PURIFICATION AND CHARACTERIZATION OF A PROTEIN MODULATOR OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ISOLATED FROM *HOMARUS AMERICANUS*

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BY

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

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To Mom, Dad, Riley, and Uncle Lloyd

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ABBREVIATIONS

CD	Circular Dichroism
DEAE	Diethylaminoethyl
DTNB	5,5'-dithiobis-N,N'-tetraacetic acid
EDTA	ethylenediamine-N,N'-tetraacetic acid
EGTA	ethylene glycol bis(B-aminoethyl ether)-N,N'-tetra-acetic acid
MBA	N,N'-methylenebisacrylamide
NMR	Nuclear magnetic resonance
ORD	Optical rotatory dispersion
PAGE	Polyacrylamide gel electrophoresis
PDE	Cyclic nucleotide phosphodiesterase (EC 3.1.4.17)
PMSF	Phenyl methyl sulfonyl fluoride
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N',-tetramethylethylenediamine
TPCK	N-tosyl-Lphenylalanylchloromethyl ketone
TNC	Troponin C
TNI	Troponin I
TNT	Troponin T

MATERIALS

Bovine heart was obtained from Burn's meats, Winnipeg.

Fresh lobster was obtained from Neptune Fisheries, Winnipeg.

5'- Nucleotriclase, cyclic 3' 5' - adenosine monophosphate, SDS were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cyanogen bromide was obtained from Pierce Chemical Co., (Rockford, Ill.).

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

Ammonium persulphate and EDTA were obtained from Fischer Scientific Co. (New Jersey).

TPCK-trypsin was obtained from Worthington Biochemicals (Freehold, N.J.).

All other chemicals were reagent grade or better.

INTRODUCTION

The protein modulator of cyclic nucleotide phosphodiesterase has been shown in recent years to be of apparent importance physiologically as a regulatory protein, however, it is also an interesting example for study for the protein chemist. It has been observed that the protein modulator is a member of a family of homologous calcium-binding proteins and it has been proposed that these proteins share a common calcium-binding domain. Study of the protein modulator, in this respect, may provide interesting information about this calcium-binding domain. The protein modulator is also interesting as an example of a cellular regulatory mechanism involving protein-protein interaction. It appears to function physiologically when it is activated as a result of calcium binding and is then capable of interacting with large protein molecules such as enzymes and perhaps other proteins.

The protein modulator is being extensively studied in several tissues and species. Comparison of the results obtained from these investigations may lead to the discovery of elements in the protein modulator structure which are essential for its calcium-binding ability or for its interaction with other proteins. It was for this reason that attempts were made to purify and characterize the protein modulator from Homarus americanus (lobster).

The strategy for the study of the protein modulator from lobster involved three parts. Firstly, the modulator

was extracted and purified from lobster muscle. Secondly, the protein was characterized in terms of traditional chemical and physical properties and these properties campared to the protein modulator isolated from other species. Finally, a large amount of modulator was prepared; subjected to tryptic digestion; and the tryptic peptides isolated, analyzed, and compared to bovine brain protein modulator tryptic peptides.

LITERATURE REVIEW

1. Introduction

Cheung (1970) and Kakiuchi et.al.,(1970) independently discovered a protein activator of the enzyme cyclic nucleotide phosphodiesterase (EC 3.1.4.17) and this has resulted in extensive research into the properties of this protein as a possibly important regulator of cyclic nucleotide levels within cells. The protein modulator was subsequently identified as a member of a class of homologous calcium-binding proteins including troponin C and the parvalbumins. Of interest were the findings that in many tissues the levels of protein modulator and PDE activities were not in parallel and indeed, some tissues appeared to have little or no PDE activity and quite substantial protein modulator activities. This indicated the possibility that the protein modulator has functions in addition to the activation of PDE and indeed subsequent findings have shown that the protein modulator may act as a ubiquitous calcium-binding protein rendering many cellular systems calcium sensitive and may be of major significance in the regulation of non-fast muscle contractile processes.

The protein modulator is associated with the activation of many enzymic systems. It is a regulator of cyclic nucleotide metabolism as an activator of a specific PDE and an adenylate cyclase. It exhibits troponin C-like activity with the Mg^{2+}dependent ATPase of skeletal muscle actomyosin in the presence of troponin I and tropomyosin. It is capable of regulating smooth muscle myosin ATPase

through its activation of myosin light chain kinase and it also activates the myosin light chain kinase of skeletal muscle myosin and other protein kinases. It stimulates unidirectional calcium transport through erythrocyte membranes by its activation of a (Ca^{2+} + Mg^{2+}) dependent ATPase. Finally, it has recently been implicated in the possible activation of two as of yet unidentified enzymes.

This protein has been demonstrated in many cell types, tissues and species by different investigators and as such has been described by many names. The protein was initially called the "protein activator of cyclic nucleotide PDE"; however, as its apparent distribution and possible functions have expanded several other names such as the "modulator protein", "calcium dependent regulator", and TNC-like protein" have been used to describe it. Recently, a new name, "calmodulin", has been proposed for the protein. Throughout this dissertation, the term "protein modulator" will be used to describe the protein.

11. Distribution and Purification of the Protein Modulator

Since the initial discovery of this non-dialyzable activator of PDE, the protein modulator has been found in every animal species and almost every tissue or cell type studied. It has been demonstrated in mammalian, avian, and amphibian brain (Cheung, 1971; Vanaman et.al., 1975) rabbit central and peripheral nerves (Vanaman et.al., 1975) bovine heart (Goren and Rosen, 1971); rat testis, thymus, and liver (Smoake et.al., 1974); human red blood cells and platelets

(Smoake et.al., 1974; McGowan et.al., 1976; Muszbek et.al., 1977); rat, bovine, and porcine adrenel medulla (Uzunov et.al., 1975; Egrie and Siegel, 1975; Kuo and Coffe, 1976a; Drabikowski et.al., 1978); electroplax of Electrophorus electricus (Childers and Siegel, 1975); many lower forms such as sea anemone, clam, earthworm, starfish, and sponge (Waisman et.al., 1975); gizzard and uterus smooth muscle (Head et.al., 1977; Drabikowski et.al., 1978); bovine neurohypophysis (Russell and Thorn, 1977); porcine coronary arteries (Wells et.al., 1975); rapidly growing normal cultured fibroblasts and those transformed by Rous sarcoma virus (Vanaman and Watterson, 1976; Watterson et.al., 1976b); C-6 glioma cells (Brostrom et.al., 1976); cultured human lymphoblastic leukemia and retinoblastoma cells and in Brown-Pierce carcinoma (Liu et.al., 1977). The protein modulator has also been demonstrated in plants (Anderson and Cormier, 1978).

The protein modulator was discovered to be protein in nature, heat stable, and very acidic with an isoelectric point of 4.3 (Cheung, 1971; Teo et.al., 1973). These properties have been utilized successfully in the purification of the protein, proceedures usually involving a heat treatment step, ammonium sulfate fractionation, some form of ion exchange chromatography, and gel filtration. A typical purification proceedure was developed by Teo et.al., (1973), while Lin et.al., (1974) utilized preparative gel electrophoresis and Pledger et.al., (1975) used isoelectric

focusing in the purification proceedure.

The protein modulator has been purified to apparent homogeneity from bovine heart (Teo et.al., 1973); bovine brain (Lin et.al., 1974; Watterson et.al., 1976a); porcine brain (Teshima and Kakiuchi, 1974; Wolff and Brostrom, 1974; Klee, 1977); rat testis (Dedman et.al.,1977; Beale et.al., 1977); porcine and bovine adrenal medulla (Egrie and Siegel, 1975; Kuo and Coffe, 1976a); chick embryo brain (Fine et.al., 1975); electroplax of Electrophorus electricus (Childers and Siegel, 1975); blood platelets (McGowan et.al. 1976; Muszbek et.al., 1977); chicken gizzard (Dabrowski et.al., 1977a); earthworm (Waisman et.al., 1978); and Renilla (Jones et.al., 1979).

