G (spacer gel buffer)	40 g sucrose 40 ml solution C H_2O → 100 ml

The following solutions were prepared immediately prior to use:

Storing buffer : 10-fold dilution of solution B.

Running buffer : 20 ml solution F + 980 ml H_2O.

Ammonium persulfate : 5 mg/ml in H_2O.

Gradient buffer : 10-fold dilution of solution C.

The separating gel (15% acrylamide, 0.4% MBA) was prepared by mixing the following solutions:

Solution A	20.3 ml
H_2O	0.7 ml
Solution D	7.5 ml
Ammonium persulfate	1.5 ml
TEMED	0.015 ml

The spacer gel (5% acrylamide, 0. 13% MBA) was prepared by mixing the following solutions:

Solution A	3.375 ml
H_2O	7.125 ml
Solution C	3.750 ml
Ammonium persulfate	0.750 ml
TEMED	0.020 ml

Protein samples were dissolved, normally at a concentration of 1 mg/ml, in solution E (sample buffer) diluted 1:1 with water. Electrophoresis was performed at 100 volts (slab gels) until the samples focused in the separating gel, and then at 300 volts until the dye front neared the bottom of the gel, or at 3 mamps per tube (tube gels). Gels were stained overnight with 0.25% Coomassie Brilliant Blue G-250 in 7.5% acetic acid and destained electrophoretically with 7.5% acetic acid.

B. Urea-PAGE

Exactly the same electrophoretic system as described above was employed, with the exception that the separating gel and the spacer gel were made 6 M in urea as follows:

Separating gel :

Solution A	20.3 ml
H_2O	0.7 ml
Solution D	7.5 ml
Urea	14.4 g
Ammonium persulfate	1.5 ml
TEMED	0.015 ml

Spacer gel :

Solution A	3.375 ml
H_2O	7.125 ml
Solution G	3.750 ml
Urea	7.2 g
Ammonium persulfate	0.750 ml
TEMED	0.020 ml

This amount of urea increases the volume of solution considerably, e.g., the volume of the separating gel mixture is increased from 30 ml to 39.5 ml by the addition of 14.4 g urea. Therefore, the concentrations of acrylamide and MBA are correspondingly lower than in non-urea-containing gels. These concentrations are :

Separating gel :	11.4% acrylamide, 0.3% MBA
Spacer gel :	3.8% acrylamide, 0.1% MBA

The sample buffer was prepared as follows :

50 ml Solution E

48 g urea

pH to 6.9

H_2O ⟶ 1000 ml

Gels were stained and destained as described above for 15% PAGE.

C. SDS-PAGE

SDS-PAGE was performed in slab or tube gels employing a discontinuous buffer system essentially as described by Swank and Munkres (1971). The following stock solutions were used :

Solution	Composition
A	30 g acrylamide 0.8 g MBA H_2O → 100 ml
B	1.5 M Tris-HCl, pH 8.8
C	10% SDS
D	0.5 M Tris-HCl, pH6.8
E (Running buffer)	3 g Tris 14.4 g glycine 1 g SDS H_2O → 1000 ml
F (Sample buffer)	0.05 M Tris 1% SDS 0.01% bromophenol blue 30% glycerol 6 M urea

The separating gel (11.4% acrylamide, 0.13% MBA) was prepared by mixing the following solutions :

Solution A	15 ml
Solution B	7.5 ml
H_2O	5.4 ml
Solution C	0.6 ml
Urea	14.4 g
Ammonium persulfate (4 mg/ml)	1.5 ml
TEMED	0.02 ml

The spacer gel (3.8% acrylamide, 0.1% MBA) was prepared by mixing the following solutions :

Solution A	5 ml
Solution D	7.5 ml
H_2O	15.7 ml
Solution C	0.3 ml
Urea	14.4 g
Ammonium persulfate	1.5 ml
TEMED	0.02 ml

Protein samples were dissolved normally at a concentration of 1 mg/ml, in solution F (sample buffer) and immersed in a boiling water bath for 10 min. Electrophoresis was performed at 25 mamps (slab gels) or 3 mamps per tube (tube gels) until the dye front neared the bottom of the gel. Gels were stained overnight with 0.25% Coomassie Brilliant Blue G-250 in 25% isopropanol, 10% acetic acid, and subsequently destained electrophoretically with 7.5% acetic acid.

D. Isoelectric focusing

Gel isoelectric focusing was performed according to the method of Wrigley, C.W. (1971) as summarized below.

Solutions

1. Acrylamide solution :

   10 g acrylamide

   0.33 g MBA

   H_2O → 50 ml

2. 40% (w/v) sucrose

3. Ammonium persulfate (100 mg/ml) - made fresh on day of use.

4. Electrode solutions :

   5% (v/v) ethylenediamine

   5% (v/v) phosphoric acid

Sample preparation

Modulator (100 μg) was dissolved in 2.3 ml H_2O.

Gel preparation

The precedure below is designed for six 0.6 x 16 cm tubes.

The following concentrated mixture was prepared just prior to casting the gels :

H_2O	1.87 ml
Acrylamide solution	7.0 ml
40% ampholytes (3.5-10.0)	0.7 ml

Each gel was mixed individually and contained the following :

Concentrated mix	1.5 ml
40% sucrose	0.5 ml
Sample in H_2O	2.3 ml

Finally, 20 µl of ammonium persulfate solution were added, mixed and the gel cast. The gels were overlaid with water and allowed to polymerize for at least 1 h.

Electrofocusing

The gels were fitted into an apparatus allowing for proper cooling, e.g., BioRad electrophoresis chamber. The bottom electrode compartment was filled with 5% ethylenediamine (cathode) and the top with 5% phosphoric acid (anode). The cooling chamber was connected to a Lauda circulating water bath set to maintain a temperature of 4° C. The apparatus was connected to an Ortec Pulsed Constant power supply and the following power settings were used (all at 0.5 microfarads) :

Voltage	Time	Pulses per sec
50 V	0.5 h	50
100 V	1.0 h	50
150 V	overnight	50
300 V	0.5 h	150

Staining and destaining

Gels were stained for 1-2 h in 0.2% bromophenol blue in ethanol : water : acetic acid (50 : 45 : 5). Destaining was achieved by diffusion employing several changes of ethanol : water : acetic acid (30 : 65 : 5).

pH gradient determination

The gels were sliced into 2 mm fractions which were collected in small tubes. 0.5 ml water at 4° C was added to each and left for 15 min in the cold. The pH of each fraction was measured in the cold room using a microelectrode.

VI. Cyanogen bromide cleavage

Cleavage of the protein modulator and chemically modified derivatives with cyanogen bromide was achieved by the method of Gross and Witkop (1961) as modified by Steers et al, (1965). The protein (0.5 mg) was dissolved in 1 ml of 70% (v/v) aqueous formic acid containing 2 mg of cyanogen bromide. The reaction mixture was kept at room temperature in a stoppered tube in the dark for 40 h. Deionized water (at least 4 volumes) was then added and the mixture was lyophilized, redissolved in water, and again lyophilized.

VII. Performic acid oxidation

Performic acid oxidation was performed according to Stegemann (1958). Protein (1 mg) was dissolved in 220 µl of performic acid (1 ml of formic acid mixed with 0.1 ml of 30% hydrogen peroxide and kept in a stoppered tube for 15 min at room temperature) and after 15 min at room temperature 1 ml of deionized water was added and the mixture lyophilized. The residue was dissolved in 0.5 ml of deionized water and again lyophilized prior to acid hydrolysis and amino acid analysis.

VIII. Acid hydrolysis and amino acid analysis

Samples containing 0.02 to 0.1 µmoles of protein were hydrolyzed with 6 N HCl, containing 50 µl of 5% (w/v) aqueous phenol per ml to protect tyrosine against destruction (Howard and Pierce, 1969), at 110° C in sealed, evacuated tubes for 22 h. Samples were dried in a vacuum desiccator prior to amino acid analysis which was carried out in a Spinco 120/139 amino acid analyzer as outlined in the Spinco manual.

IX. Protein and peptide determination by the ninhydrin method after alkaline hydrolysis

For the determination of proteins or peptides, the ninhydrin reaction depends on the presence of free amino groups. Therefore, ninhydrin colour development is normally preceded by alkaline hydrolysis which generates free α-amino groups. The method of Hirs et al, (1956) was utilized. To each protein or peptide sample (100 µl-300 µl in aqueous solution) was added 1 ml of 2.5 N NaOH. The tubes were placed in an open water bath and incubated for 2.5 h at 90° C. The samples were then cooled and 1 ml of 30%(v/v) acetic acid added to bring the pH to about 5. Ninydrin reagent (2 ml of a solution of 250 ml 4 N sodium acetate, pH 5.5, +20 g ninhydrin +3 g hydrindantin +750 ml of peroxide-free methylcellosolve) was added and the samples incubated for 20 min in a boiling water bath. After cooling the samples quickly in cold water, the tubes were shaken vigorously for 1 min to oxidize excess ninhydrin. 5 ml of 50% (v/v) ethanol were added and the absorbances of the samples read against distilled water at 570 nm in a Beckman Model 25 spectrophotometer.

X. Circular dichroism

Circular dichroism (CD) measurements were made with a Cary Model 6001 circular dichroism attachment to a Cary 60 recording spectropolarimeter in accordance with previously described methodology (Oikawa et al, 1968). These studies were performed in collaboration with Dr. C.M. Kay at the University of Alberta. The concentration of modulator used in the CD studies was measured by ultraviolet absorption employing a molar extinction coefficient, $E_{1\ cm,\ 275\text{-}278\ nm}$ (1%), of 1.9 (Stevens et al, (1976).

Troponin I concentration was determined by the method of Lowry et al, (1951). Modulator-troponin I complexes were formed by mixing modulator and troponin I in 1 : 1 molar ratio. Prior to mixing, the proteins were dialyzed against 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH7.5.

XI. Calcium ion concentration

In studies involving the effects of incremental addition of Ca^{2+} on the far-UV CD spectrum of the modulator and various chemically modified derivatives, the level of free Ca^{2+} ions in solution was adjusted by means of an EGTA-containing buffer, employing principles discussed by Perrin and Dempsey (1974). From Schwarzenbach et al, (1957), the stepwise equilibrium constants for the deprotonation of EGTA, $pK_{a1} = 2.0$, $pK_{a2} = 2.65$, $pK_{a3} = 8.85$, and $pK_{a4} = 9.46$, and the association constant for Ca^{2+}, $pK_{Ca} = 10.97$, were obtained, enabling accurate determination of the level of free Ca^{2+} ions in solution.

XII. Analysis of Ca^{2+} titration data

In studies involving the effects of incremental addition of Ca^{2+} on the far-UV CD spectrum of the native and various chemically modified derivatives of the modulator, the titration data were analyzed by the method of Hincke et al, (1978). The Ca^{2+} titration data were first normalized on a 0 to 1 scale by calculating f, the fraction of the total conformational change elicited by Ca^{2+}, in accordance with the following equation :

$$f = \frac{\left[\Theta\right]_{222}^{Ca^{2+}} - \left[\Theta\right]_{222}^{EGTA}}{\left[\Theta\right]_{222}^{Ca^{2+},max} - \left[\Theta\right]_{222}^{EGTA}} \qquad (1)$$

where $\left[\theta\right]_{222}^{Ca^{2+}}$ is the ellipticity value at 222 nm elicited by a given calcium concentration, $\left[\theta\right]_{222}^{Ca^{2+}, max}$ is the maximum ellipticity value which could be elicited by Ca^{2+}, and $\left[\theta\right]_{222}^{EGTA}$ is the ellipticity value at 222 nm in the absence of Ca^{2+}.

A computer program was then used to perform a nonlinear curve fitting iterative procedure which fit the data to an equation of the form :

$$f = \sum_i \frac{n_i K_i [Ca^{2+}]}{1 + K_i [Ca^{2+}]} \; ; \; i = 1,2 \text{ or } 3 \qquad (2)$$

and extracted the values of n_i and K_i . Here n_i is the proportion of the total conformational change due to the i^{th} class of Ca^{2+} dependent transitions, K_i is the apparent association constant of the i^{th} class of binding sites, and Ca^{2+} is the concentration of free Ca^{2+} calculated from the EGTA parameters as described in "General Experimental Procedures".

Equation (2) describes the binding of small molecules to proteins and is based upon the simplifying assumption that the binding sites in any class are equivalent, independent and non-interacting (Edsall and Wyman, 1958). If there is more than one class of binding sites with different affinities for Ca^{2+}, i is increased to equal the number of different classes.

In practice, it is often simple to decide from the shape of the Ca^{2+} titration curve which value of i is most appropriate. The extent to which a fit minimizes the mean standard error is also used in this assessment.

EXPERIMENTAL

I. Comparison of bovine heart and bovine brain modulator protein

A. Introduction

The early stages of the studies described herein relating to the protein modulator were performed with modulator purified from bovine heart. While this work was in its early stages, Vanaman et al, (1975) reported on the physical and chemical characterization of a troponin C-like Ca^{2+}-binding protein from brain. The results described below show that these two proteins are identical. In view of the much greater abundance of the protein in brain relative to heart, all subsequent investigations were carried out using the brain modulator protein and results previously obtained with the bovine heart modulator were verified with the brain protein. The two proteins were simultaneously compared with rabbit skeletal muscle troponin C.

B. Experimental procedure

Digestion with trypsin and peptide mapping

Protein (1 mg) was dissolved in 100 μl of 0.1 M ammonium bicarbonate, 0.1 mM EGTA. The solution was saturated with nitrogen and 5 μl of TPCK-trypsin (16 mg/ml in 0.1 M ammonium bicarbonate) were added; the tube was covered, and the sample mixed and incubated at 37° C for 2 h. The digestion was stopped by the addition of 10 μl of 0.2 N HCl. A sample (50 μl) of the digest was applied to Whatman No. 3MM paper and subjected to two-dimensional peptide mapping. High voltage electrophoresis was performed in a Savant electrophoresis tank at pH 4.7 as described by Tan and Stevens (1971) with methyl green (1%) as a marker (Stevenson, 1971). Descending chromatography was carried out in the other dimension using butanol : pyridine : acetic acid : water (120 : 80 : 24 : 96) as the solvent

After drying, the peptide spots were detected with the ninhydrin-collidine reagent (Margoliash and Smith, 1962).

In order to obtain enough peptide for amino acid analysis, the material obtained from 4-5 mg of protein was subjected to peptide mapping; the maps were stained with 0.01% ninhydrin in 95% ethanol at room temperature and the spots were cut out, washed in ether, and eluted first with 10% aqueous acetic acid and then with 50% aqueous pyridine. The material was taken to dryness in a vacuum desiccator, hydrolyzed in 6 N HCl at 110° C for 22 h, and subjected to amino acid analysis.

C. Results and Discussion

Fig. 14 shows the tryptic peptide maps of bovine heart modulator protein (activator), bovine brain troponin C-like calcium-binding protein (TCLP), and rabbit skeletal muscle troponin C (TN-C). The peptide maps of the heart modulator and TCLP are virtually identical. Furthermore, purified TCLP was found to have the same specific activity in stimulating modulator-deficient PDE as the bovine heart modulator. Therefore, these two proteins are probably identical. The amino terminus of the bovine heart modulator was found to be blocked, since the protein was not degraded by the Edman procedure on a Beckman automatic sequenator; a similar phenomenon was observed with brain TCLP (Watterson et al, 1976). Brain TCLP was shown to contain one residue per mole of an unusual amino acid (later identified as trimethyllysine) (Watterson et al, 1976); Dr. T.C. Vanaman (Duke University) kindly analyzed a sample of bovine heart modulator protein and showed that it also contains one residue per mole of the same unusual amino acid.

While heart modulator and brain TCLP are indistinguishable, a purified sample of rabbit skeletal muscle troponin C failed to activate

TRYPTIC PEPTIDE MAPS

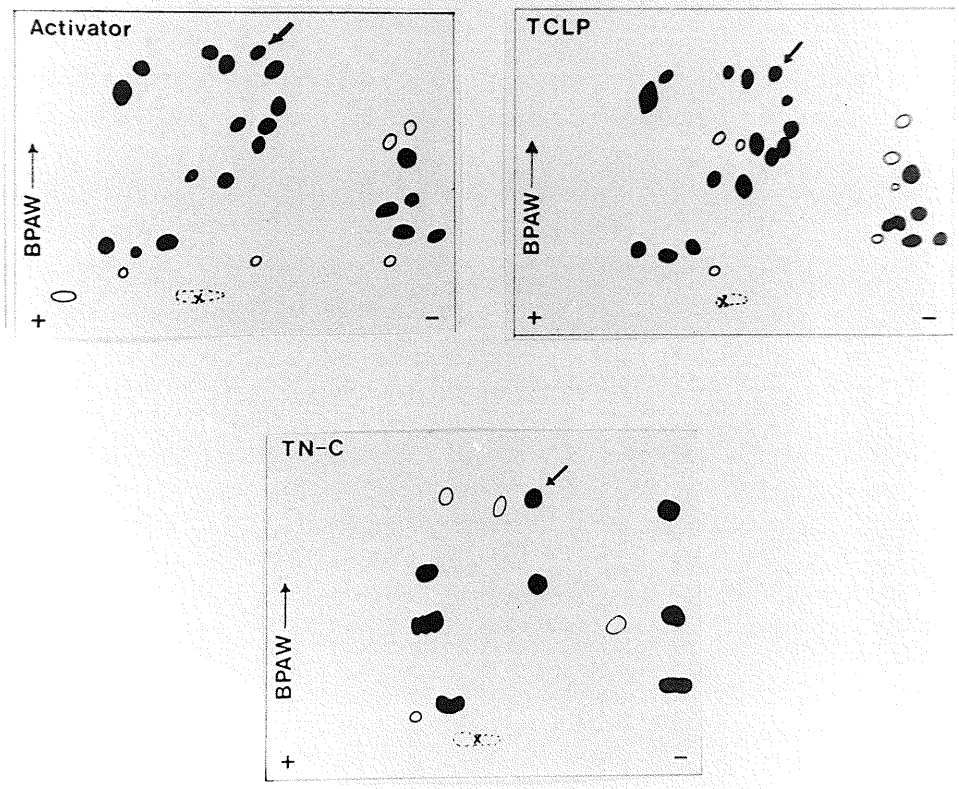


Figure 14

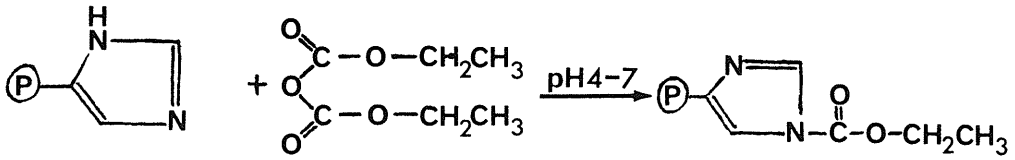
Tryptic peptide maps of bovine heart protein modulator (activator), bovine brain protein modulator (troponin C-like calcium-binding protein, TCLP), and rabbit skeletal muscle troponin C (TN-C). BPAW, butanol : pyridine : acetic acid : water (120 : 80 : 24 : 96, v/v). The arrows indicate a peptide with identical amino acid composition in all three proteins.

PDE and the tryptic peptide map of troponin C is surprisingly different from that of the other two proteins (Fig. 14). All the major peptides from both the modulator and troponin C maps were eluted and their amino acid compositions determined. Only the peptide indicated by the arrow in Fig. 14 has an identical amino acid composition in modulator and troponin C; it corresponds to residues 38-44 in the troponin C sequence (Collins et al, 1973) : -Glu-Leu-Gly-Thr-Val-Met-Arg-. This peptide was found to have the same sequence in bovine brain TCLP (Vanaman et al, 1977). It appears, therefore, that bovine heart modulator protein and bovine brain TCLP are identical and quite distinct from troponin C.

II. Histidine Modification

A. Introduction

Diethylpyrocarbonate reacts principally with amino and imidazole functions of proteins (Means and Feeney, 1971). It has been shown to be relatively specific for the chemical modification of accessible histidine residues in proteins at pH 6 or above (Grousselle et al, 1973; Vincent et al, 1975). Treatment of a protein with this reagent converts reactive histidine residues to N-carbethoxyhistidine :



imidazole group

diethylpyrocarbonate

N-carbethoxyhistidine

$+ CH_3CH_2OH + \uparrow CO_2$

Since this product is not stable to acid hydrolysis, the extent of histidine modification by diethylpyrocarbonate cannot be quantitated by amino acid analysis after acid hydrolysis. However, N-carbethoxyhistidine residues in proteins absorb light at 242 nm, which enables continuous monitoring of the modification reaction and, from the molar extinction coefficient of 3,200 M^{-1} cm^{-1} at 242 nm for N-carbethoxyhistidine residues in proteins, (Ovadi et al, 1967), the extent of histidine modification is readily calculated. N-carbethoxyhistidine residues have a relatively short half-life (55 h at pH 7 and 25° C), so that investigations of the effects of such modifications must be performed rapidly.

B. Experimental procedure

The method of Grousselle et al, (1973) was used to carbethoxylate the sole histidine residue of the protein modulator with diethylpyrocarbonate.

Protein modulator (1 mg/ml) containing endogenous Ca^{2+} [a] was incubated at 23° C in 0.1 M phosphate buffer, pH 6.0, with or without 2 mM EGTA, in the sample and reference cuvettes of a Coleman-Hitachi Model 124 double beam spectrophotometer equipped with a Model 165 recorder. The reaction was initiated by the addition, to the sample cell, of a fresh, concentrated solution of diethylpyrocarbonate (58.8 mM in absolute ethanol) to give a final reagent concentration of 0.588 mM; an equal volume of absolute ethanol was added to the reference cell and the formation of the carbethoxy-modulator was monitored by recording the increase in absorbance at 242 nm. The final concentration of ethanol in both sample and reference was <1%. Aliquots of the reaction mixture were withdrawn at selected time intervals and assayed for modulator activity. The extent of histidine modification was calculated from the absorbance using a value of 3,200 M^{-1} cm^{-1} as the molar extinction coefficient, at 242 nm, of N-carbethoxyhistidine residues in proteins (Ovadi et al, 1973).

C. Results and Discussion

The kinetics of carbethoxylation of the sole histidine residue of the protein modulator in the presence and absence of Ca^{2+} are illustrated in Fig. 15. Clearly, this histidine residue can be fully modified by carbethoxylation both in the presence and absence of Ca^{2+}. The kinetics of the reaction are pseudo-first-order, as expected. The rate

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[a] Protein modulator isolated as described in "General Experimental Procedures" is saturated with endogenous Ca^{2+} as evidenced by the fact that this modulator exhibits maximal activation of PDE without addition of Ca^{2+} ions. The carbethoxylation reaction is carried out in phosphate buffer and addition of exogenous Ca^{2+} would result in the precipitation of insoluble calcium phosphate salts.

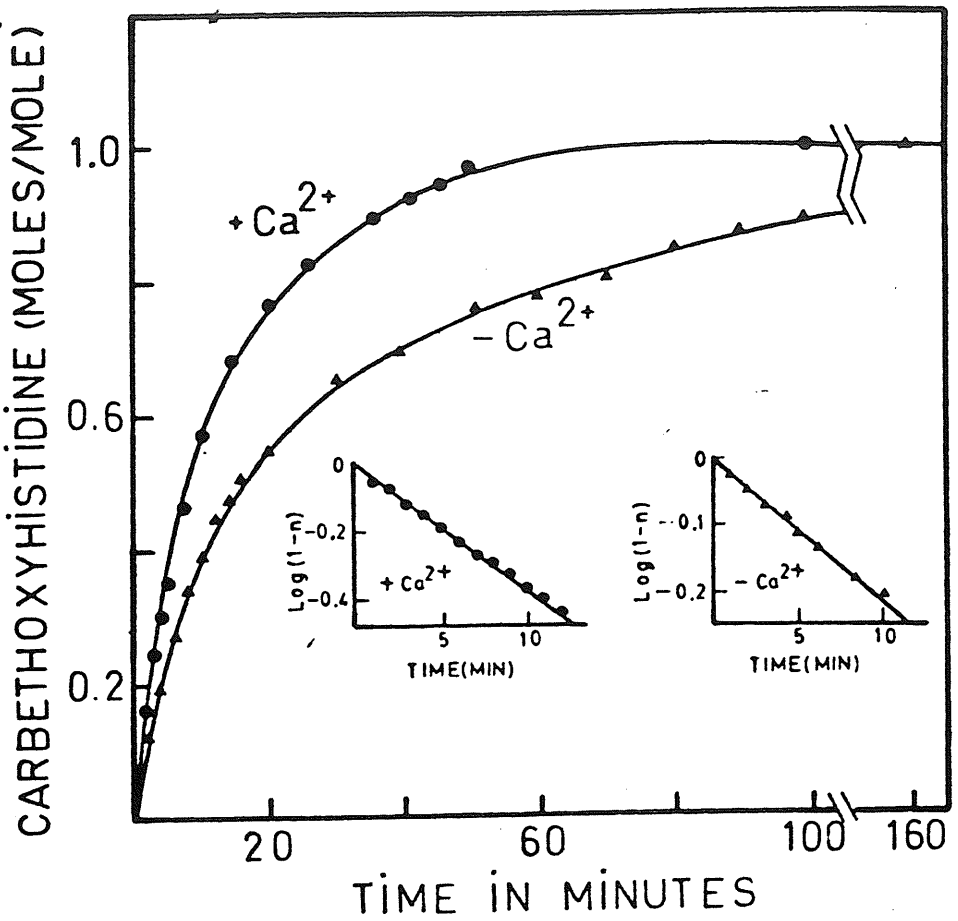


Figure 15

Kinetics of carbethoxylation of the histidine residue of the protein modulator by diethylpyrocarbonate. The protein modulator (1 mg/ml) containing endogenous Ca^{2+} was incubated at 23° C, pH 6.0, with 0.588 mM diethylpyrocarbonate in the absence (●—●) or presence (▲—▲) of 2 mM EGTA. The carbethoxyimidazole content was calculated from the absorbance at 242 nm using a molar extinction coefficient of 3200 M^{-1} cm^{-1}. The inset illustrates the pseudo-first-order nature of the reaction; n is the number of carbethoxyhistidine residues in the modulator.

of reaction of the histidine residue of the modulator with diethylpyrocarbonate is different in the presence than in the absence of Ca^{2+}, reflecting the conformational change which occurs as the modulator binds Ca^{2+}: the half-time for carbethoxylation in the presence of Ca^{2+} was 8 min., whereas, in the absence of Ca^{2+}, it was 15.5 min., indicating that the imidazole ring becomes more accessible to or more reactive with diethylpyrecarbonate as the modulator protein binds Ca^{2+}.

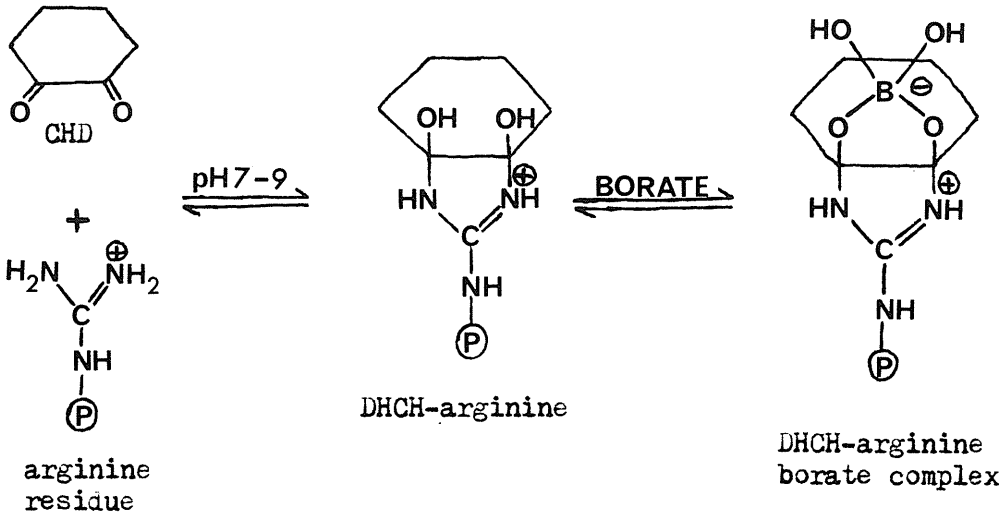
Assay of modulator activity at various time intervals during the carbethoxylation reaction revealed that this chemical modification of the single histidine residue is without effect on the ability of the modulator to stimulate PDE. It appears, therefore, that the sole histidine residue is not involved in the interaction between the protein modulator and PDE.

III. Arginine Modification

A. Introduction

Until very recently no suitable method was available for the selective modification of arginine residues in proteins under mild conditions. For example, treatment of proteins with benzil, cyclohexanedione, nitromalondialdehyde in strong alkali, or malonaldehyde in 10 N HCl resulted in quantitative modification of arginine residues, but the harsh conditions involved often led to a number of side reactions and to irreversible changes. Alternatively, modification with 2,3-butanedione, glyoxal, phenylglyoxal, and cyclohexanedione can be performed under relatively mild conditions, but, unfortunately, all of these procedures may result in significant side reactions with α- or ϵ-amino groups. An additional problem is caused by the multiple products arising when butanedione or cyclohexanedione is used for arginine modification.

These problems have largely been overcome since the publication of a method of achieving reversible modification of arginine residues (Patthy and Smith, 1975). This method involves treatment of the protein with 1,2-cyclohexanedione (CHD) at pH 8 to 9 in borate. Under the mild conditions specified, CHD reacts only with arginine residues, which are converted quantitatively to a single product, N^7,N^8-(1,2-dihydroxycyclohex-1,2-ylene)-L-arginine (abbreviated DHCH-arginine), which is stable in acidic solutions and borate buffers:



Upon hydrolysis in 6 N HCl at 110° for 24 h, DHCH-arginine is destroyed. There is an 18-20% regeneration of arginine; the remainder is converted to unknown basic products that are not eluted from the amino acid analyzer under normal conditions. However, if acid hydrolysis is carried out in the presence of excess mercaptoacetic acid, DHCH-arginine is converted to a neutral product which is eluted after the aromatic amino acids on the short column of the amino acid analyzer. Since under these conditions no arginine regeneration occurs, the extent of arginine modification is determined by measurement of the difference in arginine content of the protein before and after the modification following hydrolysis in the presence of mercaptoacetic acid.

The specificity and mild conditions of this method make it ideally suited for the identification of functional arginine residues in proteins.

B. Experimental procedure

Selective modification of arginine residues of the protein modulator was achieved by treatment with 1,2-cyclohexanedione according to Patthy and Smith (1975). To a solution of the protein modulator (5 mg/ml in 0.2 M sodium borate, pH 9.0, containing either 2 mM $CaCl_2$ or 2 mM EGTA) was added an equal volume of a 15 mM solution of 1,2-cyclohexanedione. The reaction mixture was incubated at 37° C and at appropriate time intervals aliquots were withdrawn for assay of modulator activity. After 24 h incubation, the reaction was terminated by addition of an equal volume of cold 5% (v/v) acetic acid; the sample was lyophilized and then acid hydrolyzed in the presence of excess mercaptoacetic acid (Patthy and Smith, 1975) prior to amino acid analysis to determine the extent of arginine modification. A control modulator was treated in identical fashion with the exception that the modifying agent was omitted.

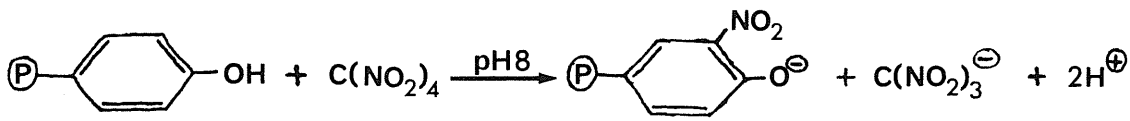
D. Results and Discussion

The degree of arginine modification was estimated by the decrease in arginine content in acid hydrolyzates of the modified modulator as revealed by amino acid analysis. Both in the presence and absence of Ca^{2+}, incubation of the modulator protein with 1,2-cyclohexanedione resulted in the modification of 4 out of a total of 6 arginine residues per mole. This degree of arginine modification was without effect on the activity of the modulator. Since this modification involves the introduction of 4 bulky dihydroxycyclohexyl groups into the modulator molecule, yet has no effect on its capacity to stimulate PDE, it is apparent that the modified arginine residues are not involved in the interaction between the modulator and PDE.

IV. Tyrosine Modification

A. Introduction

Tyrosine residues in proteins can be chemically modified by treatment with tetranitromethane, which converts reactive tyrosine residues to 3-nitrotyrosine:



tyrosine residue	tetranitro-methane	3-nitrotyrosine	nitroformate anion

The degree of nitration of tyrosine residues may be determined by measurement of the absorbance at 428 nm using a value of 4100 M^{-1} cm^{-1} for the molar extinction coefficient of 3-nitrotyrosine (Riordan et al, 1967), and also by amino acid analysis following acid hydrolysis since 3-nitrotyrosine is an acid-stable derivative. 3-Nitrotyrosine elutes from the long column of the amino acid analyzer as a discrete peak after phenylalanine.

Nitration with tetranitromethane affects not only tyrosyl groups but also cysteinyl residues. However, the nitration of tyrosyl residues does not proceed at pH 6 where cysteinyl residues continue to be oxidized. This variance in pH dependence of oxidation vs. nitration can be employed to differentiate between them (Sokolovsky et al, 1966). Of course, since the protein modulator contains no cysteine, this oxidative side reaction is of no consequence in studies involving nitration of the modulator.

Several instances have been reported of inter- and intra-molecular

cross-linking occurring as a side reaction during nitration with tetranitromethane (Means and Feeney, 1971); these cross-linkages appear to be between tyrosine residues. It is recommended always to check for the possible occurrence of this side reaction during nitration; this is conveniently and rapidly achieved by, e.g., polyacrylamide gel electrophoresis in the presence of SDS.

B. Experimental procedure

1. Nitration

Nitration of tyrosine residues was achieved by the method of Sokolovsky et al, (1966). The protein modulator (final concentration 1 mg/ml) was incubated at 23° C in 0.05 M Tris-HCl, pH 8.0, containing 1 M NaCl and 2 mM $CaCl_2$ or 2 mM EGTA in the reference and sample cuvettes of a Coleman-Hitachi Model 124 double beam spectrophotometer equipped with a Model 165 recorder. Nitration was initiated by the addition of a concentrated solution of tetranitromethane (60 mM in absolute ethanol) to the sample to give a final reagent concentration of 0.6 mM; an equal volume of absolute ethanol was added to the reference cell and the reaction was monitored by recording the absorbance at 428 nm. The final concentration of ethanol in both sample and reference was <1%. Aliquots were removed at appropriate time intervals for assay of protein modulator activity and the degree of nitration of the tyrosine residues was calculated from the absorbance at 428 nm using a value of 4100 M^{-1} cm^{-1} for the molar extinction coefficient of 3-nitrotyrosine (Riordan et al, 1967). After completion of the reaction, the reaction mixture was dialyzed extensively against water to remove excess reagent, lyophilized, and acid hydrolyzed prior to amino acid analysis; the amount of 3-nitrotyrosine in these samples was calculated from amino acid analysis using

a sample of authentic 3-nitrotyrosine in a standard run.

2. Occurrence of intermolecular cross-linking

A sample of protein modulator was incubated with tetranitromethane in the presence of Ca^{2+} as described above for 5 h, at which time reaction was complete; a control modulator was treated in identical fashion with the exception that tetranitromethane was omitted from the reaction mixture. Reaction was terminated by extensive dialysis against water to remove excess reagent. Aliquots were withdrawn from sample and control before initiation and upon completion of the 5 h incubation for: (1) assay of modulator activity, (2) acid hydrolysis and amino acid analysis, and (3) SDS-PAGE in the presence of urea.

C. Results and Discussion

1. Effects of Nitration

The protein modulator contains two tyrosine residues per mole. The kinetics of nitration of these residues by tetranitromethane are shown in Fig. 16. In the presence of Ca^{2+}, stoichiometric modification of both tyrosine residues was achieved, whereas, in the absence of Ca^{2+}, 1.5 residues of 3-nitrotyrosine were formed per mole of modulator as revealed by both spectrophotometric methods and amino acid analysis. Nitration in the presence of a sixfold higher concentration of tetranitromethane for 24 h in the absence of Ca^{2+} led to the formation of only 1.6 moles of 3-nitrotyrosine per mole of protein as estimated by amino acid analysis. This incomplete nitration in the absence of Ca^{2+} is probably due to the formation of intermolecular cross-linkages involving tyrosine residues; the fact that such cross-linking occurs as a minor side reaction during nitration of the protein modulator in the presence of Ca^{2+} is discussed below.

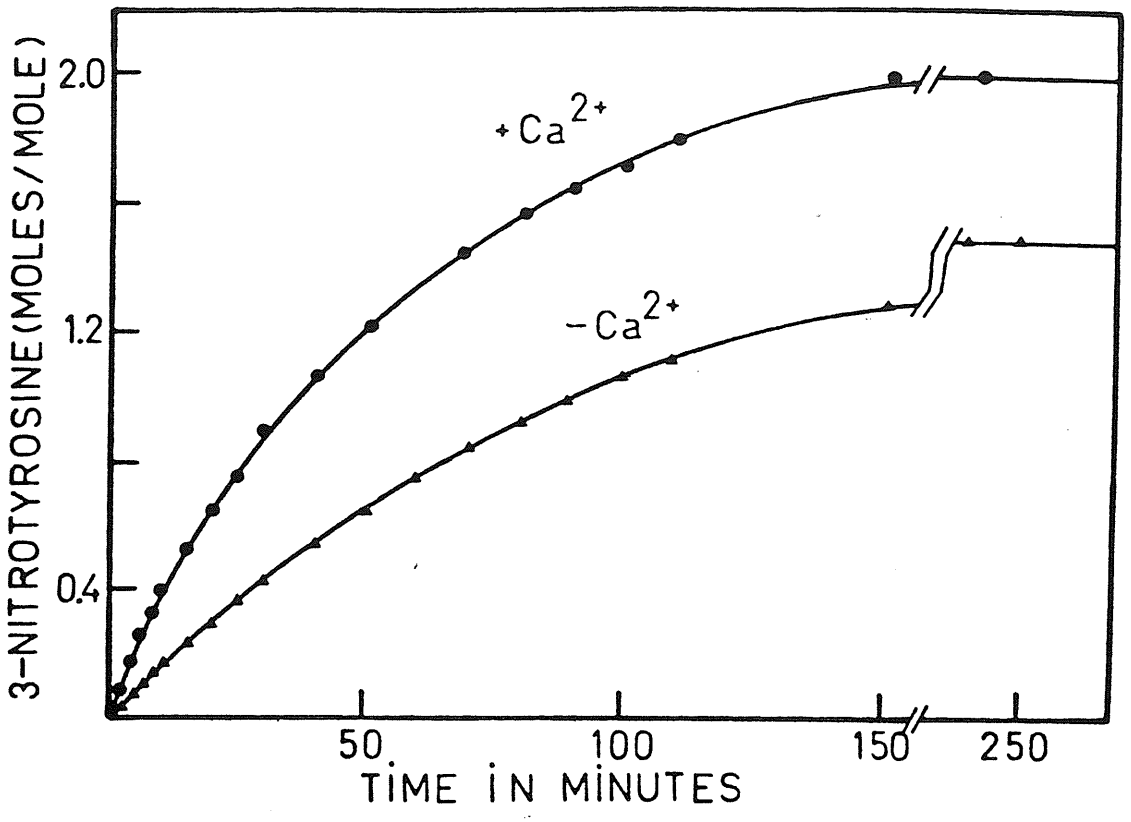


Figure 16

Kinetics of nitration of the protein modulator. The modulator (1 mg/ml) was incubated at 23° C, pH 8.0, with tetranitromethane (0.6 mM) in the presence of 2 mM Ca^{2+} (●——●) or 2 mM EGTA (▲——▲). The nitrotyrosine content was estimated from the absorbance at 428 nm using a molar extinction coefficient of 4100 M^{-1} cm^{-1}.

Clearly, the rate of nitration was greater in the presence of Ca^{2+}, indicating that one or possibly both tyrosine residues are more accessible to the modifying agent in the active, i.e., Ca^{2+}-bound, form of the modulator. Both in the presence and absence of Ca^{2+} the progress curve for nitration of the modulator appears to be monophasic, indicating that the reactivity of the two tyrosine residues differs by less than an order of magnitude. As indicated in the "Literature Review", Klee (1977a) showed, by spectrophotometric titration studies, that one tyrosine residue of the protein modulator is apparently buried within the hydrophobic interior of the protein and is not significantly affected by the conformational changes accompanying Ca^{2+} binding, whereas the second tyrosine residue, which is partially buried in the absence of Ca^{2+}, becomes more exposed as the protein binds Ca^{2+}.

Whether in the presence or absence of Ca^{2+}, modification of the tyrosine residues of the protein modulator by nitration with tetranitromethane was found to have no effect on its ability to stimulate PDE, indicating that maintenance of the integrity of either tyrosine residue is not essential for the expression of modulator activity.

These observations concerning the effects of nitration of the protein modulator were subsequently confirmed by Richman and Klee (1977) who observed that the apparent first order rate constant for the nitration reaction was ~5-fold higher in the presence than in the absence of Ca^{2+}. Furthermore, nitration of 1.7 moles of tyrosine per mole of modulator in the presence of Ca^{2+} did not significantly alter the ability of the modulator to activate PDE.

2. Occurence of intermolecular cross-linking

As mentioned earlier, there have been several reports of the occurrence of inter-and intramolecular cross-linking as a side reaction during nitration with tetranitromethane (Means and Feeney, 1971). In a later series of experiments, the possible occurrence of this side reaction during nitration of the protein modulator in the presence of Ca^{2+} was investigated. The protein modulator was nitrated as described above and the product was examined by SDS-PAGE in the presence of urea (Fig. 17). While modulator controls (slots 1 and 7) and the sample before nitration (slot 3) exhibit single bands of apparent molecular weight 37,000 [a], the nitrated modulator (slot 5) demonstrates an additional minor band of apparent molecular weight 67,000 which is probably a cross-linked dimer. This electrophoretic analysis clearly establishes that intermolecular cross-linking does indeed occur as a side reaction during nitration of the protein modulator. As judged on the basis of relative staining intensity, the side reaction is relatively minor.

Assays of modulator activity revealed that, while the control maintained 100% activity throughout the incubation period, the nitrotyrosyl modulator exhibited only 88.5% the activity of the native protein.

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[a] It is well established that the true molecular weight of the native protein modulator is approximately 17,000; the reason for the anomalous behaviour of this protein on SDS-PAGE in the presence of urea is unknown. However, it is noteworthy that several groups have reported similar behaviour upon gel filtration of the modulator and obtained molecular weights which ranged from 27,000-67,500 (Kakiuchi and Yamazaki, 1970; Cheung, 1971; Wolff and Siegel, 1972; Teo et al, 1973; Brooks and Siegel, 1973; Lin et al, 1974).