In many of these instances, the protein modulator was described as a "TNC-like protein". This is the result of the structural homology of the two proteins and the situation is further confused by the ability of the protein modulator to exhibit TNC-like properties such as: (1) the restoration of calcium sensitivity to muscle actomyosin ATPase in the presence of tropomyosin and TNT (Amphlett et.al., 1976; Dedman et.al., 1977a); (2) the ability to form a urea-stable complex with TNI (Dedman et.al., 1977a; Drabikowski et.al., 1978); (3) the ability to form a complex with TNT (Dedman et.al., 1977a; Drabikowski et.al., 1978); and (4) the Ca^{2+}-dependent change in mobility on alkaline urea-PAGE (Head and Mader, 1975a; Amphlet et.al., 1976; Kobayashi and Field, 1978).

Recently it has been demonstrated that the two proteins may be differentiated on the basis of three properties (Drabikowski et.al., 1978): (1) the difference in mobility on SDS-PAGE due to different molecular weights; (2) the difference in mobility of the complex with TNT in the presence of Ca^{2+}, and (3) the ability of the protein modulator, but not TNC, to stimulate PDE activity. In this way, the "TNC-like proteins" isolated from chick embryo brain (Fine et.al., 1975), smooth muscle (Head et.al., 1977), adrenal medulla (Kuo and Coffee, 1976a), and platelets (McGowan et.al., 1976) were identified as being identical to the protein modulator (Drabikowski et.al., 1978). Recently, as well, Dedman et.al., (1978b) have produced monospecific antibodies for rat testis and bovine brain protein modulator which do not interact with rabbit muscle TNC or carp parvabumin. In the future this should aid in the differentiation of the protein modulator and TNC.

Purified preparations from bovine brain (Wolff and Siegel, 1972), bovine adrenal medulla (Brooks and Siegel, 1973), and electroplax from Electrophorus electricus (Childers and Siegel, 1975) appear to contain 1 mole phosphate for every mole protein. Wolff and Brostrom 1974) have indicated that indeed the phosphoproteins isolated from porcine brain, bovine adrenal medulla, and bovine testis are the protein modulator. Whether the presence of phosphate in these modulators is due to contamination is yet to be determined.

The tissue distribution of the protein modulator in the adult rat has been studied (Smoake et.al., 1974) indicating a high specific activity in testis and brain; much smaller specific activities in thymus, epididymal fat pads, and adrenal glands; and very small specific activities in kidney, bone marrow, and liver. Smoake et.al., (1974) also studied the subcellular distribution of the protein modulator in human blood platelets and rat liver parenchymal cells. Seventy percent of the protein modulator in platelets was present in the soluble fraction whereas in liver cells 44% was soluble, 16% was present in the nuclear fraction, 12% was present in the microsomal-membrane fraction, and there was no protein modulator detected in mitochondria. Gnegy et.al.,(1976) recently found that 48% of the protein modulator in rat brain was particulate while 52% was particulate in rat adrenal medulla with 42% being soluble. They also demonstrated an increased soluble fraction of protein modulator upon stimulation with cAMP dependent protein kinase even in the presence of cycloheximide, indicating the release of protein modulator presumably stored in particulate fractions.

These data all show that the protein modulator which activates cyclic nucleotide PDE is present in all animal species studied, in every tissue and almost every cell type studied and in both soluble and particulate fractions of these cells. The following information on the physical and chemical properties of the protein modulator will indicate

the great degree to which the structure of the protein modulator has been conserved over evolution. It would thus appear that the protein modulator must serve some basic function(s) in animal cells.

111. Physical Properties

A. Molecular Weight

Many values for the molecular weight of the protein modulator have been determined, ranging from 40,000 in the initial study of the rat brain modulator (Cheung, 1971) to 14,500 in subsequent studies of rat brain modulator (Lin et.al., 1974). It appears that the molecular weight determinations obtained by gel filtration studies (Cheung, 1971; Lin et.al., 1975) and by SDS-PAGE in the presence of urea (Walsh, 1978) give values for the molecular weight of the protein modulator of approximately double those obtained by other techniques. The reason for this is as yet unknown. The reported values for the molecular weight obtained by other techniques are all around 17,000 daltons, falling within a range between 14,000 for the modulator from the electroplax of Electrophorus electricus (Childers and Siegel, 1975) and 19,200 for the modulator from bovine heart (Teo et.al., 1973). The protein modulator from bovine heart has since been shown to have a molecular weight of 16,800 (Wang et.al.,1975). Subsequently, the protein modulator from bovine brain has been sequenced and the molecular weight as determined by the amino acid sequence is approximately 17,000 (Vanaman et.al.,1977).

Thus the molecular weight of the modulator isolated from various sources remains relatively constant. It is interesting to note, however, that on SDS-PAGE, the protein modulator isolated from bovine brain does not coelectrophorese with the modulator isolated from rat testis (Dedman et.al., 1977b) despite the fact that the two proteins differ by only four amino acid residues (Vanaman et.al., 1979; Dedman et.al., 1978). It has also been reported that the protein modulator isolated from bovine neurohypophysis (Russell and Thorn, 1977) exists as a dimer, while all other reports of the protein modulator isolated from various sources indicate that these proteins exist as single polypeptide molecules.

B. Ultraviolet Absorption Spectrum

Protein molecules, in general, have absorption maxima at 280 nm mainly due to the presence of tryptophan within the protein, however, the protein modulator, TNC, and the parvalbumins all show a very different absorption pattern. They have considerable vibrational structure in the region 250 - 280 nm with absorption peaks at approximately 253, 259, 265, 268, and 276 nm and a shoulder at 282 nm. All of the protein modulators isolated to date exhibit these unusual optical properties, characteristic of proteins which contain no tryptophan and also have high phenylalanine to tyrosine ratios (Wang et.al., 1975; Stevens et.al., 1976; Liu and Cheung, 1976; Watterson et.al., 1976a; Kuo and Coffee, 1976a; Childers and Siegel, 1976; Klee, 1977;

Dabrowska et.al., 1977a; Waisman et.al., 1978).

C. Isoelectric Point

The protein modulator is an acidic protein and thus the isoelectric pH is relatively low. Reported values for the IpH of the protein modulator isolated from various sources all fall within a range around the value 4.0. For example: 4.3 in bovine brain (Cheung, 1971; Lin et.al., 1974; Childers and Siegel, 1975); 4.1 in bovine heart (Wang et.al., 1975); 3.9 in rat testis (Dedman et.al.,1977b); and 4.3 in the electroplax from Electrophorus electricus (Childers and Siegel, 1976).

1V. Chemical Properties

A. Amino Acid Composition

The analyses of the amino acid composition of the protein modulator show some interesting and unusual findings which along with other properties of the protein (UV spectra, Ca^{2+}-binding capacity, and etc.) led to the discovery of the homology of the modulator to TNC and the parvalbumins. The initial studies (Teo et.al., 1973; Lin et.al., 1974) and all subsequent reports show the modulator to have: (1) an unusually high content of aspartic and glutamic acid residues relative to the basic amino acids (35% of the protein is made up of acidic residues); (2) no cysteine; (3) no tryptophan; and (4) a high phenylalanine to tyrosine ratio. The latter two properties are in confirmation of the unusual ultraviolet spectral properties of the protein. All sub-

sequent reports indicate that the composition of the modulator remains remarkably constant in all species and tissues (Teo et.al., 1973; Lin et.al., 1974; Childers and Siegel, 1975; Head and Mader, 1975a; Kuo and Coffee, 1976a; Dedman et.al., 1977b; Waisman et.al., 1978; Jones et.al., 1979).

Liu and Cheung (1976) determined the amount of NH_3 in hydrolyzates of bovine brain modulator on an amino acid analyzer and concluded from these findings that 16 of the total 54 acidic residues are derived from glutamine and asparagine. Walsh and Stevens (1977) determined the total free carboxyl group content of bovine heart modulator by measuring the increase in glycine methyl ester in the presence of a water-soluble carbodiimide and concluded that 40 of the total 55 acidic residues were derived from aspartic and glutamic acid and thus 15 of the acidic residues were derived from glutamine and asparagine. Kuo and Coffee (1976a) found a very different amide content for the modulator from bovine adrenal medulla but the reason for this is unknown. They reported that only 5 of a total 47 acidic residues were derived from glutamine and asparagine.

Watterson et.al.,(1976a) reported the presence of an unidentified, ninhydrin positive compound in bovine brain protein modulator hydrolyzates which was not present in TNC. Vanaman and Watterson (1976) found this compound to be present in bovine, porcine, rabbit, rat, and chicken brain hydrolyzates and later identified it as trimethyllysine located in position 115 in the primary sequence of bovine

brain protein modulator (Vanaman et.al., 1977). Jackson et. al. (1977) independently identified trimethyllysine in rat testis modulator utilizing nuclear magnetic resonance and the fact that the unidentified compound coeluted on an amino acid analyzer with trimethyllysine. Klee (1977) detected this amino acid in porcine brain and Dedman et.al. (1978) identified trimethyllysine at position 115 in the primary sequence of rat testis modulator. Trimethyllysine was also found in the modulator from Renilla (Jones et.al., 1979).

B. Absence of Non-protein Components

Early in the investigations of the protein modulator it was found to be protein in nature; the activity being destroyed by proteases, while the activity was maintained after treatment with RNAase and DNAase (Cheung, 1971). Wang and co-workers (Teo et.al., 1973) suggested that the protein modulator from bovine heart contained bound carbohydrate when they demonstrated that the protein stained on polyacrylamide gels with periodate - Schiff reagent. They later reported (Wang et.al., 1975), however, that the purified protein modulator did not stain with periodate-Schiff reagent and they assumed the earlier positive report was due to contamination. Lin et.al. (1974) reported that the protein modulator from bovine heart did not stain with periodate-Schiff reagent and similarly, Kuo et.al. (1976a) reported that the modulator from adrenal medulla did not stain with periode-Schiff reagent.

C. End Group Analysis

Lin et.al. (1974), using the dansyl chloride method, determined that the amino terminal amino acid of bovine brain protein modulator was valine. Kuo and Coffee (1976a) determined the amino terminus of bovine adrenal medulla modulator to be isoleucine; also by the dansyl chloride proceedure. However, Watterson et.al. (1976a) with bovine brain modulator and Stevens et.al. (1976) with bovine heart modulator indicated that the amino terminus was probably blocked as they found it impossible to degrade the protein with the Edman degradation in an automatic sequenator. Watterson et.al. (1976a) could detect no ∝-dansyl amino acids using the dansyl chloride technique and Vanaman et.al. (1977) sequenced bovine brain protein modulator and demonstrated the amino terminus to be acetylated. Dedman et.al. (1977b, 1978) also found the amino terminus of rat testis modulator to be acetylated. The reason for the discrepancies in these studies is as yet unknown.

D. Amino Acid Sequence

The amino acid sequence of the bovine brain protein modulator was determined by Vanaman et.al. (1977) and is shown in appendix 1. The sequence was determined by the analysis of peptides derived from the protein by a number of different proceedures. Tryptic peptides were obtained following the digestion of the performic acid oxidized protein or of the unmodified protein in the presence of EGTA. The amino acid sequence of the peptides were de-

termined by automated or manual Edman degradation of intact or thermolysin, chymotrypsin or cyanogen bromide subfragments of the individual peptides. The order of the individual tryptic peptides was 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 confirmed by studies of chymotryptic peptides prepared from the performic acid oxidized protein. It was noted that the Asp-Lys-Asp sequence at residues 20 - 22 and 93 - 95, are relatively resistant to trypsin cleavage as is the trimethyllysine at position 115. On the other hand, tryptic cleavage was observed to occur between the methionine residues at position 71 and 72 and following the Met-Met sequence at positions 144 and 145.

Dedman et.al. (1978) determined the partial amino acid sequence of rat testis protein modulator utilizing tryptic peptides of the intact protein molecule and thermolytic or chymotryptic subfragments of some of the peptides. The order of peptides was determined utilizing cyanogen bromide peptides of the intact protein. They also noted that no tryptic cleavage occurred in the Asp-Lys-Asp sequence at positions 20-22.

The rat testis modulator and bovine brain modulator appear to differ only in four residues. This is in confirmation of previous indications that the structure of the protein modulator is highly conserved.

E. Bivalent Cation Binding Properties

The calcium-binding capacity of the protein modulator was discovered initially as a result of the positive effect the modulator has on the Ca^{2+}activation of PDE (Kakiuchi and Yamazaki, 1970; Teo and Wang, 1973). Bovine heart protein modulator (Teo and Wang, 1973) was subsequently found to bind $^{45}Ca^{2+}$ and equilibrium binding studies indicate that the protein contains two sets of binding sites, one site with a dissociation constant of $3x\ 10^{-6}M$ and two sites with a dissociation constant of $10.2 \times 10^{-5}M$. Liu et.al. (1974) investigated the Ca^{2+}-binding properties of bovine brain modulator and found that atomic absorption spectrophotometric measurements of the highly purified protein indicate only one Ca^{2+} bound per molecule modulator whereas no Mg^{2+}, Mn^{2+}, Co^{2+}, or Zn^{2+} are detectable. Equilibrium binding studies (Liu et.al., 1974; Lin et.al., 1974; Liu and Cheung, 1976) indicate that there are four Ca^{2+}-binding sites; 3 sites with a dissociation constant of $3.5 \times 10^{-6}M$ and 1 site with a dissociation constant of $1.8 \times 10^{-5}M$. Vanaman and co-workers have also studied the Ca^{2+}-binding properties of bovine brain modulator. In 1975 (Vanaman et. al.), they reported that there were only two equal binding sites ($K_D = 6x\ 10^{-6}M$) while in 1976 (Watterson et.al., 1976a), with equilibrium dialysis studies, they reported the presence of Ca^{2+}-binding sites; 2 sites with dissociation constants of $1.0 \times 10^{-6}M$ and 2 sites with dissociation constants of $8.6 \times 10^{-4}M$. Wolff et.al. (1977)

also using equilibrium dialysis but determining the concentrations of cations with atomic absorption spectrophotometry, eliminated the difficulties inherent in techniques utilizing chelators or $^{45}Ca^{2+}$. They also found four Ca^{2+}-binding sites in bovine brain modulator but they indicated that 3 of the sites had dissociation constants of 2×10^{-7}M and that the remaining site had a dissociation constant of 1×10^{-6}M. Bovine adrenal medulla protein modulator is reported to have 2 Ca^{2+}-binding sites with dissociation constants of 2×10^{-5}M (Kuo and Coffee, 1976a). Rat testis protein modulator appears to have four equivalent Ca^{2+}-binding sites ($K_D = 2.4 \times 10^{-6}$) (Dedman et.al., 1977b), and three equivalent Ca^{2+}-binding sites ($K_D = 2.1 \times 10^{-5}$M) were reported for the modulator isolated from the electroplax of Electrophorus electricus (Childers and Siegel, 1975).

Wolff et.al. (1977) also noted two sets of binding sites for Mn^{2+} in the protein modulator. Three moles of Mn^{2+}bound per mole of modulator with a dissociation constant of 1.2×10^{-6}M while 1 mole bound per mole modulator with a dissociation constant of 4.0×10^{-6}M. Furthermore, the binding of Ca^{2+} and Mn^{2+} was competitive. This is in confirmation of earlier studies by Liu et.al. (1974) where they found that Mn^{2+} present at concentrations ten times that of Ca^{2+}decreases the Ca^{2+}-binding by 40% and equilibrium dialysis indicates that Mn^{2+} binds to the modulator with a dissociation constant 10 times larger than that for

Ca^{2+}.

Wolff et.al. (1977) also identified Mg^{2+}-binding sites which have dissociation constants much greater than those for Ca^{2+} or Mn^{2+}. Dedman et.al. (1977b) indicate that high concentrations of Mg^{2+} do not compete for the Ca^{2+}-binding sites of rat testis modulator. The free concentration of Mg^{2+} in brain was found to be 1mM and apparently remains relatively constant (Veloso et.al.,1973). Wolff et.al. (1977) studied the Ca^{2+} and Mn^{2+} binding of bovine brain modulator at 1mM Mg^{2+} and interestingly, found that only the 3 high affinity sites bound the cations while the low affinity site was not available for binding. They also found that 1 mole Mg^{2+} was bound per mole protein modulator. Early studies on bovine heart protein modulator indicate that 1mM Mg^{2+} protects the protein modulator against inactivation at $4^{o}C$ (Teo et.al.,1973), however, Lin et.al. (1974) found no difference in bovine brain modulator stability in the presence of 1mM Mg^{2+} or 1mM EDTA.