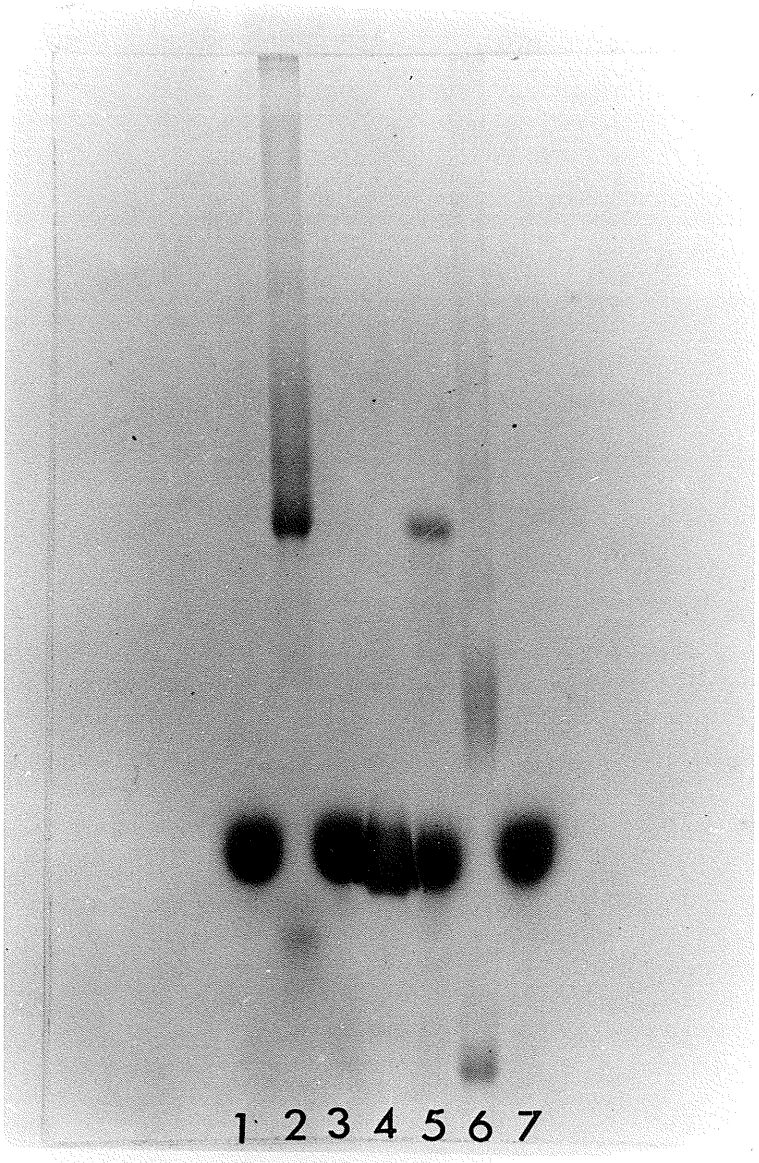


Figure 17

Demonstration of the occurrence of intermolecular cross-linking during nitration of the protein modulator by SDS-PAGE in the presence of urea according to the method of Swank and Munkres (1971). 1: native modulator, untreated (50 µg); 2: bovine serum albumin, M W 67,000 (10 µg) plus cytochrome c, M W 12,500 (10µg); 3: modulator before nitration (50 µg); 4: carboxypeptidase A, M W 35,000 (10 µg); 5: modulator after nitration (50 µg); 6: chymotrypsinogen A, M W 25,000 (10 µg) plus ovalbumin, M W 45,000 (10 µg); 7: control modulator (50 µg).

Amino acid analysis indicated that, while the control exhibited no modification of tyrosine residues, the nitrotyrosyl modulator exhibited nitration of 1.78 moles of tyrosine per mole of modulator. The observed loss of activity is most probably due to the occurrence of intermolecular cross-linking as a side reaction.

The cross-linked dimer was subsequently separated from the monomeric nitrotyrosyl modulator by gel filtration on Sephadex G-75 (Fig. 18). Two peaks with absorbance at 280 nm were eluted from the column. Those fractions indicated by the bars were pooled and lyophilized. Peak I was identified as the cross-linked dimer, and Peak II as the monomeric nitrotyrosyl modulator by SDS-PAGE in the presence of urea. On the basis of absorbance at 280 nm, the dimer represents approximately 8% of the total protein eluted from the column. The peaks were assayed for modulator activity: the monomeric nitrotyrosyl modulator (Peak II) was equally as active as the native protein, while the dimer (Peak I) was considerably less active, having approximately 0.1% the activity of the native protein. It appears, therefore, that the small loss of activity (~10%) that occurs upon nitration of the protein modulator is due to the formation of an essentially inactive cross-linked dimer, and that the nitrotyrosyl modulator, in which both tyrosine residues are nitrated, retains the capacity of the native protein to stimulate PDE. The failure to detect this small loss of activity earlier (Walsh and Stevens, 1977; Richman and Klee, 1977) was probably due to the fact that small losses observed were attributed to experimental error inherent in the assay of modulator activity.

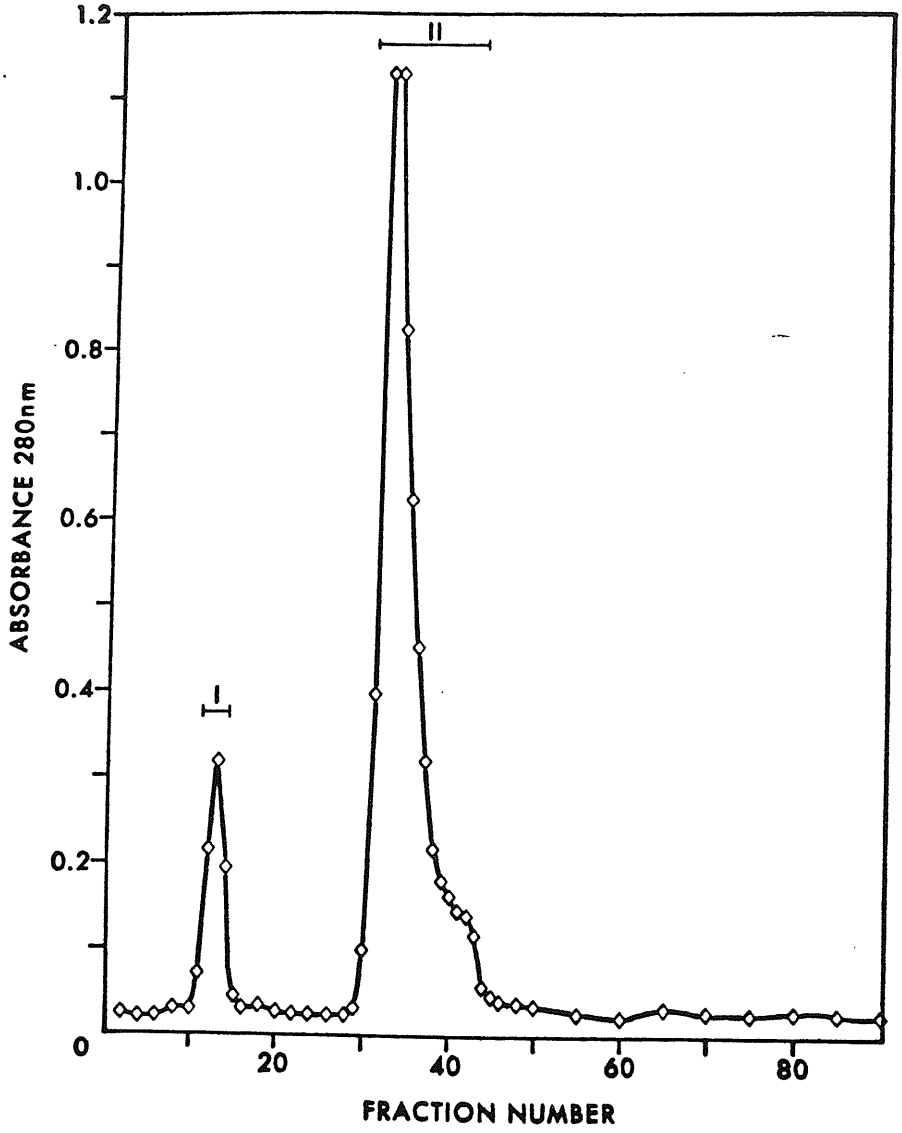


Figure 18

Separation of monomeric, nitrotyrosyl modulator from the cross-linked dimeric by-product of the nitration reaction by gel filtration. A sample of nitrated protein modulator (15 mg) in 3.5 ml of water was applied to a Sephadex G-75 column (2.5 x 90 cm) equilibrated with water and fractions (6.4 ml) were collected at a flow rate of 27.0 ml/h. The absorbance at 280 nm of selected fractions was measured in a Beckman Model 25 Spectrophotometer.

3. Circular dichroism

3.1 Effect of Ca^{2+}

Circular dichroism provides a direct method for determining the gross conformational properties of a protein in solution and is particularly useful for detecting changes in conformation of a protein brought about, for example, by ligand binding or chemical modification of amino acid residues. Circular dichroic spectrophotometry has been widely used to demonstrate the occurrence of a conformational change in the protein modulator upon binding Ca^{2+} (see Literature Review). In this, and other studies discussed later, the effects of Ca^{2+} binding on the conformation of modified modulator species was examined by circular dichroism and compared with the corresponding effects on the native protein, which are presented in the following section.

3.1.1 Native modulator

Fig. 19A shows the far-ultraviolet circular dichroism spectra of the native protein modulator in the presence and absence of Ca^{2+}. Negative maxima at 221 nm and 206.5 nm are indicative of substantial α-helical structure. The magnitude of the ellipticity at both wavelengths is markedly decreased upon removal of Ca^{2+}, indicating a decrease in α-helical structure. In the presence of Ca^{2+}, the $\left[\theta\right]_{221\ nm}$ is $-14{,}700 \pm 300$ deg cm^2 $dmole^{-1}$, whereas, in the absence of Ca^{2+}, the $\left[\theta\right]_{221\ nm}$ is $-12{,}000 \pm 300$ deg cm^2 $dmole^{-1}$. The band at 206.5 nm undergoes a similar change, but of smaller magnitude, the $\left[\theta\right]_{206.5\ nm}$ in the presence of Ca^{2+} being $-14{,}800 \pm 300$ deg cm^2 $dmole^{-1}$ and that in the absence of Ca^{2+} $-13{,}000 \pm 300$ deg cm^2 $dmole^{-1}$. Readdition of excess calcium chloride completely reversed the changes elicited by EGTA. Calculations of helical content from the observed ellipticities by the

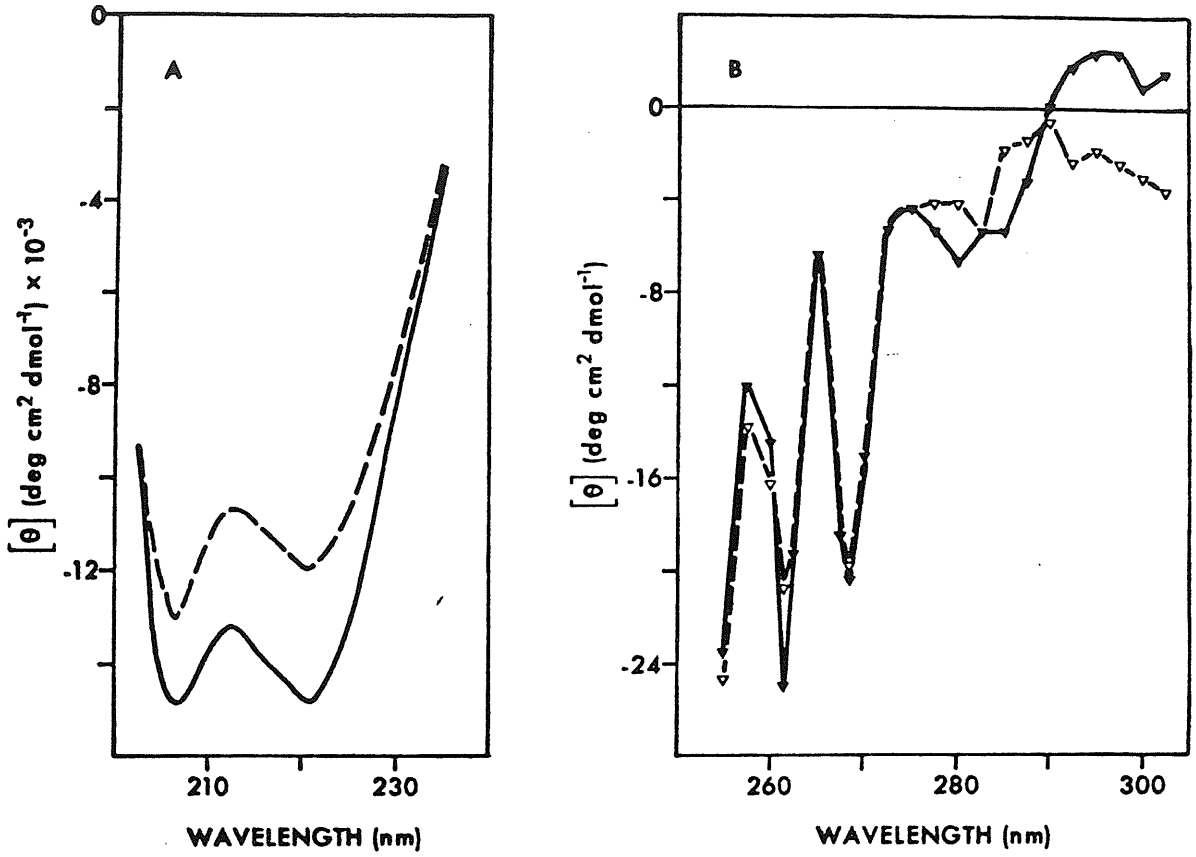


Figure 19A — Far-UV CD spectra of native bovine brain protein modulator in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.5, without (- - -) or with (——) 6 mM $CaCl_2$. Protein concentration was 1.05 mg/ml and the path length was 0.0501 cm.

Figure 19B — Near-UV CD spectra of native protein modulator in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.5, without (▽- -▽) or with (▼——▼) 6 mM $CaCl_2$. Protein concentration was 3.75 mg/ml and the path length was 1 cm.

method of Fasman (1976) revealed that the native protein, in the presence of Ca^{2+}, exhibits 49% α-helical content; removal of Ca^{2+} ions is accompanied by a reduction in helical content to 40%. The corresponding spectra at high ionic strength (0.5M KCl) were indistinguishable from those illustrated in Fig. 19A at low ionic strength (0.15M KCl), indicating that the overall degree of secondary structure of the modulator protein is unaffected by ionic strength.

The near-ultraviolet CD spectra of the protein modulator in the presence and absence of Ca^{2+} are shown in Fig. 19B. The negative dichroic bands at 261.5 nm and 268.5 nm are due to phenylalanine. Apparently, the near-UV CD spectrum is relatively insensitive to calcium binding: Ca^{2+} ions cause only a slight sharpening of the ellipticity bands below 270 nm, with no alteration in their wavelength position; the small contribution from the tyrosine residues is slightly affected by Ca^{2+} and appears as the shallow trough above 270 nm.

Figs. 19A and B indicate that Ca^{2+} binding to the protein modulator induces a conformational change in the molecule with a substantial increase in helical content, while the environments of those aromatic residues which contribute to the CD spectrum do not appear to be significantly perturbed by the binding of Ca^{2+}. The CD spectra of the modulator protein bear striking similarities to those of rabbit skeletal muscle troponin C (Murray and Kay, 1972), consistent with the fact that the two proteins exhibit considerable structural homology.

3.1.2 Nitrotyrosyl modulator

A sample of protein modulator was nitrated as described under "Experimental Procedure" and the cross-linked dimer was separated from the monomeric nitrotyrosyl modulator by gel filtration on

Sephadex G-75 as described above. The CD spectra of nitrotyrosyl modulator and native modulator were recorded as described under "General Experimental Procedures". Fig. 20A illustrates the far-UV CD spectra of nitrotyrosyl modulator in the presence and absence of Ca^{2+}. The spectra resemble very closely the far-UV CD spectra of the native protein (Fig. 19A), implying that the overall secondary structure of nitrotyrosyl modulator is very similar to that of the native protein. Furthermore, the effect of Ca^{2+} ions on the secondary structure of nitrotyrosyl modulator mimics their effect on the native molecule: the calculated helical contents for nitrotyrosyl modulator in the presence and absence of Ca^{2+} are 46% and 38%, respectively, in close agreement with the estimates for the native protein (49% and 40%, respectively). These observations are consistent with the fact that the nitrated modulator shares the following functional properties with the native protein: (1) it is as effective as the native modulator in activating PDE (see IVC1), (2) it exhibits a Ca^{2+}-dependent change in mobility on urea-PAGE (see VC3), and (3) it interacts with troponin I in the presence of Ca^{2+} to form a urea-stable complex (see VC3).

The near-UV CD spectra of nitrotyrosyl modulator in the presence and absence of Ca^{2+} are shown in Fig. 20B. The region from 265 nm to 250 nm does not differ significantly from native modulator with respect to the basic band shapes and wavelengths, consistent with the fact that the phenylalanine residues are not affected by the modification and the average degree of secondary structure is the same in nitrotyrosyl modulator as in the native molecule. However, the molar ellipticities of nitrotyrosyl and native modulator are quite different, the Ca^{2+}-free spectrum of the nitrotyrosyl derivative having the opposite sign to that of the native protein in the near ultraviolet. In the presence of Ca^{2+},

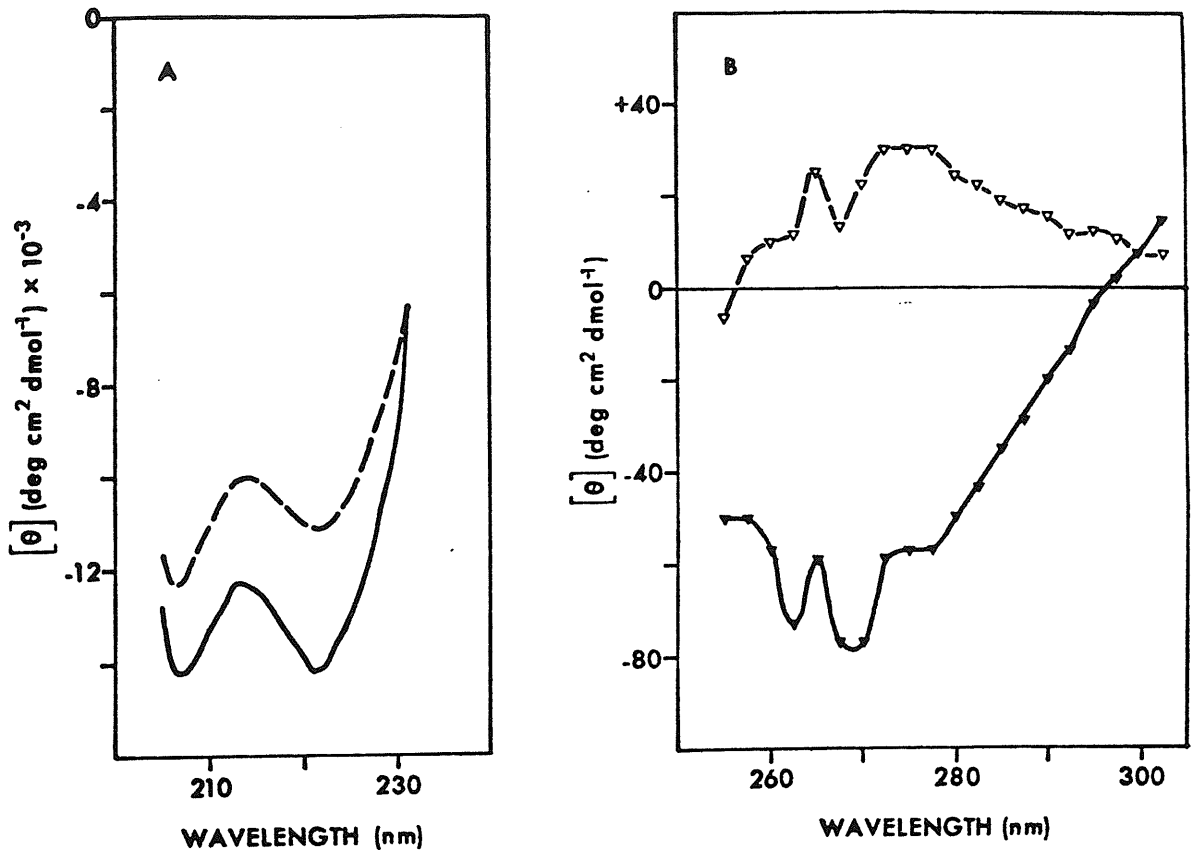


Figure 20

A: Far-UV-CD spectra of nitrotyrosyl protein modulator in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.5, without (----) or with (——) 5 mM $CaCl_2$. Protein concentration was 0.62 mg/ml and the path length was 0.0501 cm.
B: Near-UV CD spectra of nitrotyrosyl protein modulator in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.5, without (▽—▽) or with (▼—▼) 4 mM $CaCl_2$. Protein concentration was 2.5 mg/ml and the path length was 1 cm.

a weak broad positive band is apparent above 300 nm which may be ascribed to the nitrotyrosyl residues since native modulator does not have significant optical activity in this region.

3.2 Interaction with troponin I

The technique of circular dichroic spectrophotometry provides a powerful tool for studying protein-protein interactions provided the complex formation is accompanied by a conformational change. The feasibility of this approach has been successfully demonstrated in studies of the interaction of troponin C with troponin I (McCubbin et al, 1974). The following sections describe the results of such CD studies of the interaction between native and nitrotyrosyl modulator and troponin I.

3.2.1 Native modulator

Fig. 21 illustrates typical far-UV CD spectra of an equimolar mixture of native protein modulator and troponin I in the absence (A) and presence (B) of Ca^{2+}. Also shown are theoretical curves for an equimolar mixture of modulator and troponin I, in the presence and absence of Ca^{2+}, calculated from the ellipticity values of modulator and troponin I alone and assuming no conformational change occurs upon mixing. In the absence of Ca^{2+}, the observed ellipticity, $\left[\theta\right]_{221\ nm}$, is $-7,900 \pm 300$ deg cm^2 $dmole^{-1}$; addition of Ca^{2+} causes a decrease in ellipticity to $-9,500 \pm 300$ deg cm^2 $dmole^{-1}$. These values compare with the calculated values of $\left[\theta\right]_{221\ nm}$ in a 1:1 molar complex of modulator and troponin I, in the absence and presence of Ca^{2+}, of $-8,000$ deg cm^2 $dmole^{-1}$ and $-9,200$ deg cm^2 $dmole^{-1}$, respectively. These observations indicate that there is no significant difference between the observed and the calculated spectra either in the presence or absence of Ca^{2+}, suggesting that no interaction occurs between the modulator protein

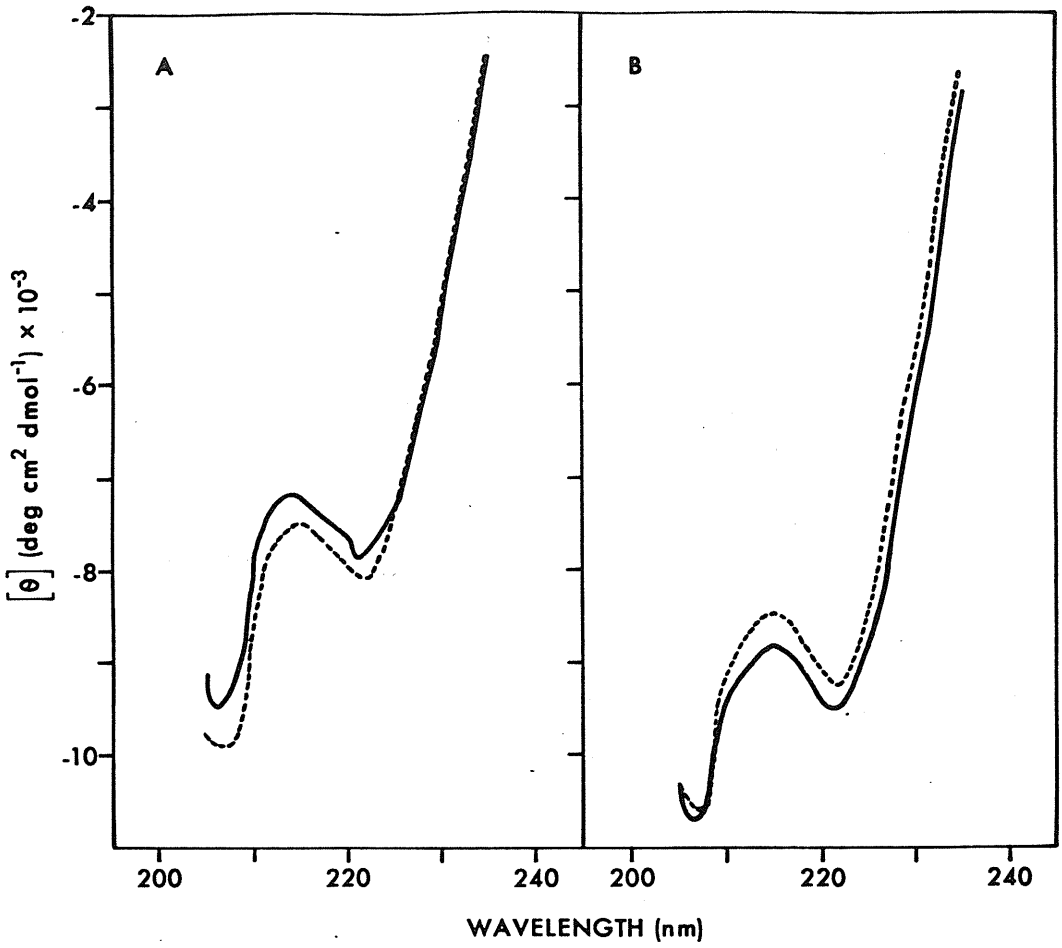


Figure 21

Calculated (----) and observed (——) far-UV CD spectra of an equimolar mixture of troponin I and native protein modulator in 0.15 M KCl, 25 mM Tris, 3 mM EGTA, pH 7.5, without (A) or with (B) 4 mM $CaCl_2$.

and troponin I either in the presence or absence of Ca^{2+}, or, alternatively, that interaction does occur but is not accompanied by a significant conformational change. However, it is noteworthy that the difference between the observed and theoretical curves in Fig. 21B is at the limit of experimental error, suggesting that interaction may indeed occur in the presence of Ca^{2+}. It has been shown, also by circular dichroism (McCubbin et al, 1974), that skeletal muscle troponin C and troponin I interact in the presence of Ca^{2+}. Furthermore, it is known from electrophoretic studies in the presence of urea (see VC3) that the native modulator does interact with troponin I in the presence of Ca^{2+}.

3.2.2 Nitrotyrosyl modulator

The possible occurrence of complex formation between nitrotyrosyl modulator and troponin I was also investigated by circular dichroism; the results are depicted in Fig. 22. In the absence of Ca^{2+} (A) there was no significant difference between observed and calculated ellipticity values (observed $[\theta]_{222\ nm} = -7,900 \pm 300$ deg cm^2 dmole^{-1} and calculated $[\theta]_{222\ nm} = -8,100 \pm 300$ deg cm^2 dmole^{-1}), whereas, in the presence of Ca^2 (B) the observed ellipticity values ($[\theta]_{222\ nm} = -9,600 \pm 300$ deg cm^2 dmole^{-1}) were significantly more negative than the calculated ellipticities ($[\theta]_{222\ nm} = -8,900 \pm 300$ deg cm^2 dmole^{-1}), suggesting a net gain in apparent α-helix content occurring upon interaction between the Ca^{2+}-nitrotyrosyl modulator complex and troponin I. This observation, in the light of the structural and functional similarities between the nitrotyrosyl modulator and native modulator, supports the belief that the native modulator, in the presence of Ca^{2+}, does indeed interact with troponin I. Nitrotyrosyl modulator, like the native protein, interacts with troponin I in the presence but not absence of Ca^{2+} to

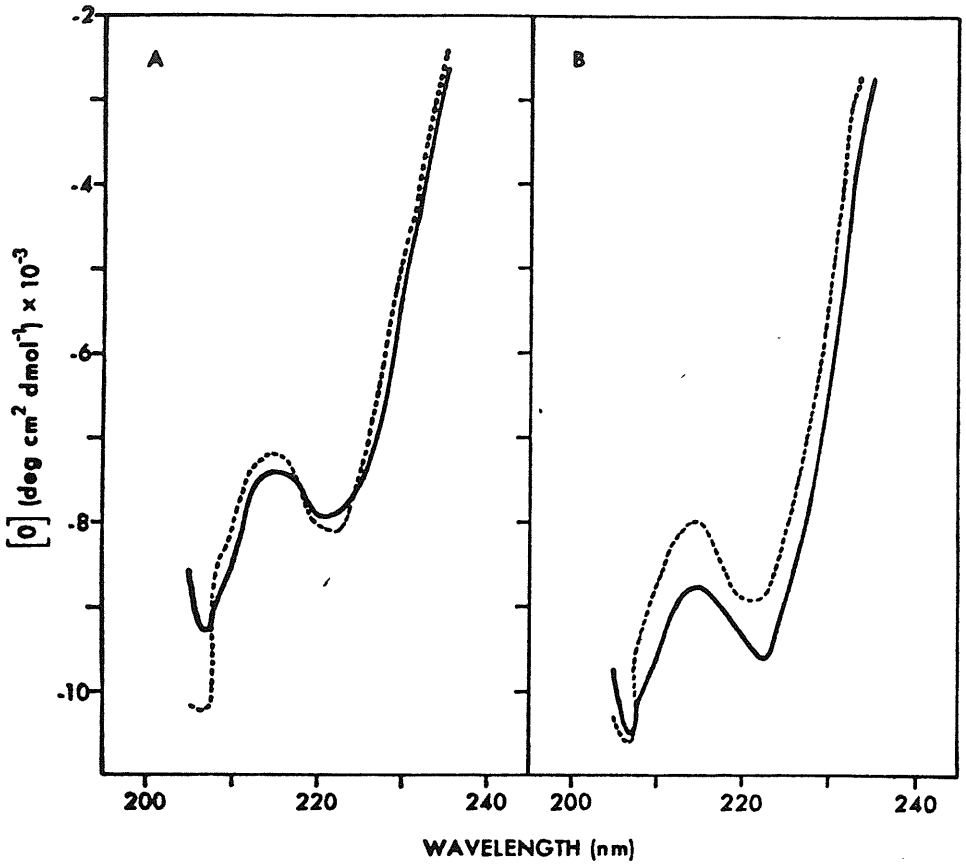


Figure 22 Calculated (·····) and observed (——) far-UV CD spectra of an equimolar mixture of troponin I and nitrotyrosyl protein modulator in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.5, without (A) or with (B) 4 mM $CaCl_2$.

form a urea-stable complex as evidenced by urea-PAGE studies (see VC3).

V. Triple Modification : Tyrosine, Arginine and Histidine

A. Introduction

We have seen that carbethoxylation of the sole histidine residue, nitration of both tyrosine residues, or modification of 4 of a total of 6 arginine residues by treatment with 1,2-cyclohexanedione has no effect on the ability of the protein modulator to stimulate PDE, suggesting that the integrity of these residues is not essential for the expression of modulator activity. These observations suggested the possibility of preparing a derivative of the modulator protein in which all these functional groups were chemically modified and studying the functional properties of such a novel derivative.

B. Experimental procedure

1. Tyrosine modification

Modification of tyrosine residues of the native protein was achieved by nitration with tetranitromethane according to Sokolovsky et al, (1966). The protein modulator (final concentration 1 mg/ml) was incubated at 23° C in 0.05 M Tris-HCl, pH 8.0, containing 1 M NaCl and 2 mM $CaCl_2$. Nitration was initiated by the addition of a concentrated solution of tetranitromethane (60 mM in absolute ethanol) to the sample to give a final reagent concentration of 0.6 mM. The reaction mixture was incubated at 23° C for 5 h at which time reaction was completed (see IV,1). The nitrated sample was dialyzed extensively against water to remove excess reagent and lyophilized.

As mentioned earlier, intermolecular cross-linking occurs as a side reaction during nitration of the modulator protein. In view of the fact that this side reaction is relatively minor, it was deemed

unnecessary to remove the cross-linked dimeric by-product before continuing the triple modification, but simply to be aware of its presence when interpreting experimental results.

2. Arginine modification

Selective modification of arginine residues of the nitrotyrosyl modulator was achieved by treatment with 1,2-cyclohexanedione according to Patthy and Smith, (1975). To a solution of nitrotyrosyl modulator (5 mg/ml in 0.2 M sodium borate, pH 9.0, containing 2 mM $CaCl_2$) was added an equal volume of a 15 mM solution of 1,2-cyclohexanedione in the same buffer. The reaction mixture was incubated at 37° C for 24 h at which time reaction was terminated by addition of an equal volume of cold 5% (v/v) acetic acid. The nitrated, arginine-modified modulator was dialyzed extensively against water and lyophilized.

3. Histidine modification

The sole histidine residue of the double-modified modulator was carbethoxylated with diethylpyrocarbonate essentially according to Grousselle et al, (1973). The double-modified modulator (1 mg/ml) was incubated at 23° C in 0.1 M PIPES, pH 6.0, containing 4 mM $CaCl_2$ in the sample and reference cuvettes of a Coleman-Hitachi Model 124 double beam spectrophotometer equipped with a Model 165 recorder. The reaction was initiated by addition, to the sample cell, of a fresh, concentrated solution of diethylpyrocarbonate (58.8 mM in absolute ethanol) to give a final reagent concentration of 0.588 mM; an equal volume of absolute ethanol was added to the reference cell and the formation of carbethoxyhistidine was monitored by recording the increase in absorbance at 242 nm. The extent of histidine modification was calculated from the absorbance using a value of 3,200 M^{-1} cm^{-1} as the molar extinction coefficient,

at 242 nm, of N-carbethoxyhistidine residues in proteins.

Aliquots were withdrawn from reaction mixtures before and after each stage of the modification procedure for the following determinations: (1) assay of protein modulator activity, (2) acid hydrolysis and amino acid analysis, (3) electrophoresis in urea containing polyacrylamide slab gels in the presence and absence of Ca^{2+}, and (4) determination of interaction with bovine cardiac troponin I by urea-PAGE.

C. Results and Discussion

1. Characterization

The extents of nitration of tyrosine residues and modification of arginine residues with 1,2-cyclohexanedione were estimated by amino acid analysis, and carbethoxylation of histidine was quantitated by measurement of the increase in absorbance at 242 nm corresponding to the formation of N-carbethoxyhistidine. The results are depicted in Table V. It is apparent that slightly less than two tyrosine residues per mole were nitrated in the first stage of the triple modification; this non-stoichiometric modification probably reflects the formation of a small percentage of cross-linked dimer as a by-product of the reaction (see IVC2).

Subsequent treatment of the nitrotyrosyl modulator with 1,2-cyclohexanedione resulted in the further modification of 5 arginine residues. It was demonstrated previously (IIIC) that treatment of the native modulator with 1,2-cyclohexanedione under conditions identical to those described above for the modification of arginine residues of the nitrotyrosyl modulator resulted in the loss of 4 of a total of 6 arginine residues per mole. The modification of an additional arginine residue

TABLE V. Effect of triple modification on the stimulation of phosphodiesterase by the protein modulator

Types of residues modified	Number of residues modified[a]			Residual activity
	Tyr[b]	Arg[b]	His[c]	(%)
None	0	0	0	100
Tyrosine	1.78	0	0	88.5
Tyrosine + Arginine	1.81	5.06	0	82.7
Tyrosine + Arginine + Histidine	1.82	4.93	0.93	81.3

[a] Native modulator contains 2 tyrosines, 6 arginines and 1 histidine residue (Stevens et al, 1976; Watterson et al, 1976; Vanaman et al, 1977). Analyses of the derivatives showed that only these three residues were affected by our chemical modification.

[b] The extents of tyrosine and arginine modification were quantitated by acid hydrolysis and amino acid analysis.

[c] The extent of histidine modification was quantitated spectrophotometrically by measuring the increase in absorbance at 242 nm.

in the nitrated protein may reflect a conformational change occurring upon nitration of the modulator which makes this arginine residue accessible to the modifying agent, or, alternatively, the extent of arginine modification achieved in 24 h may reflect the ratio of modifying agent to protein: the concentration of nitrotyrosyl modulator utilized in these experiments was somewhat less than that of the native modulator (IIIB), whereas the reagent concentration was the same in each case. The latter alternative appears more likely since no significant difference was observed, by the technique of circular dichroic spectrophotometry, in the overall conformation of the native modulator and its nitrotyrosyl derivative (see IVC3). It is clear from Table V that the nitrotyrosine residues are stable to the conditions of arginine modification.

The final stage of the triple modification involved treatment with diethylpyrocarbonate and, as indicated in Table V, this effected stoichiometric modification of the sole histidine residue as desired. The kinetics of carbethoxylation of this histidine residue in the double-modified modulator were essentially identical to the kinetics of carbethoxylation of this residue in the native protein in the presence of Ca^{2+} (see IIC) suggesting that the tyrosine and arginine modifications, in spite of introducing bulky side chains into the molecule, do not affect the accessibility of the histidine residue to diethylpyrocarbonate. The results of amino acid analysis of the triple-modified modulator (Table V) reveal that modified tyrosine and arginine residues are stable to the conditions of carbethoxylation. Amino acid analyses at each stage of the triple modification procedure also revealed that none of the treatments had effect on any residues other than tyrosine, arginine and histidine.

It is apparent, therefore, that the first stage of the triple

modification involves nitration of 2 tyrosine residues, the second stage modification of 5 arginine residues, and the third stage carbethoxylation of the sole histidine residue.

2. Effect on PDE-stimulating activity

Aliquots of the appropriate reaction mixtures were withdrawn before addition of the modifying agent and again upon completion of the modification reaction for assay of modulator activity. The results are depicted in Table V. The initial stage of the triple modification, nitration of both tyrosine residues, is accompanied by a decrease in modulator activity of approximately 10%. This loss of activity is due to the formation of a small amount of cross-linked dimer in a minor side reaction (see IVC2). The monomeric, nitrated modulator retains full modulator activity. The subsequent modification of 5 arginine residues of the nitrotyrosyl modulator did not result in any further significant loss of modulator activity. Similarly, carbethoxylation of the sole histidine residue of the double-modified derivative caused no further loss of modulator activity. A control modulator subjected to identical experimental conditions, with the exception that modifying agents were omitted, retained full modulator activity throughout.

It is interesting that the introduction of several bulky substituents, namely 2 nitro groups, 5 dihydroxycyclohexyl groups, and one carbethoxy group, results in a modulator derivative which retains the capacity of the native protein to activate PDE.

3. Effect on troponin C-like activities

As mentioned in the "Literature Review", the protein modulator and troponin C are homologous Ca^{2+}-binding proteins and share many common physical and chemical properties. It was, therefore, interesting to

investigate the effect of triple modification on the troponin C-like activities of the modulator protein. Thus, the triple-modified and intermediate species were examined for: (1) a Ca^{2+}-dependent change in electrophoretic mobility in urea-containing polyacrylamide gels, and (2) Ca^{2+}-dependent interaction with troponin I in the presence of 6 M urea.

3.1 Effect on Ca^{2+}-dependent change in electrophoretic mobility

It is a well known property of troponin C that its mobility in urea-containing polyacrylamide gels is significantly affected by Ca^{2+} (Perry et al, 1973; Drabikowski et al, 1977a). The protein modulator has been observed to exhibit a similar Ca^{2+}-dependent change in mobility (Amphlett et al, 1976; Drabikowski et al, 1977b). Thus, the mobility of the native protein in the presence of Ca^{2+} is markedly faster than its mobility in the absence of Ca^{2+}. This fact is illustrated in Fig. 23A: it can be seen that in the presence of Ca^{2+} (slot 1) the native modulator migrates faster than the native protein in the absence of Ca^{2+} (slot 2). The reason for this apparently anomalous behaviour will be discussed later.

As can be seen from slots 3 and 4 in Fig. 23A the nitrotyrosyl modulator, like the native protein, exhibits a Ca^{2+}-dependent change in mobility, albeit less marked than that of the native protein. The faint band of slower mobility corresponds to the dimeric side product produced in the nitration reaction (see IVC2). It is apparent from slots 5-8 in Fig. 23A that neither the double- nor the triple-modified modulator exhibits a Ca^{2+}-dependent change in mobility.

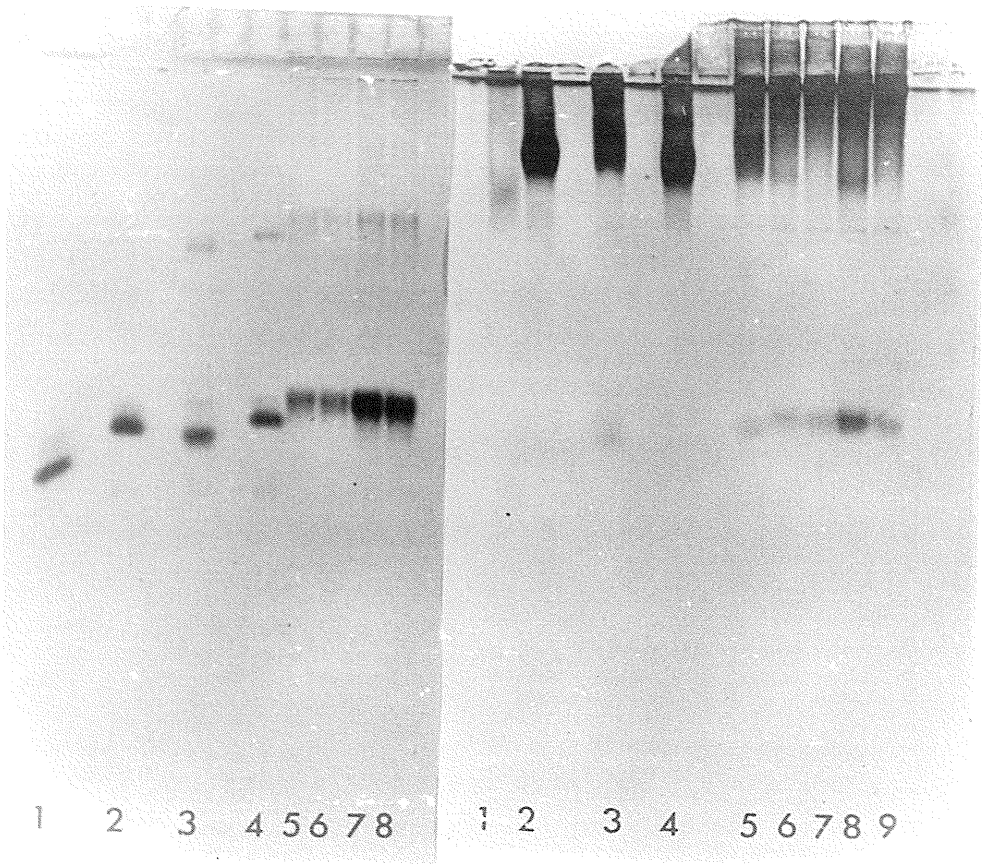


Figure 23

A: The effect of triple modification on the Ca^{2+}-dependent change in electrophoretic mobility of the modulator on urea-polyacrylamide gels. Electrophoresis was performed by the method of Davis (1964) in the presence of 6 M urea as described in "General Experimental Procedures" VB. Protein samples were dissolved in sample buffer containing 6 M urea and either 4 mM $CaCl_2$ (slots 1, 3, 5 and 7) or 4 mM EGTA (slots 2, 4, 6 and 8). Approximately 10^2 µg of protein were applied to each slot. 1 and 2: native modulator; 3 and 4: nitrotyrosyl modulator; 5 and 6: double-modified modulator; 7 and 8: triple-modified modulator

B: The effect of triple modification on the Ca^{2+}-dependent interaction of the modulator with troponin I as monitored by urea-polyacrylamide slab gel electrophoresis according to the method of Davis (1964). Modulator samples were incubated with an approximately 2-fold weight excess of bovine cardiac troponin I in the sample buffer containing 6 M urea and either 4 mM $CaCl_2$ (slots 2, 4, 6 and 8) or 4 mM EGTA (slots 3, 5, 7 and 9). 1: troponin I alone; 2 and 3: native modulator; 4 and 5: nitrotyrosyl modulator; 6 and 7: double-modified modulator; 8 and 9: triple-modified modulator.

3.2 Effect on interactions with troponin I

It was originally reported by Perry et al, (1972) that troponin C, in the presence of Ca^{2+}, forms a complex with troponin I which is stable in 6 M urea. A similar property was demonstrated for the brain modulator protein (Amphlett et al, 1976; Drabikowski et al, 1977b). Therefore, urea-PAGE serves as a useful tool for monitoring the interaction of various modulator species, e.g., chemically modified derivatives, with troponin I. Fig. 23B shows the results of such investigations of interactions between the various modulator species and troponin I. Slot 1 shows troponin I alone. Slot 2 represents a mixture of native protein modulator and excess troponin I in the presence of Ca^{2+}; no band corresponding to the free modulator is apparent, but rather a band with much slower mobility, corresponding to a modulator-troponin I complex is evident. In the absence of Ca^{2+} (slot 3) no such complex is formed, as indicated by the absence of a band with slow mobility and the presence of a band corresponding to the free modulator. The situation in the case of the nitrotyrosyl modulator resembles that of the native protein. Thus, interaction occurs between nitrotyrosyl modulator and troponin I in the presence (slot 4) but not in the absence (slot 5) of Ca^{2+}. In the case of the double-modified modulator, no interaction with troponin I occurs either in the presence (slot 6) or absence (slot 7) of Ca^{2+}. A similar situation prevails in the case of the triple-modified derivative, i.e., no interaction with troponin I occurs either in the presence (slot 8) or absence (slot 9) of Ca^{2+}.

These observations correlate exactly with the observed effects of Ca^{2+} on the mobility of the modified species in urea-containing polyacrylamide gels, i.e., those species which exhibit a Ca^{2+}-dependent

change in mobility in urea gels also interact with troponin I in the presence of Ca^{2+} to form a complex which is stable in 6 M urea, whereas species whose mobility is unaffected by Ca^{2+} do not form urea stable complexes with troponin I.

4. Effect of EGTA on PDE stimulation

From these studies of the effects of triple modification on the PDE-stimulating activity and troponin C-like activities of the modulator protein, it is apparent that, while the nitrotyrosyl modulator exhibits both a Ca^{2+}-dependent change in electrophoretic mobility in urea-containing polyacrylamide gels and interaction with troponin I in the presence of Ca^{2+}, the double- and triple-modified derivatives exhibit neither of these properties. On the other hand, all 3 modulator derivatives stimulate PDE as effectively as the native protein. These observations suggest that arginine modification, following tyrosine nitration, disrupts the site for interaction with troponin I, but not that for interaction with PDE. Alternatively, the double- and triple-modified derivatives, due to the incorporation of several bulky groups, may be locked into an active conformation which is no longer dependent on Ca^{2+} for activity. If the latter were the case, the PDE-stimulating activity of the double- and triple-modified modulators would be unaffected by EGTA. Fig. 24 shows the effect of EGTA on the stimulation of PDE activity by the triple-modified modulator. Clearly, the triple-modified modulator is dependent on Ca^{2+} for expression of PDE-stimulating activity, disproving the possibility of a locked conformation. Identical results were obtained with the double-modified modulator, nitrotyrosyl modulator and native modulator indicating that the activities of all these species are Ca^{2+}-dependent. It is apparent from these observations that the

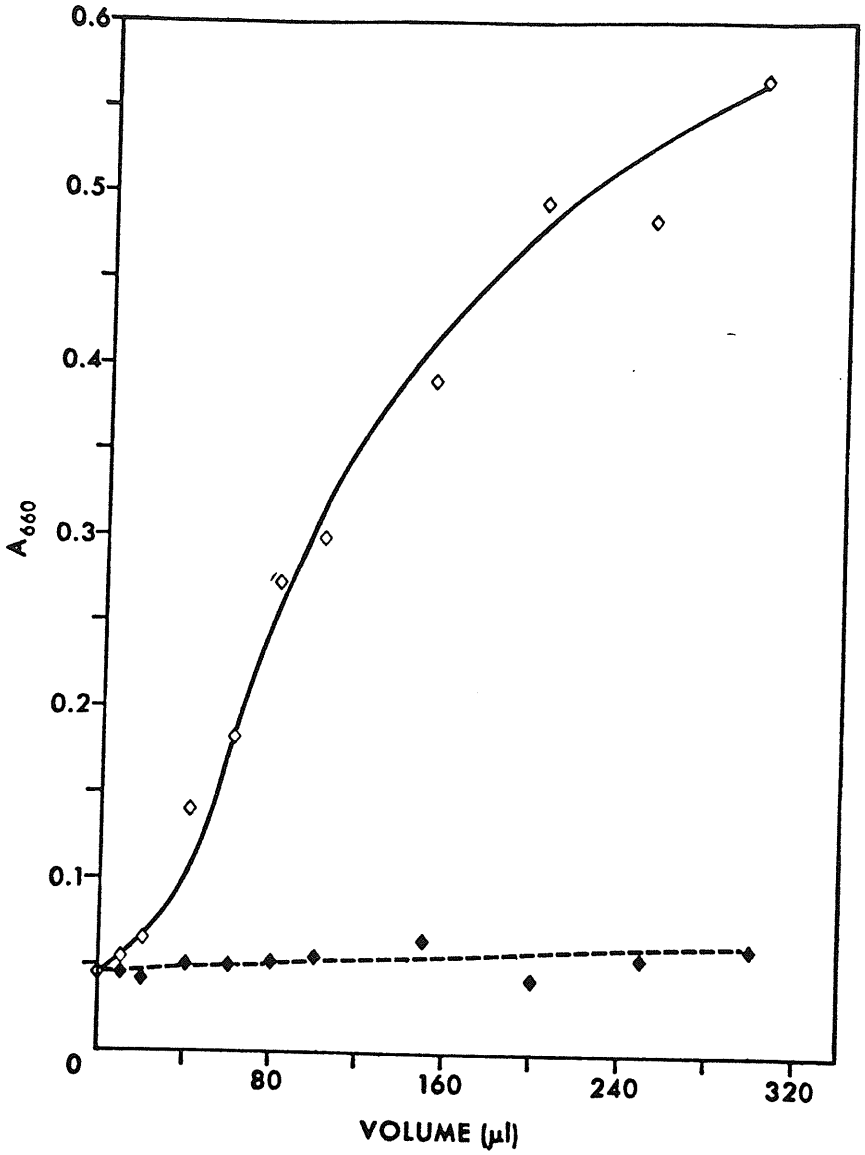


Figure 24

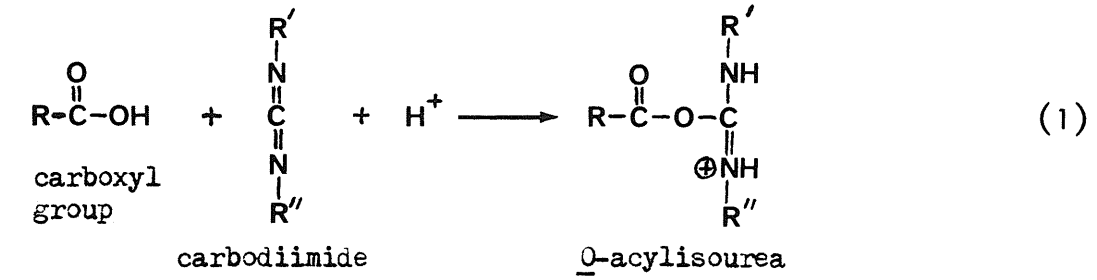
The effect of EGTA on the stimulation of PDE activity by the triple-modified modulator. Aliquots of a solution of the triple-modified derivative were assayed for modulator activity as previously described ("General Experimental Procedures" IV. in the presence of 0.125 mM Ca^{2+} (◇—◇) or 0.5 mM EGTA (◆—◆).

sites for interaction with troponin I and PDE within the modulator molecule are distinct.

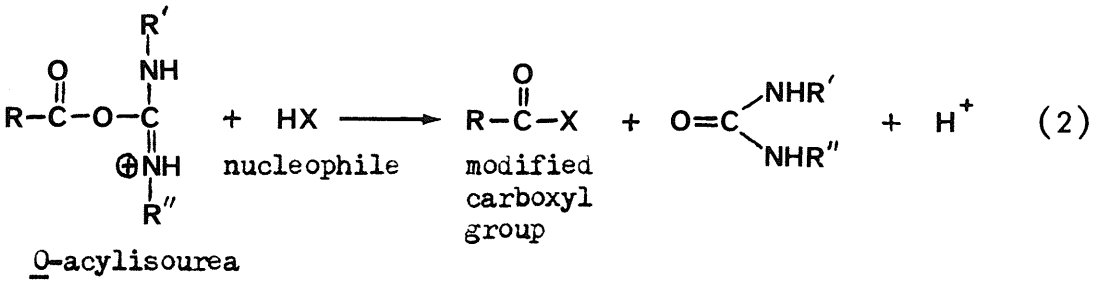
VI. Carboxyl Group Modification

A. Introduction

The most widely used method for the selective chemical modification of carboxyl functions in proteins is that of Hoare and Koshland (1967) which involves activation of the carboxyl group by a water-soluble carbdiimide (Equation (1)) and the subsequent reaction of the activated carboxyl group with a nucleophile such as glycine methyl ester (Equation (2)):

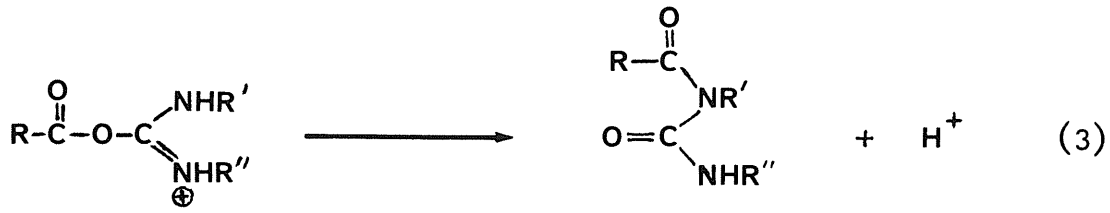


(1)



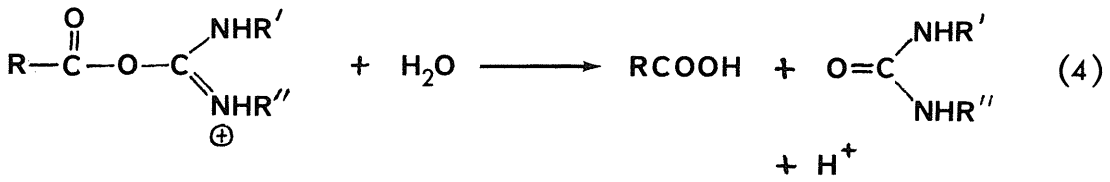
(2)

Competing reactions occur, necessitating careful choice of reaction conditions to minimize the formation of unwanted side products. For example, the O-acylisourea may rearrange to give the N-acylurea (Equation (3)), and may react with water to regenerate the carboxyl group (Equation (4)):



(3)

O-acylisourea N- acylurea

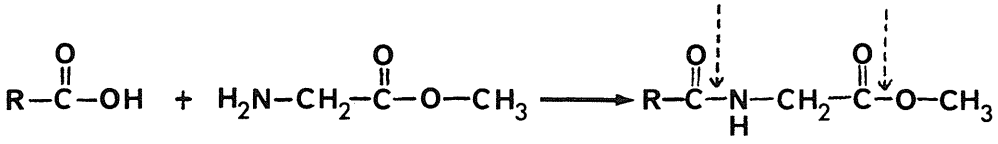


(4)

O-acylisourea

The hydrolysis apparently proceeds at a rate at least 20 times that of the rearrangement. In any one cycle, therefore, a small amount of N-acylurea would be formed, but in reactions involving an excess of carbodiimide the carboxyl function would be regenerated and could react with further carbodiimide. Careful choice of reaction conditions, particularly the nature and concentration of the nucleophile, can eliminate the rearrangement to form the N-acylurea. Thus, it is desirable to utilize a nucleophile which is capable of reacting rapidly with the O-acylisourea and so effectively competes with the rearrangement. Such suitable nucleophiles include glycine methyl ester, glycine ethyl ester, taurine, norleucine methyl ester and aminomethanesulfonic acid.

This method can be used for the quantitative determination of free carboxyl groups in a protein and for the selective modification of accessible carboxyl functions with a view to assessing their functional role(s). In determination of the total free carboxyl group content of a protein, the protein is treated with a water-soluble carbodiimide and appropriate nucleophile (e.g. glycine methyl ester) under denaturing conditions (high concentrations of urea or guanidine hydrochloride). All free carboxyl groups will, therefore, be blocked by glycine methyl ester:



carboxyl group | glycine methyl ester | blocked carboxyl group

Extensive dialysis can be used to remove excess glycine methyl ester and subsequent acid hydrolysis cleaves the derivative at the positions indicated by the arrows above releasing glycine quantitatively. Hence, the increase in glycine content of the acid hydrolyzate, as estimated by amino acid analysis, is a measure of the total free carboxylic acid content of the molecule.

Carboxyl group modification may also be performed under non-denaturing conditions in order to achieve selective blockage of a small number of reactive carboxyl groups. The mild reaction conditions avoid ambiguities caused by denaturation. Furthermore, the reaction provides great versatility since both carbodiimide and nucleophile can be varied. Thus, for example, the benzylcarbodiimide has an aromatic side chain which might attract it preferentially to the active site of enzymes operating on hydrophobic substrates.

In addition to rearrangement to form the N-acylurea and hydrolysis to regenerate the carboxyl group, another important side reaction often occurs during carboxyl group modification by this method, viz., inter- and intramolecular cross-linking of protein molecules. This side reaction arises from the fact that proteins contain nucleophilic side chains, e.g., amino functions, which compete with the exogenous nucleophile in displacement of the carbodiimide from the activated carboxyl group. The importance of this side reaction in studies of the role of carboxyl groups in protein function has been emphasised recently by Timkovich (1977) who demonstrated the occurrence of covalent polymerization during carbodiimide-promoted carboxyl group modification of cytochrome c, myoglobin, ribonuclease and lysozyme.

B. Experimental procedure

1. Determination of total free carboxyl content

The total free carboxyl group content of the protein modulator was determined by the method of Hoare and Koshland (1967). The protein was dissolved (1 mg/ml) in 3 ml of 1 M glycine methyl ester in 7.5 M urea. The pH was adjusted to 4.75 with 1 M HCl and the reaction initiated by the addition of 0.1 ml of 3 M 1-ethyl-3-dimethylaminopropyl-carbodiimide (EDC) in 7.5 M urea to give a final reagent concentration of 0.1 M. The reaction mixture was incubated at 25° C and the pH maintained at 4.75 by addition of 1 M HCl using a Radiometer pH-stat. Aliquots of 1 ml were withdrawn from the reaction mixture after 1,2 and 4 h of incubation and added to 5 ml of 1 M sodium acetate, pH 4.75, to quench the reaction with the diimide. The samples were then dialyzed extensively against 1 mM HCl in the cold and lyophilized prior to acid hydrolysis and amino acid analysis. The extent of carboxyl group modification was estimated from the increase in glycine content of the modulator.

2. Carboxyl group modification in the native protein

For modification of carboxyl groups in the native protein, the modulator (4 mg) was dissolved in 8 ml of 0.25 M glycine methyl ester hydrochloride containing 2 mM $CaCl_2$ or 2 mM EGTA and the solution was adjusted to pH 4.75 with 0.2 M NaOH. The reaction was initiated by the addition of solid EDC to a final concentration of 1.44 mM. The reaction mixture was incubated at 25° C and the pH maintained at 4.75 using the pH-stat with 0.1 M HCl. Aliquots of the reaction mixture were withdrawn at suitable time intervals, diluted several-fold in 1 M sodium acetate, pH 4.75, to quench the reaction with the diimide, and dialyzed

extensively against 1 mM HCl to remove excess reagents, prior to: (1) assay of modulator activity, (2) acid hydrolysis and amino acid analysis, and (3) SDS-PAGE in the presence of urea. Two control experiments were also performed in which the modulator protein was treated in identical manner to that described above except that the glycine methyl ester was omitted from one control and the carbodiimide was omitted from the other.

C. Results and Discussion

1. Total free carboxyl content

The acid hydrolyzate of the protein modulator contains 25 aspartic acid and 30 glutamic acid resudues for a total of 55 acidic residues (Stevens et al, 1976). The total free carboxyl group content of the modulator was determined as described above. Reaction for 1, 2 and 4 h gave values of 38, 40 and 41 carboxyl groups, respectively. This value of approximately 40 free carboxyls and 15 amides agrees, within experimental error, with the values (38 and 16) obtained by Liu and Cheung (1976), who obtained their values by estimating the amide content of the protein by determining on the amino acid analyzer the NH_3 liberated from the protein by hydrolysis in 2 N HCl at 100° C.

Elucidation of the amino acid sequence of the bovine brain modulator protein (Vanaman et al, 1977) revealed the presence of 23 Asp plus Asn and 27 Glu plus Gln residues in the molecule, values slightly lower than those published previously (Stevens et al, 1976; Liu and Cheung, 1976; Watterson et al, 1976). Amide assignments have not, as yet, been completed.

2. Carboxyl group modification in the native protein

Fig. 25 shows the effect of carboxyl group modification in the presence and absence of Ca^{2+} on the capacity of the modulator protein to activate PDE. Amino acid analyses revealed that, in the presence or absence of Ca^{2+}, complete loss of modulator activity occurred concomitant with the modification of 22 carboxyl groups per mole; in the presence of Ca^{2+}, modification of 2 carboxyl groups occurred within the first minute, with only an 8% loss in activity. Clearly, the loss of modulator activity is faster in the absence than in the presence of Ca^{2+}, reflecting the conformational change occurring in the modulator as it binds Ca^{2+}. Control reactions in the presence and absence of Ca^{2+} in which only the carbodiimide was omitted from the reaction mixture retained full activity throughout the incubation period of 60 min; furthermore, these samples exhibited no increase in glycine content after acid hydrolysis, indicating the complete removal of the glycine methyl ester in the dialysis step.

The other control experiment involved treatment of the modulator protein, both in the presence and absence of Ca^{2+}, with the water-soluble carbodiimide but omitting glycine methyl ester from the reaction mixture. Fig. 26 shows that such treatment in the presence of Ca^{2+} results in a gradual loss of modulator activity. A similar effect was observed in the absence of Ca^{2+}, the rate of loss of activity being slightly faster in this case. This carbodiimide-induced loss of activity is probably the result of two simultaneously occurring processes: (1) rearrangement of the O-acylisourea to form the stable N-acylurea, thereby, effecting carboxyl group modification, and (2) inter- and intramolecular cross-linking between activated carboxyl and amino groups (Caraway and Koshland,

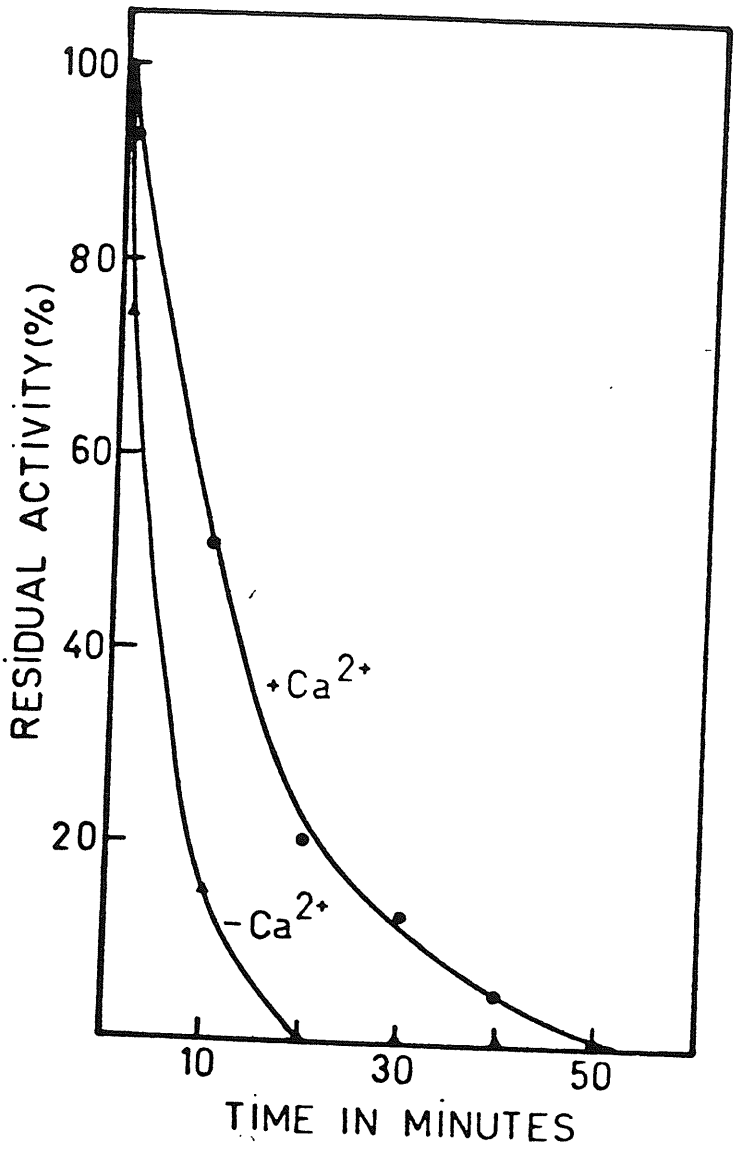


Figure 25

Time course of inactivation of protein modulator by blocking of the carboxyl groups with glycine methyl ester. The modulator (0.25 mg/ml) was incubated at 25° C, pH 4.75, with 0.25 M glycine methyl ester, 1.44 mM EDC in the presence of 2 mM $CaCl_2$ (●—●) or 2 mM EGTA (▲—▲).

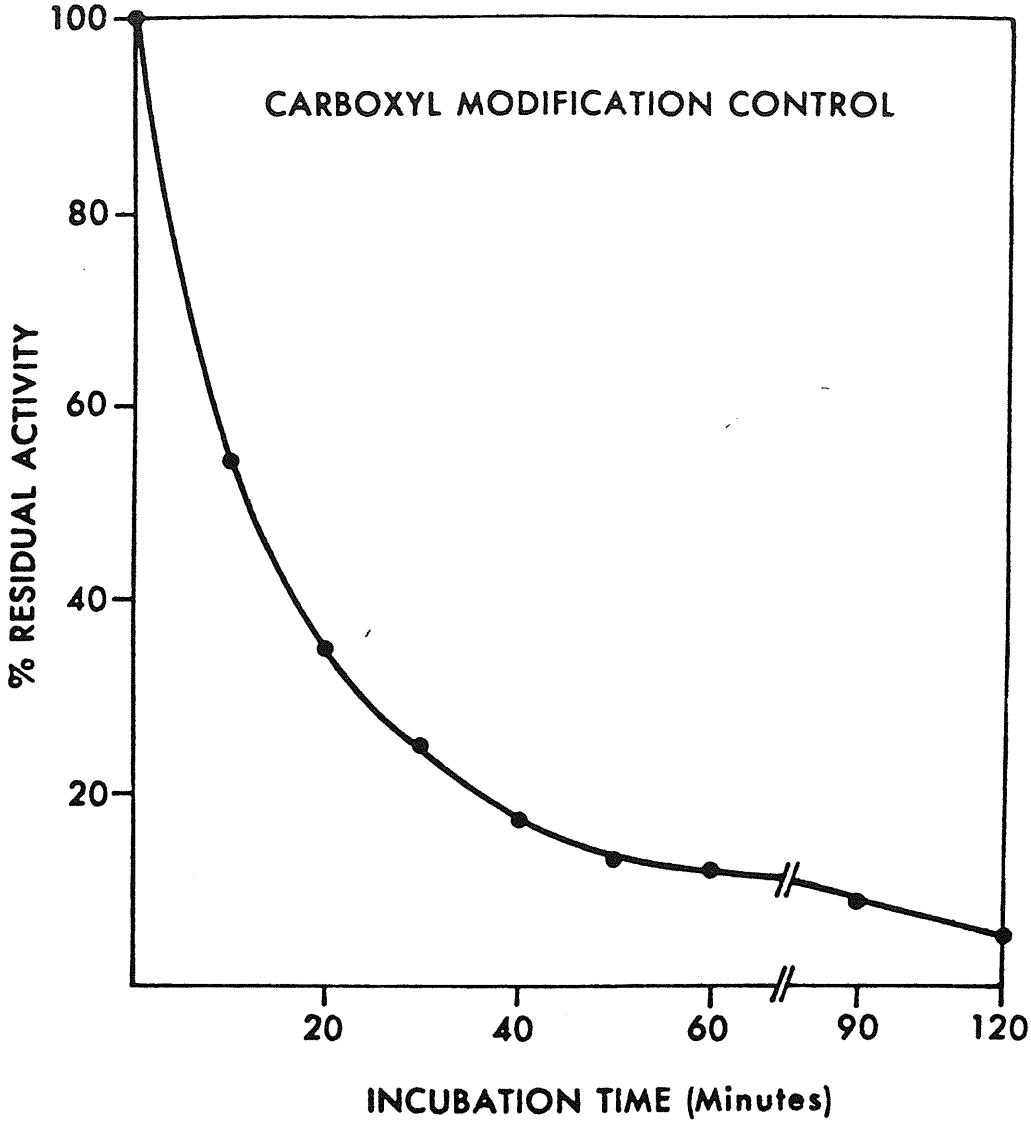


Figure 26

Time course of inactivation of protein modulator by treatment with EDC alone. The modulator (0.25 mg/ml) was incubated at 25° C, pH 4.75, with 1.44 mM EDC in the presence of 2 mM $CaCl_2$.

1972). Fig. 27A demonstrates that the carbodiimide, in the absence of glycine methyl ester, does indeed promote the formation of intermolecular cross-linkages. The native protein modulator before addition of EDC exhibits a single faint band on SDS-PAGE in the presence of urea (slot 1, Fig. 27A). Incubation with EDC results in the rapid disappearance of this band and the appearance of a diffuse staining region of slow mobility which is visible after 10 min. incubation (slot 2) and becomes more intense with prolonged incubation (slots 4, 5, 7, 8, 10, 11, 12). Slots 3, 6 and 9 represent molecular weight markers. As had been observed previously (see IVC2) the modulator migrates as if it has a molecular weight of 35,500 in this electrophoretic system, i.e., it behaves as if it were a dimer. The diffuse banding pattern of the polymers seen in Fig. 27A is in agreement with the observations of Timkovich (1977) who reported similar diffuse banding of the polymeric species produced upon incubation of various test proteins with carbodiimide. This spreading can be attributed to the presence of a heterogeneous population within the classes of dimers, trimers, etc. Furthermore, the cross-linked polypeptides are probably randomly and multiply branched, and hence bind SDS in varying amounts.

It is apparent, therefore, that carbodiimide, in the absence of added nucleophile, promotes intermolecular cross-linking of the protein modulator and such polymerization results in loss of modulator activity. In light of this information, it was necessary to assess the importance of this side reaction in the actual carboxyl group modification experiment, i.e., it was necessary to determine whether polymerization occurred to a significant extent when the protein modulator was treated with both carbodiimide and glycine methyl ester. The reaction conditions

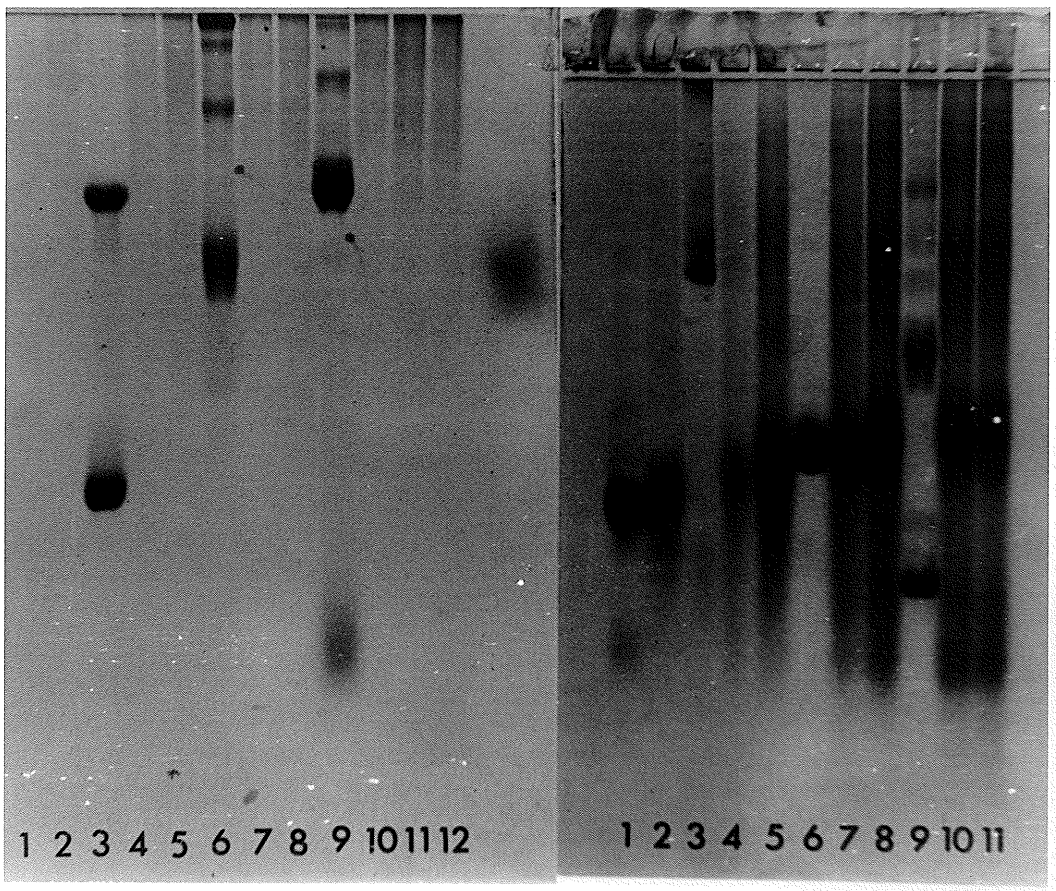


Figure 27 SDS-PAGE in the presence of urea by the method of Swank and Munkres (1071) as described in VC.

A: Demonstration of intermolecular cross-linking upon treatment of the modulator with EDC alone. The gel shows aliquots (10 μg protein) withdrawn from the reaction mixture at the following times: 1: 1 min; 2: 10 min; 4: 20 min; 5: 30 min; 7: 40 min; 8: 50 min; 10: 60 min; 11: 90 min; 12: 120 min; 3: chymotrypsinogen A, M W 25,000 (10 μg) plus pepsin, M W 35,000 (10 μg); 6: ovalbumin, M W 45,000 (10 μg) plus γ-globulin, M W 160,000 (10 μg); 9: bovine serum albumin, M W 67,000 (10 μg) plus cytochrome c, M W 12,500 (10 μg).
B: Demonstration of intermolecular cross-linking in the actual carboxyl group modification. The gel shows aliquots (50 μg) withdrawn from the reaction mixture at the following times: 1: 0 min; 2: 10 min; 4: 20 min; 5: 30 min; 7: 40 min; 8: 60 min; 10: 90 min; 11: 120 min; 3: bovine serum albumin (10 μg) plus cytochrome c (10 μg); 6: carboxypeptidase A, M W 35, 000 (10 μg); 9: ovalbumin (10 μg) plus chymotrypsinogen A (10 μg).

utilized in the actual carboxyl group modification experiment were chosen to minimize the likelihood of intermolecular cross-linking, i.e., the concentration of carbodiimide was minimal and the molar ratio of glycine methyl ester to protein was very high. In order to assess the importance of the polymerization side reaction, aliquots withdrawn at appropriate time intervals from the actual carboxyl group modification experiment were examined by SDS-PAGE in the presence of urea (Fig. 27B). Slot 1 represents the native protein; a predominant band of molecular weight ~30,000 is apparent. Incubation with carbodiimide and glycine methyl ester results in slight retardation of this band (slot 2) with progressively greater retardation with prolonged incubation (slots 3, 5, 6, 8, 9, 10, 11). Polymerization is not apparent until 30 min incubation (slot 5) and becomes progressively more pronounced. Thus, after 120 min. incubation (slot 11) at least 4 distinct bands are visible with considerable spreading. These bands include one of apparent molecular weight approximately 18,000 which is also evident in the native protein (slot 1) and probably represents the monomer. Slots 3, 6 and 9 represent molecular weight markers.

It is evident, therefore, that intermolecular cross-linking does not occur to a significant degree within the first 30 min incubation of the modulator protein with carbodiimide and glycine methyl ester; by this time, approximately 85% of modulator activity has been lost. Hence, while carbodiimide-promoted cross-linking may account for a small amount of the observed loss of modulator activity, this loss is predominantly accounted for by the chemical modification of free carboxyl groups in the protein molecule.

Chemical modification of a large number of carboxyl groups is

required for the complete loss of modulator activity. This suggests that the loss of activity is due to a gross conformational change in the molecule, resulting from the introduction of several bulky substituents, rather than the blockage of a small number of specific carboxyl groups which are involved in the interaction with PDE.

Similar conclusions were reached by Liu and Cheung (1976) using glycine ethyl ester as nucleophile. They observed rapid modification of 5 carboxyl groups of the protein modulator with little loss of activity, followed by slower blockage of an additional 5 carboxyl groups with complete loss of modulator activity. On this basis, they suggested the existence of at least two classes of carboxyl groups in the modulator: one set of about 5 which are accessible to the modifying agent, and another set, also about 5, which are less readily available. Blockage of the easily accessible groups did not materially affect the activity of the modulator; on the other hand, blockage of the less easily available groups profoundly reduced the modulator activity. Liu and Cheung (1976) also examined the optical rotatory dispersion spectra of the native and carboxyl-modified modulators. Blockage of the first 5 carboxyl groups (the easily accessible group) caused no apparent change in the reduced mean residue rotation at 231 nm ($[m']_{231}$) relative to that of the native protein. However, when the other 5 carboxyl groups were then blocked, the $[m']_{231}$ was reduced some 20%, indicating that the modified protein assumed less helical structure, consistent with the suggestion that the loss of modulator activity upon extensive carboxyl group modification is due to a gross conformational change in the molecule. Using the technique of equilibrium dialysis, they demonstrated that the carboxyl-modified, inactive modulator retains the Ca^{2+}-binding ability

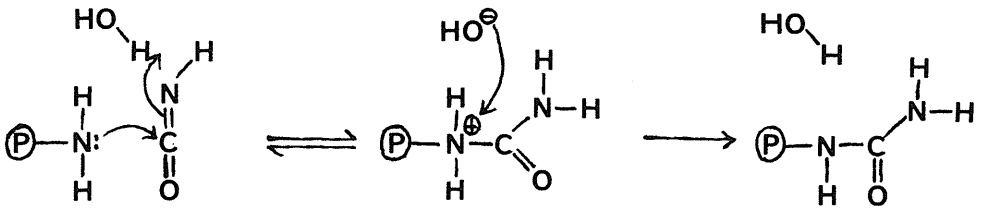
of the native protein, suggesting that the modified carboxyl groups were not involved in coordinating Ca^{2+} ions. Even when the modification was performed in the absence of Ca^{2+}, the resultant modified modulator retained the ability to bind Ca^{2+}.

VII. Lysine Modification

A. Introduction

1. Carbamoylation

A commonly used method for achieving selective modification of amino functions in proteins involves reaction with cyanate ion or cyanic acid (HNCO). Whereas cyanate reacts with α-amino, ε-amino, thiol, imidazole, phenolic hydroxyl, and carboxyl groups of proteins, only the amino group derivatives are stable at alkaline pH. The reaction with amino groups appears to involve the unprotonated amine and electrically neutral cyanic acid (Stark, 1965):



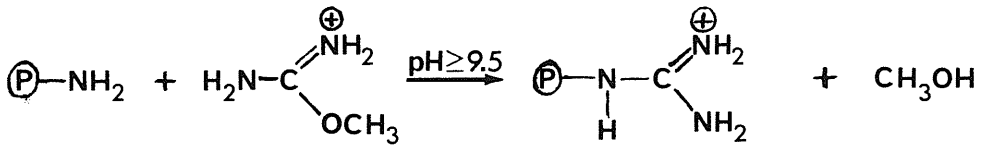
amino group cyanate

As a result of their lower pK values, α-amino groups react at neutral pH approximately 100 times more rapidly than do ε-amino groups (Stark, 1965). The reaction of lysine residues with cyanate converts them into homocitrulline which enables quantitation of the extent of lysine modification by determining the homocitrulline content of the protein by amino acid analysis after acid hydrolysis; homocitrulline elutes from the long column of the analyzer just prior to valine. However, it must be recognised that homocitrulline, while quite stable under normal conditions,

is partially degraded to lysine during acid hydrolysis (17-30% in 6 N HCl at 110° C for 22 h), (Stark, 1972).

2. Guanidination

Lysyl residues may be converted to the homoarginine derivative by treatment of proteins with O-methylisourea at alkaline pH:



ε-amino group — O-methylisourea — homoarginine

Unfortunately, conditions necessary to carry out the reaction require a high pH, unsuitable for many proteins. α-Amino groups react much more slowly with O-methylisourea than do ε-amino groups.

The guanidino derivative obtained upon treatment with O-methylisourea is strongly basic and, therefore, like amino groups, is protonated in the physiological pH range. Hence, this modification retains the positive charge of the modified lysine residue enabling one to distinguish with less ambiguity between simply the need to maintain a positive charge and the specific necessity of lysine residues for the expression of physiological activity.

Homoarginine, produced upon reaction of ε-amino groups with O-methylisourea, is stable to acid hydrolysis and may be quantitated by amino acid analysis; it elutes from the amino acid analyzer shortly after arginine.

B. Experimental procedure

1. Carbamoylation

Selective modification of amino groups of the protein modulator was achieved by the method of Stark (1972). To a solution (5.0 mg/ml in 0.05 M Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$ or 2 mM EGTA) of the modulator was added an equal volume of 0.4 M potassium isocyanate in the same buffer. The reaction mixture was incubated at 23° C. Aliquots were withdrawn at appropriate time intervals, the reaction quenched by addition to an equal volume of 1 M glycine and the reaction mixture dialyzed extensively against water prior to : (1) assay of modulator activity, (2) acid hydrolysis and amino acid analysis, and (3) electrophoresis on 15% polyacrylamide slab gels. The extent of carbamoylation was estimated from the residual lysine and the newly formed homocitrulline as determined by amino acid analysis. Homocitrulline in acid hydrolyzates was quantitated from a standard amino acid analysis employing authentic homocitrulline.

2. Guanidination

Amino groups in the protein modulator were also modified by guanidination by the method of Habeeb (1972). Protein modulator containing endogenous Ca^{2+} [a] was dissolved (5 mg/ml) in 0.05 M sodium

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[a] Protein modulator isolated as described in "General Experimental Procedures" is saturated with endogenous Ca^{2+} as evidenced by the fact that this modulator exhibits maximal activation of PDE without addition of Ca^{2+} ions. The guanidination reaction is carried out in phosphate buffer and addition of exogenous Ca^{2+} would result in the precipitation of insoluble calcium phosphate salts.

phosphate, pH 11, with or without 2 mM EGTA . Guanidination was initiated by the addition of an equal volume of 1 M O-methylisourea in the same buffer. The reaction mixture was incubated at 23° C and aliquots were withdrawn at selected intervals, the reaction quenched by rapid freezing and the mixture lyophilized prior to : (1) assay of modulator activity, and (2) acid hydrolysis and amino acid analysis.

C. Results and Discussion

1. Carbamoylation

Since the modulator is believed to contain a blocked amino terminal (see Literature Review XIB) reaction with potassium isocyanate under the conditions used should be specific for the carbamoylation of lysine residues to yield homocitrulline (Stark, 1972). Fig. 28 shows the time course of inactivation of the modulator protein by carbamoylation. In the absence of Ca^{2+}, both the sample containing the reagent and the control sample were completely inactive after 15 days incubation, indicating that the modulator is unstable under those conditions even in the absence of reagent. However, it is apparent from Fig. 28 that the initial loss of activity in the absence of Ca^{2+} is much more rapid in the sample containing the potassium isocyanate than in the control sample. As can be seen in Table VI, amino acid analysis indicates that considerable lysine modification accompanies the loss of activity observed in the absence of Ca^{2+}. These observations, therefore, indicate that modification of one or more lysine residues results in a decrease of modulator activity.

The observation is even more striking if the reaction is carried out in the presence of Ca^{2+} (Fig. 28, Table VI) since in this case the

TABLE VI. Carbamoylation of protein modulator[a]

Reaction time (days)	Sample + Ca^{2+} (mol/mol)			Sample - Ca^{2+} (mol/mol)		
	Lysine	Homocitrulline	Total	Lysine	Homocitrulline	Total
0	8.06	0.0	8.06	8.99	0.0	8.99
2	6.53	2.32	8.85	5.28	3.60	8.88
7	5.60	3.32	8.92	3.15	5.89	9.04
15	2.41	5.80	8.21	2.26	6.30	8.56

[a] The lysine and homocitrulline contents were determined after acid hydrolysis and the values have not been corrected for possible hydrolysis of homocitrulline back to lysine (Stark, 1972).

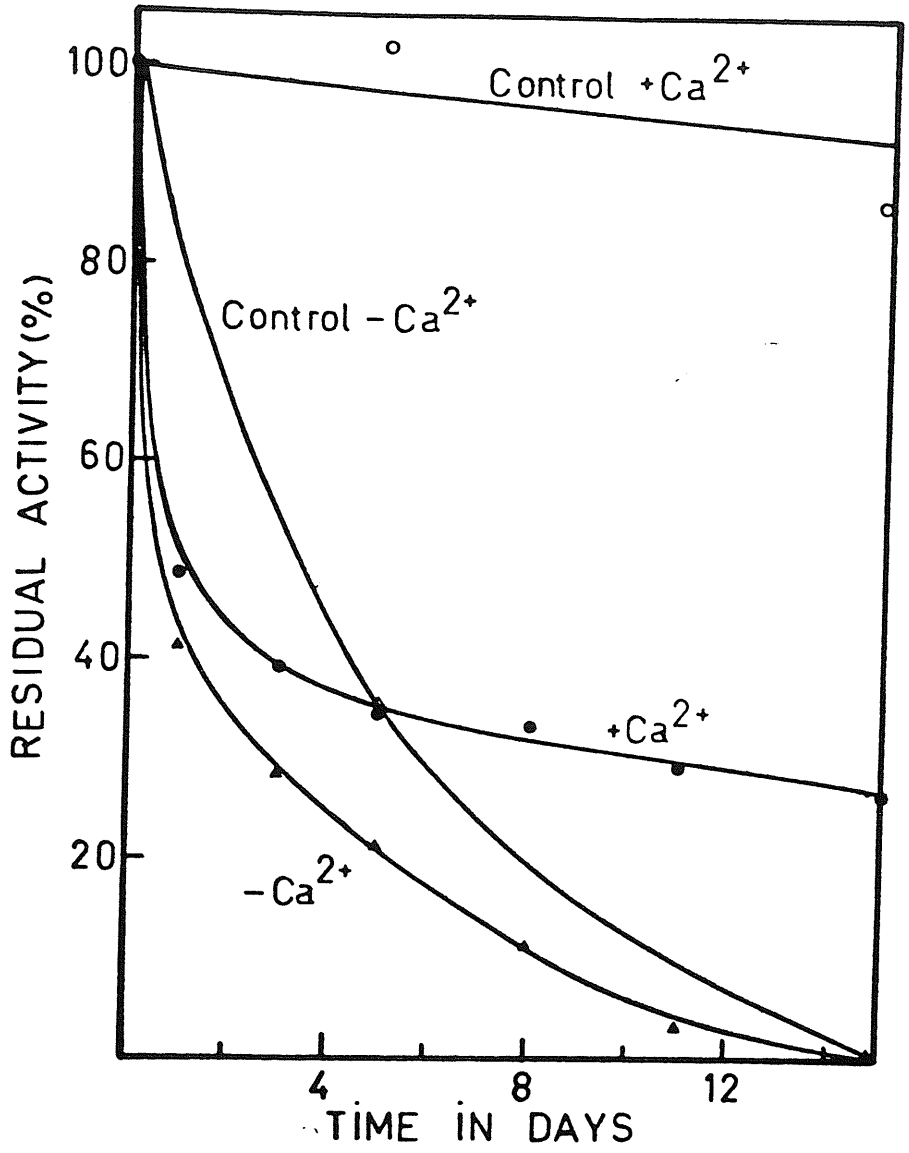


Figure 28

Time course of inactivation of protein modulator by carbamoylation. The modulator (2.5 mg/ml) was treated with potassium isocyanate (0.2 M) at 23° C, pH 7.5, in the presence of 2 mM Ca^{2+} (●—●) or 2 mM EGTA (▲—▲). Open symbols represent controls treated the same way, except that potassium isocyanate was omitted.

control sample retains full activity over the entire time period, whereas the sample incubated with isocyanate shows a substantial initial decrease in modulator activity which levels out at 30-35% of the original value after 4 days of incubation. Thus, reaction of modulator with isocyanate in the presence of Ca^{2+} results in modification of 1 or 2 lysine residues with a concomitant 60-70% decrease in modulator activity, but further modification of the remaining lysine residues does not result in a further decrease in activity. If one takes into account the fact that the values in Table VI should be corrected for some hydrolysis of homocitrulline back to lysine during acid hydrolysis before amino acid analysis (17-30%, according to Stark, 1972), it can be concluded that, after 15 days incubation, essentially all the lysine residues have been modified; such a sample still retains ~30% of its original activity.