These results all indicate that the protein modulator does indeed bind Ca^{2+} and that the binding of Mn^{2+} and Mg^{2+} are probably not physiologically important, however, there are many apparent discrepancies in the reported number of binding sites and also in the affinities of the various binding sites for Ca^{2+}. The discrepancies reported for the protein modulator isolated from different tissues and species could be legitimate differences in the Ca^{2+}-binding properties of the proteins, however, many discrepancies were

also found in the data reported for the bovine brain modulator obtained in different laboratories. It seems likely that the majority of the discrepancies are due to the different techniques utilized by the various laboratories.

F. Ca^{2+} Induced Conformational Changes in the Modulator

The protein modulator must bind Ca^{2+} in order to be active and evidence derived from tryptic inactivation studies, tyrosine fluorescence changes, circular dichroism spectral analysis, optical rotatory dispersion studies, sedimentation velocity studies, chemical modification studies and etc., indicate that the binding of Ca^{2+} induces a conformational change in the modulator.

The first piece of supporting evidence was described by Ho et.al. (1975) as they found a difference in the susceptibility of bovine heart protein modulator to tryptic and chymotryptic inactivation in the presence of 10uM Ca^{2+} or 0.1 mM EGTA. Ca^{2+} appears to stabilize the protein and the kinetics of these inactivation studies indicate that the protein has a dissociation constant for Ca^{2+} of 2.6×10^{-6}M; a result quite comparable with the dissociation constants previously discussed. This increase in stability was confirmed by other investigators (Wang et. al., 1975; Egrie and Siegel, 1975; Liu and Cheung, 1976; Drabikowski et.al. 1977a). Recently, Drabikowski et.al. (1977a) and Walsh and Stevens (1977) demonstrated that tryptic digestion of the modulator produced only 2 large peptides when the digestion was carried out in the presence

of Ca^{2+}, whereas digestion in the absence of Ca^{2+} was complete, producing many small peptides. The large peptides obtained in this manner are essentially devoid of PDE stimulating ability, however, they still display the TNC-like activities such as the Ca^{2+} dependent change in mobility on urea-PAGE and interaction with TNI. These results indicate that the conformation of the protein in the presence of Ca^{2+} is such that the majority of sites susceptible to tryptic attack are not available for digestion whereas in the absence of Ca^{2+}, the protein loses secondary structure and is more easily disgested.

The fluorescence of proteins at approximately 310 and 315 nm is due to tyrosine residues and can be used as an indicator of changes in the environment of these residues in the protein. Wang et.al.(1975) found that the fluorescent emission at 315 nm of bovine heart protein modulator was enhanced 30% upon the addition of Ca^{2+}. Likewise, Dedman et.al. (1977b) reported a 2.5 fold increase in fluorescent emission at 315 nm of rat testis protein modulator when the Ca^{2+} concentration was increased from 10^{-8}M to 10^{-5}M. These results indicate that a change in the conformation of the protein modulator occurs upon binding Ca^{2+} such that the environment of tyrosine residues also changes. Interestingly, it is reported that the UV spectrum of the bovine heart modulator shows only a small decrease in the presence of Ca^{2+} (Wang et.al., 1975) and the UV spectrum of bovine brain adrenal medulla is not affected at all by the

presence or absence of Ca^{2+} (Kuo and Coffee, 1976b). Klee (1977) reported a significant 8% decrease in the UV absorption spectrum of porcine brain modulator due to the addition of 0.5 mM $CaCl_2$, however, 1.5 mM $MgCl_2$ resulted in little or no changes in the spectrum.

The addition of Ca^{2+} to a urea-polyacrylamide gel electrophoresis system results in a reduced mobility of the protein modulator (Head and Mader, 1975a; Amphlett et.al., 1976; Kobayashi and Field, 1978) and increase the distribution coefficient of the modulator in gel filtration studies (Kobayashi and Field, 1978). Likewise, the removal of Ca^{2+} by EGTA was reported to result in a decreased Stokes radius of the modulator as measured by gel filtration while the addition of Ca^{2+} results in a significant increase in the sedimentation coefficient of the protein modulator from 1.5S in the absence of Ca^{2+} to 1.89S in the presence of Ca^{2+} (Kuo and Coffee, 1976b). As they demonstrated that Ca^{2+} does not cause a change in the molecular weight of the protein modulator, Kuo and Coffee conclude that the binding of Ca^{2+} to the protein modulator results in a decrease in the axial ratio of the molecule and an increase in molecular symmetry.

Optical rotatory dispersion studies carried out by Liu and Cheung (1976) indicate that the Ca^{2+}-free modulator has an α-helical content of 39%. Measurements made of the modulator in the presence of Ca^{2+} show an increase in rotatory power and an apparent α-helical content of 57%.

They also show that the Ca^{2+} concentration resulting in a half-maximum decrease of the reduced mean residue rotation at 231 nm was approximately 8uM, a value comparable to the Ca^{2+} concentration giving half-maximum activation of PDE. Thus the binding of Ca^{2+} to the protein modulator results in an increased α-helical content which appears to correspond to the activation of the modulator.

Many laboratories have similarly studied the effects of Ca^{2+} on the circular dichroic spectrum of the protein modulator. Kuo and Coffee, (1976b) found that the addition of Ca^{2+} resulted in a 14% increase in α-helical content of bovine adrenal medulla protein modulator from 29% to 43% and the further addition of a 50-fold molar excess $MgCl_2$ had no effect on the magnitude of the ellipticity bands. Klee (1977) found that procine brain protein modulator contains approximately 30-35% α-helical content in the presence of EGTA which was increased by 5-8% with the addition of Ca^{2+}. Dedman et.al. (1977b) using rat testis protein modulator calculated an α-helical content of 45% at a Ca^{2+} concentration of 10^{-8}M which was increased by 9% with the addition of Ca^{2+} to a concentration of 10^{-5}M. Walsh et.al. (appendix 1978) confirm these results in general with the protein modulator isolated from bovine brain. Using far U.V. circular dichroic spectra they indicate that in the presence of EGTA, the protein modulator exhibits 40% α-helical content whereas in the presence of Ca^{2+}it exhibits 49% α-helical content (an increase of 9%).

Wolff et.al. (1977) examined the far U.V. circular dichroic spectra of bovine brain modulator in the absence of divalent cations and found, as other experimentors have, that the protein exhibits two negative maxima at 222nm and 207nm. They calculated that, in the absence of cations, the protein modulator contains 28%α-helix; whereas adjustment of the calcium ion concentration to 25μM results in an α-helical content of 42%. These results are in general agreement with results of other workers, however, they also studied the effects of Mn^{2+} and Mg^{2+} on the circular dichroic spectra and found some interesting results. Adjustments of the cation-free modulator solution to 100μM Mn^{2+} increases the α-helical content from 28% to 40% and adjustment to 1μM Mg^{2+} increases the α-helical content from 28% to 39%. It has been previously assumed that the large conformational change associated with the binding of Ca^{2+} as detected by circular dichroism and optical rotatory dispersion studies represents the change of the modulator from an inactive to an active conformation. However, as 1mM Mg^{2+} is not sufficient to activate PDE in the presence of the protein modulator, the large increase in α-helical content as seen with CD and ORD may not represent the activation of the protein modulator. This activation may thus require only a very subtle change in conformation; a change too subtle for detection by these techniques.

Walsh (1978) recently reported data obtained from a triply modified derivative of bovine brain protein modu-

lator which would seem to partially confirm this proposal. The triple modified derivative contains one modified histidine, two modified tyrosines, and five modified arginine residues and although the derivative retains full capacity to stimulate PDE, it no longer expresses a Ca^{2+}-dependent change in electrophoretic mobility or any interaction with TNI. It would thus seem possible that this derivative, although still active, does not show considerable conformational change upon binding Ca^{2+}. It would be interesting to study the far UV circular dichroic spectra of this derivative in the presence and absence of Ca^{2+}.
Likewise, Walsh and Stevens (1977) demonstrate the inactivation of bovine brain protein modulator, in the presence of Ca^{++} by the modification of methionine residues with N-chlorosuccinimide. This inactive, modified protein modulator has an identical U.V. circular dichroic spectrum as the unmodified protein modulator in the presence of Ca^{++} indicating that although the two differ in activity, the gross conformation as indicated by the CD spectrum is nearly identical.