It is apparent from Fig. 28 that the major effect of carbamoylation on the activity of the modulator protein occurs in the first 48 h of the incubation period: in the presence of Ca^{2+}, approximately 55% of modulator activity is lost with modification of about 2 lysine residues per mole of modulator. Consequently, this period of the reaction was examined in more detail. Fig. 29 shows a more detailed analysis of the time course of inactivation during the first 48 h. Aliquots of the reaction mixture withdrawn at selected time intervals were also subjected to electrophoresis on 15% polyacrylamide slab gels (Fig. 30). Carbamoylation of reactive ϵ-amino groups converts the positive charge of the reactive lysine residue to the uncharged homocitrulline. With a higher net negative charge, the modified species would be expected to migrate more rapidly towards the anode, as is indeed observed. It is apparent from Fig. 30 that increased time of incubation with isocyanate is

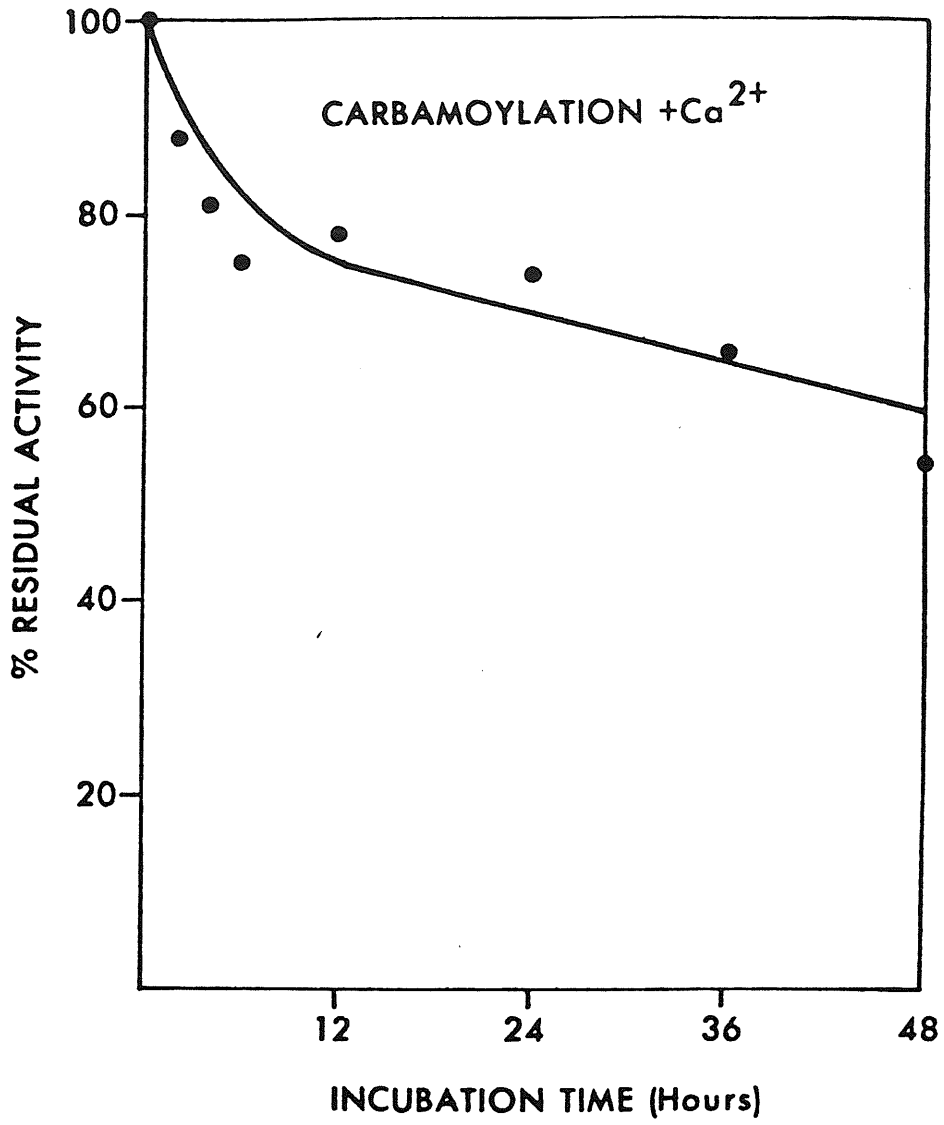


Figure 29 A more detailed analysis of the initial stages of the time course of inactivation of protein modulator by carbamoylation. The modulator was treated as described in the legend to Fig. 28 in the presence of 2 mM Ca^{2+}.

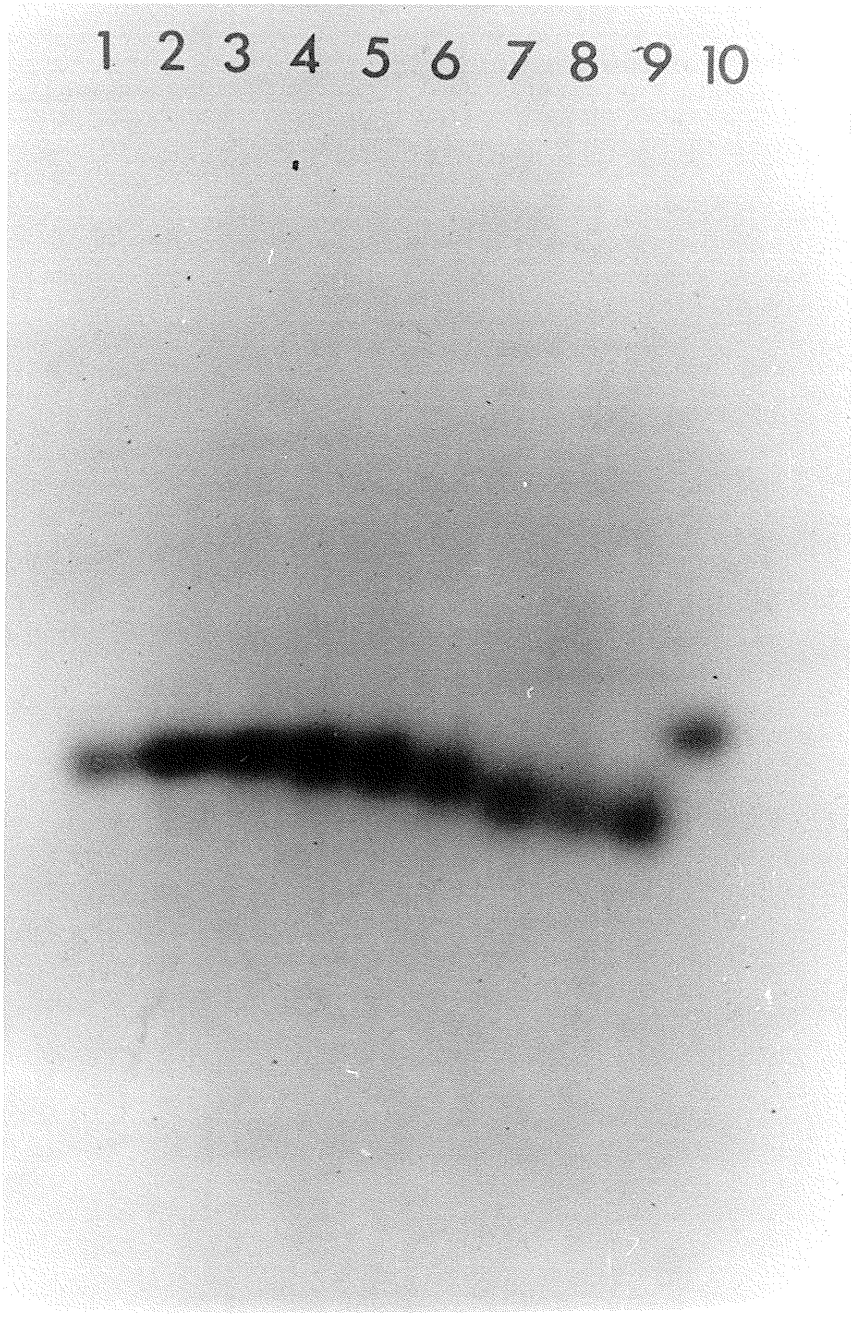


Figure 30

Monitoring of the carbamoylation reaction in the presence of Ca^{2+} (see Fig. 29) by 15% polyacrylamide slab gel electrophoresis according to the method of Davis (1964). The gel shows aliquots (10 μg) withdrawn from the reaction at the following times: 2: 0 h; 3: 2 h; 4: 4 h; 5: 6 h; 6: 12 h; 7: 24 h; 8: 36 h; 9: 48 h. Native modulator protein (10 μg) is shown in slots 1 and 10.

accompanied by increased mobility of the modified protein. At each stage of the reaction, a single species is evident on the gel suggesting the formation of a homogeneous modified derivative.

These observations indicate that carbamoylation of approximately 2 specific lysine residues occurs within 48 h of incubation with isocyanate and is accompanied by a loss of approximately 50% of modulator activity. Prolonged incubation with the modifying agent results in carbamoylation of the remaining 5 lysine residues (trimethyllysine, which elutes with lysine on amino acid analysis, is resistant to carbamoylation) with little additional loss of modulator activity. These observations implicate the involvement of one or two specific lysine residues in the expression of modulator activity.

2. Guanidination

Selective modification of the ϵ-amino group of lysine residues was also attempted by guanidination with O-methylisourea. This modification was performed with a view to assessing the functional importance of the positive charge of the ϵ-amino groups of those lysine residues which were carbamoylated previously; while carbamoylation abolishes the positive charge on the modified lysine residues, guanidination, which converts reactive lysine residues to homoarginine, retains the positive charge of the ϵ-amino group. It is reasonable to assume that those lysine residues which are accessible to isocyanate will, likewise, be accessible to O-methylisourea. Consequently, if one were to observe guanidination of accessible lysine residues with retention of modulator activity, this would be suggestive of the requirement of the positive charge of the ϵ-amino group of lysine residues for the interaction between modulator protein and PDE. This was found to be the

case, as shown in Table VII. Both in the presence and in the absence of Ca^{2+}, the activity of guanidinated modulator is not significantly different from that of control samples even though 2-3 lysine residues have been modified. It is clear from the controls that the modulator is unstable under the conditions of incubation, especially in the absence of Ca^{2+}. This observation is consistent with other reports (Cheung, 1971) that the modulator protein is unstable at high pH.

TABLE VII. Guanidination of protein modulator

Reaction time (days)	Residual activity (%)				Lysine (mol/mol)	
	$+Ca^{2+}$		$-Ca^{2+}$			
	Control[a]	Sample	Control[a]	Sample	$+Ca^{2+}$	$-Ca^{2+}$
0	100	100	100	100	9	9
7	81	79	32	42	7	7
20	68	75	0	0-25	6	7

[a] Controls are the same as the samples except that O-methylisourea was omitted.

VIII. Methionine Modification

A. Carboxymethylation

1. Introduction

Haloacetates are the most widely used protein alkylating agents. Under various conditions they have been shown to react with sulfhydryl, imidazole, thioether, and amino groups of proteins. With rather harsh treatment, phenolic side chains may also react. One instance has been cited of reaction of a carboxyl group of ribonuclease T1 with iodoacetate but this has been attributed to the unusual reactivity of that particular group (Takahashi et al, 1967). The reactions of haloacetates with proteins have been reviewed by Gurd (1972).

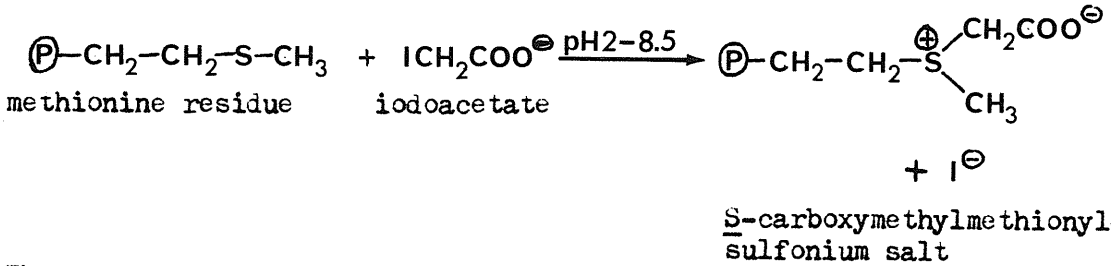
Sulfhydryl groups are the most reactive of the many nucleophilic groups found in proteins which are known to react with haloacetates. Reactivity increases with pH since the anion is the reactive species. Carboxymethylation of sulfhydryl groups cannot occur in the protein modulator due to the lack of cysteine.

Haloacetates react considerably more slowly with imidazole groups of proteins than with sulfhydryl groups. Reaction with imidazole groups, which occurs above pH 5.5, can proceed in two steps to a 1,3-dicarboxymethyl derivative. This derivative and each of the two monocarboxymethyl derivatives are stable compounds which can be isolated after acid hydrolysis and quantitated by conventional amino acid analysis (Gundlach et al, 1959; Stark and Stein, 1964). Upon amino acid analysis, 1-carboxymethyl histidine elutes near proline, 3-carboxymethylhistidine near cystine, and 1,3-dicarboxymethylhistidine near aspartic acid.

Amino groups of proteins react with haloacetates at high pH, where they are unprotonated, but again the rate is considerably less

than the rate of sulfhydryl group modification. The reaction can proceed in two steps via ε-monocarboxymethyllysine to the dicarboxymethyl derivative. Both derivatives are stable to acid hydrolysis and can be quantitated by amino acid analysis : ε-monocarboxymethyllysine elutes from the amino acid analyzer near methionine, and ε-dicarboxymethyllysine elutes near aspartic acid.

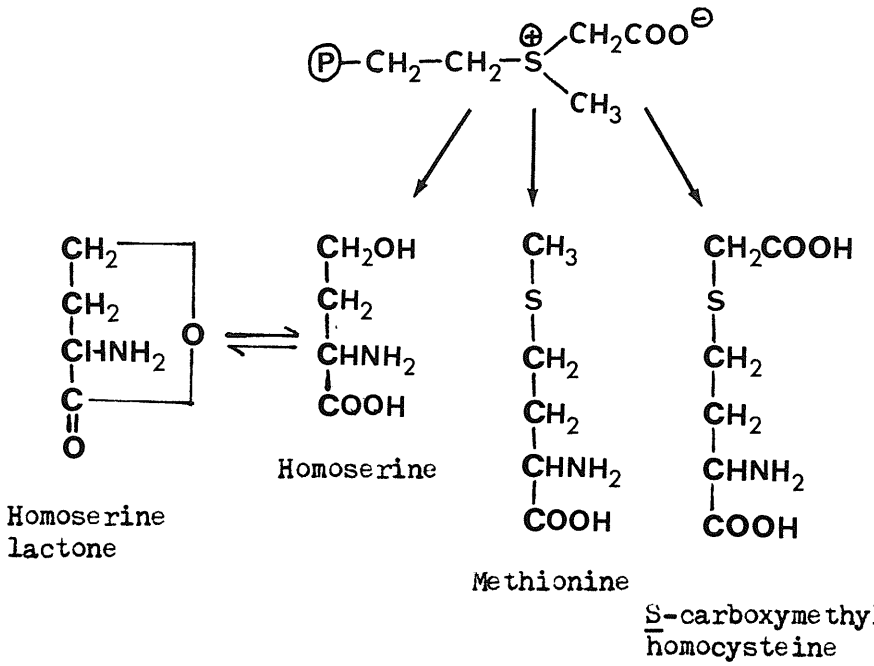
Haloacetates react with methionyl residues of proteins over a wide range of pH (Gundlach et al, 1959). This fact enables specific modification of methionine residues to be attained in many cases by performing the reaction at low pH where other reactive groups are protonated, i.e., pH 2 to 3. Reaction of thioether groups of proteins with iodoacetate occurs as follows :



methionine residue iodoacetate

S-carboxymethylmethionyl-sulfonium salt

The product of the reaction is destroyed during acid hydrolysis, but its three decomposition products are all measurable by amino acid analysis (Gundlach et al, 1959):



S-carboxymethylhomocysteine elutes from the amino acid analyzer near proline, homoserine near glutamic acid, and homoserine lactone near ammonia. This complex pattern of decomposition with regeneration of methionine complicates the quantitation of the extent of carboxymethylation of methionine residues of proteins. Treatment of the carboxymethylated protein with performic acid prior to its hydrolysis can be used to distinguish between that methionine which is regenerated and that remaining after carboxymethylation (Vithayathil and Richards, 1960). S-carboxymethylmethioninesulfonium is not affected by this treatment; the methionine content of the modified protein is, therefore, obtained from the amount of methionine sulfone detected by amino acid analysis (it elutes from the analyzer immediately after aspartic acid), and the observed methionine is attributed to the breakdown of its sulfonium derivative.

Haloacetamides are often slightly more reactive with proteins than are the corresponding acids. The resulting different ionic properties of the modified protein may or may not be advantageous insofar as the modification affects the properties of the protein. In some instances, the negative charge of haloacetates appears to enhance reactivity with a particular group by directing it to a positively charged centre. On the other hand, residues which may be unreactive with haloacetates due to repulsion of the alkylating agent by a neighbouring negative charge may, in some instances, be successfully modified by treatment with the corresponding haloacetamide.

2. Experimental procedure

2.1 Modification : effect on activity

Selective modification of methionine residues of the protein modulator was attempted by carboxymethylation with iodoacetic acid and iodoacetamide by the method of Gundlach et al, (1959). The modulator (5 mg/ml) was dissolved in 0.2 M sodium acetate, pH 5.5, containing either 2 mM $CaCl_2$ or 2 mM EGTA. Reaction was initiated by the addition of an equal volume of a freshly prepared 15 mM solution of iodoacetic acid (or iodoacetamide) in the same buffer to yield a 54-fold molar excess of alkylating agent over protein. The reaction mixture was incubated at 37° C in the dark. Aliquots of the reaction mixture were withdrawn at selected time intervals and the reaction terminated by addition to an equal volume of 80 mM cysteine. Part of the mixture was assayed for modulator activity and the remainder was dialyzed extensively against water to remove excess reagent and lyophilized prior to : (1) performic acid oxidation followed by acid hydrolysis and amino acid analysis, and (2) urea-PAGE. Carboxymethylation was also carried out

in a similar fashion at pH 2.5 (0.05 M potassium hydrogen phthalate-HCl) and at pH 6.9 (0.1 M phosphate buffer) for studies of the time course of inactivation.

2.2 Characterization

2.2.1 Carboxymethylation

In a separate experiment, modulator (5 mg/ml) was dissolved in 0.2 M sodium acetate buffer, pH 5.5, containing 4 mM $CaCl_2$. Reaction was initiated by addition of an equal volume of 15 mM iodoacetic acid solution containing 3.2 μCi of 1-^{14}C-iodoacetic acid in the same buffer. The reaction mixture was incubated in the dark at 37° C. Aliquots of the reaction mixture were withdrawn at times 24 h and 168 h and the reaction quenched by addition to an equal volume of 80 mM cysteine. The resultant mixtures were dialyzed extensively against water and lyophilized.

2.2.2 Tryptic digestion

The lyophilized residues were dissolved in 200 μl of 0.1 M ammonium bicarbonate, saturated with nitrogen and 10 μl of TPCK-trypsin (16 mg/ml of 0.1 M ammonium bicarbonate) were added. The reaction mixture was incubated at 37° C for 2 h, at which time the digestion was terminated by addition of 20 μl of 0.2 N HCl with mixing. Half the sample (110 μl) was applied to each of two Whatman 3 MM paper chromatograms and subjected to two-dimensional peptide mapping, using methyl green (1%) as marker to standardize the maps, as described in IB.

2.2.3 Autoradiography

The maps were cut to size, stapled to sheets of X-ray film in the dark room, placed in lightproof holders and kept in the dark for 3-4 days. The films were developed and the positions of labeled peptides on the peptide maps identified by lining up the X-ray

film with the chromatogram and observing the locations of dark spots on the film.

2.2.4 Elution of labeled peptides

The labeled spots were cut out of the peptide maps and the peptides eluted first with 10% aqueous acetic acid and then with 50% aqueous pyridine. The eluted peptides were dried in a vacuum desiccator and oxidized with performic acid prior to acid hydrolysis and amino acid analysis.

3. Results and Discussion

3.1 Effect on modulator activity

Fig. 31 illustrates the time course of inactivation of the protein modulator by carboxymethylation at pH 5.5 in the presence of Ca^{2+}. Identical results were obtained when the reaction was performed at pH 2.5 and pH 6.9. The loss of activity was slightly less rapid when the modification was performed in the presence of EGTA. It is apparent from Fig. 31 that the inactivation occurs in two phases : an initial rapid phase which is accompanied by the loss of approximately 60% of modulator activity (24 h), and a second slower phase in which the remaining activity is lost (168 h). Controls treated in an identical fashion, with the exception that the reagent was omitted, retained full activity throughout this period.

The extent of methionine modification at each stage of the reaction was determined by amino acid analysis of the performic acid oxidized protein (Fig. 31). Amino acid analysis revealed that the only residues affected by alkylation were methionine residues; hence, no reaction occurred with residues of histidine or lysine which are known to yield acid-stable carboxymethyl derivatives. It is apparent from

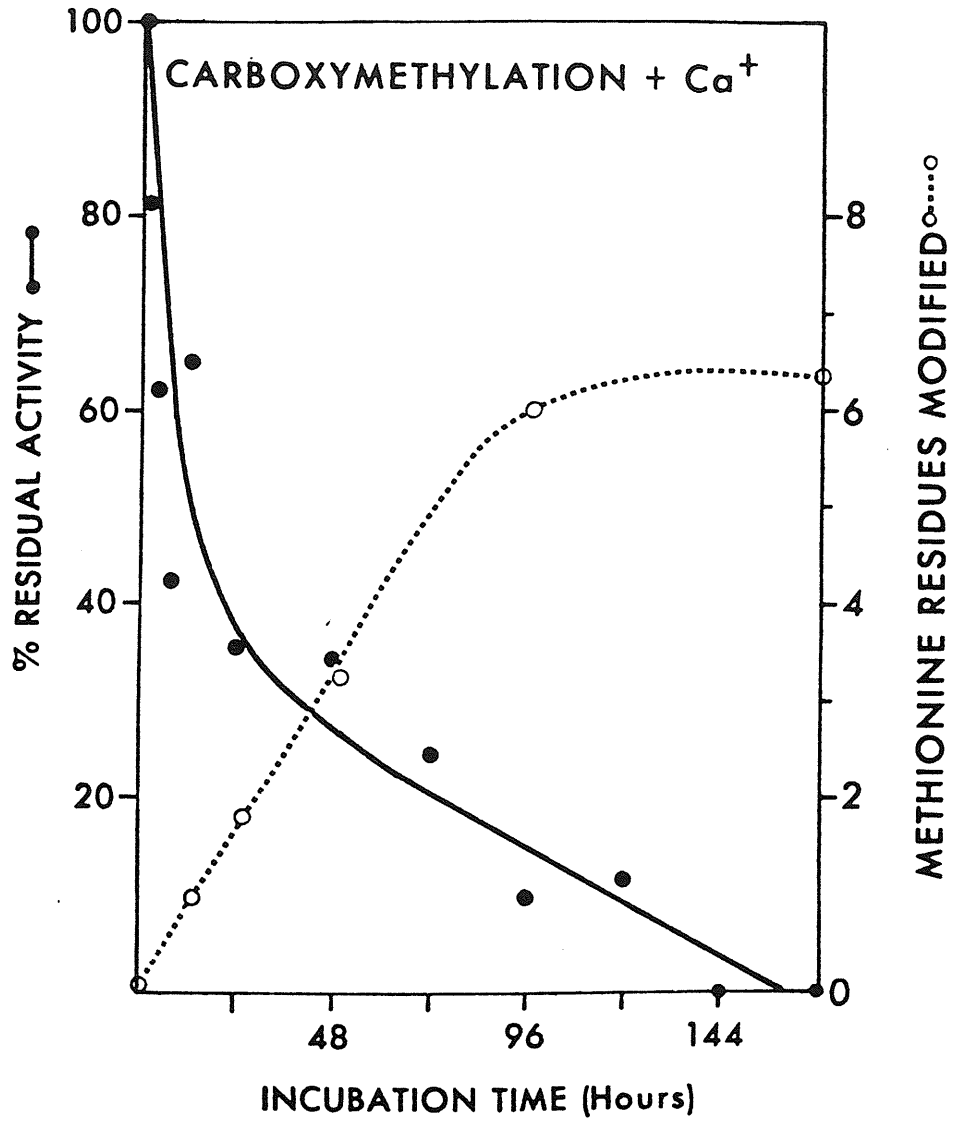


Figure 31

Time course of carboxymethylation of protein modulator. The modulator (5 mg/ml) was treated with iodoacetic acid (7.5 mM) at 37° C, pH 5.5, in the presence of 2 mM Ca^{2+}. Aliquots were withdrawn from the reaction mixture at the indicated times for assay of modulator activity (●——●) and determination of the extent of methionine modification (o---o) as described in "Experimental" VIIIA2 2.1.

Fig. 31 that the initial rapid loss of modulator activity is accompanied by the modification of approximately 2 methionine residues per mole; complete loss of modulator activity occurs concomitant with the carboxymethylation of approximately 6 of a total of 9 methionine residues.

3.2 Urea-PAGE

The carboxymethylation reaction in the presence of Ca^{2+} was also monitored by electrophoresis on polyacrylamide slab gels in the presence of 6 M urea, as shown in Fig. 32. After 24 h incubation with iodoacetate (slot 4), the native band and 3 or 4 other bands of slower mobility are apparent. With prolonged incubation, the native band and the bands of intermediate mobility gradually disappear in sequence (slots 5-9) while the band of slowest mobility increases in intensity, until at 168 h (slot 9) it is the predominant species visible. There appears to be a definite transition from the rapidly moving, native protein via 2 or 3 intermediates to the slowly migrating, inactive species. The native modulator in the presence (slot 1 and 2) and absence (slots 11 and 12) of Ca^{2+} are included for comparison.

3.3 Characterization

A sample of modulator was treated in a separate experiment with 1-^{14}C-iodoacetic acid so that modified methionine residues contained a ^{14}C label. Modulator incubated in this way for 24 h and 168 h was digested with trypsin and subjected to peptide mapping. The locations of peptides containing the ^{14}C label, and, therefore, modified methionine residues, were identified by autoradiography. These peptides were eluted from the chromatograms and oxidized with performic acid, to convert unmodified methionine residues to methionine sulfone, and subjected to acid hydrolysis and amino acid analysis.

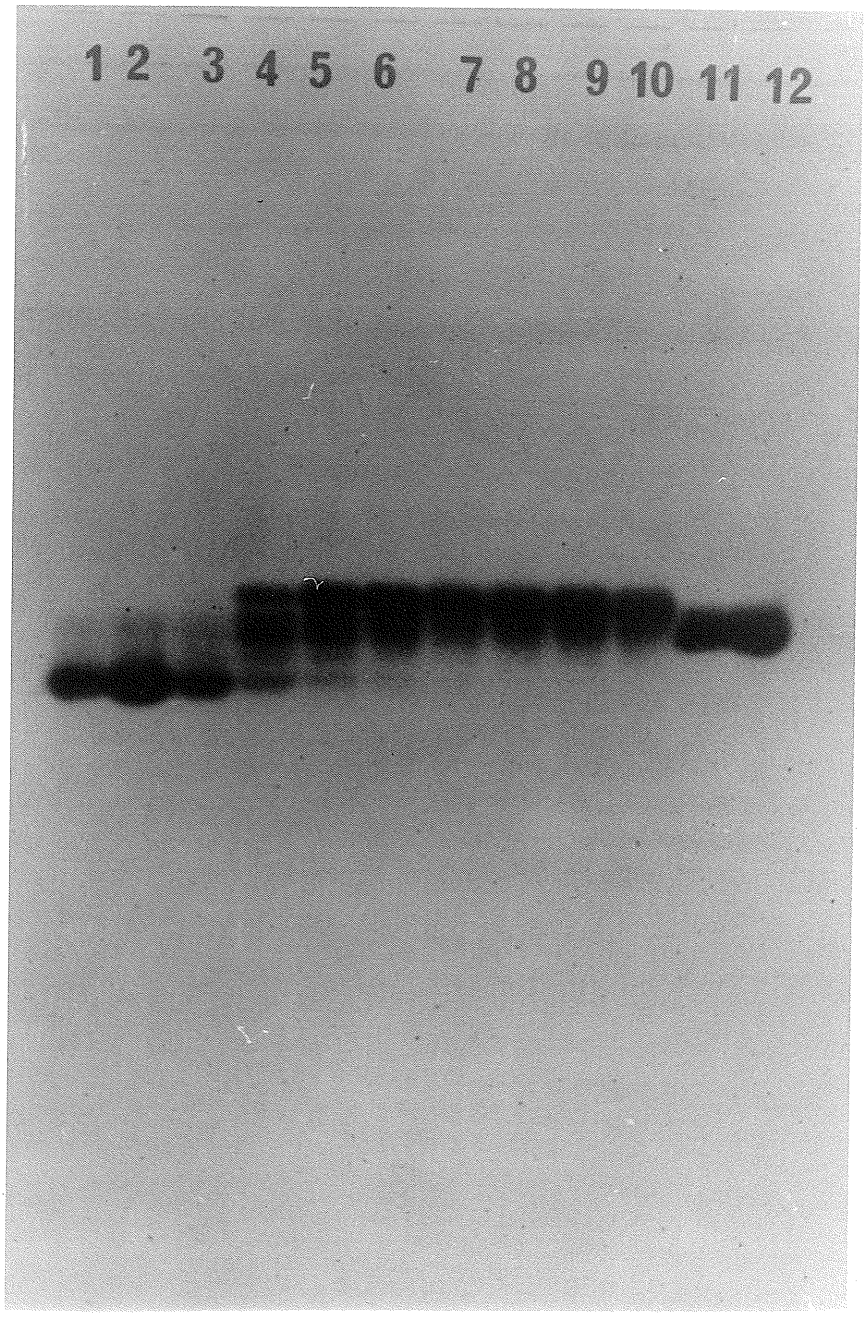


Figure 32

Monitoring of the carboxymethylation reaction in the presence of Ca^{2+} (see Fig. 31) by 15% PAGE in the presence of 6 M urea according to the method of Davis (1964). The gel shows aliquots (10 µg) withdrawn from the reaction mixture at the following times: 3: 0 h; 4: 24 h; 5: 48 h; 6: 72 h; 7: 96 h; 8: 120 h; 9: 144 h; 10: 168 h. Native modulator in the presence of 4 mM Ca^{2+} is shown in slots 1 (20 µg) and 2 (30 µg); native modulator in the presence of 4 mM EGTA is shown in slots 11 (20 µg) and 12 (30 µg).

Examination of the autoradiogram obtained from the tryptic digest of the 24 h modulator sample revealed a single intense dark spot (denoted tryptic peptide 1, Tp 1) and 3 fainter spots (denoted Tp 2, Tp 3 and Tp 4). A peptide map of control modulator (not treated with iodoacetate) was stained with ninhydrin-collidine to visualize the peptides. No spots were seen in the locations of the labeled peptides obtained from the modified modulator. This facilitated the isolation of labeled peptides from unmodified contaminants. The amino acid compositions of the labeled peptides are shown in Table VIII; also shown are the regions of modulator sequence (see Appendix II) which correspond most closely to the calculated compositions. Tp 1, which provided the most intense spot on the autoradiogram, corresponds to residues 31-37 in the primary sequence. This peptide contains a single methionine residue, Met 36, which, therefore, must be carboxymethylated. Tp 2 corresponds to residues 127-148 in the native molecule. This peptide contains two methionine residues, Met 144 and Met 145. The fact that methionine sulfone was detected in the acid hydrolyzate of the peptide suggests that one of these methionine residues is not carboxymethylated. Vanaman et al, (1977) observed that unusual tryptic hydrolysis occurs following the Met 144-Met 145 sequence in the native protein. The amino acid composition of Tp 2 indicates that this peptide bond (Met 145-Thr 146) is not cleaved by trypsin in carboxymethylated modulator. This is presumably due to the modification of Met 145; conversion of this residue to the carboxymethyl derivative apparently prevents the unusual tryptic cleavage at Met 145-Thr 146. Hence, it appears that carboxymethylation of Met 145 but not Met 144 occurs.

Tp 3 migrates very rapidly towards the cathode during the

TABLE VIII. Amino acid compositions of ^{14}C-labeled peptides obtained by tryptic digestion of modulator incubated with 1-^{14}C-iodoacetic acid.

Amino acid	Tp 1	Residues 31-37 [a]	Tp 2	Residues 127-148 [a]	Tp 3	Residues 76-77 [a]	Tp 4	Residues 107-126 [a]	Tp 5	Residues 38-74 [a]
Lysine	0.30	-	0.70	1	1.00	1	1.08	1[b]	1.59	-
Histidine	-	-	-	-	-	-	trace	1	-	-
Arginine	1.05	1	0.39	-	-	-	0.29	1	1.58	1
Aspartic acid	0.30	-	4.01	4	0.50	-	3.28	3	5.30	7
Threonine	0.94	1	1.36	1	-	-	1.89	2	3.06	3
Serine	0.43	-	0.49	0	0.32	-	0.59	-	0.85	1
Glutamic acid	1.01	1	5.47	5	0.36	-	4.54	4	6.88	6
Proline	-	-	0.57	0	-	-	-	-	-	2
Glycine	1.65	1	2.03	2	0.69	-	2.03	1	3.77	3
Alanine	0.24	-	2.21	2	-	-	0.39	-	1.00	3
Valine	0.89	1	1.04	2	-	-	1.75	2	1.80	1
Methionine[c]	-	1	0.26	2	-	1	-	2	trace	3
Isoleucine	-	-	1.00	1	-	-	1.00	1	1.67	2
Leucine	1.00	1	1.00	0	-	-	1.97	2	2.80	3
Tyrosine	-	-	0.19	1	-	-	-	-	-	-
Phenylalanine	-	-	1.01	1	-	-	-	-	-	2
Methionine sulfone[d]	-	-	+	-	-	-	+	-	+	-

[a] From Vanaman et al, (1977).

[b] This represents a residue of trimethyllysine (Vanaman et al, 1977).

[c] Methionine values in the tryptic peptides are reduced due to modification of methionine residues.

[d] Could not quantitated due to overlap with aspartic acid, but its presence is indicated by +. Methionine sulfone represents unmodified methionine.

electrophoretic stage of peptide mapping. This fact, together with its calculated amino acid composition, suggests that Tp 3 is a very small positively charged peptide. The best alignment that can be made is with residues 76-77 in the sequence, i.e., Met 76-Lys 77. Hence, Met 76 appears to be carboxymethylated.

Tp 4 has an amino acid composition which indicates that it corresponds to residues 107-126. This peptide contains the sole residues of histidine and trimethyllysine, and also contains two methionine residues: Met 109 and Met 124. The acid hydrolyzate of Tp 4 contained methionine sulfone, indicating the presence of unmodified methionine in Tp 4. These observations suggest that either Met 109 or Met 124 is carboxymethylated with iodoacetate.

Examination of the autoradiogram of the peptide map derived from modulator which had been incubated with 1-^{14}C-iodoacetic acid for 168 h (i.e., to the stage of complete inactivation) revealed the presence of 5 intense dark spots: 4 of these spots corrseponded to Tp 1 - Tp 4 and an additional dark spot, Tp 5, which was not observed on the 24 h autoradiogram. The amino acid composition of Tp 5 is shown in Table VIII. This peptide correlates most closely with residues 38-74 which sequence contains 3 methionine residues: Met 51, Met 71 and Met 72. The presence of methionine sulfone in the acid hydrolyzate again indicates that the peptide contains unmodified methionine. Again Vanaman et al, (1977) demonstrated an unusual cleavage at the peptide bond between Met 71 and Met 72. The amino acid composition of Tp 5 suggests that this peptide bond is not cleaved in carboxymethylated modulator. Met 71 and Met 72 are at the end of a helical section close to the random coil linking Ca^{2+}-binding sites II and III while Met 51 is in a helical region close to

a junction with a Ca^{2+}-binding loop (see "Concluding Remarks"); hence, Met 71 and 72 are probably exposed and accessible to iodoacetate, whereas Met 51 appears to be buried within the hydrophobic interior of the molecule and inaccessible to iodoacetate. These observations suggest that Met 71 and Met 72 are carboxymethylated, while Met 51 is not.

Therefore, complete loss of modulator activity appears to occur upon carboxymethylation of Met 36, Met 71, Met 72, Met 76, Met 145 and either Met 109 or Met 124. This analysis of ^{14}C-labeled modified peptides indicates that 6 specific amino acid residues are carboxymethylated with iodoacetate; this is consistent with the results of amino acid analyses, given in IVA3 3.1, which showed that complete loss of modulator activity was accompanied by modification of approximately 6 methionine residues.

It should be noted that the results of the autoradiographic study presented in this section were obtained in a single experiment. The interpretation of these results was complicated by the apparently equal accessibility of so many methionine residues to the modifying agent. Clearly, one cannot implicate all these methionine residues in the interaction between the protein modulator and PDE. With a view to simplifying interpretation, selective modification of methionine residues in the modulator was attempted by two other methods, as presented in the following sections.

B. Mild Oxidation

1. Introduction

A mild specific modification development by Shechter et al, (1975) enables attainment of selective oxidation of methionine residues in nonsulfhydryl proteins by treatment with mild oxidizing reagents, such

as N-chlorosuccinimide (NCS) or chloramine-T, at neutral or slightly alkaline pH. In studies with model proteins, they demonstrated that such treatment with chloramine-T resulted in the oxidation of cysteine to cystine, in addition to oxidation of methionine to methionine sulfoxide, but no other amino acid was modified; with NCS, trytophans were oxidized as well.

Based on studies with bovine α-lactalbumin, bovine α-chymotrypsin, bovine pancreatic ribonuclease, and hen egg-white lysozyme (Shechter et al, 1975) and chicken ovoinhibitor (Shechter et al, 1977), it appears that NCS at pH 7.0-8.5 is capable of distinguishing between 3 main classes of methionine residues in proteins : (1) exposed methionines are those residues which can be oxidized with 1-3 equivalents of NCS per methionine residue, (2) buried methionine residues resist oxidation with up to 100 equivalents of NCS per residue, and (3) partially exposed methionine residues can be oxidized with 10-20 equivalents per residue. Chloramine-T, due to its aromatic moiety, is capable (when used in high concentrations) of penetrating into hydrophobic regions of a protein, causing oxidation of buried methionine residues.

The extent of oxidation of methionine residues is determined by treatment of the oxidized protein with cyanogen bromide which cleaves at methionine residues, converting them to homoserine (plus the lactone), but leaves methionine sulfoxides intact. During acid hydrolysis of the oxidized and cyanogen bromide-treated protein, methionine sulfoxides are reduced back (due to the inclusion of dithioerythritol in the hydrolysis mixture) and determined as methionines by amino acid analysis, while the amount of homoserine (and its lactone) corresponds to that of the unoxidized methionine in the original sample.

The fact that the modulator protein lacks both cysteine and tryptophan makes mild oxidation ideally suited for investigating the role(s) of methionine residues in the expression of modulator activity.

2. Experimental procedures

2.1 Mild oxidation

2.1.1 Analytical scale

Selective oxidation of methionine residues of the protein modulator was achieved by treatment with NCS (and chloramine-T) essentially as described by Shechter et al, (1975). The modulator (0.5 mg/ml) was dissolved in 0.1 M Tris-HCl, pH 8.5, containing 4 mM $CaCl_2$ or 4 mM EGTA. Oxidation was initiated by the addition of a freshly prepared, concentrated solution of NCS (10 mM) to yield a 3-fold molar excess of NCS over methionine residues. The reaction mixture was incubated at 23° C. Aliquots of the reaction mixture were withdrawn at appropriate time intervals and the reaction was terminated by addition to an equal volume of 40 mM methionine. Part of the mixture was assayed for modulator activity, and the remainder was dialyzed extensively against water and lyophilized prior to : (1) amino acid analysis after cyanogen bromide digestion and acid hydrolysis in the presence of 0.2 M dithioerythritol, and (2) urea-PAGE.

2.1.2 Preparative scale

From the results of the experiments involving mild oxidation on an analytical scale, it was apparent that modification both in the presence and absence of Ca^{2+} led to complete loss of modulator activity. In order to characterize the modified derivatives in detail, samples of modulator protein were oxidized on a preparative scale as described above starting with about 20 mg protein. Modulator

inactivated by oxidation in the presence of Ca^{2+} (denoted oxidized modulator$_{Ca^{2+}}$) was prepared by incubating the protein with NCS as described above for 90 min, at which time reaction was terminated by the addition of an equal volume of 40 mM methionine; the mixture was then dialyzed extensively against water and lyophilized. Modulator inactivated by oxidation in the absence of Ca^{2+} (denoted oxidized modulator$_{EGTA}$) was prepared by incubating the protein with NCS as described above for 15 min, at which time reaction was terminated and the sample treated as described above.

Oxidized modulator$_{Ca^{2+}}$ was subjected to the following studies :

(1) analysis of troponin C-like activities,

(2) analysis of $^{45}Ca^{2+}$-binding properties by gel filtration (see below),

(3) analysis of cyanogen bromide peptides by electrophoresis,

(4) isolation and amino acid composition analysis of the cyanogen bromide peptide containing the modified methionine residues (see below), and

(5) circular dichroism studies.

Oxidized modulator$_{EGTA}$ was subjected to analyses (1) to (3) above.

2.2 $^{45}Ca^{2+}$-binding studies

Oxidized modulator$_{Ca^{2+}}$, oxidized modulator$_{EGTA}$, and the corresponding controls were dissolved in 20 mM Tris, 1 mM EGTA, pH 7.5, at a concentration of 1.5 mg/ml and dialyzed against 4 litres of the same buffer to remove endogenous Ca^{2+}. The samples were then dialyzed against two changes (4 litres each) of 20 mM Tris, 1 mM magnesium acetate, pH 7.5, to remove EGTA. The dialyzed samples were incubated with $^{45}CaCl_2$ (1 μCi per 1.5 mg protein at a $CaCl_2$ concentration of 0.0875 mM) at room temperature for 45 min. The samples were then applied to a Sephadex

G-25 column (1.5 x 90 cm) previously equilibrated with 20 mM Tris, 1 mM magnesium acetate, pH 7.5, and elution achieved with the same buffer. 2 ml fractions were collected at a flow rate of 100 ml/h. Selected fractions were assayed for protein by the ninhydrin method after alkaline hydrolysis (Hirs et al, 1956; see "General Experimental Procedures"), and ^{45}Ca was determined by scintillation counting in a Beckman LS-250 liquid scintillation counter.

2.3 Isolation of modified cyanogen bromide peptide

Electrophoretic analysis of cyanogen bromide digests of native and oxidized modulator protein (see below) revealed that a single peptide, which contains all the oxidized methionine residues, obtained from oxidized modulator$_{Ca}2+$ is not obtained by cyanogen bromide digestion of the native protein. This peptide was isolated as follows:

A sample of oxidized modulator$_{Ca}2+$ (15 mg) was digested with cyanogen bromide as described under "General Eperimental Procedures". The digest was subjected to preparative gel electrophoresis employing 15% polyacrylamide slab gels in batches of 1 mg of digest. The first gel was stained and destained in the usual way to check the efficiency of the separation, and the Rf value of the modified peptide was determined and found to be 0.59, in agreement with previously obtained values. The location of the modified peptide in all subsequent preparative gels was identified by this Rf value and this portion of each gel was cut out. The gel slices were cut into 1 mm cubes and homogenized in a minimum volume of 0.1 M Tris-HCl, pH 8.1 in order to elute the peptide from the gel. The homogenate was stirred overnight and then centrifuged at 27,000 g for 30 min. The supernatant was decanted into a lyophilization flask and the pellet was resuspended in a minimum volume of 0.1 M Tris-HCl,

pH 8.1 and centrifuged as before. The resultant supernatant was combined with the first supernatant, and the mixture was lyophilized. The residue was dissolved in 3.5 ml of water and applied to a Sephadex G-25 column (2.5 x 90 cm) previously equilibrated with deionized water and elution achieved with deionized water. 6.4 ml fractions were collected at a flow rate of 108 ml/h. The absorbance at 280 nm of each fraction was measured in a Beckman Model 25 spectrophotometer. The peptide eluted between fractions 38-48, which coincided with the void volume as determined in an independent filtration using blue dextran. These fractions were pooled and lyophilized.

The residue, shown to be homogeneous by 15% PAGE, was dissolved in 5 ml of water. Aliquots of 0.5, 1.0 and 1.5 ml were lyophilized in hydrolysis tubes and acid hydrolyzed in the presence of 0.2 M dithioerythritol prior to amino acid analysis in the usual manner.