Kuo and Coffee (1976b) also studied the near UV circular dichroic spectrum of bovine adrenal medulla protein modulator and found that the spectrum was relatively insensitive to Ca^{2+}-binding. They concluded from this that phenylalanine does not play a significant role in Ca^{2+}-binding. Other workers (Wolff et.al., 1977; Klee, 1977), on the other hand, have indicated a significant change in

the environment of tyrosine and phenylalanine residues. Wolff et.al. (1977) found with bovine brain modulator that the binding of Ca^{2+} results in the appearance of a negative maximum of 282nm and the difference U.V. spectrum has maxima at 280, 269, and 258nm. They concluded from this that tyrosine and phenylalanine make contributions in Ca^{2+} binding. Klee (1977) also found that the U.V. difference spectrum indicates a significant alteration in the environment of these two residues.

Chemical modification studies also demonstrate that the protein modulator undergoes conformational changes upon binding Ca^{2+} as differences in the rate and extent of modification occur in the presence and absence of Ca^{2+} (see section VI).

V. Homologous Group of Calcium-Binding Proteins

Calcium has been implicated as a regulatory agent in many cellular processes (see review Berridge, 1975) including muscle contraction, microtubule assembly, stimulus-secretion coupling, platelet aggregation, and the regulation of many enzymes. Indeed, because of this fact, Rasmussen (Rasmussen, 1970; Rasmussen et.al.,1972) suggests that calcium should be given second messenger status similar to the cyclic nucleotides.

The role of calcium can presumably be effected through calcium-binding proteins which in turn regulate various cellular systems. The binding of calcium to these proteins would depend on the localization of the proteins within the

cell, the proteins' relative affinities for calcium, and the concentration of calcium present within the cell at any given time. In most cells, the concentration of calcium varies between approximately 10^{-6}M and 10^{-8}M (Kretsinger, 1976), while extracellular Ca^{2+}concentrations are very much higher (10^{-3}). Most of the intracellular calcium-binding proteins have dissociation constants within these intracellular limits and, presumably, if the dissociation constants were outside these limits, then calcium could not function as a regulatory agent for them. Many calcium-binding proteins have been discovered,some with very specific and well understood functions and others, with functions as yet unidentified.

The protein modulator, as has been discussed, is a calcium-binding protein and this may afford it an important role in cellular regulation in its own right or it may act as a central control agent in the interaction of the two second messenger systems mediated by Ca^{2+} and/or the cyclic mucleotides.

A. Parvalbumins

The parvalbumins, a class of low molecular weight, adidic, water soluble, calcium binding proteins, which were initially found in the white muscle of lower vertebrates, have not as yet had a physiological function determined. They have been demonstrated in the skeletal muscle of higher vertebrates including turtle, chicken, rabbit, and man (Lehky et.al., 1974; Pechère, 1974) and are known

to be primarily muscle proteins which are not, however, present in all muscle types (Baron et.al., 1975). These proteins display an unusual ultraviolet spectrum as a result of the high phenylalanine to tyrosine ratio (Lehky et.al, 1974) and the spectra are very similar to the spectrum for TNC and that previously described for the protein modulator. The amino acid sequence has been determined for parvalbumins isolated from pike (Frankenne et.al., 1973), hake (Capony et. al., 1973), carp (Coffee and Bradshaw, 1973), frog (Capony et.al., 1975), and rabbit (Engield et.al., 1975; Capony et. al., 1976) and the comparison of these sequences indicates that they are homologous proteins. The tertiary structure of carp parvalbumin has been elucidated by x-ray crystallography (Kretsinger et.al., 1971; Kretsinger and Nockolds, 1973; Hendrickson and Karle, 1973; Moews and Kretsinger, 1975) and it was noted that the molecule contains six regions of α-helix labeled A through F. One of the two calcium ions is bound in the loop connecting helix C to helix D while the other calcium ion is bound in the loop connecting helix E to helix F and the two sites are related to each other by a two fold symmetry axis. The coordination of both the CD Ca^{2+} and EF Ca^{2+} can be represented by an octahedron and the ligands involved are well described for the parvalbumins (Kretsinger, 1976).

B. Ca^{2+}-binding Proteins Involved in Myosin ATPase Reg'n

Myosin ATPases are indirectly modulated by calcium. In all muscle types, regulation is achieved by controlling

the interaction of actin and myosin; the differences lie in the location of the sites where this regulation occurs. Two types of regulatory systems have been demonstrated. The regulation can occur through the actin bound tropomyosin - troponin complex of thin filaments or through the myosin light chains associated with myosin heavy chains of thick filaments. Indeed, most invertebrates possess a myosin-linked control system along with an actin-linked system (Lehman and Szent-Gyorgyi, 1975). Lobster muscle myosin, however, appears to have a solely actin linked regulatory system (Lehman et.al., 1972). Both troponin C of the troponin complex and myosin light chains are homologous to the parvalbumins and to the protein modulator.

1. TROPONIN C

The control of Actomyosin by troponin represents a complex regulatory system (Ebashi and Endo, 1968; Greaser and Gergely, 1971; Weber and Murray, 1973; Ebashi, 1974; Kretsinger, 1976). Troponin is composed of three subunits; TNT (mol.wt. 37,000) attaches the subunit complex to tropomyosin; TNI (mol.wt. 23,000) inhibits actin-myosin interaction by maintaining tropomyosin in a blocking position on the actin strands; and TNC (mol.wt. 18,000) binds Ca^{2+} and releases the TNI inhibition. TNC is a low molecular weight, acidic (pI = 4.1 - 4.3) protein which has UV spectral properties similar to the parvalbumins and the protein modulator (Murray and Kay, 1972; Van Eerd and Kawasaki, 1973; Watterson et.al., 1976a). TNC appears to have a limited

distribution similar to the parvalbumins in that it has been demonstrated in vertebrate and some invertebrate skeletal and cardiac muscles only (Kendrick-Jones et.al., 1970; Head and Mader, 1975a; Drabikowski et.al., 1978). Head et.al. (1977) could not demonstrate any TNI - TNC complex formation by affinity chromatography in invertebrates and some investigators (Lehky et.al., 1974) feel that smooth muscle ATPase is solely myosin regulated. Indeed, many proteins from non-muscle cells and invertebrate muscle which were originally thought to be TNC have subsequently been identified as the protein modulator (see section II)

Collins et.al. (1973) studied the amino acid sequence of rabbit skeletal muscle TNC and found it to be homologous to carp and hake parvalbumins. They also recognized four EF hand regions in the amino acid sequence and concluded that TNC has evolved by gene replication. Kretsinger and Barry (1975) subsequently presented a three-dimensional model of TNC and likewise proposed four EF hand regions for the molecule which are homologous to each other. They proposed that the first and second EF hand regions are paired to one another by a two-fold axis similar to the CD and EF loops of the parvalbumins and the third and fourth EF hand regions are also paired in this manner.

TNC thus appears to have four calcium binding sites not only as demonstrated by binding studies but by x-ray crystallography studies as well. Two of the sites bind

only Ca^{2+} (pK_D = 6.7) while the remaining two sites bind Ca^{2+} (pK_D = 8.7) and Mg^{2+} (pK_D = 3.5) (Potter and Gergely, 1975; Kretsinger, 1976; Collins et.al., 1977). Recently, however, Hincke et.al. (1978) have indicated that while bovine skeletal TNC has two sets of binding sites, bovine cardiac muscle TNC has four equivalent binding sites. This indicates a difference in the Ca^{2+}-binding affinity of TNC depending on the tissue of origin, a result also found with the protein modulator. Subsequently, van Eerd and Takahashi (1976) have demonstrated that the four binding regions of bovine cardiac muscle TNC are all homologous to each other and to the EF hands of other troponins and parvalbumins.