3. Results and Discussion

3.1 Oxidation in the presence of Ca^{2+}

3.1.1 Effect on PDE-stimulating activity

Fig. 33 illustrates the effects of mild oxidation of the protein modulator with NCS in the presence of Ca^{2+}. Such treatment results in a rapid loss of modulator activity with concomitant modification of methionine residues. From the inset in Fig. 33, the activity appears to decrease linearly as a function of the number of methionine residues oxidized, and complete loss of activity correlates with oxidation of 4 methionine residues per mole of modulator. A control modulator treated simultaneously in identical manner, with the exception that NCS was omitted from the reaction mixture, retained full modulator activity and exhibited apparent modification of one methionine residue during

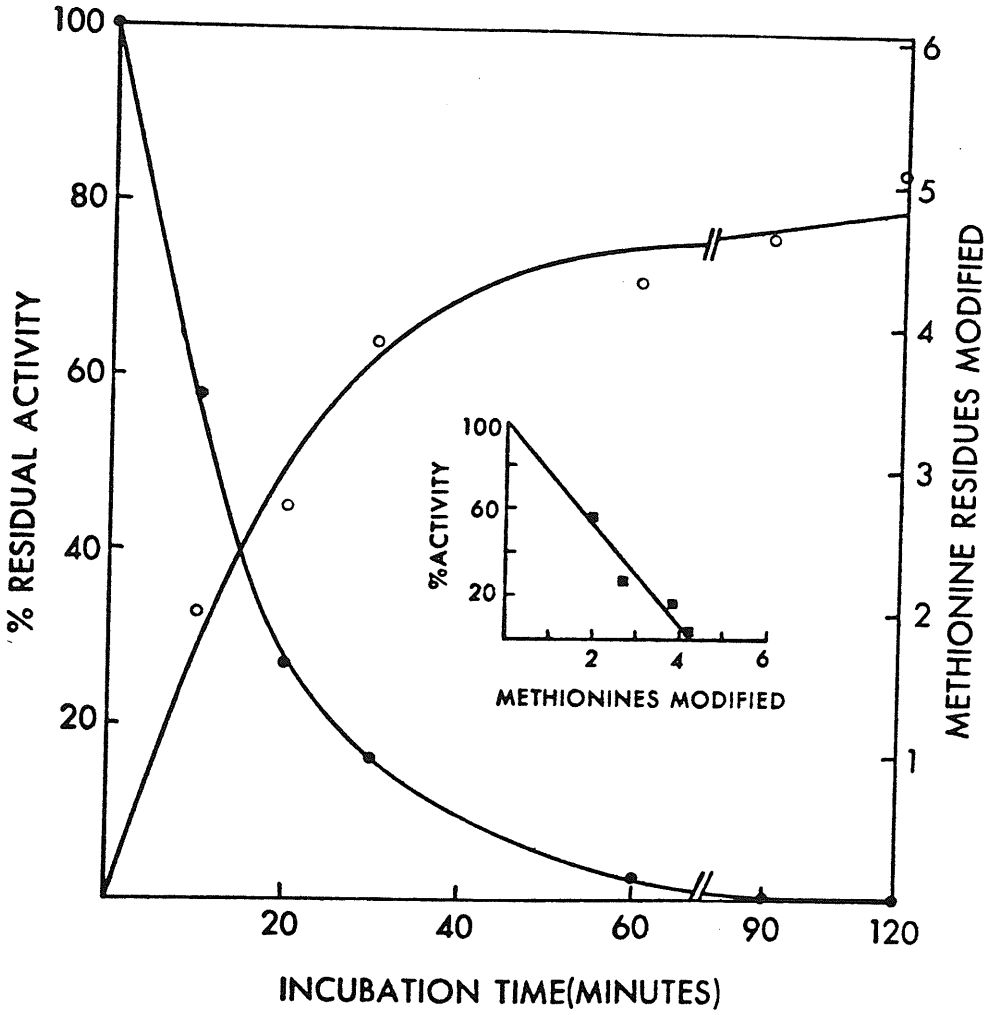


Figure 33

Time course of mild oxidation of the protein modulator with NCS in the presence of Ca^{2+}. The modulator (0.5 mg/ml) was treated with NCS (3 equivalents per methionine residue) at 23° C, pH 8.5, in the presence of 4 mM Ca^{2+}. Aliquots were withdrawn from the reaction mixture at the indicated times for assay of modulator activity (●——●) and determination of the extent of methionine modification (o——o) as described in "Experimental" VIIIB1. The inset (■——■) illustrates the relationship between loss of activity and modification of methionine residues.

the course of the incubation period of 120 min; this is due to the fact that the Met 109-Thr 110 peptide bond is resistant to cyanogen bromide cleavage in the native modulator (see IVB3 3.1.5). Complete loss of modulator activity, therefore, occurs concomitant with the oxidation of either 3 or 4 methionine residues per mole.

3.1.2 Urea-PAGE

The oxidation was also monitored by discontinuous polyacrylamide slab gel electrophoresis in the presence of urea as shown in Fig. 34. The mobility of the modulator decreases as a function of time of incubation with NCS (slot 1-8), and the mobility of the inactive modulator resembles that of the native modulator in the absence of Ca^{2+}. Since mild oxidation converts reactive methionine residues to methionine sulfoxide without a change in charge, one would not expect this modification to affect the mobility of the protein in this electrophoretic system. The fact that the oxidized, inactive modulator comigrates with the native modulator in the absence of Ca^{2+} suggests the possibility that mild oxidation abolishes, or dramatically reduces, the affinity of the molecule for Ca^{2+}.

3.1.3 Phosphodiesterase- and Ca^{2+}-binding properties of oxidized modulator

The loss of modulator activity resulting from mild oxidation could be due to various reasons: the oxidized modulator may no longer bind to phosphodiesterase, the oxidized modulator may have lost its Ca^{2+}-binding ability, or an ineffective complex may be formed between phosphodiesterase and oxidized modulator. In order to distinguish between these possibilities, the ability of the oxidized modulator to interact with phosphodiesterase and to bind Ca^{2+} ions was

Figure 34

Monitoring of the mild oxidation reaction in the presence of Ca^{2+} (see Fig. 33) by 15% PAGE in the presence of 6 M urea according to the method of Davis (1964). The gel shows aliquots (20 µg) withdrawn from the reaction mixture at the following times: 1: 0 min; 2: 10 min; 3: 25 min; 4: 40 min; 5: 60 min; 6: 90 min; 7: 120 min; 8: 240 min. Native modulator (10 µg) in the presence of 4 mM $CaCl_2$ (slot 9) and 4 mM EGTA (slot 10) are also shown.

examined. A competition assay with native modulator was utilized in order to determine whether or not the oxidized modulator interacts with phosphodiesterase. To a constant amount of native modulator (sufficient to give ~50% maximal stimulation of phosphodiesterase) were added increasing amounts of oxidized modulator and the effects on the stimulation of phosphodiesterase activity observed by assaying modulator activity as described in "General Experimental Procedures". The results are depicted in Fig. 35. If the oxidized, inactive modulator retains the ability to interact with phosphodiesterase, one would expect it to compete with the native modulator; such competition would result in a decrease in PDE activity ($A_{660\ nm}$) with increasing oxidized : native modulator ratio. On the other hand, if the oxidized, inactive modulator has lost the ability to bind to phosphodiesterase, one would expect to see no competition with the native protein and observe no effect on PDE activity (A_{660}) with increasing oxidized : native modulator ratio. The latter effect was observed (Fig. 35), indicating that the oxidized, inactive modulator does not interact with phosphodiesterase.

The Ca^{2+}-binding properties of the oxidized, inactive modulator were also investigated. The ability of the inactive modulator to bind $^{45}Ca^{2+}$ was assessed by removing endogenous Ca^{2+} from the modified protein with EGTA and dialyzing out the Ca^{2+}-EGTA and excess EGTA; the protein was then incubated with $^{45}CaCl_2$ and the mixture passed through a Sephadex G-25 column, as described in IVB2 2.2. Fractions eluted from the column were assayed for ninhydrin-positive material after alkaline hydrolysis and ^{45}Ca determined by scintillation counting (Fig. 36). While a peak of ^{45}Ca eluted with the native protein as expected (Fig. 36B), no ^{45}Ca eluted coincident with the oxidized modulator (Fig. 36A). This suggests

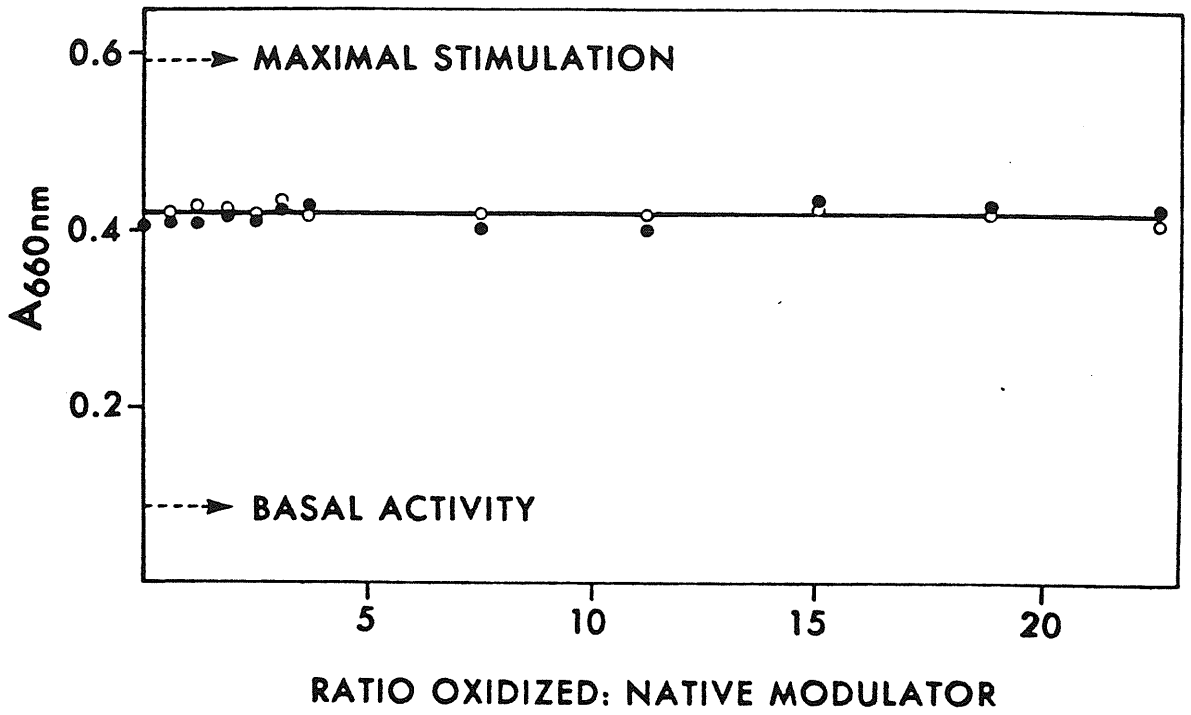


Figure 35

Assay of competitive binding of oxidized modulator and native modulator to PDE. Increasing amounts of modulator oxidized in the presence of Ca^{2+} (●——●) and absence of Ca^{2+} (o——o) were assayed for modulator activity in the presence of a fixed amount (~1 unit) of native modulator protein as described in "General Experimental Procedures" IV.

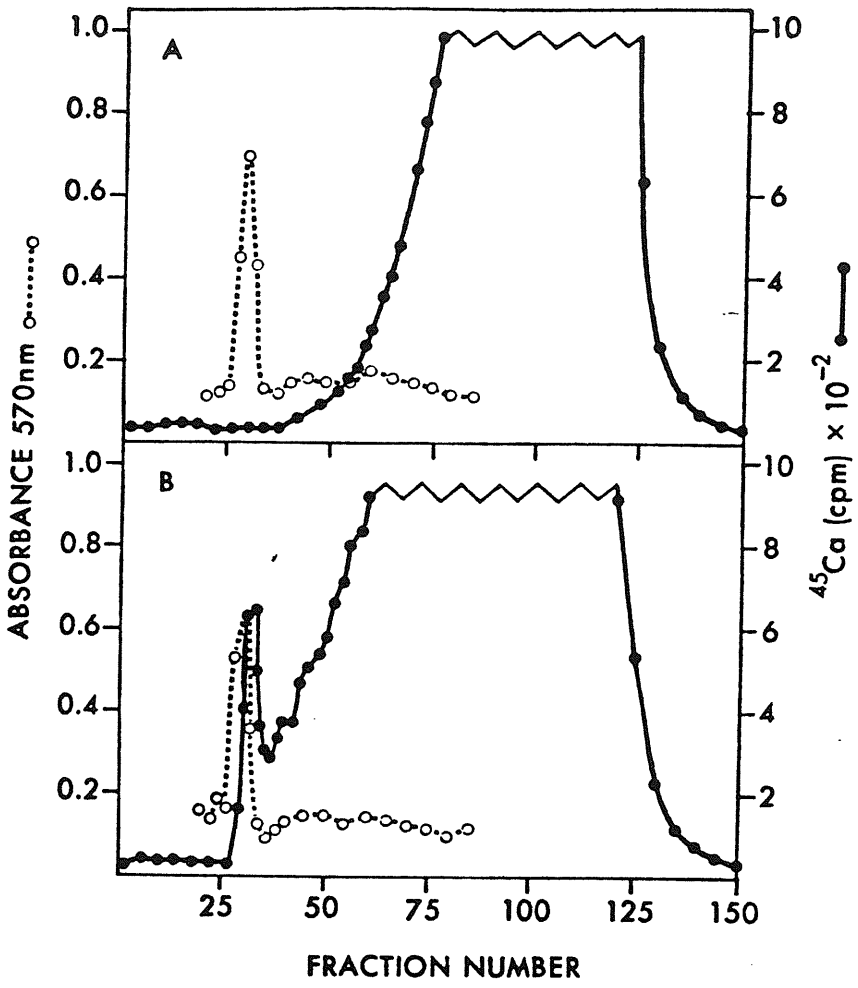


Figure 36

Determination of $^{45}Ca^{2+}$-binding by oxidized (A) and native modulator (B) by gel filtration. Protein samples (1.5 mg) were incubated with 1 µCi of $^{45}CaCl_2$ (8.75×10^{-5} M) and applied to a Sephadex G-25 column (1.5 x 90 cm) equilibrated with 20 mM Tris, 1 mM magnesium acetate, pH 7.5. 2 ml fractions were collected and assayed for protein by the ninhydrin method after alkaline hydrolysis (o---o) and ^{45}Ca was determined by scintillation counting (●—●) as described in "General Experimental Procedures" IX.

that the Ca^{2+}-binding affinity of the oxidized modulator is abolished or drastically reduced, consistent with the electrophoretic studies described earlier.

3.1.4 Effect on troponin C-like activities

As indicated in VC3, the modulator protein and troponin C both exhibit a Ca^{2+}-dependent change in mobility on urea-PAGE and interact with troponin I in the presence of Ca^{2+} to form a urea-stable complex. The effect of mild oxidation of the modulator protein on these so-called troponin C-like activities was investigated, i.e., oxidized $\text{modulator}_{Ca^{2+}}$ was examined for a Ca^{2+}-dependent change in electrophoretic mobility in urea-containing polyacrylamide gels, and Ca^{2+}-dependent interaction with troponin I in the presence of 6 M urea. Fig. 37A illustrates the fact that the native modulator in the presence of Ca^{2+} (slot 1) has significantly greater mobility than the native protein in the absence of Ca^{2+} (slot 2). On the other hand, the mobility of the oxidized modulator (slots 3 and 4) is unaffected by Ca^{2+}.

Fig. 37B shows the results of electrophoretic analyses of possible interactions with troponin I. Slot 1 shows troponin I alone. Slot 2 represents a mixture of native modulator protein and excess troponin I in the presence of Ca^{2+}. No band corresponding to the free modulator is apparent, but rather a band with much slower mobility, corresponding to a modulator-troponin I complex is evident. In the absence of Ca^{2+} (slot 3) no such complex is formed as indicated by the absence of a band with slow mobility and the presence of a band corresponding to the free modulator. In the case of the oxidized modulator, no complex formation occurs either in the presence (slot 6) or absence (slot 7) of Ca^{2+}.

It appears, therefore, that mild oxidation of the protein

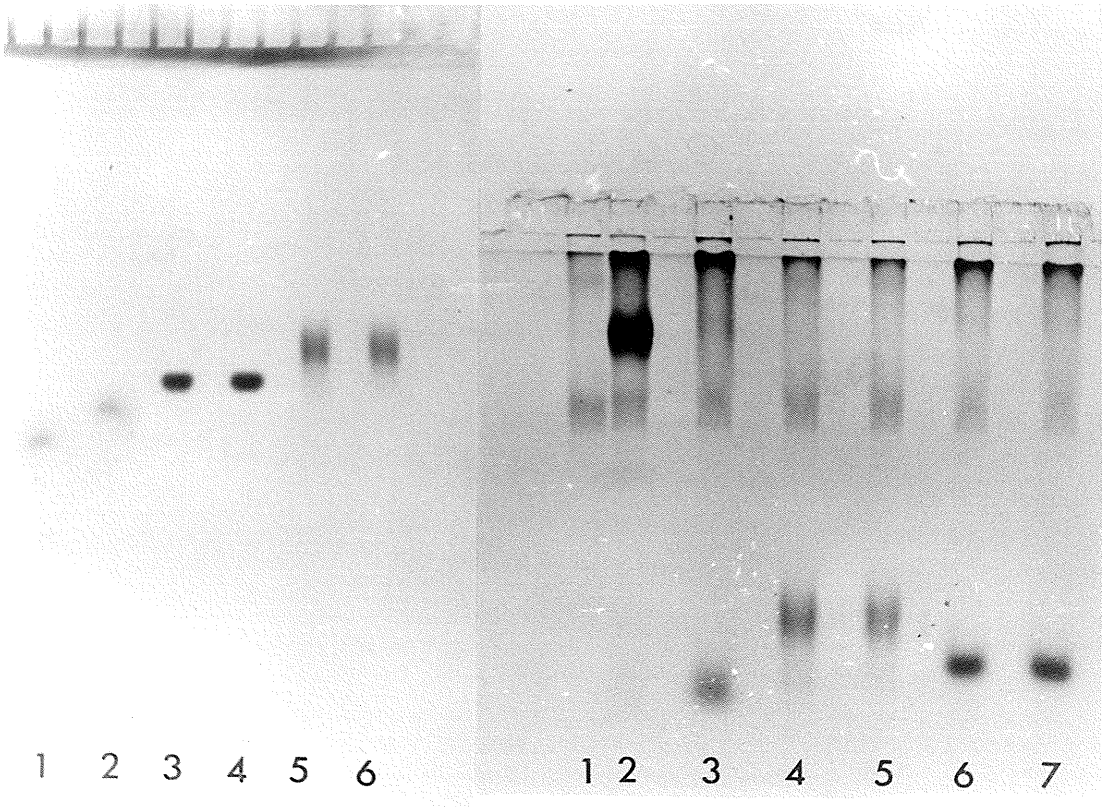


Figure 37 Effect of mild oxidation and alkylation on troponin C-like activities of the protein modulator. A: Effect on the Ca^{2+}-dependent change on urea-PAGE as described in "General Experimental Procedures" VB. Protein samples were dissolved in sample buffer containing 6 M urea and either 4 mM $CaCl_2$ (slots 1, 3 and 5) or 4 mM EGTA (slots 2, 4 and 6). 5 μg of protein were applied to slot 1 and 2 and 10 μg to slots 3-6. 1 and 2: native modulator; 3 and 4: oxidized modulator; 5 and 6: alkylated modulator. B: Effect on the Ca^{2+}-dependent interaction of the modulator with troponin I, monitored by urea-PAGE as described in "General Experimental Procedures" VB. Modulator samples were incubated with an approximately 2-fold weight excess of bovine cardiac troponin I in the sample buffer containing 6 M urea and either 4 mM $CaCl_2$ (slots 2, 4 and 6) or 4 mM EGTA (slots 3, 5 and 7). 1: troponin I alone; 2 and 3: native modulator; 4 and 5: alkylated modulator; 6 and 7: oxidized modulator.

modulator in the presence of Ca^{2+} results in the loss of troponin C-like activities of the molecule in addition to the loss of phosphodiesterase-stimulating activity.

3.1.5 Identification of oxidized methionine residues

Mild oxidation with NCS under the conditions used converts exposed methionine residues to methionine sulfoxide. Chemical cleavage of proteins is conveniently achieved at methionine residues by treatment with cyanogen bromide, but this agent does not cleave proteins at methionine sulfoxide residues (Shechter et al, 1975). This fact provided a sound basis from which to attempt identification of the oxidized methionine residues in the primary sequence of the protein modulator. Initially, the electrophoretic patterns on 15% polyacrylamide slab gels of cyanogen bromide digests of the modulator after different times of incubation with NCS were examined (Fig. 38). Ten distinct peptide bands are visible in the digest of the native protein (slots 1-3); after 60 min incubation with NCS (slot 7), which results in totally inactive material, the peptide pattern no longer changes and only 6 peptides are apparent, 5 of which correspond to peptides derived from the native protein and one of which is a new band. This pattern is compatible with the modification of 4 methionine residues only if these oxidized residues are in a sequence uninterrupted by unmodified methionines.

The new peptide containing the modified methionine residues was subsequently isolated by preparative slab gel electrophoresis and subjected to acid hydrolysis in the presence of dithioerythritol and amino acid analysis. The amino acid composition of this peptide is presented in Table IX; also shown is the region of modulator sequence which corresponds most closely to this calculated composition.. It appears,

TABLE IX. Amino acid composition of methionine-oxidized cyanogen bromide peptide of the protein modulator.

	Amino acid composition [a]					
Amino acid	1	2	3	Average	Integer	Residues 52-124 [b]
Lysine	4.67	4.36	4.53	4.52	4-5	4
Histidine	0.70	0.54	0.57	0.60	1	1
Arginine	3.23	3.32	3.30	3.28	3	4
Aspartic acid	11.80	11.92	12.02	11.91	12	13
Threonine	5.10	5.01	5.09	5.07	5	5
Serine	1.82	1.92	2.00	1.91	2	2
Glutamic acid	15.17	15.00	15.15	15.11	15	11
Proline	trace	trace	trace	trace	0-1	1
Glycine	5.85	5.75	5.76	5.79	6	5
Alanine	5.79	5.70	5.72	5.74	6	5
Cysteine	0.00	0.0	0.0	0.0	0	0
Valine	4.00	4.00	4.00	4.00	4	4
Methionine	3.00	2.76	3.17	2.98	3	5[c]
Isoleucine	3.83	3.90	3.94	3.89	4	4
Leucine	4.53	4.60	4.62	4.58	4-5	4
Tyrosine	1.15	1.17	1.17	1.16	1	1
Phenylalanine	3.78	3.79	3.88	3.82	4	4

[a] The results represent amino acid analyses of 3 independent hydrolyses of the peptide.

[b] Values taken from the amino acid sequence as determined by Vanaman et al, (1977).

[c] This value includes one residue which was converted to homoserine (plus the lactone) in the cyanogen bromide peptide.

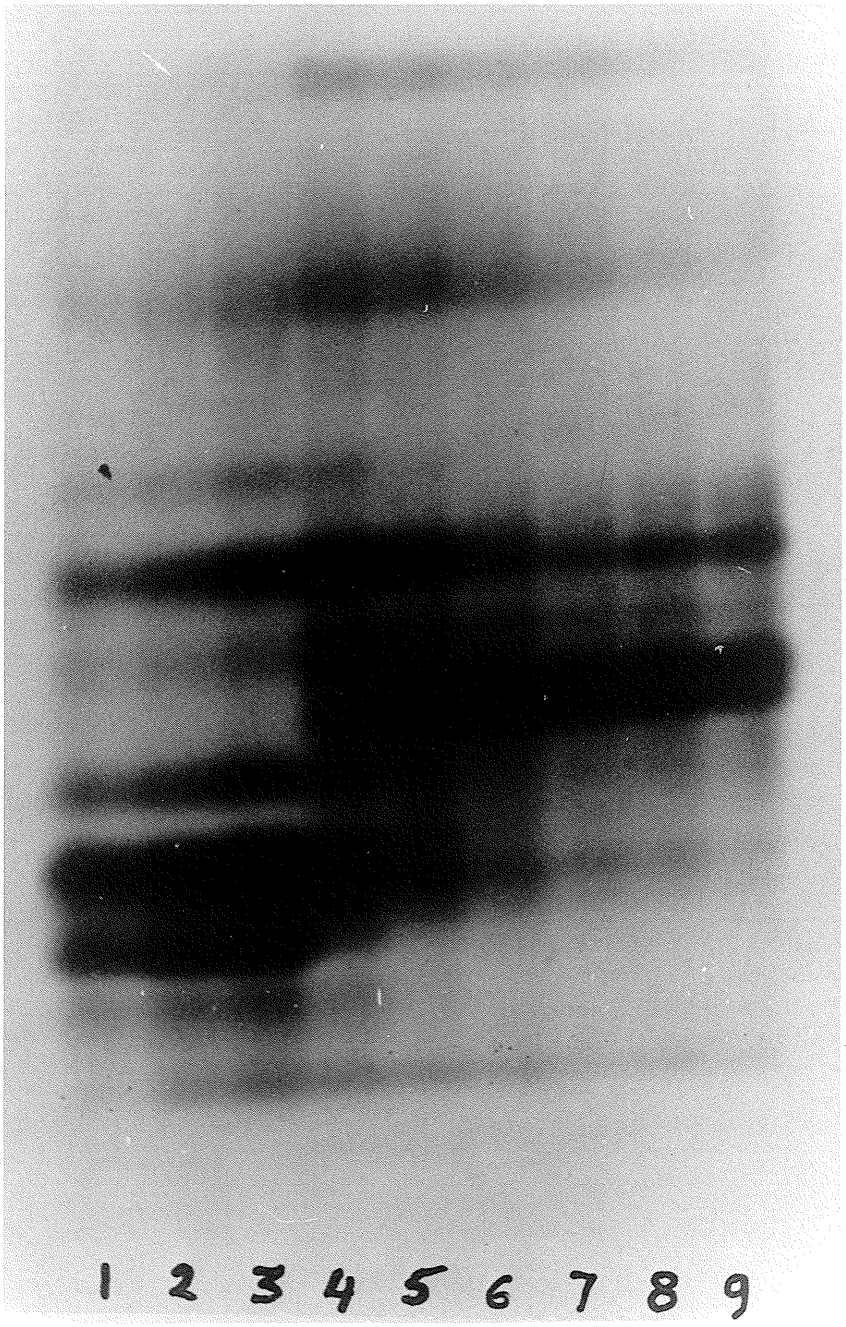


Figure 38

Monitoring of the mild oxidation reaction in the presence of Ca^{2+} (see Fig. 33) by electrophoretic analysis (15% PAGE) of cyanogen bromide digests. 1 and 2: digests of native modulator; 3-9: digests of modulator incubated with NCS for the following periods of time: 0, 10, 20, 30, 60, 90 and 120 min, respectively. Approximately 100 µg of protein were applied to each slot.

therefore, that this modified peptide corresponds to residues 52-124 in the modulator sequence. This region of the sequence contains 5 methionine residues which are located in positions 71, 72, 76, 109 and 124. Residue 124 must not have been oxidized since cleavage with cyanogen bromide took place at this position; on the other hand residues 71, 72, 76 and 109 appear to have been oxidized since no cleavage with cyanogen bromide occurred at these residues. These observations are consistent with the results given earlier which showed that complete loss of modulator activity occurred concomitant with the oxidation of 4 methionine residues per mole. Furthermore, electrophoretic studies predicted these oxidized methionines to occur in a sequence uninterrupted by unmodified methionines, as is indeed the case.

The Met 109-Thr 110 peptide bond appears to be resistant to cyanogen bromide cleavage in the native modulator (C. Klee, personal communication); this finding is consistent with our observation that cyanogen bromide digestion of the native modulator followed by acid hydrolysis in the presence of dithioerythritol consistently yields approximately 1 residue per mole of methionine - if quantitative cleavage occurred at all methionine peptide bonds, no free methionine would be detected in the acid hydrolyzate. Why this Met-Thr peptide bond should be resistant to cyanogen bromide cleavage is unclear; presumably it is due to some influence by neighbouring residues since a Met-Thr peptide bond at position 145-146 is readily cleaved by cyanogen bromide. In view of this unusual resistance to cyanogen bromide cleavage, the possibility arises that Met 109 may not be susceptible to mild oxidation with NCS. Due to lack of further information at the present time, it is possible only to conclude that Met 109 may, or may not, be oxidized by exposure

to NCS.

In summary, treatment of the protein modulator in the presence of Ca^{2+} with 3 equivalents of NCS per methionine residue results in specific oxidation of methionine residues in positions 71, 72 and 76, and possibly 109, accompanied by a loss of PDE-stimulating activity and troponin C-like activities. These methionine residues must be exposed on the surface of the native molecule in the presence of Ca^{2+}, the remaining methionine residues being partially or completely buried within the hydrophobic interior of the protein.

3.1.6 Circular dichroism studies

3.1.6.1 Effect of Ca^{2+} on CD spectra

The far-UV CD spectra of oxidized modulator in the presence and absence of Ca^{2+} are shown in Fig. 39A. The spectrum observed in the presence of Ca^{2+} is essentially indistinguishable from that of the native protein in the presence of Ca^{2+} (Fig. 19) indicating that the average degree of secondary structure in the native molecule is unaffected by the oxidation of surface methionine residues. Calculations of amount of secondary structure estimated from the observed ellipticities indicate that the oxidized modulator in the presence of Ca^{2+} has 50% helical content and 13% β-sheet structure, compared with values of 49% and 11%, respectively, for the native protein. It is conceivable that the oxidized modulator, upon binding of Ca^{2+}, may assume a conformation different from that of the native protein in the presence of Ca^{2+} but with the same proportions of helix, β-sheet and random structure; however, in view of the fact that the oxidation of methionine residues is an extremely mild chemical modification, with incorporation of a single oxygen atom per modified residue, it appears more likely that the conformation of the

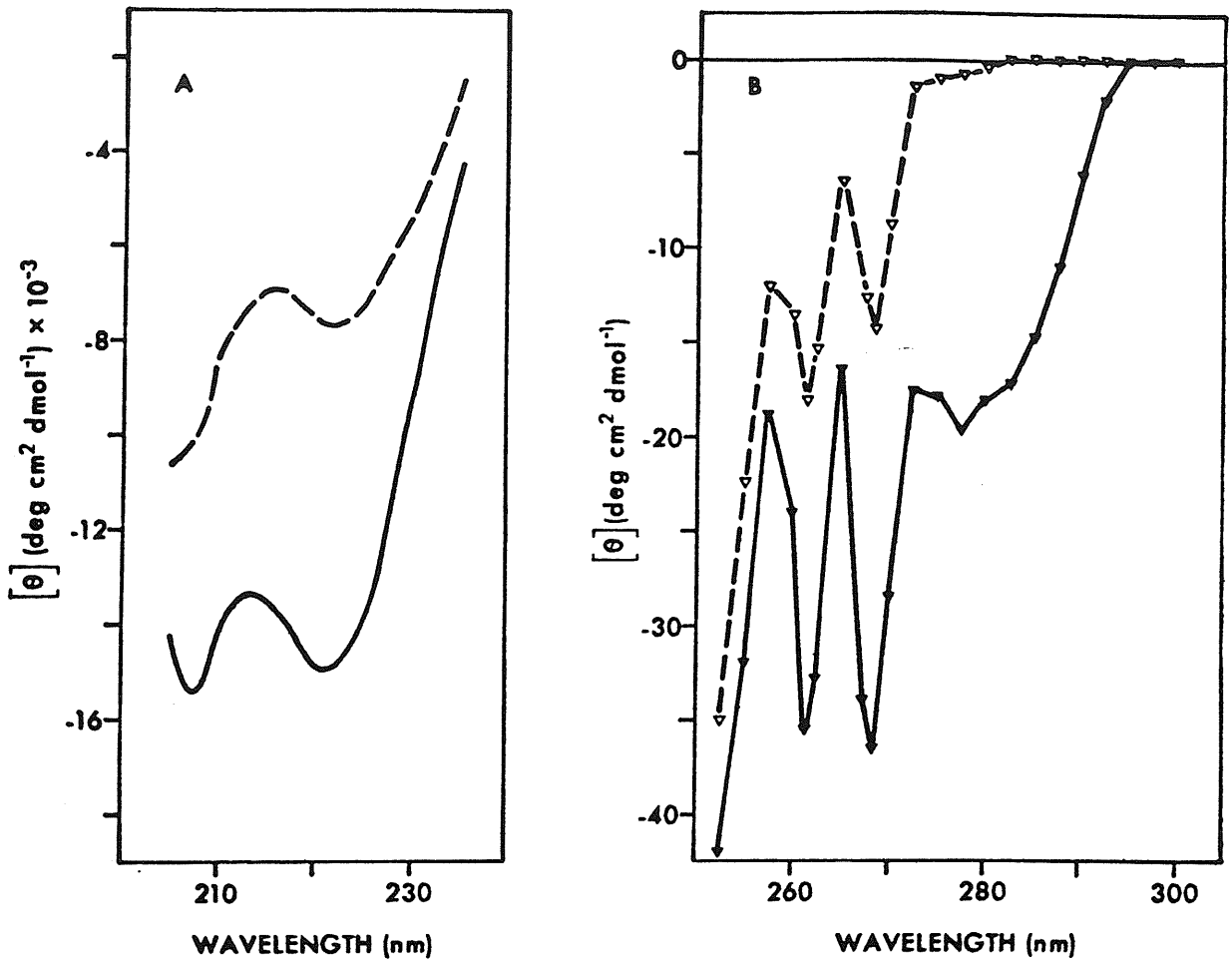


Figure 39

A: Far-UV-CD spectra of oxidized modulator protein in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.6, without (- - - -) or with (———) 3 mM $CaCl_2$. Protein concentration was 1.17 mg/ml and the path length was 0.0501 cm.

B: Near-UV CD spectra of oxidized modulator protein in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.6, without (▽- -▽) or with (▼——▼) 3 mM $CaCl_2$. Protein concentration was 4.13 mg/ml and the path length was 1 cm.

molecule is not affected by such treatment. Therefore, while oxidation of surface methionine residues has no significant effect on the conformation of the molecule, such treatment does destroy the ability of the modulator to bind to and activate phosphodiesterase, suggesting that one (or more) of these methionine residues is involved in the interaction between the modulator protein and phosphodiesterase.

It is clear from Fig. 39A that removal of Ca^{2+} ions from the oxidized modulator has considerable effect on the conformation of the molecule: the helical content decreases substantially from 50% to 26%, and the β-sheet content decreases slightly from 13% to 10%.

Comparison of Fig. 19 with Fig. 39 indicates that the shape and rotational strength of the Ca^{2+}-free native modulator CD spectrum are different from those of the oxidized modulator. The oxidized modulator in the absence of Ca^{2+} exhibits 26% helical content, while the Ca^{2+}-free native modulator exhibits 40% helical content. This suggests that the oxidized modulator exists in a "more inactive" conformation than the native protein in the absence of calcium ions. It may be that the native protein in the absence of Ca^{2+} assumes a "primed" conformation such that Ca^{2+} ions can fit readily into their binding sites with an increase in helical content from 40% to 49%. On the other hand, the oxidized modulator does not assume this "primed" conformation in the absence of Ca^{2+}, having 26% helical content. However, the oxidized modulator does attain the native conformation upon binding Ca^{2+} ions. It is possible that in going from the Ca^{2+}-free to the Ca^{2+}-bound state the oxidized modulator passes through a conformational state similar to that of the native protein in the absence of Ca^{2+}.

The conformational change induced in the oxidized modulator by

Ca^{2+} is also reflected in the near-UV CD spectra (Fig. 39B). The contributions from phenylalanine at 261.5 nm and 268.5 nm are considerably enhanced in the presence of Ca^{2+}. The small tyrosine contribution is affected by Ca^{2+} and appears as the shallow trough above 270 nm. Consistent with the conclusions from the far-UV CD spectra, the near-UV CD spectrum of the oxidized modulator in the presence of Ca^{2+} resembles very closely that of the native modulator in the presence of Ca^{2+} (see Fig. 19), indicating that the microenvironment of both phenylalanine and tyrosine residues is preserved upon oxidation of surface methionine residues.

The Ca^{2+} binding ability of the oxidized modulator was previously examined by a gel filtration technique (see VIIIB 3.1.6.3). No binding of $^{45}Ca^{2+}$ to the oxidized modulator could be demonstrated by this technique and it was concluded that the Ca^{2+}-binding affinity of the oxidized modulator was either abolished or dramatically reduced relative to the native molecule. The latter conclusion must be the case since it is clear from Fig. 39 that the oxidized modulator, at high Ca^{2+} concentration, does bind Ca^{2+}. This conclusion was verified subsequently by Ca^{2+} titration studies which are discussed below.

3.1.6.2 CD titration studies

As discussed earlier, it is well established that the native modulator protein undergoes considerable changes in conformation upon binding Ca^{2+} ions. Circular dichroism is a sensitive detector of this conformational change. Therefore, the Ca^{2+}-binding properties of the native modulator and chemically modified derivatives of the modulator can be suitably investigated by studying the effect of incremental addition of Ca^{2+} on the far-UV CD spectrum of the protein. Such a technique can identify different classes of Ca^{2+}-binding sites within

the molecule and yield the corresponding dissociation constants for Ca^{2+}; it is particularly well suited for comparing the Ca^{2+}-binding properties of a chemically modified form of the modulator with those of the native molecule. Similar CD titration studies have been performed with native bovine cardiac troponin C and skeletal muscle troponin C (Hincke et al, 1978).

Fig. 40 illustrates the effects on the conformation of addition of $CaCl_2$ in increments to the native and oxidized modulator. The fraction of completion of the CD spectral change, f, is plotted against the negative logarithm of the free Ca^{2+} concentration in solution, pCa. The solid curve in Fig. 40 is a best fit computer-calculated theoretical titration curve for the native modulator calculated assuming the native protein possesses two classes of Ca^{2+}-binding sites with apparent dissociation constants for Ca^{2+} of $1.89 \pm 0.19 \times 10^{-7}$M and $4.03 \pm 2.28 \times 10^{-4}$M (see "General Experimental Procedures"). The dashed line in Fig. 40 represents a best fit computer-calculated theoretical curve for the oxidized modulator calculated assuming the oxidized protein possesses two classes of Ca^{2+}-binding sites with apparent dissociation constants for Ca^{2+} of $4.25 \pm 1.54 \times 10^{-10}$M and $3.83 \pm 0.46 \times 10^{-4}$M. It is unlikely, however, that a class of Ca^{2+}-binding sites with $K_d = 4.25 \times 10^{-10}$M truly exists in the oxidized modulator; if such a class was present, binding of ^{45}Ca to the oxidized modulator would have been detected in the experiments described in VIIIB3 3.1.3.

Ca^{2+} binding to the high affinity sites ($K_d = 1.89 \times 10^{-7}$M) of the native modulator causes 80% of the observed CD spectral change, the remainder being elicited by binding of Ca^{2+} to the low affinity site(s) with apparent dissociation constant 4×10^{-4}M. The CD titration curve

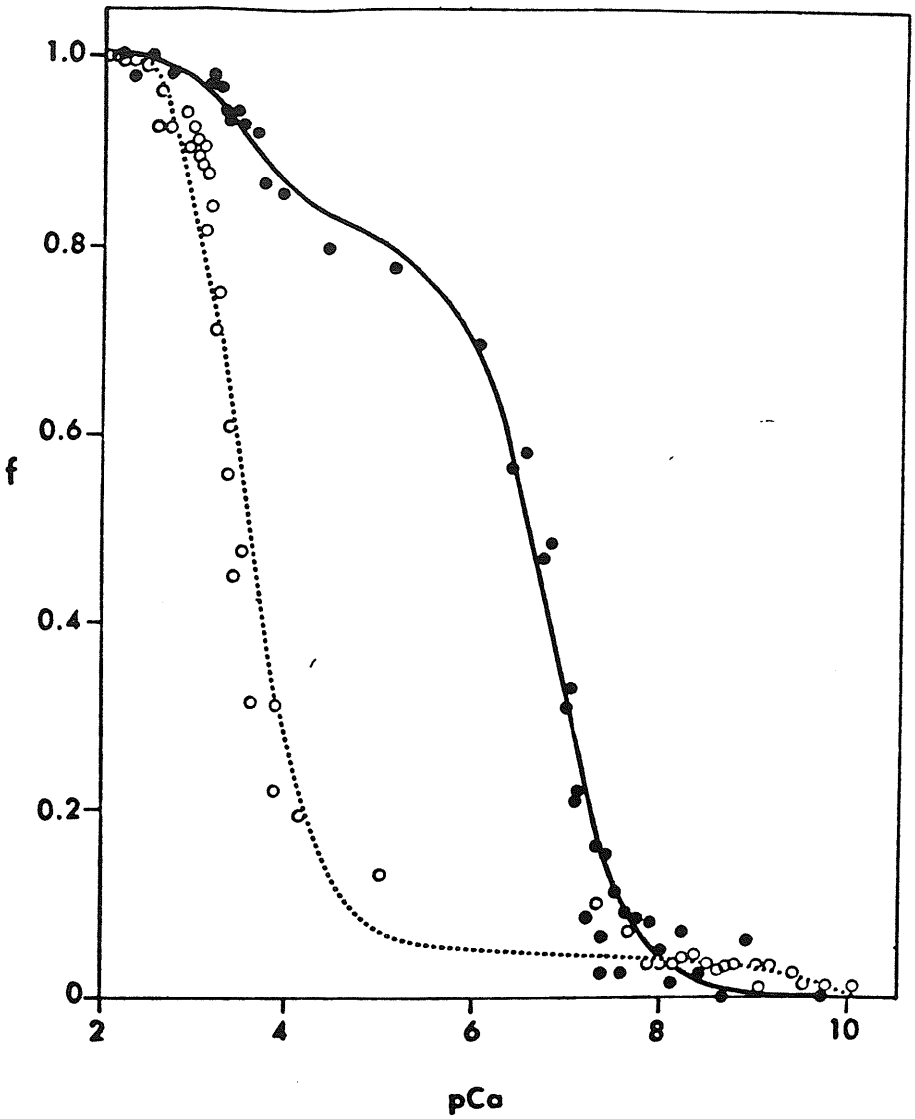


Figure 40

CD calcium titration of the conformational change in native protein modulator (●), oxidized protein modulator (○). Protein was dissolved in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.47. $CaCl_2$ was added incrementally and $[\Theta]_{222\ nm}$ recorded after each addition. f is the fraction of completion of the conformational change, and pCa = -log $[Ca^{2+}]$. The lines represent computer-calculated theoretical titration curves which best fit the data points.

for the oxidized modulator demonstrates that the Ca^{2+}-binding properties of this modified form of the protein are distinctly different from those of the native molecule. That the affinity of the oxidized modulator for Ca^{2+} ions is reduced by ~3 orders of magnitude relative to that of the native protein is due to the fact that the Ca^{2+}-induced spectral change involves a transition from 26% to 50% helical content for the oxidized modulator, but only from 40% to 49% helical content for the native protein (see VIIIB3 3.1.6.1). The difference in Ca^{2+} affinity exhibited by the oxidized and native modulator reflects the conformational differences between the Ca^{2+}-free states of the protein and not any conformational difference between the Ca^{2+}-bound states.

As indicated in "General Experimental Procedures", the theoretical curves in Fig. 40 are constructed on the assumption that the binding sites in any class are equivalent, independent and non-interacting. The theoretical curves in Fig. 40 are not steep enough for the data in the region above pCa~6. It is possible that a better fit of these portions of the curves would be obtained by assuming cooperativity in the state function change.

From the CD studies described above it is apparent that both the far-UV and the near-UV CD spectra of the oxidized modulator in the presence of Ca^{2+} are indistinguishable from the corresponding spectra of the native protein, suggesting that mild oxidation does not have any significant effect on the overall conformation of the molecule. Hence, the loss of activity upon mild oxidation of exposed methionine residues is due to the modification of one (or more) specific methionine residues which are involved in the binding interaction between modulator and PDE. The fact that $^{45}Ca^{2+}$ binding to the oxidized modulator was not demonstrated by

gel filtration was due to the fact that the Ca^{2+} concentration in the incubation medium (8.75×10^{-5}M) was below the dissociation constant of oxidized modulator for Ca^{2+} (3.83×10^{-4}M); while this Ca^{2+} concentration was adequate to demonstrate binding of $^{45}Ca^{2+}$ to the native protein, it was too low to demonstrate such binding to the oxidized derivative.