2. MYOSIN LIGHT CHAINS

Molluscan muscle and the muscle of some primitive invertebrates exemplify myosin-linked regulation of myosin ATPase activity (Lehman et.al., 1972; Kendrick-Jones et. al., 1970) while rabbit muscle is a typical example of actin-linked regulation through troponin - tropomyosin interactions. Molluscan thin filaments do not contain significant amounts of troponin and do not bind calcium or sensitize rabbit myosin ATPase while thin filaments of rabbit, arthropod, and annelid muscle bind Ca^{2+} and do confer calcium sensitivity to rabbit myosin ATPase. The calcium sensitivity of molluscan myosin depends on an EDTA extractable light chain which, however, does not appear to bind Ca^{2+} itself (Lehman et.al., 1972; Szent-Gyorgy et.al., 1973).

In spite of the fact that rabbit skeletal muscle myosin is actin regulated, it contains four light chains and two heavy chains. The two heavy chains contain the ATPase and actin-combining activity while the function of light chains have not been adequately studied as yet (Kretsinger, 1976). Two types of light chains (Weeds and McLachlan, 1974; Collins, 1976), alkali light chains which do not bind Ca^{2+} and DTNB light chains which bind 1 mole Ca^{2+} per molecule, have been demonstrated in rabbit myosin. The alkali light chains are thought to be important in the ATPase activity as they cannot be removed without total loss of this activity. Removal of the DTNB light chain, on the other hand, has no apparent effect on the ATPase activity but DTNB light chains functionally replace the mollusc EDTA light chains (Kendrick-Jones, 1974). Alexis and Gratzer (1978) have shown that DTNB light chains show an increased α-helical content, considerable changes in intrinsic fluorescence, and large changes in Stokes radius upon binding Ca^{2+}; all indicating a change in conformation upon binding Ca^{2+} similar to that seen in the protein modulator and TNC. The amino acid sequence of rabbit skeletal muscle alkali light chains has been studied (Frank and Weeds, 1974; Weeds and McLachlan, 1974; Tufty and Kretsinger, 1975), their homology to TNC and carp parvalbumin noted, and four EF hand regions identified despite the fact that alkali light chains do not bind Ca^{2+}. Collins (1976) compared the sequence of rabbit muscle DTNB light chains, alkali

light chains, EDTA light chains, TNC, and carp parvalbumins and found them to be homologous proteins each with four homologous regions. He predicted that all four calcium-binding domains of alkali light chains have lost their calcium-binding capacity and three of the domains of DTNB light chains have likewise lost the ability to bind Ca^{2+}. He proposes, as have others, that all these proteins have evolved from a single Ca^{2+}-binding protein containing 1 EF hand region.

C. THE PROTEIN MODULATOR

Wang et.al. (1975) first suggested the similarity between the mechanism of the protein modulator activation of PDE and TNC regulation of actmyosin. They noted (Wang et.al., 1975; Stevens et.al., 1976) that both processes involved Ca^{2+}-binding proteins which have similar molecular weights, UV absorption spectra, electrophoretic mobilities, and amino acid compositions. Despite the fact that the two proteins have very dissimilar tryptic peptide maps, one identical tryptic peptide was isolated from both proteins. Watterson et.al. (1976a) also compared the physical and chemical properties of bovine brain protein modulator and TNC and noted that both proteins have blocked amino termini and similar UV absorption spectra, amino acid compositions, and calcium-binding properties. They also reported that the primary sequence of selected tryptic peptides of the two proteins were very similar or indeed identical. Dedman et.al. (1977b) compared the physical and

chemical properties of rat testis protein modulator and TNC and also concluded that the two proteins are probably homologous. Troponin C thus has a blocked amino terminus (Watterson et.al., 1976a; Wilkinson, 1976), shows conformational changes upon binding calcium (Drabikowski et.al., 1977b; Hincke et.al., 1978), is more resistant to tryptic cleavage in the presence of calcium (Drabikowski et.al., 1977b), interacts with TNI (Head and Mader, 1975a; Head et. al., 1977; Walsh and Stevens, 1978), and shows a decreased mobility on urea-PAGE in the presence of EGTA (Head and Perry, 1974; Drabikowski et.al., 1977b). These are all properties which TNC shares with the protein modulator.

The homology of the two proteins is confirmed when the complete amino acid sequence of bovine brain protein modulator (Vanaman et.al., 1977) and the partial amino acid sequence of rat testis protein modulator (Dedman et.al., 1978) were completed and compared to the known sequences of TNC. In fact, these comparisons indicate that the homology between TNC and the protein modulator is much greater than the homology between any of the other members of this family of proteins. By aligning the sequence of the bovine brain protein modulator so that residue 1 corresponds to residue 9 of cardiac TNC of residue 8 in skeletal muscle TNC, maximum homology to both TNC's is maintained throughout the entire linear sequence by introducing only two small gaps in the modulator sequences. They indicate that 114 of the total 148 positions shared by the modulator and TNC are

comparable. With rat testis modulator, alignment of residue 1 with residue 8 of rabbit skeletal muscle TNC provides at least 50% homology of the two proteins with the introduction of only 1 small gap in the modulator sequence.

As mentioned previously, the amino acid sequence of TNC can be divided into 4 homologous calcium-binding domains. The amino acid sequence of the modulator also possesses internal homology. All four 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 domain (residue 117 - 148). The level of internal homology in the protein modulator appears to be greater than that observed within the TNC's.

The predicted three-dimensional structure of TNC as proposed by Kretsinger and Barry (1975) has been applied with minor alterations to the protein modulator,(Walsh, 1978). All four domains within the modulator contain potential calcium binding sites with calcium liganding residues in the appropriate positions. Likewise, the regions of sequence adjacent to the Ca^{2+}-binding loops contain the appropriate hydrophobic residues necessary for the helices thought to be essential in the formation of functional Ca^{2+}-binding loops.

The proposal that the three-dimensional structures

of the modulator and TNC are very similar was substantiated by the comparison of the peptide fragments obtained with controlled tryptic digestion of the protein modulator (Walsh et. al., 1977) with those obtained in a similar fashion from TNC (Drabikowski et.al, 1977b). These studies show that cleavage by trypsin in both proteins, in the presence or absence of Ca^{2+}, occurs in homologous regions. Furthermore, the ability of the protein modulator to replace TNC and render skeletal muscle another Mg^{2+}-dependent ATPase calcium sensitive is another excellent indicator of their structural homology and three dimensional similarity.

V1. Implication of Specific Amino Acids in the Function of the Modulator

Chemical modification studies provide an excellent tool for studying the Ca^{2+} binding domain of this protein and its interaction with other proteins such as with PDE. These studies are also interesting as evidence of the conformational change which occurs in the protein modulator upon binding Ca^{2+} as the rate or extent of modification usually differs depending on whether modification proceedures are carried out in the presence or absence of Ca^{2+}.

A. Carboxyl Groups

As was previously discussed, Liu and Cheung (1976) demonstrated, utilizing optical rotatory dispersion studies, that bovine brain protein modulator takes on a more helical structure when it binds Ca^{2+}. They also demonstrated the

presence of two functionally distinct sets of carboxyl groups in the modulator. Five carboxyl groups are modified rapidly with glycine ethyl ester or methoxyamine and the resultant modified modulator exhibits no change in the mean residue rotation and only a small loss in the ability to activate PDE. On the other hand, modification of an additional 5 carboxyl groups requires considerably more time and the resultant modified protein exhibits a 20% decrease in the mean residue rotation (i.e. conversion to a structure with less α-helical content) and is completely incapable of activating PDE. The carboxyl group modified, inactive modulator still retains the ability to bind Ca^{2+} as demonstrated by equilibrium dialysis, indicating that the modified carboxyl groups are not involved in coordinating Ca^{2+}. Even when the modification was performed in the absence of Ca^{2+}, the modified modulator retains the ability to bind Ca^{2+}.

Walsh and Stevens (1977) modified carboxyl groups of the protein modulator isolated from bovine heart and bovine brain with a proceedure involving both carbodiimide and glycine methyl ester. They show that modification of 2 carboxyl groups occurs in the first minute with a loss of only 8% modulator activity and it requires the modification of 22 carboxyl groups before a complete loss in modulator activity is observed. They conclude from their own work and that of Liu and Cheung (1976), that there are a few easily accessible carboxyl groups in the protein modulator,

modification of which results in very little loss of activity; while the remainder of the carboxyl groups are presumably located in the interior of the protein and modification of these residues results in gross conformational changes in the modulator and the concommitant loss in activity. Two sets of carboxyl groups have also been identified in TNC (Hincke et.al., 1978).