In view of the fact that the Ca^{2+}-binding affinity of the oxidized modulator was so greatly reduced relative to that of the native protein, the possibility arose that the observed loss of activity upon oxidation was due to the presence of insufficient Ca^{2+} concentrations in the assay medium. This possibility was discounted by increasing the Ca^{2+} concentration in the modulator assay medium from 0.125 mM to 5.625 mM, a concentration well above that required to elicit the full conformational change in the oxidized modulator as evidenced by CD studies. The oxidized modulator was still ineffective in activating phosphodiesterase and did not compete with native modulator, giving results identical to those depicted in Fig. 35 in the competition assay. The possibility that high Ca^{2+} concentrations displaced essential Mg^{2+} ions from the phosphodiesterase was also discounted by raising the Mg^{2+} concentration in the assay medium in accord with the increase in Ca^{2+} concentration; furthermore, no effect on the basal activity of the phosphodiesterase was observed by increasing Ca^{2+} alone. The oxidized modulator, therefore, is inactive in stimulation of PDE purely because it no longer binds to the enzyme.

3.1.6.3 Interactions with troponin I

The results of CD studies of the interaction of native modulator with troponin I in the presence and absence of Ca^{2+} were summarized in IVC3 3.2.2. This section is concerned with similar studies involving the oxidized modulator.

An equimolar mixture of oxidized modulator and TN-I in the absence of Ca^{2+} (Fig. 41A) yielded a far-UV CD spectrum significantly different from the theoretical spectrum calculated on the assumption that no interaction occurs. Thus, the observed $[\theta]_{222\ nm}$ in the absence of Ca^{2+} was $-6,300 \pm 300$ deg cm^2 $dmole^{-1}$, compared with the calculated value of $-5,500 \pm 300$ deg cm^2 $dmole^{-1}$. Clearly, interaction occurs between the oxidized modulator and troponin I in the absence of Ca^{2+}, and this complex formation is accompanied by a net gain in apparent helix content. On the other hand, in the presence of Ca^{2+}, (Fig. 41B) the observed and calculated CD spectra are indistinguishable, indicating that the oxidized modulator does not interact with troponin I in the presence of Ca^{2+}. It is apparent from the electrophoretic studies in VIIIB3 3.1.4 that the oxidized modulator does not form a urea-stable complex with troponin I either in the presence or absence of Ca^{2+}. Hence, the complex formed between oxidized modulator and troponin I in the absence of Ca^{2+} (Fig. 41A) is dissociated in 6 M urea.

3.2 Oxidation in the absence of Ca^{2+}

3.2.1 Effect on PDE-stimulating activity

Fig. 42 shows that treatment of the protein modulator with NCS in the absence of Ca^{2+} results in a rapid loss of modulator activity with concomitant modification of methionine residues. Comparison with Fig. 33 indicates that both the rate of methionine oxidation and the rate of inactivation are more rapid in the absence than in the presence of modulator-bound Ca^{2+}; this presumably reflects the conformational change which occurs in the protein modulator as it binds Ca^{2+} ions. From the inset in Fig. 42, complete loss of activity in the absence of Ca^{2+} correlates with oxidation of 3 methionine residues per mole of modulator.

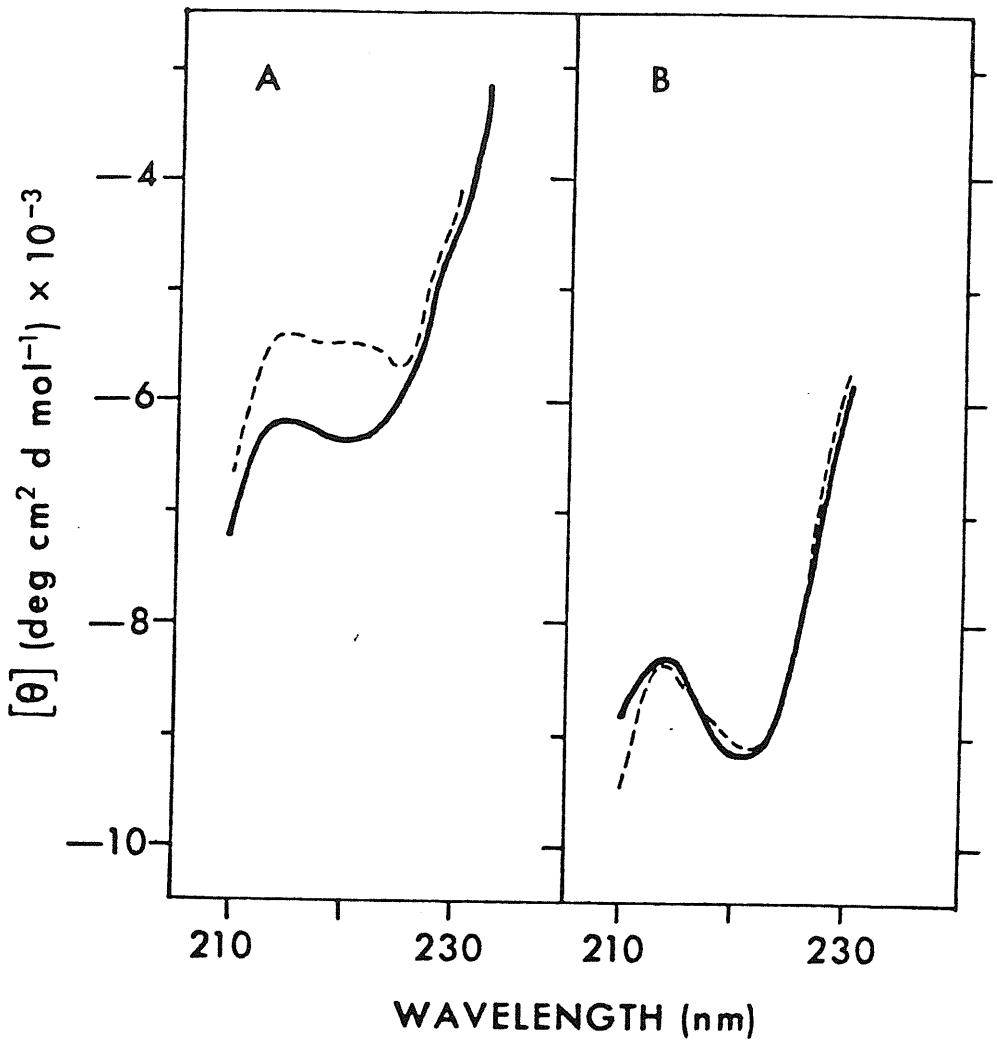


Figure 41 Calculated (----) and observed (——) far-UV CD spectra of an equimolar mixture of troponin I and oxidized protein modulator in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.5, without (A) or with (B) 4 mM $CaCl_2$.

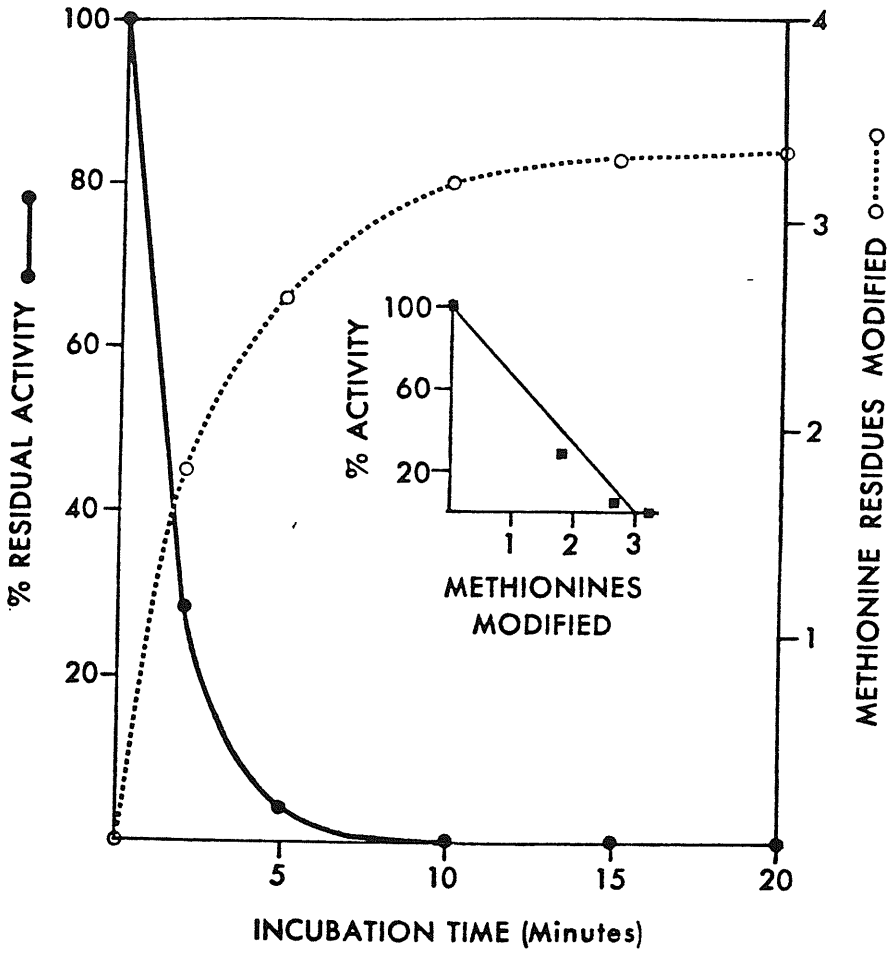


Figure 42

Time course of mild oxidation of the protein modulator with NCS in the absence of Ca^{2+}. The modulator (0.5 mg/ml) was treated with NCS (3 equivalents per methionine residue) at 23° C, pH 8.5, in the presence of 4 mM EGTA. Aliquots were withdrawn from the reaction mixture at the indicated times for assay of modulator activity (●——●) and determination of the extent of methionine modification (○- - -○) as described in "Experimental" VIIIB1. The inset (■——■) illustrates the relationship between loss of activity and modification of methionine residues.

A control modulator treated simultaneously in an identical manner, with the exception that NCS was omitted from the reaction mixture, retained full modulator activity and exhibited no modification of methionine residues during the course of the incubation period of 60 min.

3.2.2 PAGE

The oxidation was also monitored by discontinuous polyacrylamide slab gel electrophoresis in the presence of urea (not shown). At the time of complete loss of activity (10 min), two distinct bands were apparent on the gel, indicating that inactivation was not achieved by modification of 3 specific methionine residues, but rather that several methionines are susceptible to oxidation and, hence, are exposed on the outer surface of the molecule. This conclusion was substantiated by analysis of the cyanogen bromide peptides obtained from modulator incubated for different lengths of time with NCS in the absence of Ca^{2+} (Fig. 43). The pattern obtained is clearly more complex than that obtained in the presence of Ca^{2+} (Fig. 38), reflecting the accessibility of several methionine residues. For instance, after 10 min incubation with NCS, at which stage the modulator is inactive, approximately 10 peptide bands are apparent (slot 5), 5 of which co-migrate with peptides derived from the native protein (slots 1, 2 and 9). Furthermore, prolonged incubation with NCS results in further changes in the cyanogen bromide peptide pattern (slots 6-8), indicative of continued modification of methionine residues. This was confirmed by amino acid analysis: for example, after 60 min incubation with NCS, 5.2 methionine residues were modified.

3.2.3 Phosphodiesterase- and Ca^{2+}-binding properties of oxidized modulator

A sample of modulator inactivated by treatment

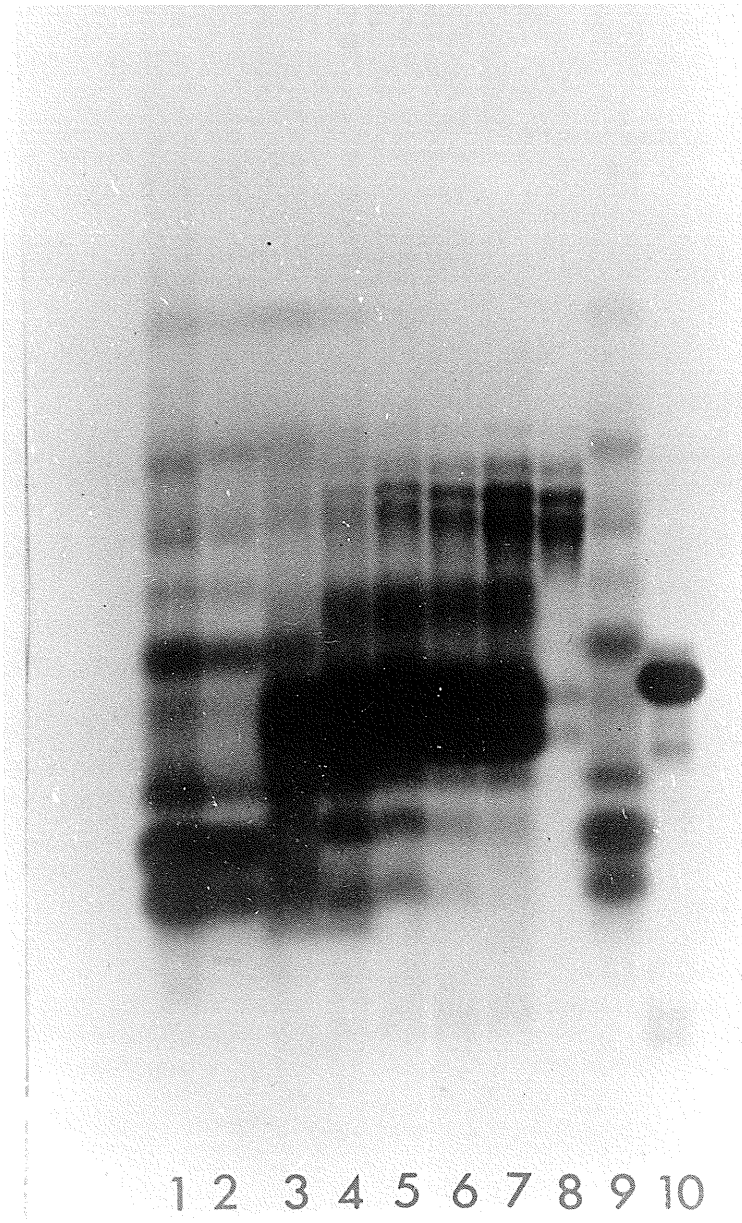


Figure 43

Monitoring of the mild oxidation reaction in the absence of Ca^{2+} (see Fig. 42) by electrophoretic analysis (15% PAGE) of cyanogen bromide digests. 1 and 9: digests of native modulator; 2-8: digests of modulator incubated with NCS for the following periods of time: 0, 2, 5, 10, 15, 20 and 60 min, respectively; 10: undigested native modulator. Approximately 100 μg of protein were applied to each of slots 1-9, and 50 μg to slot 10.

with NCS in the absence of Ca^{2+} was examined for its ability to bind to phosphodiesterase and to bind Ca^{2+} ions. Fig. 35 shows that this oxidized modulator has lost the ability to interact with phosphodiesterase. Ca^{2+}-binding ability was investigated by gel filtration technique employing $^{45}Ca^{2+}$. Results identical to those depicted in Fig. 36 were obtained, i.e., the oxidized modulator did not bind Ca^{2+} ions under the conditions used.

3.2.4 Comparison of cyanogen bromide peptides

The pattern of cyanogen bromide peptides obtained from oxidized modulator reflects the disposition of methionine residues in the molecule. Comparison by PAGE of the cyanogen bromide peptides obtained by digestion of the modulator oxidized in the presence and absence of Ca^{2+} will indicate whether or not the same methionine residues are accessible to oxidation in the presence and absence of Ca^{2+}. The results of such a comparative electrophoretic study are depicted in Fig. 44. The native modulator is represented in slots 1 and 6, and a cyanogen bromide digest of the native protein in slots 2 and 5. Slot 3 represents the cyanogen bromide digest of modulator oxidized in the presence of Ca^{2+}, and slot 4 a similar digest after oxidation in the absence of Ca^{2+}. Clearly, there is no similarity in the banding patterns seen in slots 3 and 4, indicating that the disposition of methionine residues in the molecule is markedly affected by the binding of Ca^{2+}. While 3 or 4 methionine residues are exposed on the surface of the modulator in the presence of Ca^{2+}, several methionine residues are exposed when Ca^{2+} is removed from the molecule; this is consistent with the observation that removal of Ca^{2+} ions is accompanied by a considerable loss of secondary structure. No attempt was made to identify which methionines are oxidized in the

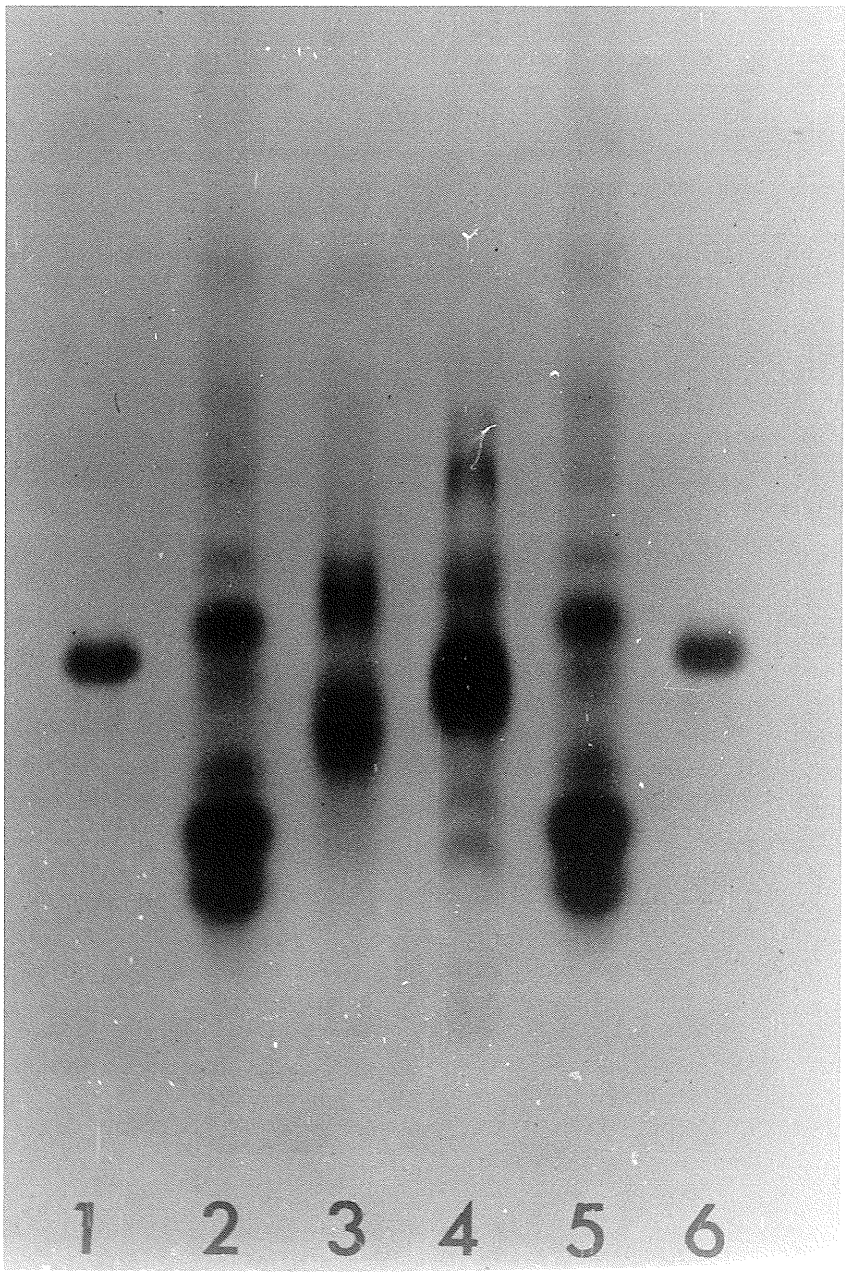


Figure 44

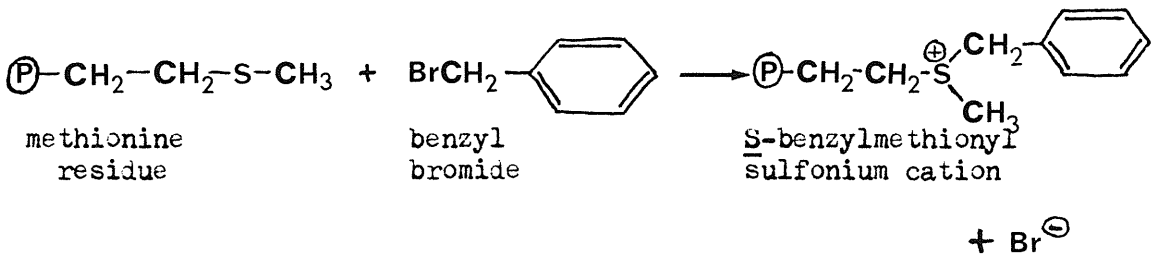
Comparison by 15% PAGE of cyanogen bromide peptides of modulator oxidized in the presence and absence of Ca^{2+} as described in the text. 1 and 6: undigested native modulator (10 μg); 2 and 5: cyanogen bromide digests (100 μg) of native modulator; 3: cyanogen bromide digest of modulator oxidized in the presence of Ca^{2+}; 4: cyanogen bromide digest of modulator oxidized in the absence of Ca^{2+}.

absence of Ca^{2+}.

C. Alkylation

1. Introduction

Rogers et al, (1976) demonstrated that, at pH values near neutrality, methionine and cysteine are alkylated by benzyl bromide considerably more quickly than any other amino acid; for example, the alkylation rates relative to methionine were found to be 200: ≤ 0.03 : ≤ 0.03 : ≤ 0.02 for unprotonated sulfhydryl, histidine, tryptophan, and protonated sulfhydryl, respectively. Consequently, in non-sulfhydryl proteins or at acidic pH, treatment with benzyl bromide is relatively specific for alkylation of methionine residues. Alkylation presumably occurs by a mechanism similar to that of carboxymethylation with iodoacetate:



methionine residue | benzyl bromide | S-benzylmethionyl sulfonium cation

As in the case of the corresponding carboxymethyl derivative, the S-benzylmethioninesulfonium salt is degraded to a number of products on acid hydrolysis. A suitable method for determining the extent of methionine alkylation in a protein treated with benzyl bromide involves performic acid oxidation of the modified protein, whereupon unmodified methionine residues are converted to methionine sulfone, whereas alkylated methionines

are unaffected. Methionine sulfone is then resistant to acid hydrolysis and is readily quantitated by amino acid analysis; it elutes from the amino acid analyzer immediately after aspartic acid. The colour value for methionine sulfone is the same as that for aspartic acid (Spackman et al, 1958).

It was also reported by Rogers et al, (1976) that alkylation of free methionine by benzyl bromide is more than 50 times faster than alkylation by iodoacetate; this difference in rate can be even greater for methionine residues in proteins. In view of the fact that carboxymethylation of the protein modulator was a very slow reaction, alkylation with benzyl bromide provided an alternative method for achieving the selective modification of methionine residues with the potential for more rapid reaction yielding more readily interpretable results.

2. Experimental procedure

2.1 Analytical scale

Alkylation of the modulator protein with benzyl bromide was performed essentially according to the method of Rogers et al, (1976). The modulator (1 mg/ml) was dissolved in 0.1 M PIPES, pH 6.8, containing either 4 mM $CaCl_2$ or 4 mM EGTA. Reaction was initiated by the addition of a freshly prepared concentrated solution of benzyl bromide (340 mM in Sequanal grade acetone) to a final concentration of 11.9 mM. The reaction mixture was incubated at 23° C. Aliquots were withdrawn at suitable time intervals and added to an equal volume of 50 mM methionine to quench the reaction. Part of the mixture was assayed for modulator activity and the remainder was dialyzed extensively against water to remove excess reagent and lyophilized prior to : (1) performic acid oxidation followed by acid hydrolysis and amino acid analysis, and (2) urea-PAGE.

2.2 Preparative scale

A sample of modulator protein (15 mg) was incubated with benzyl bromide in the presence of Ca^{2+} for 30 min as described above. The reaction was terminated by addition of an equal volume of 50 mM methionine and the mixture dialyzed extensively against water and lyophilized. The alkylated modulator so obtained was subjected to the following studies:

(1) analysis of troponin C-like activities,

(2) analysis of cyanogen bromide peptides by electrophoresis, and

(3) circular dichroism studies.

3. Results and Discussion

Studies of the effects of carboxymethylation of methionine residues (see VIIIA) were complicated by the slow rate of reaction. Rogers et al, (1976) pointed out that alkylation of methionine residues in proteins with benzyl bromide takes place much more rapidly than carboxymethylation with iodoacetic acid. Consequently, the effects of treatment of the protein modulator with benzyl bromide were investigated.

3.1 Effect on PDE-stimulating activity

Fig. 45 illustrates the effects of alkylation of the protein modulator with benzyl bromide in the presence of Ca^{2+}. The reaction occurs in two phases: an initial rapid loss of >90% of modulator activity, which occurs concomitant with the modification of approximately 3 methionine residues, followed by a slower second phase during which the remaining activity is lost with alkylation of a further methionine residue. The biphasic nature of the reaction is clearly seen in the inset in Fig. 45. A control modulator treated simultaneously in identical manner, with the exception that benzyl bromide was omitted from the reaction mixture, retained full modulator activity and exhibited no modification of

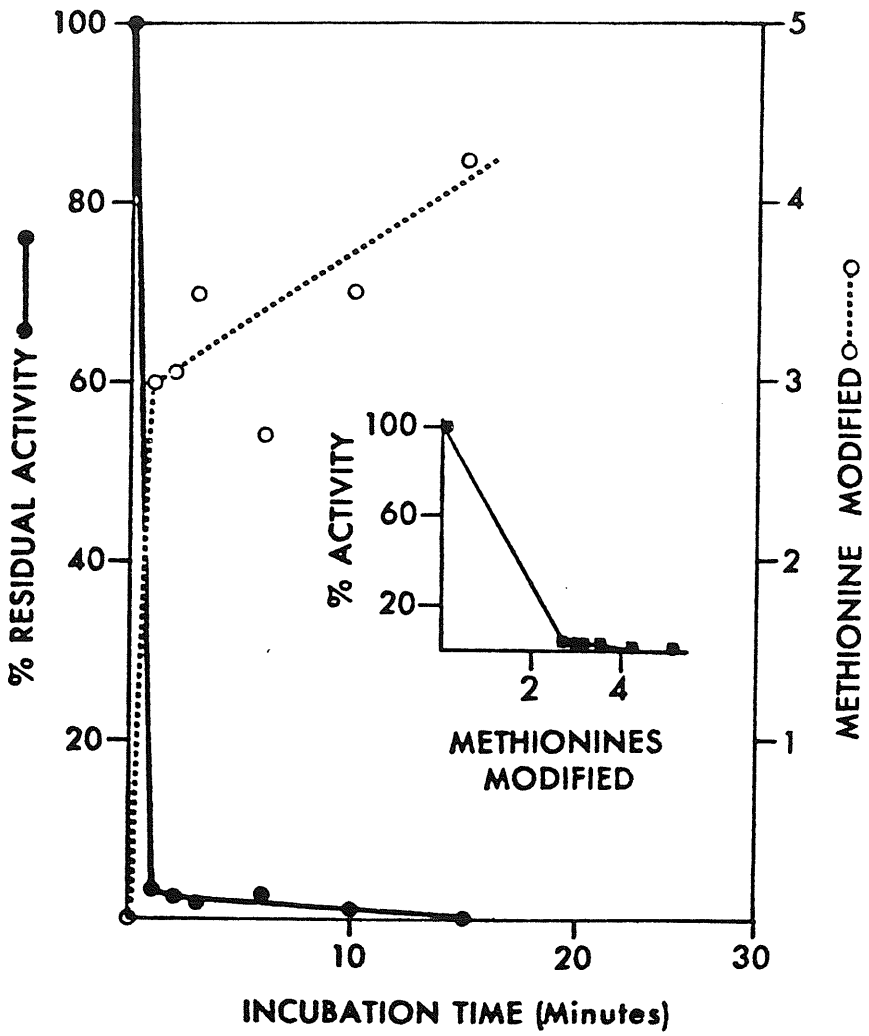


Figure 45

Time course of alkylation of the protein modulator with benzyl bromide in the presence of Ca^{2+}. The modulator (1 mg/ml) was treated with benzyl bromide (11.9 mM) at 23° C, pH 6.8, in the presence of 4 mM $CaCl_2$. Aliquots were withdrawn from the reaction mixture at the indicated times for assay of modulator activity (●——●) and determination of the extent of methionine modification (o --o) as described in "Experimental" VIIIC1. The inset (■——■) illustrates the relationship between loss of activity and modification of methionine residues.

methionine residues during the course of the incubation period of 30 min. Amino acid analyses revealed that no residues other than methionine were affected by the treatment with benzyl bromide, indicating that the modification is specific for methionine residues.

3.2 Urea-PAGE

The alkylation was also monitored by discontinuous polyacrylamide slab gel electrophoresis in the presence of urea, as shown in Fig. 46. The band corresponding to the native modulator (slot 1) disappears very rapidly and is replaced by 2 bands of slower mobility (slot 2). Little change in the electrophoretic pattern is apparent between 1 min (slot 2) and 15 min (slot 7) incubation with benzyl bromide. After 30 min incubation, however, a single band of yet slower mobility is observed; amino acid analysis indicated that 5.1 methionine residues were alkylated at this stage of the reaction. Alkylation with benzyl bromide converts uncharged methionine to the positively charged S-benzylmethioninesulfonium; hence, alkylated modulator species would be expected to migrate less rapidly toward the anode than does the native protein, as is indeed observed.

3.3 Phosphodiesterase-binding properties of alkylated modulator

In order to determine whether or not the alkylated modulator interacts with PDE, a competition assay with the native protein was utilized as described in VIIIB3 3.1.3 for oxidized modulator. Alkylated modulator protein was prepared as described under "Experimental procedure". To a constant amount of native modulator (sufficient to give ~50% maximal stimulation of PDE) were added increasing amounts of alkylated modulator and the effects on the stimulation of PDE activity obeerved by assaying modulator activity, as described in "General Experimental Procedures".

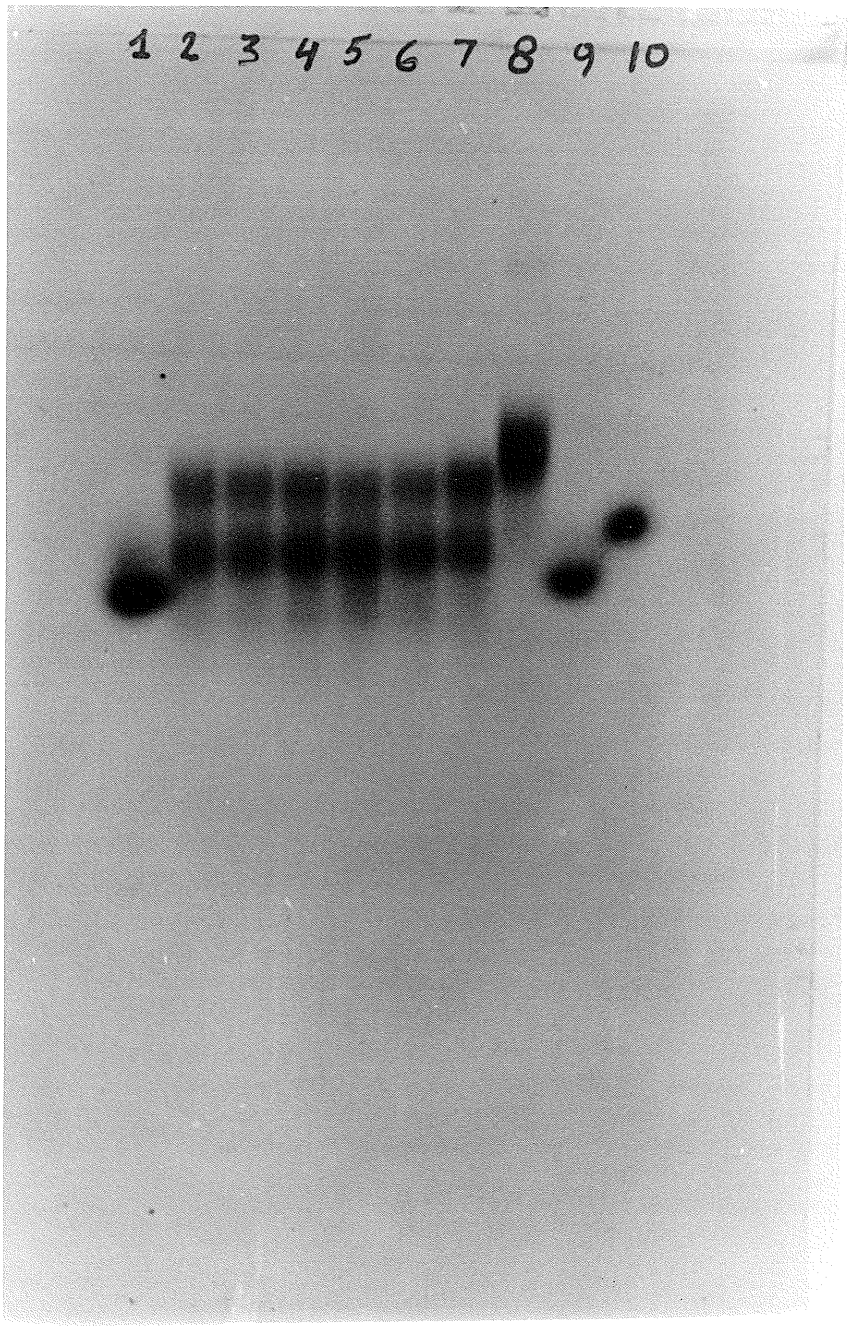


Figure 46

Monitoring of the alkylation reaction in the presence of Ca^{2+} (see Fig. 45) by 15% PAGE in the presence of 6 M urea according to the method of Davis (1964). The gel shows aliquots (20 µg) withdrawn from reaction mixture at the following times: 1: 0 min; 2: 1 min; 3: 2min; 4: 3 min; 5: 5 min; 6: 10 min; 7: 15 min; 8: 30 min. Native modulator (10 µg) in the presence of 4 mM $CaCl_2$ (slot 9) and 4 mM EGTA (slot 10) are also shown.

As shown in Fig. 47, increasing ratios of alkylated: native modulator have no effect on the stimulation of PDE by the standard amount of native protein, indicating that the alkylated modulator does not compete with the native protein for the modulator binding site on the PDE. Therefore, the alkylated, inactive modulator does not interact with PDE.

3.4 Effect on troponin C-like activities

The alkylated modulator was examined for a Ca^{2+}-dependent change in mobility on urea-PAGE and for Ca^{2+}-dependent interaction with troponin I in the presence of urea at the same time as similar studies were carried out with oxidized modulator (see VIIIB3 3.1.4). The results are depicted in Fig. 37. It is apparent from Fig. 37A that the mobility of the alkylated modulator (slots 5 and 6) is unaffected by Ca^{2+}. Furthermore, the alkylated modulator does not interact with troponin I in the presence of urea (Fig. 37B) either in the presence (slot 6) or absence (slot 7) of Ca^{2+}.

3.5 Circular dichroism studies

3.5.1 Effect of Ca^{2+} on CD spectra

The far-UV CD spectra of alkylated modulator in the presence and absence of Ca^{2+} (Fig. 48A) demonstrate a Ca^{2+}-induced conformational change in the molecule: in the presence of Ca^{2+}, helical content was calculated to be 34% with 14% β-sheet structure; removal of Ca^{2+} reduced these values to 20% and 10.5%, respectively. The conformation of the alkylated modulator in the presence of Ca^{2+} is significantly different from that of the native molecule (Fig. 19A), suggesting that the introduction of a number of bulky benzyl groups alters the conformation of the molecule. While this may be the cause of the loss of modulator activity upon alkylation, it does not rule out the possibility that the

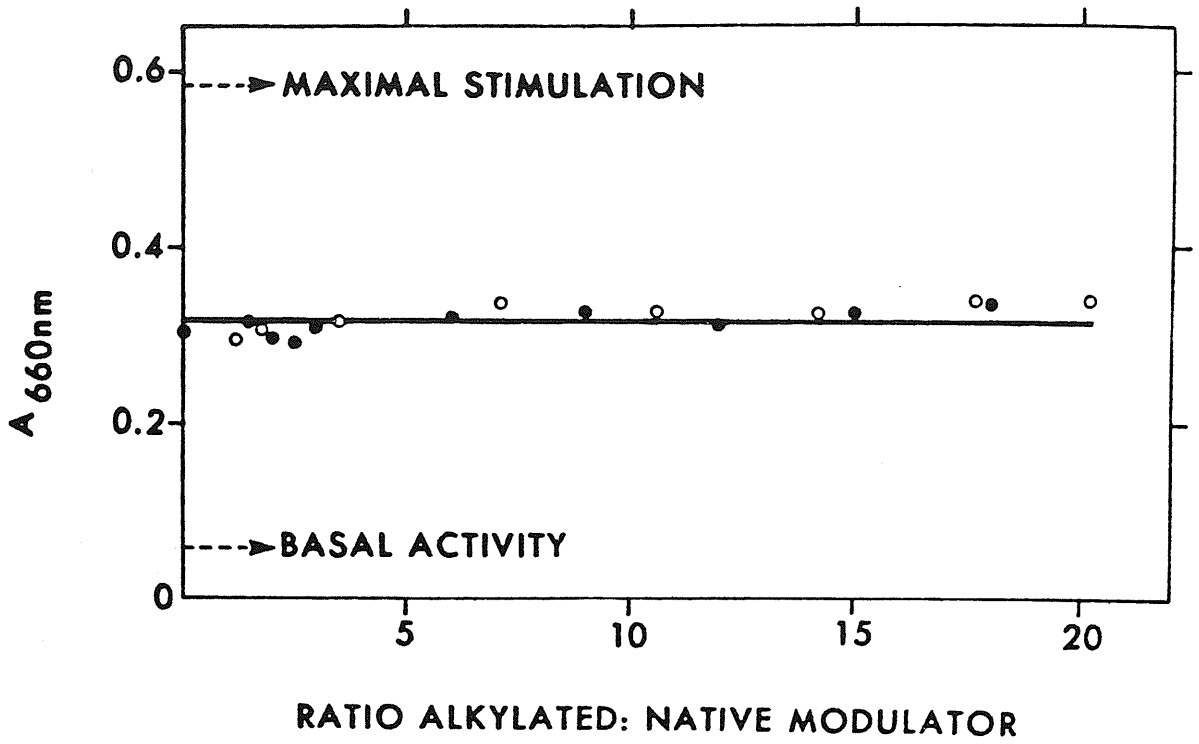


Figure 47

Assay of competitive binding of alkylated modulator and native modulator to PDE. Increasing amounts of modulator alkylated in the presence (●——●) and absence (○——○) of Ca^{2+} were assayed for modulator activity in the presence of a fixed amount (~1 unit) of native modulator protein as described in "General Experimental Procedures" IV.

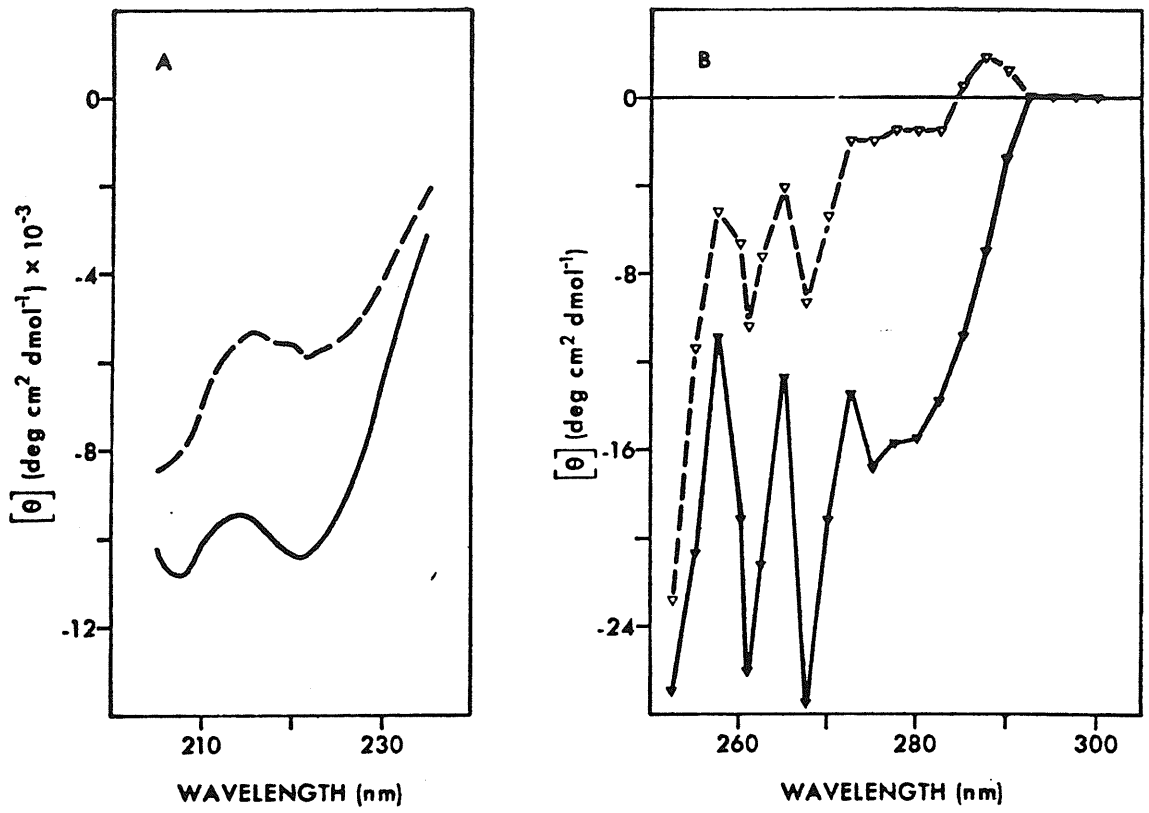


Figure 48

A: Far-UV CD spectra of alkylated modulator protein in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.6, without (- - - -) or with (———) 3 mM $CaCl_2$. Protein concentration was 1.41 mg/ml and the path length was 0.0501 cm.
B: Near-UV CD spectra of alkylated modulator protein in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.6, without (▽- -▽) or with (▼———▼) 3 mM $CaCl_2$. Protein concentration was 4.36 mg/ml and the path length was 1 cm.

observed loss of activity is due to alkylation of one or more methionine residues which are involved in the interaction between the protein modulator and phosphodiesterase.

The near-UV CD spectra (Fig. 48B) again show considerable sharpening of the phenylalanine bands at 261.5 nm and 267.5 nm and the appearance of a shallow trough around 280 nm (due to tyrosine) upon binding of Ca^{2+}. The spectrum obtained in the presence of Ca^{2+} closely resembles that of the native molecule in the presence of Ca^{2+} (Fig. 19B).

3.5.2 CD titration studies

The conformational change occurring as the alkylated modulator binds Ca^{2+} was examined in detail by investigating the effects of incremental addition of Ca^{2+} ions on the far-UV CD spectrum of the alkylated modulator, as was done previously with the native and oxidized modulator (see VIIIB3 3.1.6.2). Fig. 49 illustrates the CD Ca^{2+} titration curve obtained for the alkylated modulator. The solid curve is a best fit computer-calculated theoretical titration curve for the alkylated modulator calculated assuming the alkylated protein possesses two classes of Ca^{2+}-binding sites with apparent dissociation constants for Ca^{2+} of $7.50 \pm 5.73 \times 10^{-9}$M and $3.19 \pm 0.44 \times 10^{-4}$M. Again it is most unlikely that the alkylated modulator possesses a high affinity class of binding sites with $K_d = 7.50 \times 10^{-9}$M. Comparison with Fig. 40 indicates that the Ca^{2+}-binding properties of the alkylated modulator are very similar to those of the oxidized modulator, but quite distinct from those of the native protein.