B. Amino Groups

Walsh and Stevens (1977) found that while modification of carboxyl groups results in little change in modulator activity, carbamoylation of the modulator with isocyanate results in the modification of 1 - 2 lysine residues and a concommitant 60 -70% loss in modulator activity. Further modification of lysine residues results in no further loss in activity. In the absence of Ca^{2+}, carbamoylation proceeds much faster.

Selective modification of α-amino groups by guanidination with o-methylisourea results in the modification of lysine with the retention of positive charge. Modification of 2 - 3 lysine residues in the protein modulator, in this manner (Walsh and Stevens, 1977) whether in the presence or absence of Ca^{2+} has no effect on the activity of the modulator although some difficulty in data interpretation was indicated. They suggest this may indicate that the presence of a positive charge at the location in the sequence of these lysine residues and not the presence of the lysine residue per se, is important for modulator activity. The

effect may be in Ca^{2+}-binding or in the actual interaction of the modulator with PDE.

C. Histidine Residues

Carbethoxylation of the sole histidine residue of bovine heart protein modulator was performed by treatment of the protein with diethylpyrocarbonate while monitoring modification spectrophotometrically at 242nm (Walsh and Stevens, 1977). The histidine residue is fully modified in the presence or absence of Ca^{2+}, however, the rates of modification vary depending on the calcium concentration. This modification results in no change in the ability of the modulator to stimulate PDE and thus this residue does not appear to be directly involved either in the coordinating of Ca^{2+} or in the interaction with PDE. This histidine residue was also found to be unusually resistant to carboxymethylation (Walsh and Stevens, 1977).

D. Arginine Residues

Treatment of bovine heart protein modulator with 1, 2 - cyclohexanedione in borate buffer, pH 9.0, in the presence or absence of Ca^{2+}, results in the modification of 4 of the total 6 possible arginine residues (Walsh and Stevens, 1977). This degree of arginine modification has no effect on the ability of the modulator to activate PDE and thus at least 4 of these residues are apparently not involved in either Ca^{2+}-binding or in the interaction with PDE.

E. Tyrosine Residues

Nitration of the protein modulator by tetranitromethane, performed in the presence of Ca^{2+}, results in the modification of both tyrosine residues (Walsh and Stevens, 1977). This modification has no effect on the ability of the modulator to stimulate PDE which is rather surprising as a tyrosine residue in TNC apparently coordinates one of the bound calcium ions and is in close proximity to a second bound Ca^{2+} (Kretsinger and Barry, 1975). The nitration performed in the absence of Ca^{2+} results in the modification of only 1.5 residues of tyrosine and the modulator retains its activity (Walsh and Stevens, 1977).

Richman and Klee (1978) reported similar results with nitration experiments finding that in the absence of Ca^{2+}, tyrosine 99 is predominately modified. In a previous paper they also indicate that the 2 tyrosyl residues certainly have different microenvironments (Klee, 1977).

Recently it was reported that the limited nitration in the absence of Ca^{2+} is due to the formation of intermolecular x-linkages involving tyrosine residues; a relatively inactive dimer being the result (Walsh, 1978). The far UV circular dichroism spectrum of the monomeric nitrotyrosyl derivative of the protein modulator closely resembles the spectrum of the native modulator thus confirming that the overall secondary structures are indeed very similar.

F. Triple Modification

The modification of histidine, arginine, or tyrosine independently thus has no effect on the activity of the protein modulator. Walsh and Stevens (1978) have also produced a triple-modified derivative (one histidine, two tyrosines, and five arginine residues modified) which still retains the ability to stimulate PDE, however, the double modified derivative (two tyrosines and 5 arginines modified) and the triple modified derivative possess none of the TNC-like activities of the modulator including the Ca^{2+} effected mobility change in urea-PAGE and the ability to form a urea stable complex with TNI. It is interesting that the introduction of several bulky groups into the protein in this way does not effect its ability to stimulate PDE.

G. Methionine

Carboxymethylation studies were undertaken by Walsh and Stevens (1977) to specifically modify the sole histidine residue present in the modulator. They found however, that methionine residues are modified with this proceedure rather than the histidine residue.

Modification of methionine residues by mild oxidation with reagents such as chloramine-T or N-chlorosuccinimide was also performed by Walsh and Stevens (1977). They found that in the presence of Ca^{2+}, activity is completely lost after 60 min along with the modification of 3 methionine residues, while in the absence of Ca^{2+}, inactivation

occurs after 10 minutes and the modification of 4 methionine residues. The modulator modified in this way ($+Ca^{2+}$) does not compete for native modulator in PDE assays. This modification also results in the loss of the TNC-like activities of the modulator (Walsh, 1978; Walsh and Stevens, 1978).

Subsequently, Walsh and Stevens (1978) identified the methionine residues which are modified by the mild oxidant, N-chlorosuccinimide, in the presence of Ca^{2+}, by studying the cyanogen bromide peptides of the modified protein. They found that the methionine residues at positions 71, 72, 76, and possibly 109 in the modulator sequence are modified and that these residues appear to be on the surface of the molecule exposed to solvent.

Walsh and Stevens (1977) indicate that the methionine residues modified by mild oxidation in the presence and absence of Ca^{2+} are different. They subsequently demonstrated (Walsh and Stevens, 1978) that the modulator modified in the presence of Ca^{2+} loses the ability to activate PDE although it has an identical UV circular dichroic spectrum as the unmodified protein, indicating that modification does not significantly alter the overall conformation of the protein. Modification of the protein in the absence of Ca^{2+}, on the other hand, alters the conformation of the protein considerably. Walsh and Stevens (1978) also determined by circular dichroism titration studies, that the modification of methionine in the

presence of Ca^{2+} results in the loss of the high affinity calcium binding site (K_D = 1.89 x 10^{-7}).

H. Conclusions

Using the data obtained from chemical modification studies and from the analysis of peptides obtained from controlled tryptic digestion of the modulator, Walsh (1978) concludes that the PDE binding site is located in the region joining the second and third calcium-binding domains while the TNC-like activities of the modulator are associated with the third calcium-binding domain.

The majority of the bulky groups introduced by the triple modification proceedure are apparently located in the third calcium-binding domain (Walsh,(1978), as this results in the loss of TNC-like properties of the modulator without apparent effect on its ability to stimulate PDE. Weeks and Perry (1978) recently isolated a peptide from TNC consisting of residues 83 - 134 which retains the ability to complex with TNI and inhibits the phosphorylation by cAMP dependent protein Kinase.

Lysine 75, lys 77, met 71, met 72 and met 76 are all in close proximity to each other and have all been implicated in the ability of the modulator to activate PDE. All of these residues are on the surface of the molecule and, except for lys 77, are conserved in the structure of both the troponins and the modulator (Walsh, 1978). Walsh (1978) has suggested that lysine 75 and lysine 77 may be involved in the initial recognition process in the inter-

actions between the modulator and PDE while met 71, 72 and 76 are involved in strengthening binding once recognition has occurred. These proposals are further strengthened by analysis of the peptides obtained by limited tryptic digestion of the modulator. When digestion is performed in the presence of Ca^{2+}, cleavage occurs between lysine 75 and methionine 76 and the resulting peptides are incapable of stimulating PDE. Digestion in the absence of Ca^{2+} at low concentration of trypsin produces a peptide corresponding to residues 1 - 106 which retains significant PDE stimulating activity. Cleavage between lysine 75 and methionine 76 clearly disrupts the proposed PDE binding site, whereas the peptide from residues 1 - 106 clearly includes this proposed PDE binding site.