As was observed previously in the case of the oxidized modulator (see VIIIB3 3.1.6.2) the observed loss of activity occurring upon alkylation with benzyl bromide, which is accompanied by a reduction in Ca^{2+} affinity

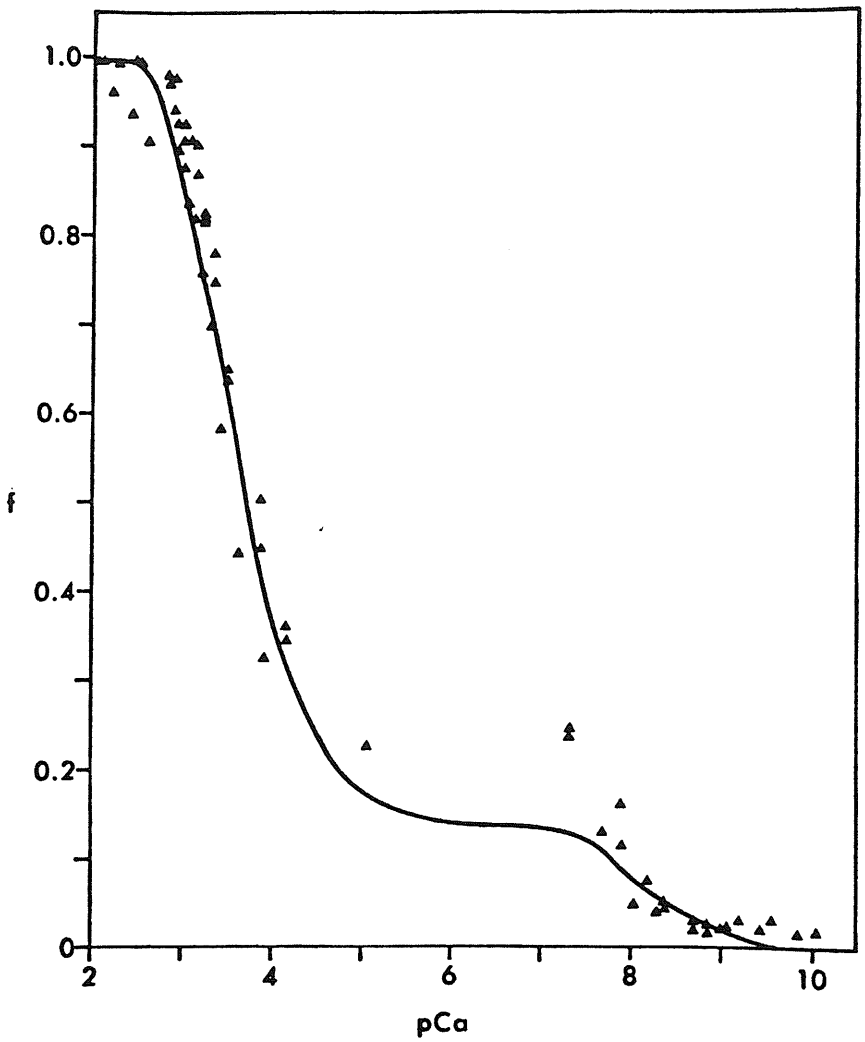


Figure 49

CD calcium titration of the conformational change in alkylated protein modulator. Protein was dissolved in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.47. $CaCl_2$ was added incrementally and $[\Theta]_{222\ nm}$ recorded after each addition (▲). f is the fraction of completion of the conformational change, and pCa = -log $[Ca^{2+}]$. The solid line represents a computer-calculated theoretical titration curve which best fits the data points.

by approximately 3 orders of magnitude relative to the native protein, may be due to the fact that insufficient Ca^{2+} concentration was maintained in the assay medium. Consequently, all experiments were repeated at high Ca^{2+} and also at high Ca^{2+} and Mg^{2+} concentrations to evaluate this possibility. Again, the alkylated modulator remained inactive and also failed to compete with the native protein in the competition assay, giving results identical to those depicted in Fig. 47.

These observations leave no doubt that the loss of modulator activity induced by alkylation is due to the fact that the modified protein no longer interacts with PDE. Whether the ability to interact with PDE is the result of blockage of a specific methionine residue(s) which is involved in the binding interaction, or is simply due to a conformational change in the molecule as a result of the introduction of a number of bulky substituents, is uncertain. The latter alternative cannot be ruled out since the far-UV CD spectrum of alkylated modulator in the presence of Ca^{2+} is significantly different from the corresponding spectrum of the native protein (compare Fig. 48A and Fig. 19A) and indicates a loss of secondary structure upon alkylation. Indeed, the alkylated modulator in the presence of Ca^{2+} exhibits less helical structure (34%) than the native protein in the absence of Ca^{2+}(40%).

3.5.3 Interactions with troponin I

Fig. 50 depicts the results of CD studies of interaction between alkylated modulator protein and troponin I. Clearly, both in the absence (A) and presence (B) of Ca^{2+}, alkylated modulator interacts with troponin I and complex formation is accompanied by a substantial conformational change with an increase in overall secondary structure. The observed $\left[\theta\right]_{222\ nm}$ in the absence of Ca^{2+} is -12,300 ± 300 deg cm^2 $dmole^{-1}$,

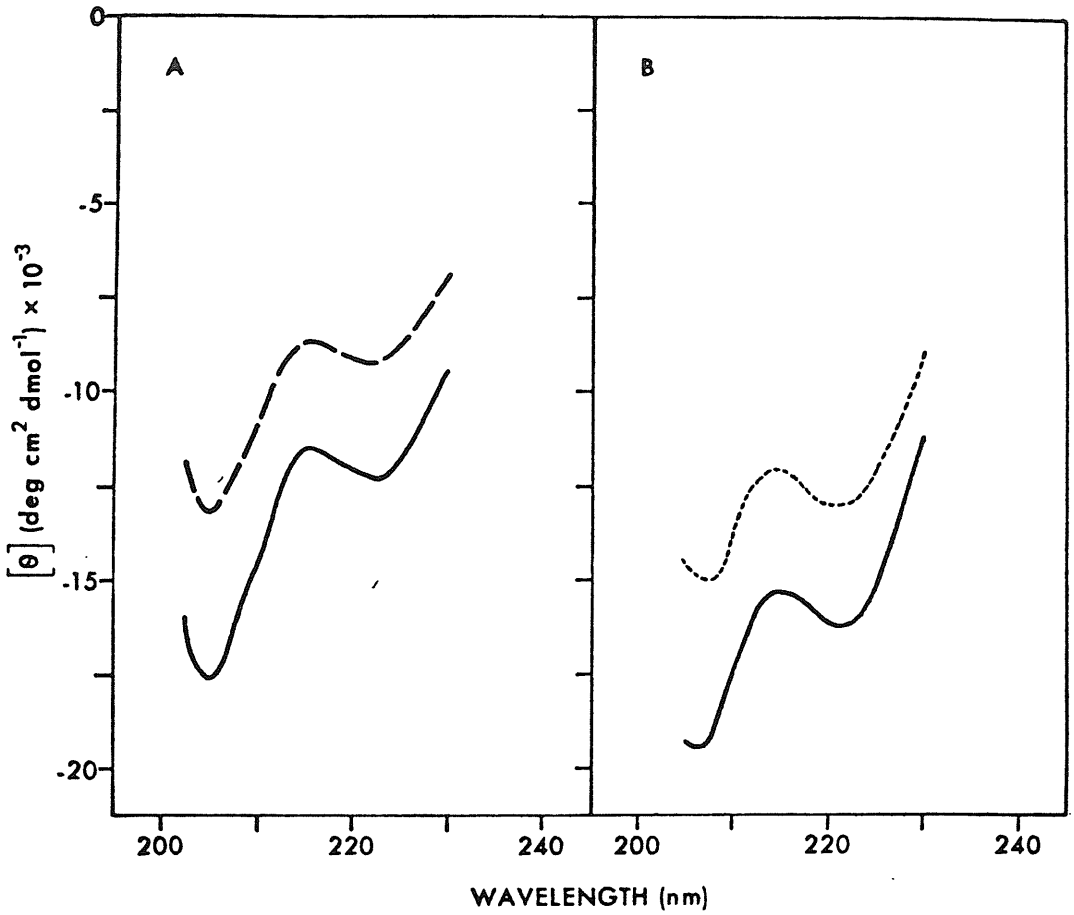


Figure 50

Calculated (- - -) and observed (——) far-UV CD spectra of an equimolar mixture of troponin I and alkylated modulator protein in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.5, without (A) or with (B) 4 mM $CaCl_2$.

while the calculated value is -9,200 ± 300 deg cm^2 $dmole^{-1}$; the observed and calculated values in the presence of Ca^{2+} are -16,300 deg cm^2 $dmole^{-1}$ and -13,000 deg cm^2 $dmole^{-1}$, respectively. Electrophoretic analysis (see VIIIC3 3.4) revealed that any complexes formed between alkylated modulator and troponin I are dissociated in 6 M urea.

3.6 Analysis of cyanogen bromide peptides

It was demonstrated by Spande et al, (1970) that methionylsulfonium salts are stable to cyanogen bromide cleavage. Therefore, cyanogen bromide will cleave an alkylated protein only at those peptide bonds involving an unmodified methionine residue. The locations of methionine alkylation can, therefore, be examined by electrophoretic analysis of the cyanogen bromide digest obtained from the alkylated protein. The results of such an analysis are shown in Fig. 51. The native modulator is represented in slot 1, and a cyanogen bromide digest of the native modulator in slot 2. Slot 3 represents a cyanogen bromide digest of the alkylated modulator. The multiple bands evident in the digest of the alkylated modulator (slot 3) are indicative of a complex pattern of modification of methionine residues. Some bands appear to co-migrate with peptides derived from the native protein, indicating the presence of methionine residues which are inaccessible to benzyl bromide in the native molecule. Amino acid analysis revealed that approximately 5 methionine residues were modified in the alkylated modulator (see VIIIC3 3.1). It is possible that this represents 5 specific methionine residues which are accessible to the modifying agent, but this cannot be stated unequivocally.

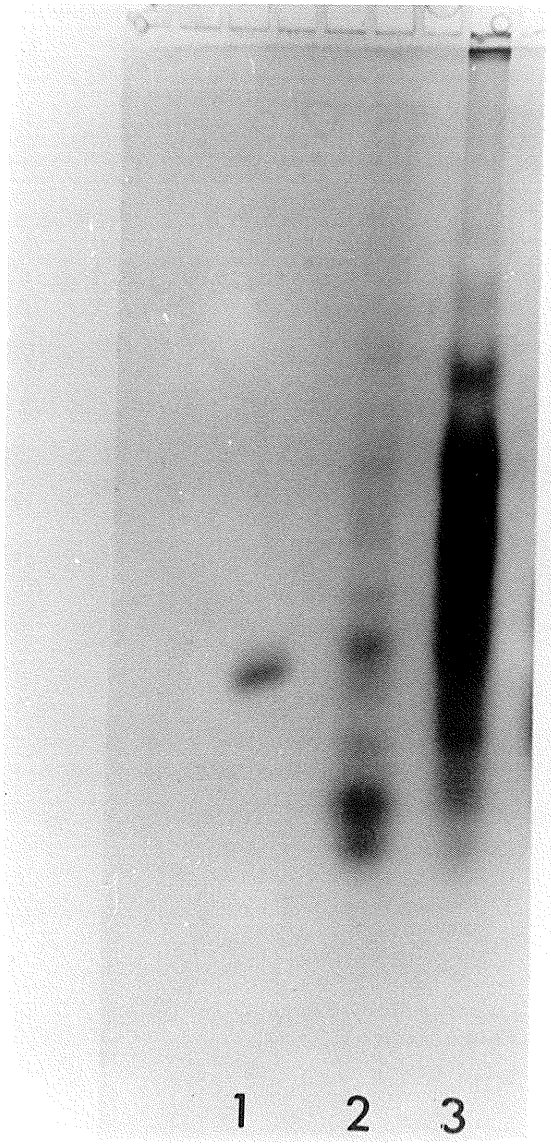


Figure 51

Analysis of cyanogen bromide peptides of alkylated modulator by 15% PAGE as described in the text. 1: undigested native modulator (10 µg); 2: cyanogen bromide digest of native modulator (100 µg); 3: cyanogen bromide digest of alkylated modulator (100 µg).

3.7 Alkylation in the absence of Ca^{2+}

3.7.1 Effect on PDE-stimulating activity

Fig. 52 shows that treatment of the protein modulator in the absence of Ca^{2+} with benzyl bromide results in a very rapid loss of >95% of modulator activity, followed by a slower loss of the remaining activity. This correlates with the very rapid modification of approximately 4 methionine residues per mole of modulator followed by a slower modification of a further 3 methionine residues. Comparison with Fig. 45, which depicts the effects of alkylation in the presence of Ca^{2+}, reveals that more methionine residues are accessible to the modifying agent in the absence of Ca^{2+}, once more presumably reflecting the conformational change which occurs in the protein modulator as it binds Ca^{2+}. A control modulator treated simultaneously in identical manner, with the exception that benzyl bromide was omitted from the reaction mixture, retained full modulator activity and exhibited no modification of methionine residues during the course of the incubation period of 30 min. Amino acid analyses again revealed that no residues other than methionine were affected by the treatment with benzyl bromide, indicating that the modification is specific for methionine residues.

3.7.2 Urea-PAGE

The alkylation in the absence of Ca^{2+} was also monitored by discontinuous polyacrylamide slab gel electrophoresis in the presence of 6 M urea. A pattern identical to that obtained as a result of alkylation in the presence of Ca^{2+} (Fig. 46) was observed (not shown). Thus, the band corresponding to the native modulator disappeared within 1 min of incubation with benzyl bromide in the absence of Ca^{2+} and was replaced by 2 bands of slower mobility. Little change in the

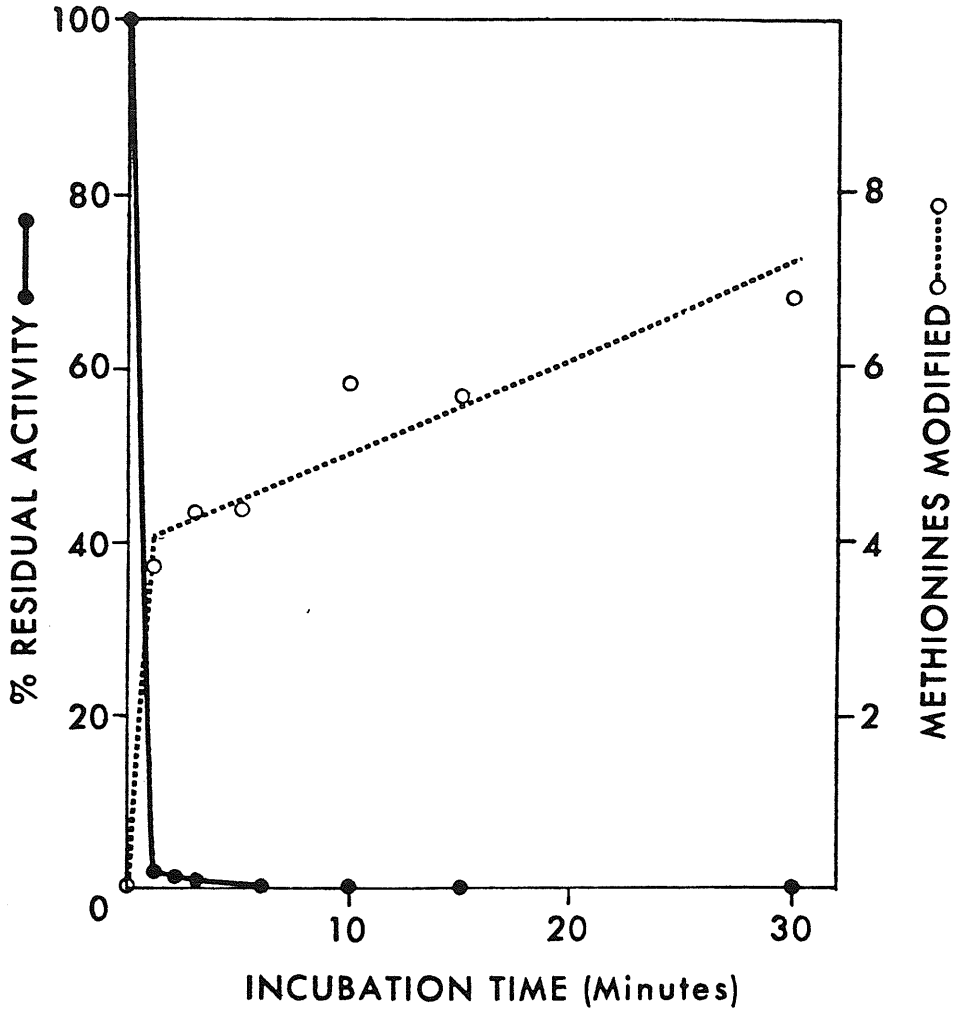


Figure 52

Time course of the alkylation of the protein modulator with benzyl bromide in the absence of Ca^{2+}. The modulator (1 mg/ml) was treated with benzyl bromide (11.9 mM) at 23° C, pH 6.8, in the presence of 4 mM EGTA. Aliquots were withdrawn from the reaction mixture at the indicated times for assay of modulator activity (●—●) and determination of the extent of methionine modification (o--o) as described in "Experimental" VIIIC.

electrophoretic pattern was observed between 1 and 15 min incubation with the alkylating agent, but after 30 min incubation a single species of yet slower mobility was apparent; amino acid analysis indicated that ~7 methionine residues were alkylated at this stage of the reaction.

3.7.3 Phosphodiesterase-binding properties

The competition assay with the native protein was utilized to determine whether or not modulator alkylated in the absence of Ca^{2+} retains the ability to interact with PDE (Fig. 47). Clearly, increasing ratios of alkylated: native modulator have no effect on the stimulation of PDE by the standard amount of native protein, indicating that the modulator alkylated in the absence of Ca^{2+} does not interact with PDE.

IX. Further CD studies of the native modulator protein

A. Effect of other divalent cations on the CD spectra of native modulator

Fig. 53 illustrates the effects of Mg^{2+} (A) and Mn^{2+} (B) on the far-UV CD spectra of the protein modulator. In the absence of divalent cations, negative maxima occur at 221 nm and 206.5 nm, with ellipticity values $[\theta]_{221\ nm}$ = -12,000 deg cm^2 $dmole^{-1}$ and $[\theta]_{206.5\ nm}$ = -12,800 deg cm^2 $dmole^{-1}$. Mg^{2+} (1.5 mM) induces a net gain in apparent helical content indicated by the more negative ellipticities, $[\theta]_{221\ nm}$ = -12,600 deg cm^2 $dmole^{-1}$ and $[\theta]_{206.5\ nm}$ = -13,200 deg cm^2 $dmole^{-1}$. This corresponds to a slight increase in helical content from 39.6% to 40.8%. Subsequent addition of excess Ca^{2+} (to a final concentration of 3 mM) elicited the full conformational change seen in Fig. 19A with Ca^{2+} alone, suggesting that Ca^{2+} displaces Mg^{2+} from the modulator.

The observed effect of Mn^{2+} on the conformation of the protein modulator (Fig. 53B) was similar to that of Mg^{2+}. The ellipticity values became slightly more negative, viz., $[\theta]_{221\ nm}$ = -12,400 deg cm^2 $dmole^{-1}$ and $[\theta]_{206.5\ nm}$ = -13,000 deg cm^2 $dmole^{-1}$, corresponding to a slight increase in helical content from 39.6% in the absence of divalent cations to 40.4% in the presence of Mn^{2+} (1.5 mM). Again, subsequent addition of Ca^{2+} to a concentration of 3 mM elicited the full conformational change see in Fig. 19A with Ca^{2+} alone, i.e., the helical content increased to 49%.

Wolff et al, (1977) observed much more pronounced effects of these divalent cations on the far-UV CD spectrum of the bovine brain modulator protein. They estimated the modulator to exhibit 28% helical content in the absence of divalent cations, increasing to 39% upon

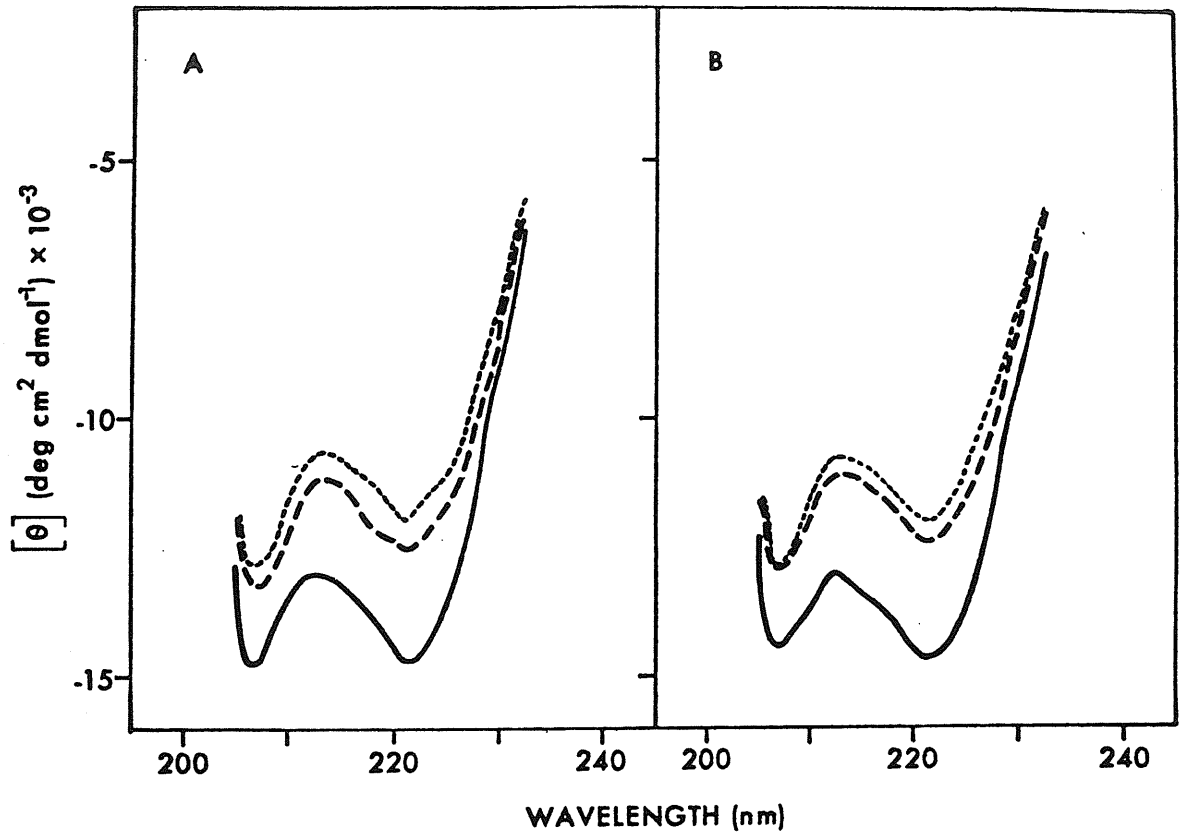


Figure 53

The effect of divalent cations on the far-UV CD spectra of the native protein modulator. Samples of the protein modulator (0.91 mg/ml) in 0.15 M KCl, 25 mM Tris, 2 mM EGTA, pH 7.5, were prepared and the spectra examined in a 0.0501 cm cell (------).
A: The effect of Mg^{2+} (1.5 mM). The CD spectrum was recorded after addition of Mg^{2+} to a final concentration of 1.5 mM (- - -). The CD spectrum was recorded again after addition of Ca^{2+} to a final concentration of 3 mM (——).
B: The effect of Mn^{2+} (1.5 mM). The CD spectrum was recorded after addition of Mn^{2+} to a final concentration of 1.5 mM (- - -) and again after addition of Ca^{2+} to a final concentration of 3 mM (——).

addition of Mg^{2+} and 40% upon addition of Mn^{2+}. Ca^{2+} binding resulted in an increase in helical content to 42%. The low helical content observed by Wolff et al, (1977) in the Ca^{2+}-free state may be due to partial denaturation of the preparation resulting in an exaggerated response to the addition of divalent cations. On the other hand, Kuo and Coffee (1976b) reported that addition of excess Mg^{2+} to Ca^{2+}-free modulator resulted in no detectable change in either the position or magnitude of the ellipticity bands in the far-UV CD spectrum. They may have been unable to detect a slight change in the spectrum similar to that seen in Fig. 53 due, perhaps, to slightly less precise measurements.

B. Effect of urea on the conformation of the modulator

It is well known that both troponin C (Perry et al, 1973) and the modulator protein (Amphlett et al, 1976) exhibit a Ca^{2+}-dependent change in electrophoretic mobility in the presence of urea, and several examples of the latter effect have been presented earlier. Thus, the mobility of either protein in the presence of 6 M urea is significantly greater in the presence of Ca^{2+} than in the absence of Ca^{2+}. This phenomenon may be due to stabilization of the conformation of the modulator by Ca^{2+} so that, in the presence of 6 M urea, the modulator remains compact and rigid, while removal of Ca^{2+} removes the stabilizing effect so that the molecule is completely unfolded in 6 M urea. Consequently, in view of the fact that high percentage acrylamide gels (commonly 15% polyacrylamide) are routinely used to monitor this Ca^{2+}-dependent change in mobility, the observed differences in mobility may be due to a sieving effect - the compact, rigid modulator in the presence of Ca^{2+} encounters relatively little resistance during its passage through the gel, whereas

the unfolded molecule in the absence of Ca^{2+} is significantly more retarded. In order to test this hypothesis, the effect of increasing concentrations of urea on the far-UV CD spectra of the native modulator in the presence and absence of Ca^{2+} was investigated (Fig. 54). Clearly, Ca^{2+} ions confer considerable stabilization against urea denaturation: at 6 M urea, the protein retains approximately 60% of the structural integrity which exists in the absence of urea. On the other hand, in the absence of Ca^{2+}, the molecule is essentially completely unfolded in the presence of 6 M urea.

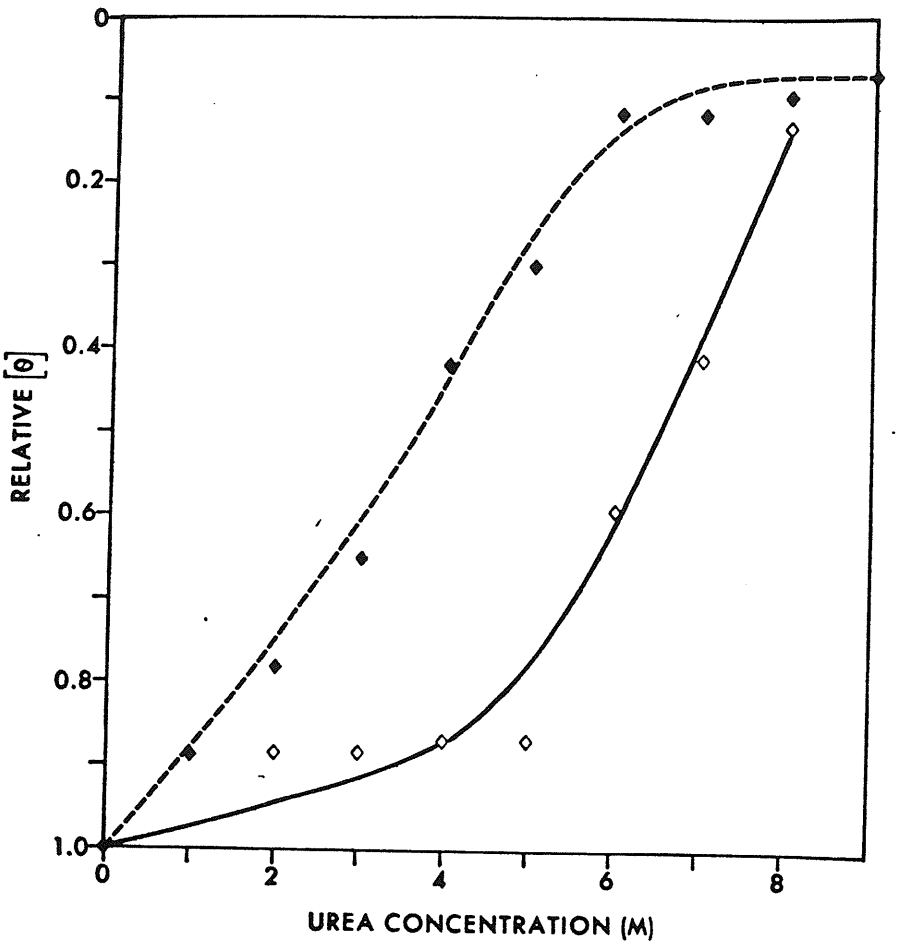


Figure 54 The effect of urea on the gross conformation of the native protein modulator. The modulator (0.541 mg/ml) was incubated with varying concentrations of urea in the presence (◇—◇) and absence (◆ -◆) of 3 mM $CaCl_2$ and ellipticity at 222 nm, $[\Theta]_{222\ nm}$, was recorded within 30 min. The change in conformation is expressed as relative $[\Theta]$, the overall change in $[\Theta]_{222\ nm}$ from zero to 9 M urea being assigned a value of 1. The theoretical value of $[\Theta]_{222\ nm}$ for a random coil is -1800 deg cm^2 $dmole^{-1}$ (Fasman, 1976) the experimentally observed $[\Theta]_{222\ nm}$ at 9 M urea was -1200 deg cm^2 $dmole^{-1}$. The path length was 0.0501 cm.

X. Controlled Enzymatic Digestion

A. Introduction

The fact that the Ca^{2+}-dependent protein modulator and troponin C share many common physical and chemical properties, and exhibit a considerable degree of sequence homology, suggests that the two calcium-binding proteins have similar three-dimensional structures. This possibility was substantiated by the observation that the modulator protein can substitute for troponin C in a reconstituted Ca^{2+}-sensitive actomyosin ATPase system (Amphlett et al, 1976; Dedman et al, 1977).

Another approach to assessing the similarities in the tertiary structures of the two proteins involves isolation and characterization of peptide fragments obtained by controlled proteolytic digestion of the two proteins. Thus, e.g., treatment of each protein with a limiting amount of trypsin would be expected to cleave each protein at a small number of accessible trypsin-sensitive peptide bonds. Isolation and characterization of the resultant tryptic fragments permits identification of the site(s) of cleavage in each molecule. Comparison of the relative locations of these cleavage points in the primary structures then sheds light on the similarity (or dissimilarity) of the tertiary structures of the proteins, i.e., if the two proteins exhibit very similar tertiary structures, one would expect the cleavage points to occur at homologous positions in the primary sequences when the proteins are aligned to give maximum sequence homology. Furthermore, study of the functional properties of the isolated peptide fragments furnishes information concerning the structural requirements for functional expression.

Since both troponin C and the protein modulator undergo substantial conformational changes upon removal of Ca^{2+}, controlled

proteolytic digestion both in the presence and absence of Ca^{2+} with isolation and characterization of the resultant peptide fragments may provide information concerning the nature of these conformational changes and also whether the conformational changes experienced by the two proteins are in any way similar.

The work described in this section was performed in collaboration with Dr. W. Drabikowski, Nencki Institute of Experimental Biology, Warsaw, Poland and has been published (Walsh et al, 1977).

B. Experimental procedure

1. Digestion with trypsin

The protein modulator (2 mg/ml) was incubated at 20° C in 20 mM ammonium bicarbonate, pH 8.2, in the presence of either 0.1 mM $CaCl_2$ or 1 mM EGTA and using an enzyme : substrate ratio of 1:60 (w/w) or 1:500 (w/w) respectively. Aliquots were withdrawn from the reaction mixture at suitable time intervals and the digestion stopped by the addition of soybean trypsin inhibitor (4 mg per mg of trypsin). The time course of digestion was monitored by electrophoresis on 8% polyacrylamide slab gels (60 mM glycine, 20 mM Tris, pH 8.5) containing 5 M urea and either 0.1 mM Ca^{2+} or 1 mM EGTA according to Davis (1964).

2. Isolation of peptide fragments

Peptide fragments obtained by controlled tryptic digestion were purified by preparative slab gel electrophoresis: the tryptic digests were subjected to electrophoresis on 8% polyacrylamide slab gels with 80 mM glycine, 20 mM Tris, pH 8.5, in the presence of 5 M urea and 1 mM $CaCl_2$. The areas of the gel occupied by peptides were cut out, the peptides were eluted, lyophilized, dissolved in a small volume and

subjected to another preparative gel electrophoresis as before with the exception that 2 mM EGTA replaced 1 mM $CaCl_2$. The peptide regions were again cut out of the gel, the peptides eluted, lyopholized, dissolved in a small volume and low molecular weight impurities originating from polyacrylamide were removed by gel filtration on Sephadex G-25. The purity of the isolated peptides was checked by urea-PAGE and SDS-PAGE.

C. Results

1. In the presence of Ca^{2+}

The time course of the controlled tryptic digestion of bovine brain protein modulator in the presence of Ca^{2+} is shown in Fig. 55A. After incubation for 1 h, the band representing the native protein has essentially completely disappeared and two major new peptide bands, named peptide I and peptide II, are visible in the gel. These two peptides were isolated and purified as described under "Experimental Procedure" and their amino acid compositions are given in Table X. Comparison of the amino acid compositions of these peptides with the amino acid sequence of the modulator protein (Vanaman et al, 1977; see Appendix II) indicates that peptide I represents residues 1-77, whereas peptide II accounts for residues 78-148; together these two peptides account for the entire protein. It had been shown earlier (Drabikowski et al, 1977b) that peptide II retains the ability of the native modulator to change its mobility on urea-PAGE as a function of Ca^{2+} concentration, and to interact with troponin I to form a urea-stable complex in the presence of Ca^{2+}; these properties were also exhibited by the carboxyl-terminal fragment obtained by controlled tryptic digestion of troponin C in the presence of Ca^{2+} (Drabikowski et al, 1977a,b).

TABLE X. Amino acid composition of the protein modulator and its tryptic fragments.[a]

	Protein Modulator[b]	Peptide I	Residues 1-77[b]	Peptide II	Residues 78-148[b]	Peptide E_1	Residues 1-106[b]	Peptide E_2	Residues 1-90[b]	Peptide E_3	Residues 107-148[b]
Lysine	8[c]	5.48	5	3.49	3[c]	6.36	6	5.52	5	1.73	2[c]
Histidine	1	0.41	0	1.42	1	-	0	-	0	0.77	1
Arginine	6	2.31	2	3.47	4	5.15	5	4.26	4	0.93	1
Aspartic acid	24	12.11	12	11.63	12	15.91	16	14.09	13	6.50	8
Threonine	12	7.23	8	3.88	4	8.44	9	8.63	9	2.78	3
Serine	4	2.31	2	2.18	2	4.01	4	3.57	3	0.54	0
Glutamic acid	26	12.25	12	14.46	14	18.22	18	17.86	17	8.85	8
Proline	2	2.11	2	-	0	trace	2	trace	2	-	0
Glycine	11	64.50[d]	8	7.04[d]	3	12.00[d]	8	13.06[d]	6	13.24[d]	3
Alanine	11	6.12	6	5.23	5	9.06	9	7.67	7	2.39	2
Valine	7	2.73	2	4.79	5	3.66	3	2.29	2	3.36	4
Methionine	9	1.86	5	5.09	4	5.07	5	5.08	5	4.08	4
Isoleucine	8	4.05	5	3.53	3	5.99	6	5.39	5	2.02	2
Leucine	9	5.91	5	3.19	4	7.18	7	6.54	6	2.15	2
Tyrosine	2	0.40	0	1.86	2	1.04	1	-	0	0.95	1
Phenylalanine	8	4.76	5	2.76	3	6.98	7	5.99	6	0.98	1

[a] The analyses were carried out as described in "General Experimental Procecures". The results are expressed as moles/mole and have not been corrected for destruction of residues during acid hydrolysis.

[b] Values taken from the amino acid sequence as determined by Vanaman et al, (1977). Protein Modulator is devoid of cysteine and tryptophan.

[c] These values include one residue of trimethyllysine, which under normal amino analysis conditions elutes together with lysine.

[d] In all of the peptides isolated the glycine value was abnormally high; this can be ascribed to the fact that the peptides were isolated by electrophoresis in the presence of Tris-glycine buffer.

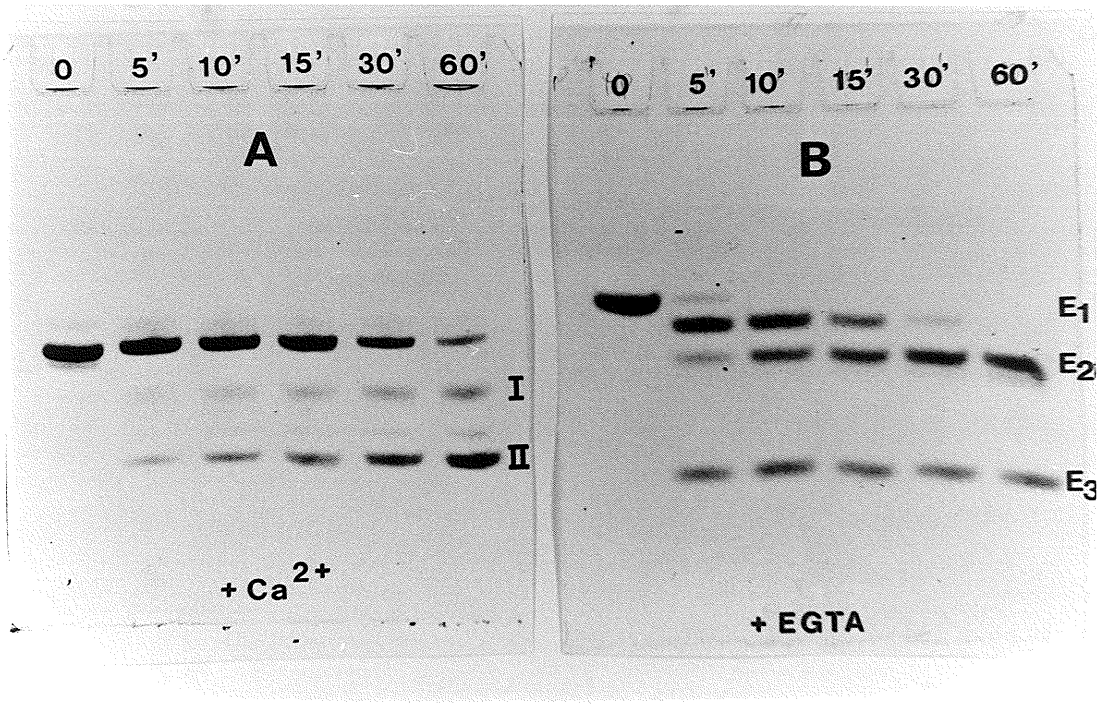


Figure 55

Time course of tryptic digestion of protein modulator in the presence of either Ca^{2+} or EGTA as described under "Experimental procedure". The time of digestion in minutes is indicated on the abscissa.
A: digestion and electrophoresis in the presence of 0.1 mM Ca^{2+}; trypsin added at a ratio of 1:60 (w/w).
B: digestion and electrophoresis in the presence of 1 mM EGTA; trypsin added at a ratio of 1:500 (w/w).

Fig. 56 shows that both peptide I and peptide II are essentially devoid of PDE-stimulating activity; peptide II appears slightly more potent than peptide I, but it can be calculated from the half-maximal stimulation figure that on a molar basis, 850 times more of peptide II than of native modulator would be required to give the same amount of stimulation. When a mixture of equimolar amounts of peptides I and II was assayed for PDE-stimulatory activity, no stimulation over and above that due to the additive effect of the two peptides was apparent.

2. In the presence of EGTA

When the protein modulator was treated in the presence of EGTA with trypsin at the same trypsin to substrate ratio, i.e., 1:60 (w/w), used in the presence of Ca^{2+}, the protein was rapidly broken down to small peptides, consistent with the fact that removal of Ca^{2+} resulted in a substantial loss of secondary structure of the modulator. However, as seen in Fig. 55B, distinct large peptide fragments can be obtained in the presence of EGTA using a trypsin to modulator ratio of 1:500 (w/w). It appears that the native molecule is first cleaved to yield peptides E_1 and E_3 and that E_1 is then further degraded to give peptide E_2 since, as a function of time, band E_1 gets weaker as band E_2 gets stronger. Peptides E_1, E_2, and E_3 were purified by preparative electrophoresis and their amino acid compositions were determined and compared with the amino acid sequence of the modulator protein (Vanaman et al, 1977); the results are depicted in Table X. Peptide E_1 represents residues 1-106, E_2 residues 1-90, and E_3 residues 107-148. Thus, E_1 and E_3 account for the entire protein and the initial peptide bond cleavage is at position 106; upon further incubation, a further 16 residues are removed from peptide E_1 (1-106) to give peptide E_2 (1-90). Peptide E_1 (1-106) exhibits a

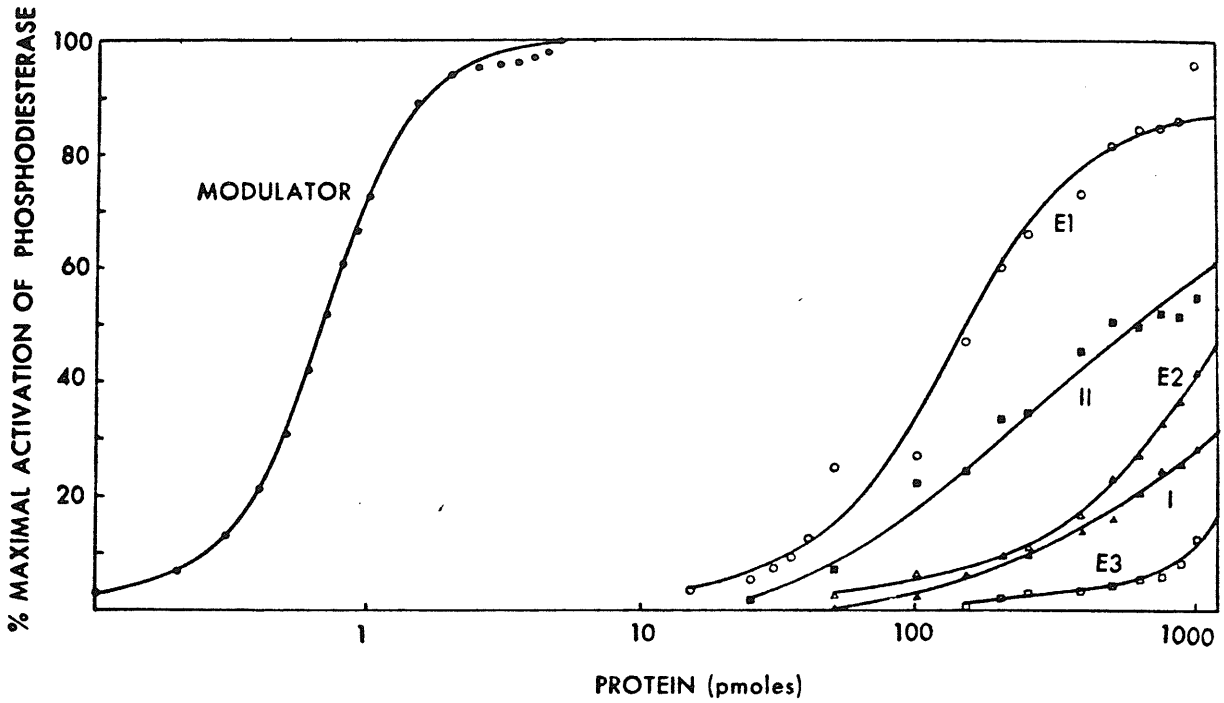


Figure 56

Activation of PDE by brain modulator protein and its tryptic fragments. Assays were performed as described in "General Experimental Procedures" IV; the amount of protein or peptide added to a given amount of modulator-deficient PDE (0.4 to 0.5 units) is plotted on the abscissa on a logarithmic scale. ●, native modulator protein; ○, peptide E_1; ■, peptide II; △, peptide E_2; ▲, peptide I; □, peptide E_3.

Ca^{2+}-dependent change in mobility upon urea-PAGE, and appears to be able to interact with troponin I. Peptide E_2 (1-90) shows only a weak mobility change and does not interact with troponin I.

Fig. 56 shows that peptides E_2 and E_3 are devoid of PDE-stimulatory activity, but that peptide E_1 (1-106) appears to have considerable residual activity; based on the half-maximal stimulation figure, one would need, on a molar basis, 220 times as much of E_1 as of native modulator to give identical stimulation. Addition of equimolar amounts of E_1 and E_3 (which account for the entire protein) to the assay mixture does not result in any stimulation over and above that brought about by E_1 alone.

D. Discussion

Fig. 57 compares the amino acid sequences of rabbit skeletal muscle troponin C and bovine brain modulator protein in the region of the tryptic cleavage points in the presence and absence of Ca^{2+}.[a] The tryptic cleavage points in troponin C are known to occur at residues 8, 84 and 88 in the presence of Ca^{2+} (Drabikowski et al, 1977a). The modulator protein is 7 residues shorter at the amino-terminal end and there is no trypsin-sensitive peptide bond at the position homologous to position 8 in troponin C. The main tryptic cleavage in the protein modulator in the presence of Ca^{2+} takes place at the Lys-Asp peptide bond

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[a] Determination of the tryptic cleavage points in troponin C was performed by Dr. Z. Grabarek in collaboration with Dr. Ptitsyn and Dr. Vinokurov at the Institute for Protein Research, Poustchino, U.S.S.R. (unpublished results).

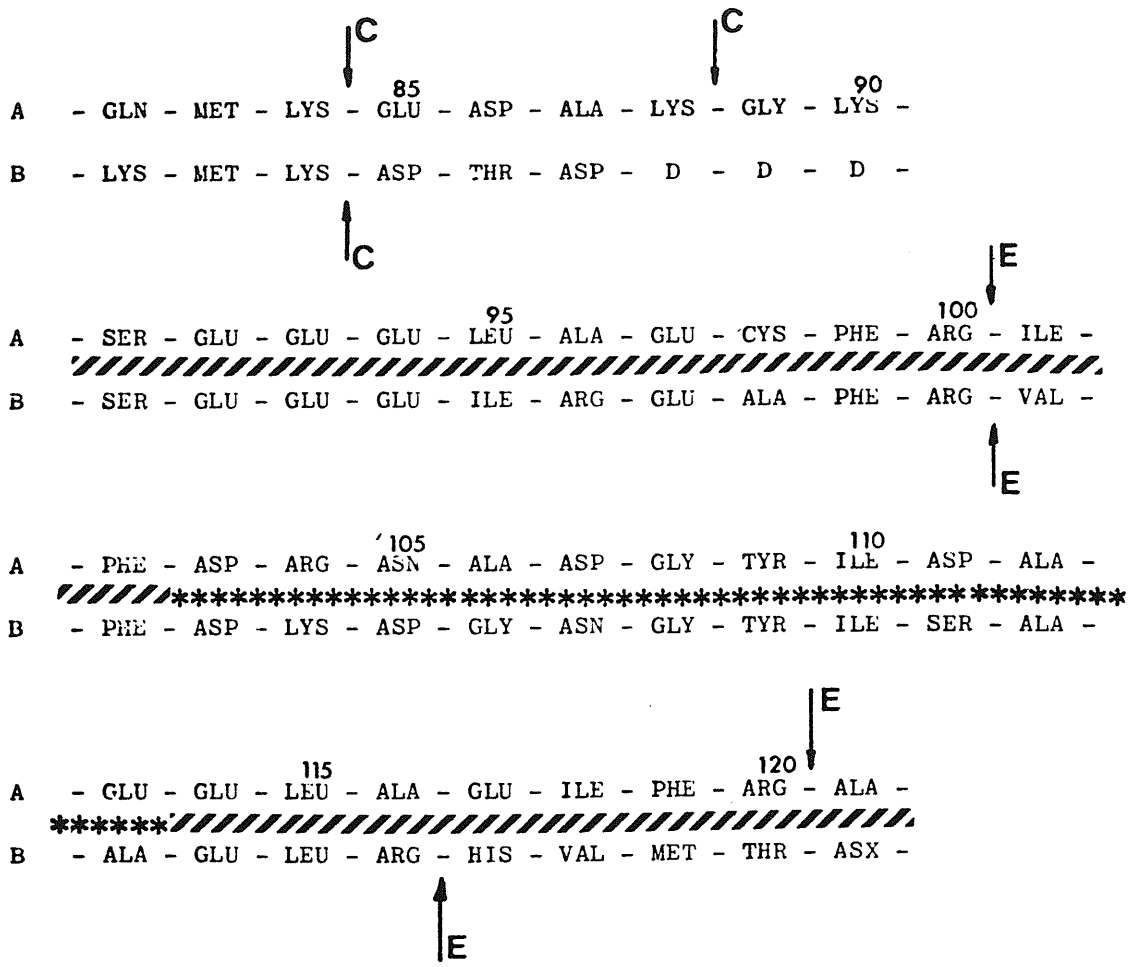


Figure 57

Amino acid sequences of bovine brain modulator protein and rabbit skeletal muscle troponin C in the region of the tryptic cleavages.
A: rabbit skeletal muscle troponin C as determined by Collins et al, (1973).
B: bovine brain modulator protein as determined by Vanaman et al, (1977).
The numbering is that for troponin C. Peptide bond cleavage points in the presence of Ca^{2+} are indicated by ↑C and those in the presence of EGTA by ↑E. **** indicates the E and F helical regions of the proposed third calcium-binding region, whereas the ////// indicate the EF Ca^{2+}-binding loop.

homologous to the Lys^{84}-Glu^{85} peptide bond in troponin C; further cleavage at a position homologous to Lys^{88} in troponin C is not possible since there is a deletion of 3 residues in that region of the modulator sequence.

In the absence of Ca^{2+}, the first tryptic cleavage in the modulator occurs at an Arg-His peptide bond and the second at an Arg-Val bond; in troponin C, the homologous Arg^{100}-Ile^{101} bond is also cleaved under similar conditions. Since, in troponin C, the position comparable to the Arg-His peptide bond in the modulator is occupied by Ala^{116}-Glu^{117}, the homologous peptide bond is thus not trypsin-sensitive; instead the peptide bond Arg^{120}-Ala^{121} is cleaved.

As discussed earlier (see "Literature Review"), Kretsinger and Barry (1975) predicted the three-dimensional structure of troponin C based on the known tertiary structure of parvalbumin and by analogy with the calcium-binding sites of parvalbumin. The predicted troponin C structure consists of 4 calcium-binding regions arranged in two pairs. Each calcium-binding region consists of a so-called "EF hand" made up of the E helix, the EF calcium-binding loop and the F helix. Because of the extensive sequence homology between protein modulator and troponin C, it is reasonable to apply the same model to the modulator. It can be seen from Fig. 57 that, in both troponin C and the protein modulator, tryptic cleavage in the presence of Ca^{2+} occurs in the short sequence connecting the second and third calcium-binding regions. On the other hand, in the absence of Ca^{2+}, cleavage in both troponin C and the modulator occurs preferentially in the sequences making up the E and F helices of the third calcium-binding region. These results confirm that both troponin C and the modulator protein undergo substantial and similar

conformational changes as a result of calcium binding. In the presence of Ca^{2+}, only regions of sequence not involved in calcium binding are susceptible to tryptic attack, whereas the reverse is true in the absence of Ca^{2+}.

The only peptides which retain the ability of the native modulator to interact with troponin I are peptide II (78-148), containing the third and fourth calcium-binding regions, and peptide E_1 (1-106) which extends from the amino-terminus through the first half of the F helix of the third calcium binding site. Leavis et al, (1977) reported that the region of the third calcium binding site in troponin C is the site of interaction with troponin I. The homology with the protein modulator is again striking. Peptide E_1 (1-106) is the only peptide which retains some, albeit very low, modulator activity; it does not contain the sole residue of trimethyllysine known to occur at position 115 (corresponding to position 125 in skeletal muscle troponin C occupied by a histidine residue) of bovine brain modulator protein (Watterson et al, 1976; Vanaman et al, 1977). It is interesting to note that peptide E_2 (1-90), which is only 16 residues shorter than E_1 (1-106) but lacks the third calcium-binding region, has completely lost the ability both to stimulate PDE and to interact with troponin I.

CONCLUDING REMARKS

The effects of the various chemical modifications on the PDE stimulating activity of the protein modulator are summarized in Table XI. Particular chemically modified derivatives of the modulator were examined in more detail and some of the properties of these derivatives are summarized in Table XII.

The conclusions derived from the studies of the Ca^{2+}-dependent protein modulator are most conveniently summarized in relation to the predicted structure of the molecule. Fig. 58 represents the detailed secondary structure of the protein modulator based on the parameters of Chou and Fasman (1974a,b) used in predicting the secondary structure of a protein from the amino acid sequence. This schematic representation, and those shown in Figs. 59 and 60, were kindly provided by Dr. C.M. Kay of the University of Alberta. These structures were designed on the basis of 11 amino acids per 3 turns of helix. By analogy with the predicted three-dimensional structure of rabbit skeletal muscle troponin C (Kretsinger and Barry, 1975) there is a distance of approximately 11Å between Ca^{2+}-binding sites I and II on the one hand, and III and IV on the other; there is also a distance of ~30Å between Ca^{2+}-binding sites I and IV.

There is a considerable degree of secondary structure apparent in the molecule, consistent with the observations from circular dichroism and other studies. The molecule exhibits 51% helical and 8% β-sheet structure based on the Chou-Fasman parameters, in close agreement with the values of 49% and 10%, respectively, obtained by CD spectrophotometric analysis of the Ca^{2+}-modulator complex. The Chou-Fasman rules for

TABLE XI. Summary of chemical modification studies

Modified function	No. of residues in native protein	No. of residues modified		Residual PDE activity (%)		Effect of Ca^{2+} on rate[a]
		$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	
His	1	1	1	100	100	+
Tyr	2	2	1.5	100	100	+
Arg	6	4	4	100	100	0
Carboxyl	40	2		90		-
		22	22	0	0	
Lys (carbamoylation)	8	2-3	4	45	35	-
		3-4	6	35	20	
Lys (guanidination)	8	2	2	80	40	-
		3	2-3	75	0	
Met (oxidation)	9	4	3	0	0	-
Met (carboxymethylation)	9	2	1	34	50	+
		6		0		
Met (alkylation)	9	3	4	3	3	0
		4	5	0	0	

[a] In the case of his, tyr and arg modifications rate refers to the rate of modification of those residues. In the other cases rate refers to the rate at which modulator activity is lost. A positive sign (+) indicates that the reaction or loss of activity is faster in the presence of Ca^{2+}, and a negative sign (-) indicates the loss of activity is slower in the presence of Ca^{2+}.

TABLE XII. Summary of properties of selected chemically modified derivatives of the protein modulator

Modulator derivative[a]	Helical content (%)		β-sheet content (%)		TN-I interaction[b]		Effect on TN-C activities		K_d for Ca^{2+} (M)
	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	change in mobility[c]	TN-I interaction[d]	
Native	49	40	10	11	-	-	+	+	1.89×10^{-7} 4.03×10^{-4}
Nitrated	46	38	14	11	+	-	+	+	
Oxidized	50	26	13	10	-	+	-	-	4.25×10^{-10} 3.83×10^{-4}
Alkylated	34	20	14	10.5	+	+	-	-	7.50×10^{-9} 3.19×10^{-4}

[a] Each derivative was formed in the presence of Ca^{2+}.

[b] Determined under non-denaturing conditions by CD.

[c] Refers to a Ca^{2+}-dependent change in electrophoretic mobility on urea-PAGE.

[d] Determined by PAGE in the presence of 6 M urea.

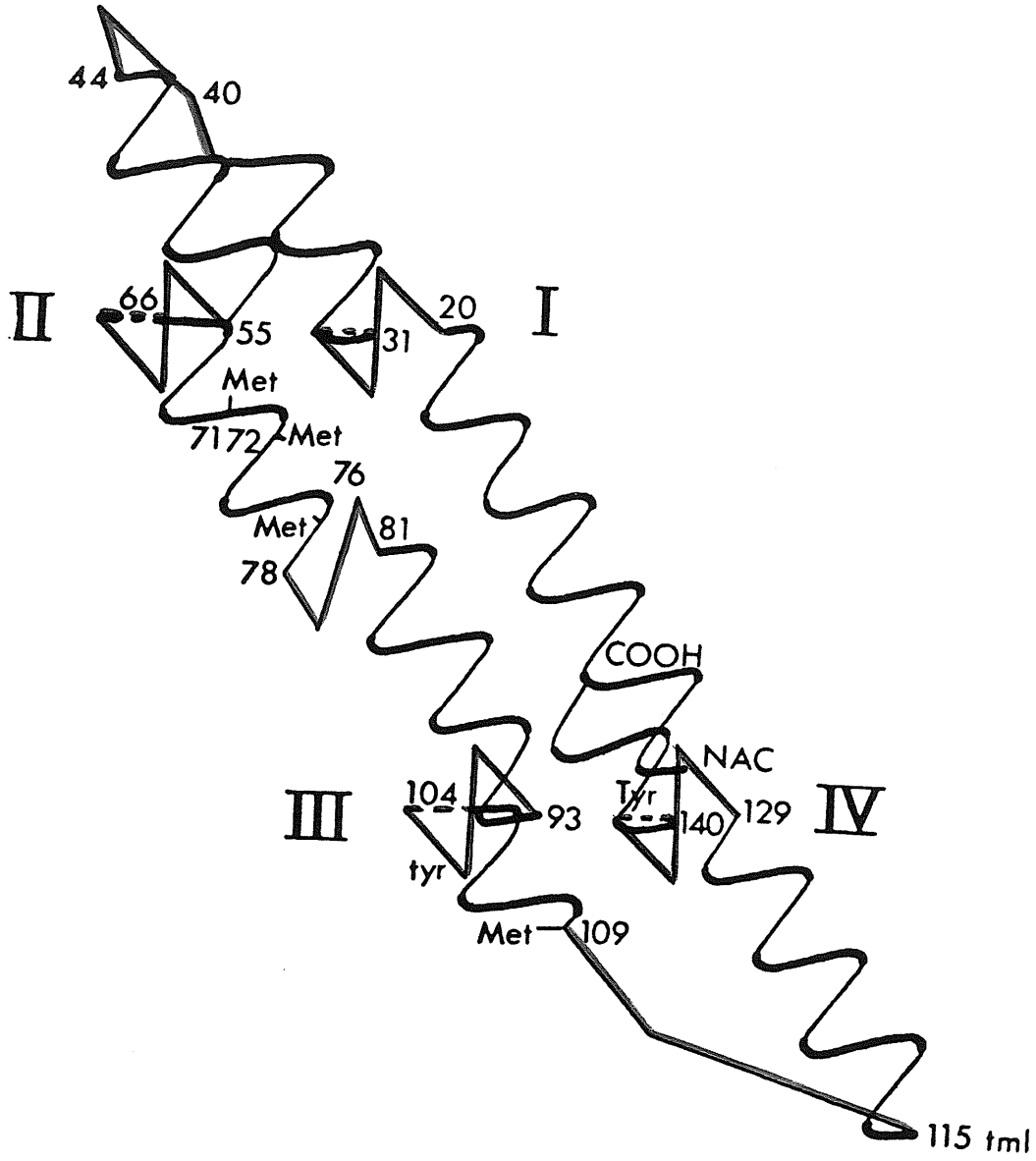


Figure 58

Schematic representation of the secondary structure of the bovine brain protein modulator as predicted from the amino acid sequence (Vanaman et al, 1977) by the method of Chou and Fasman (1974a, b). Presumed Ca^{2+}-binding sites are numbered I to IV and represented in blue. Other regions of β-turns are depicted in red. Regions of predominantly helical structure are depicted in black. The locations of certain key residues are noted.

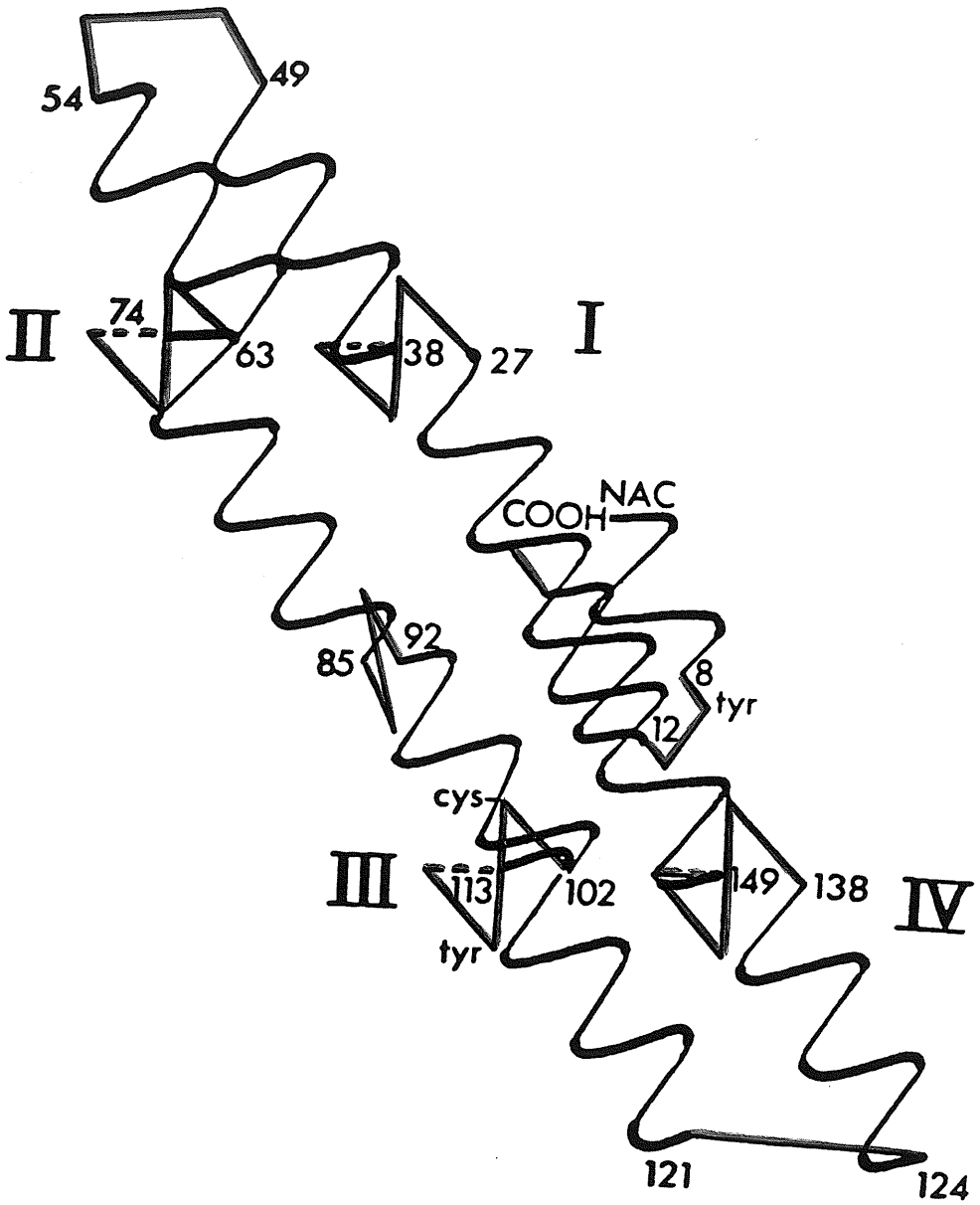


Figure 59 Schematic representation of the secondary structure of rabbit skeletal muscle troponin C as predicted from the amino acid sequence (Collins et al, 1973, 1977) by the method of Chou and Fasman (1974a,b).

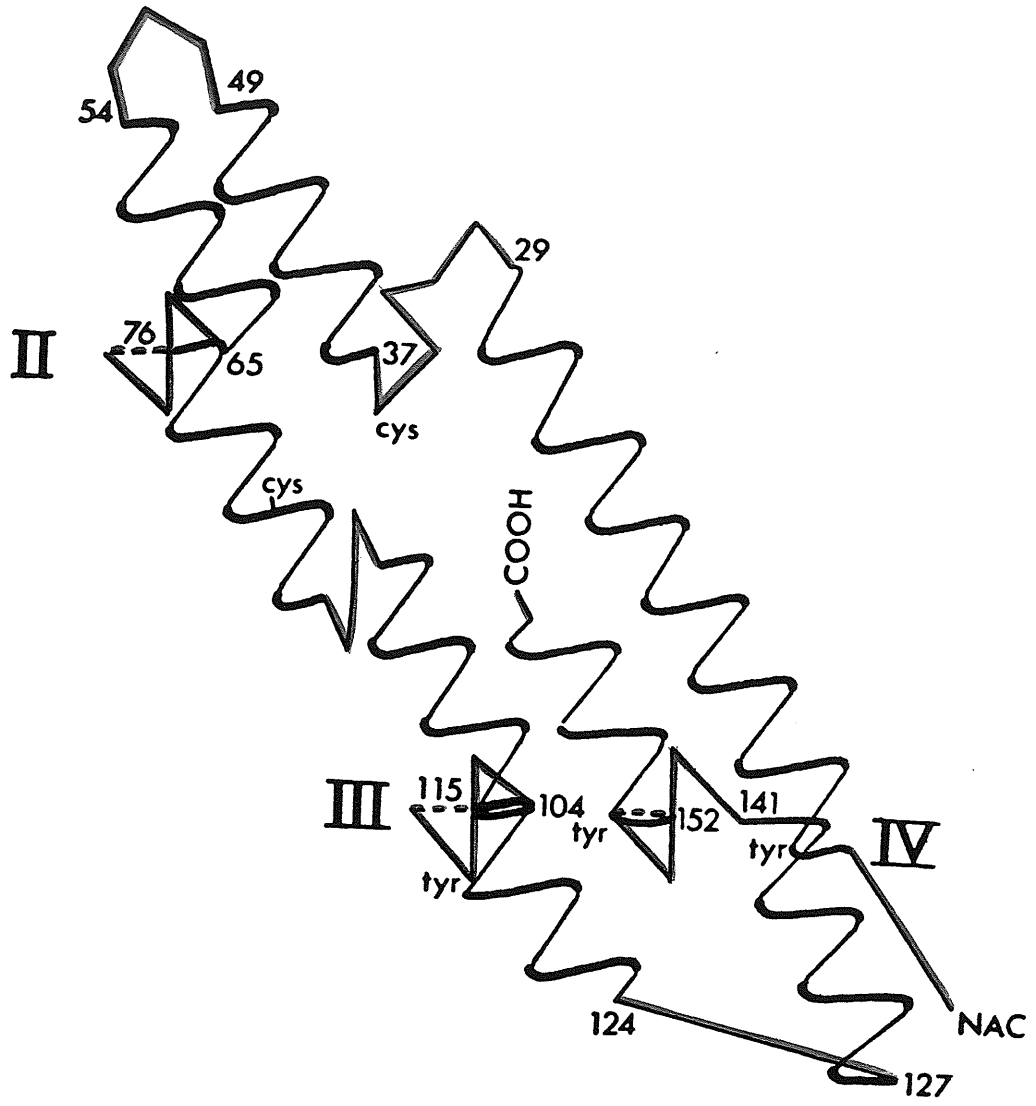


Figure 60 Schematic representation of the secondary structure of bovine cardiac troponin C as predicted from the amino acid sequence (van Eerd and Takahashi, 1975) by the method of Chou and Fasman (1974a,b).

secondary structure prediction are based on a statistical survey of the likelihood of finding each of the 20 amino acids in α-helical or β-sheet conformation. The predicted structure is that which should be found, all other things being equal. The fact that the structure of the Ca^{2+}-modulator complex as determined by CD closely resembles the predicted structure, whereas the Ca^{2+}-free modulator exhibits considerably less helical content (40%), suggests that the Ca^{2+}-free state of the modulator is constrained in some way. The constraints may be between groups which are involved in Ca^{2+} binding, so that the interactions disappear when Ca^{2+} binds. Alternatively, there may be direct ion-pair interactions between, e.g., a carboxyl group in one calcium-binding domain and an ϵ-amino group in another; these interactions might force the domains together and cause the relatively rigid α-helical regions to be shortened.

Four calcium-binding sites are clearly apparent in the modulator molecule, and each exhibits the "EF hand" structure predicted by Kretsinger, i.e.,each calcium-binding region consists of a helical region, a calcium-binding loop containing the Ca^{2+}-coordinating ligands, and another helical region. β-turns are apparent between Ca^{2+}-binding sites I and II, II and III, and III and IV. The single residue of the unusual amino acid, trimethyllysine, at position 115 occurs at the end of a rather long sequence of β-structure linking the third and fourth calcium-binding regions.

The two tyrosine residues, located at positions 99 and 138, are each in a calcium-binding loop, sites III and IV, respectively. Both of these tyrosine residues can be nitrated with tetranitromethane. Such treatment has no effect on the capacity of the modulator to stimulate PDE or on the troponin C-like activities of the molecule; furthermore,

nitration appears to have no effect on the overall conformation of the molecule, as evidenced by circular dichroism. Richman and Klee (1978) succeeded in selectively nitrating tyrosine 99 in the presence of Ca^{2+}, suggesting this residue to be more exposed, while tyrosine 138 is buried. No such selective nitration could be achieved in the absence of Ca^{2+}, indicating that both tyrosine residues exist in similar environments. These findings were consistent with earlier spectrophotometric titration studies (Klee, 1977a) which indicated one tyrosine residue (presumably Tyr 138) to be completely buried in the hydrophobic interior of the molecule and unaffected by Ca^{2+} binding, while the second tyrosine (Tyr 99) is partially buried in the absence of Ca^{2+} and becomes more exposed as the modulator binds Ca^{2+}. This is in agreement with the observation (see "Experimental") that the rate of nitration is greater in the presence than in the absence of Ca^{2+}.

The nearby histidine at position 107 appears to undergo a similar change in location upon binding of Ca^{2+}; this residue becomes more accessible to diethylpyrocarbonate as the modulator binds Ca^{2+}. Such treatment again has no effect on the PDE-stimulating activity of the modulator. Similarly, arginine residues do not appear to be essential for the expression of modulator activity since blockage of 5 of the 6 arginine residues with bulky dihydroxycyclohexyl groups had no effect on the capacity of the modulator to stimulate PDE. Three of the 6 arginine residues are located in the third calcium-binding region, at positions 86, 90 and 106.

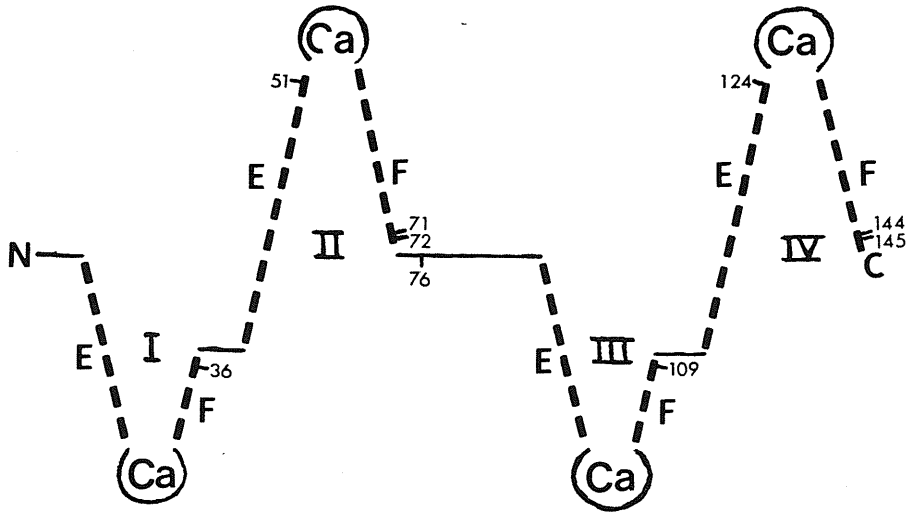
The simultaneous modification of the single histidine, both tyrosines and 5 arginines had no effect on the stimulation of PDE by the modulator, but did abolish troponin C-like properties. This triple

modification introduced a large number of bulky side chains into the molecule, particularly into the third calcium-binding region. Leavis et al, (1977) recently concluded, by studying various fragments of troponin C, that the binding site of troponin C for troponin I is located between residues 88-119, i.e., in the third Ca^{2+}-binding region. It appears likely, therefore, that troponin C-like properties of the modulator protein are abolished due to the introduction of these bulky groups into the third calcium-binding region. On the other hand, PDE-stimulating activity is unaffected by the triple modification, suggesting that the site of interaction with PDE is distinct from the site of interaction with troponin I and is not located in the third calcium-binding region.

The protein modulator contains approximately 40 free carboxyl groups, which accounts for its highly acidic nature (isoelectric pH~4.0). Attempts to achieve selective chemical modification of a small number of these free carboxyl groups with a view to assessing their functional role proved fruitless. It appears that a large number of carboxyl functions are equally accessible and modified at approximately the same rate. Complete loss of PDE-stimulating activity, both in the presence and absence of Ca^{2+}, occurred concomitant with the blockage of 22 free carboxyl groups. Such extensive modification undoubtedly disrupts the native conformation of the molecule, thereby destroying the integrity of the PDE binding domain. This conclusion was substantiated by the studies of Liu and Cheung (1976) who showed, by optical rotatory dispersion, that blockage of a number of free carboxyl groups led to unfolding of the molecule and loss of activity. Furthermore, Liu and Cheung (1976) demonstrated, using the technique of equilibrium dialysis, that carboxyl-modified, inactive modulator retains the ability to bind Ca^{2+}. This

suggests that either the carboxyl groups accessible to the modifying agent are not involved in Ca^{2+} coordination, or chemical modification of these groups makes available other carboxyl groups to bind Ca^{2+}.

Studies by carboxymethylation, mild oxidation and alkylation of the role of methionine residues in the function of the protein modulator implicated their involvement in the interaction between the modulator and PDE. To facilitate understanding of this involvement, the secondary structure of the modulator is represented diagramatically below. This schematic representation is based on a similar model used by Drabikowski et al. (1977a) to depict the secondary structure of rabbit skeletal muscle troponin C. I-IV denote the 4 EF hands, each consisting of helix E, an EF Ca^{2+}-binding loop, and helix F. The dashed lines denote regions of α-helix, and the numbers indicate the positions of methionine residues.



Mild oxidation of the protein modulator with NCS resulted in the modification of methionine residues 71, 72, 76 and possibly, 109. This modification had three major effects on the protein modulator: (1) it caused a shift in the Ca^{2+}-protein toward a state with low helical content relative to the Ca^{2+}-free native protein and a new, less asymmetric environment for phenylalanine residues; (2) it caused a decrease in Ca^{2+} affinity of ~1000-fold; and (3) it destroyed the ability of the Ca^{2+}-modulator complex to activate PDE. All the observations associated with effects (1) and (2) can be explained if we distinguish 3 conformational states of the modulator:

$$M_1 \overset{K_1}{\rightleftharpoons} M_2 \overset{K_2}{\rightleftharpoons} M_3 \overset{nCa^{2+}}{\rightleftharpoons} M_3(Ca)_n$$

where $K_1 = \frac{M_2}{M_1}$ and $K_2 = \frac{M_3}{M_2}$.

M_3 activates PDE and native modulator exists as M_2 in the absence of Ca^{2+}, i.e., $K_1 > 1$, $K_2 << 1$. Addition of Ca^{2+} to M_2 causes a shift to M_3 because Ca^{2+} binds selectively to M_3. This binding may be cooperative since there is more than one binding site; this would explain why the experimental CD Ca^{2+} titration curves in Figs. 40 and 49 are slightly steeper than the theoretical curves. Oxidation (and alkylation) of methionine residues apparently causes a shift in the $M_1 \rightleftharpoons M_2$ equilibrium in the absence of Ca^{2+} so that $K_1 < 1$. The existence of M_1 is indicated by the different CD spectra for the Ca^{2+}-free oxidized (and alkylated) modulator by comparison with Ca^{2+}-free native modulator. The alkylated modulator, probably due to the introduction of several bulky groups, cannot assume as much helical content, even in the presence of Ca^{2+}, as

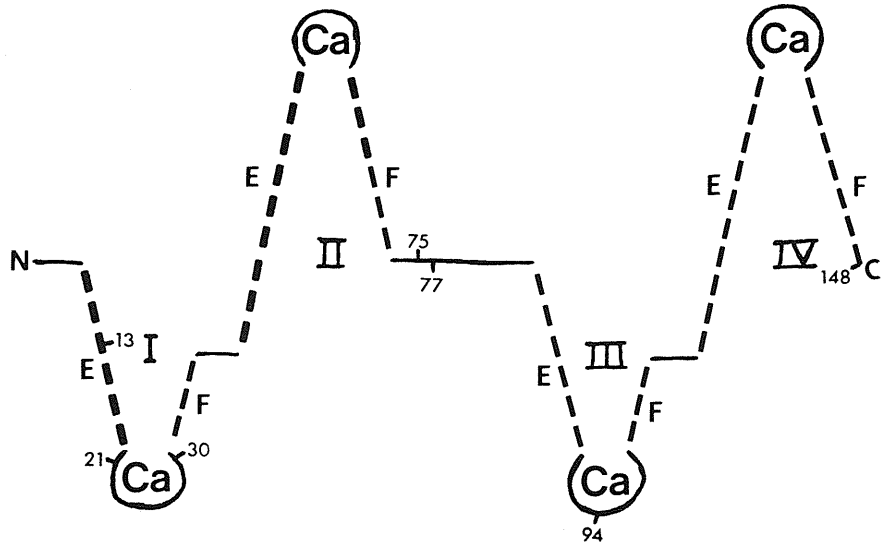
the native modulator has in the absence of Ca^{2+}.

K_1 must be << 1 for alkylated modulator and <1 for oxidized modulator; this explains why Ca^{2+} saturation occurs at much higher levels for these modulator derivatives than for the native protein. The alkylated modulator, however, is not converted to the Ca^{2+}-native modulator conformation by any amount of Ca^{2+}.

In the presence of adequate amounts of Ca^{2+}, the oxidized modulator exhibited an overall conformation, as determined by circular dichroism, which was essentially indistinguishable from that of the native molecule in the presence of Ca^{2+}. The oxidized modulator did not compete with the native protein for the modulator binding site on PDE, however, indicating that mild oxidation abolishes the interaction between the modulator and PDE. This is strong evidence that one or more of these methionine residues is involved in the binding interaction between the modulator and PDE. Furthermore, the conditions of the oxidation reaction were such that oxidation only of exposed methionine residues occurred. Therefore, Met 71, Met 72, Met 76 and possibly Met 109 are exposed on the surface of the molecule, the remaining methionines being partially exposed or buried within the hydrophobic interior of the molecule. One would expect the site involved in protein-protein interaction to be located on the surface of the molecule. It appears, therefore, that the binding site of the modulator for PDE is located in the region joining calcium-binding sites II and III, and is indeed distinct from the presumed site of interaction with troponin I (the third Ca^{2+}-binding region). It is noteworthy that mild oxidation of these exposed methionine residues also abolished troponin C-like activities, i.e., the Ca^{2+}-dependent change in mobility on urea PAGE, and the Ca^{2+}-dependent interaction with

troponin I to form a urea-stable complex; furthermore, CD studies showed that the oxidized modulator, in the presence of Ca^{2+}, does not interact with troponin I even in the absence of urea. It would be interesting to determine whether or not the oxidized modulator can replace troponin C in a reconstituted Ca^{2+}-sensitive actomyosin ATPase system, as can the native protein.

Treatment of the protein modulator with isocyanate resulted in the modification of ~2 lysine residues with a concomitant loss of ~60% of modulator activity; modification of the remaining lysine residues had no further effect on the capacity of the molecule to stimulate PDE. The fact that guanidination of 2-3 lysine residues had no effect on modulator activity suggested that the positive charge of the ε-amino group of 1 or 2 of the lysine residues carbamoylated with isocyanate is required for full expression of modulator activity. If one examines the distribution of lysine residues (denoted by the numbers in the diagram below) in the modulator molecule:



it is apparent that 5 are located in regions of substantial secondary structure; the remaining 2 residues (Lys 75 and Lys 77) are located in the region joining calcium-binding sites II and III and are very close to Met 71, Met 72 and Met 76 which are apparently an integral part of the PDE binding domain. Furthermore, these 2 lysine residues are exposed on the surface of the molecule and would, therefore, be expected to be readily accessible to isocyanate and O-methylisourea. It is reasonable to postulate that Lys 75 and Lys 77 are the lysines carbamoylated with isocyanate and that this results in the observed decrease in modulator activity. It is widely believed that protein-protein interaction involves an initial recognition which occurs via charged side chains and provides an initial relatively weak interaction, followed by the formation of a strong intermolecular binding which occurs via hydrophobic residues (Chothia and Janin, 1975). It is conceivable that Lys 75 and/or Lys 77, and perhaps other neighbouring side chains, are involved in the initial recognition process and that, once weak binding occurs via ionic interactions, the binding is strengthened by the formation of hydrophobic bonds involving any one or more of Met 71, 72 and 76. In this context, it is interesting to compare this region of the modulator sequence with the homologous region in rabbit skeletal muscle and bovine cardiac troponin C (see Appendix II). Met 71, Met 72 and Met 76 are conserved in all 3 proteins; so too is Lys 77 (in modulator numbering). The only difference between the modulator and the TN-C molecule lies with Ala 73 and Lys 75. This lysine residue is replaced by cysteine in bovine cardiac TN-C and by glutamine in rabbit skeletal muscle TN-C; hence, neither TN-C has a positive charge in this position. It is conceivable that neutralization of this positive charge on Lys 75 upon carbamoylation

causes the observed decrease in modulator activity because it interferes with the recognition process.

Figs. 59 and 60 are representations of the secondary structures of rabbit skeletal muscle TN-C and bovine cardiac TN-C, respectively, for purposes of comparison with the modulator protein (Fig. 58). These structures were also calculated from the known amino acid sequences using the method of Chou and Fasman (1974a,b). Clearly the 3 molecules are remarkably similar. One notable feature is the predicted absence of calcium-binding site I in bovine cardiac TN-C.

The studies involving characterization of the fragments obtained by controlled tryptic digestion of the protein modulator and comparison with similar fragments obtained from rabbit skeletal muscle troponin C, clearly establish that these proteins possess similar three-dimensional structures, the sites of tryptic cleavage occurring in homologous positions. Furthermore, removal of Ca^{2+} from each molecule followed by controlled tryptic digestion results in cleavage at different peptide bonds, yet again the sites of cleavage are in homologous locations in the 2 proteins. Therefore, not only are modulator and TN-C structurally similar, but they undergo a similar change in conformation on removal of Ca^{2+} ions. An interesting corollary to this work is that the single site of cleavage of the protein modulator in the presence of Ca^{2+} occurs at the Lys 75-Met 76 peptide bond. This is the region of the proposed PDE binding domain; the resulting fragments are essentially devoid of PDE-stimulating activity. The only modulator fragment with significant PDE-stimulating activity, albeit very low relative to the native protein, was obtained by digestion in the absence of Ca^{2+} and corresponded to residues 1-106 and, therefore, included the proposed PDE binding domain. Clearly the

interaction between the protein modulator and PDE requires strict maintenance of the tertiary structure of the PDE binding domain on the modulator and structural integrity of the functional groups comprising the domain.

The studies described herein have shed some light on the mechanism whereby PDE is regulated by protein-protein interaction via the Ca^{2+}-dependent protein modulator, the conformational change which takes place in the modulator as it binds Ca^{2+} enabling it to perform so many tasks, and the structural homology between the modulator and troponin C of both rabbit skeletal muscle and bovine cardiac muscle. It is hoped that this will provide a prototype for similar studies of the mechanism of regulation of other enzymes and enzyme systems, e.g., adenylate cyclase, myosin light chain kinase and erythrocyte (Ca^{2+}-Mg^{2+}) ATPase, which are known to be subject to modulator-mediated regulation and which may well contain a common modulator-binding domain. Elucidation of the detailed three-dimensional structure of the protein modulator will aid greatly in understanding its mode of action; the studies described, in conjunction with the known amino acid sequence, should facilitate interpretation of X-ray crystallographic data of both the active and inactive forms of the modulator.

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APPENDIX I

Comparison of amino acid compositions of bovine heart and bovine brain modulator

Amino acid	Bovine heart[a]	Bovine brain[b]
Lysine	9[c]	9[c]
Histidine	1	1
Arginine	6	7
Aspartic acid	25[d]	24[d]
Threonine	12[d]	12[d]
Serine	3[d]	5[d]
Glutamic acid	30	29
Proline	2	2
Glycine	12	12
Alanine	12[e]	12[e]
Cysteine	0[e]	0[e]
Valine	9	8
Methionine	9	10
Isoleucine	8	8
Leucine	10	10
Tyrosine	2	2
Phenylalanine	9[f]	8[f]
Tryptophan	0[f]	0[f]

[a] From Stevens et al, (1976).

[b] From Watterson et al, (1976).

[c] The values for lysine include 1 residue of trimethyllysine which does not separate from lysine in the routine amino acid analysis.

[d] Values obtained by extrapolation to zero time.

[e] No carboxymethylcysteine was detected in the hydrolyzate of the reduced carboxymethylated protein.

[f] Tryptophan was absent as judged by spectral analysis.

APPENDIX II

```
                                                                  10                                                  20
A.  Ac met ASP asp ile tyr lys ala ALA val glu GLN LEU THR GLU GLU GLN lys asn GLU PHE LYS ALA ALA PHE ASP IIE PHE val
B.                                Ac(ala,asx)GLN LEU THR GLU GLU GLN IIE ALA GLU PHE LYS glu ALA PHE SER LEU PHE ASP
C.       Ac ASP thr gln gln ala glu ALA arg ser tyr LEU SER GLU GLU met IIE ALA GLU PHE LYS ALA ALA PHE ASP MET PHE ASP

                                                                                  40
leu gly ALA GLU asp gly cys IIE SER THR LYS GLU LEU GLY lys VAL MET ARG MET LEU GLY GLN ASN PRO THR pro GLU GLU LEU GLN
lys ASP GLY ASX GLY THR     IIE THR THR LYS GLU LEU GLY THR VAL MET ARG ser LEU GLY GLN ASN PRO THR glu ala GLU LEU GLX
ala ASP GLY gly GLY ASP     IIE SER val LYS GLU LEU GLY THR VAL MET ARG MET LEU GLY GLN THR PRO THR lys GLU GLU LEU ASP
50                                                                                70
GLU MET IIE ASP GLU VAL ASP GLU ASP GLY SER GLY THR VAL ASP PHE ASP GLU PHE LEU VAL MET MET VAL ARG cys MET LYS ASP ASP
ASX MET IIE ASN GLU VAL ASP ala ASP GLY ASX GLY THR IIE ASP PHE pro GLU PHE LEU thr MET MET ALA ARG lys MET LYS ASP thr
ala IIE IIE GLU GLU VAL ASP GLU ASP GLY SER GLY THR IIE ASP PHE GLU GLU PHE LEU VAL MET MET VAL ARG gln MET LYS GLU ASP
80                                                                                100
SER LYS GLY LYS SER GLU GLU GLU LEU SER ASP leu PHE ARG MET PHE ASP LYS ASN ALA ASP GLY TYR IIE ASP leu GLU GLU LEU LYS
asp             SER GLU GLU GLU IIE arg GLU ala PHE ARG VAL PHE ASP LYS ASP GLY ASN GLY TYR IIE SER ALA ala GLU LEU ARG
ALA LYS GLY LYS SER GLU GLU GLU LEU ALA GLU cys PHE ARG IIE PHE ASP ARG ASN ALA ASP GLY TYR IIE ASP ALA GLU GLU LEU ala
                110                                                               120                                 130
ile MET LEU gln ALA THR GLY GLU thr IIE THR GLU ASP ASP IIE GLU GLU LEU MET LYS ASP GLY ASP LYS ASN ASN ASP GLY ARG IIE
his VAL MET thr asx leu GLY GLU tml LEU THR ASP GLU GLU VAL ASP GLU MET IIE ARG GLU ALA ASN ile ASP gly ASP GLY glx VAL
glu IIE PHE arg ALA SER GLY GLU his VAL THR ASP GLU GLU IIE GLU ser LEU MET LYS ASP GLY ASP LYS ASN ASN ASP GLY ARG IIE
                140
ASP TYR ASP GLU PHE LEU GLU PHE MET lys GLY VAL GLU COOH
ASX TYR GLX GLX PHE VAL GLN MET MET thr ALA lys COOH
ASP PHE ASP GLU PHE LEU lys MET MET glu GLY VAL GLN COOH
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Amino acid sequences of bovine brain modulator protein and muscle TN-Cs. A. Bovine cardiac muscle TN-C taken from van Eerd and Takahashi (1976). B. Bovine brain modulator protein taken from Vanaman et al, (1977). C. Rabbit skeletal TN-C taken from Collins (1976a). Residues shown in capital letters are identical or functionally conservative replacements; those in lower case are assumed to be nonconserved. The numbers refer to positions in the bovine brain modulator protein. Trimethllysine is abbreviated as tml. The presumed Ca^{2+}-binding sites are enclosed in boxes, and some of the residues of the modulator protein which were chemically modified are underlined.