V11: The Protein Modulator in Cyclic Nucleotide Metabolism

In 1958 Rall and Sutherland first demonstrated, purified and characterized cAMP as an intermediary of the epinephrine induced hyperglycemia in liver. (Rall and Sutherland, 1958; Sutherland and Rall, 1958). Since the initial discovery, cAMP has been demonstrated throughout the animal kingdom and in microorganisms and it appears to play a key regulatory role in many cellular processes in most mammalian tissues (Jost and Rickenber, 1971). Cyclic GMP is the only other cyclic nucleotide which has been detected in cells and it also has been demonstrated throughout the animal kingdom and in prokaryotes (Goldberg et.al., 1973); Goldberg and Hoddox, 1977). Most of the studies to date

indicate that cAMP and cGMP are regulatory agents which act in opposition to each other. Because of the apparent importance of the cyclic nucleotides, much research has been performed not only in their effects *per se*, but also on the regulation of their production and hydrolysis. In this way the study of the protein modulator is deemed important.

A. Cyclic Nucleotide Phosphodiesterases

The cyclic nucleotide phosphodiesterases (PDE) catalyzes the reaction whereby cAMP and/or cGMP are hydrolyzed. These reactions appear to be irreversible (Cheung *et.al.*,1974) and this seems to be the only mechanism in cells whereby the cyclic nucleotides are degraded. Thus the kinetic properties, distribution and regulation of these enzymes may be as important in controlling cyclic nucleotide levels as those of adenylate cyclase and guanylate cyclase. Indeed, recently it has been demonstrated that some PDE's even respond to hormonal stimulation (Wells and Hardman, 1977) although it is unknown whether this feature of the enzyme is physiologically important.

PDE activity was first demonstrated by Sutherland and Rall (1958) and the enzyme was subsequently partially purified from rabbit brain by Drummond and Parrott-Yee (1961) and from bovine heart by Butcher and Sutherland (1962). PDE activity has since been demonstrated in tissues throughout the animal kingdom and in many bacterial strains (Appleman, 1973). Subcellular distribution studies indicate that the majority of PDE is present in soluble fractions, however,

significant amounts are also present in particulate fractions (Appleman, 1973). Coulson and Kennedy (1971) and Thompson et.al. (1976) have even demonstrated the presence of PDE in the nuclear membrane.

The existence of multiple forms of PDE was first demonstrated in rat brain (Brooker et.al., 1968) and has since been demonstrated in many tissues, where kinetically distinct PDE's have been separated (Appleman, 1973; Wells and Hardman, 1977). The various forms of the enzyme differ in kinetic properties, subcellular distribution, molecular weights, and substrate specificities but two of the forms, one soluble and one particulate, appear to be similar in many tissues. The soluble enzyme referred to as the high-Km enzyme, has a greater affinity for cGMP than for cAMP. The particulate enzyme, referred to as the low-Km enzyme, is specific for cAMP and appears to show negative cooperativity. Lymphocytes and monocytes are the only known examples of cells which seem to contain only one enzyme form (Thompson et.al., 1976). Monocytes seem to have only a high affinity enzyme similar to the low-Km enzyme of other tissues while lymphocytes contain an enzyme with a higher affinity for cAMP than any enzyme preparation yet reported. PDE's which are specific for cGMP have also been demonstrated (Goldberg and Haddox, 1977; Nasu et.al., 1978). Although many of the enzyme forms are quite distinct, there have also been reports of the possible interconversion of several enzyme forms in some tissues (Thompson et.al.,

1976; Pichard and Cheung, 1976).

Thus, the PDE control of cyclic nucleotide levels appears to be regulated by many mechanisms including allosteric modulation, isoenzymes, enzyme localization, and protein-protein interaction (Teo et.al., 1973).

B. Protein Modulator Activation of PDE

Cheung (1969) reported a loss of bovine brain PDE activity during purification which was partially restored by incubating the preparation with snake venom. It was subsequently demonstrated that a factor(s) was present in the venom which activates the partially purified enzyme but not the crude preparation. This factor was found to be non-dialyzable, insensitive to DNAase or RNAase attack, and was only moderately pH and heat sensitive. Cheung subsequently demonstrated the existence in bovine brain, of a protein modulator which restores full activity to the partially purified enzyme (Cheung, 1970; Cheung, 1971). A protein modulator dependent PDE was likewise demonstrated in bovine heart (Teo and Wang, 1973; Goren and Rosen, 1971) and rat brain (Kakiuchi and Yamazaki, 1970).

The existence of multiple forms of PDE in bovine heart was demonstrated by Beavo et.al.(1970) and subsequently, Ho et.al. (1976) separated two forms of soluble PDE from bovine heart, one of which was protein modulator and Ca^{2+}-sensitive. Liu and Cheung (1976) have shown that, at least in bovine brain, the particulate PDE is not activated by the protein modulator. Since this time a protein modulator activatable

PDE has been purified 5000-fold in bovine heart using the protein modulator in the purification proceedure (Ho et. al., 1977). Also, a modulator dependent PDE has been purified 400-fold from bovine brain utilizing affinity chromatography with the protein modulator bound to Sepharese 4B (Klee and Krinks, 1978).

The modulator activatable enzyme is capable of hydrolyzing both cAMP and cGMP, however, some investigators believe that it is more active with cGMP as substrate than with cAMP as substrate (Goren and Rosen, 1971; Kakiuchi et. al., 1973; Ho et.al., 1976). The Ca^{2+} and modulator sensitive PDE purified 5000-fold by Ho et.al. (1977) has a greater affinity for cGMP than for cAMP, however, it has a Vmax for cAMP which is 3 times greater than for cGMP. Lin et.al. (1974) indicate that this preferential hydrolysis depends on the substrate concentration. They demonstrate that at mM substrate concentrations, the enzyme hydrolyzes cAMP best while at uM concentrations cGMP appears to be the better substrate. As intracellular cyclic nucleotide concentrations are in the uM range, it would appear that this enzyme is predominately a cGMP enzyme, however, it is possible that localized concentrations of cyclic nucleotides might be increased to effectively mM levels in the vicinity of the enzyme, in which case it could operate as a cAMP enzyme.

The protein modulator enhances the hydrolysis of both cAMP and cGMP by this enzyme. Goren and Rosen (1971)

reported that the protein modulator activates the enzyme by decreasing the Km for cAMP without influencing the Vmax while Cheung (1971) and Ho et.al. (1976) indicate that not only does the protein modulator decrease the Km for cAMP by 90% but it increases the Vmax for cAMP approximately 5-fold. Studies of a highly purified PDE from rat brain (Uzunov, 1976b) reveal that the modulator increases the affinity for cAMP 4-fold without affecting the Vmax, however, the Vmax of the modulator dependent enzyme was found to be several fold higher than other molecular forms of the enzyme. Ho et.al. (1976) also showed that the enhancement of cGMP hydrolysis by the modulator is affected through a decreased Km with no change in Vmax being apparent.

Lin et.al. (1974) showed that although the protein modulator stimulates the hydrolysis of both cyclic nucleotides, it preferentially stimulates cAMP hydrolysis and also that the stimulation is greatest at low substrate concentrations. This has led some investigators to believe that the basal activity of the enzyme functions predominately in the hydrolysis of cGMP whereas the Ca^{2+} and protein modulator dependent activity functions in the hydrolysis of cAMP.

The protein modulator activatable enzyme is dependent on the protein modulator and Ca^{2+} for maximum activity, however, it has a basal activity which is not altered by Ca^{2+} or the protein modulator (Teo and Wang,(1973). Most investigators have been unable to isolate the basal and activatable activities

into separate enzyme forms, however, recently Nasu et.al. (1978) have reported the separation of a Ca^{2+} and protein modulator dependent and independent cGMP PDE from porcine heart. The dependent enzyme preferentially hydrolyzes cGMP is inhibited by cAMP, and exhibits no basal activity in the presence of EGTA whereas the independent form is not affected by cAMP or Ca^{2+}. They have concluded that the independent form represents the basal activity previously reported for protein modulator activatable enzymes. This ability to separate the enzyme into two distinct forms may eliminate some of the difficulties previously encountered in the characterization of the enzyme.

The activation of PDE is mutually dependent on the presence of the protein modulator and Ca^{2+}. Chelation of Ca^{2+} with EGTA to levels of free calcium much below 1 uM renders the modulator inactive and conversely Ca^{2+} alone will not activate the enzyme (Kakiuchi et.al., 1973; Teo and Wang, 1973; Lin et.al., 1974; Wickson et.al., 1975; Waisman et.al., 1978a).

At least two mechanisms can explain the mutual dependence of Ca^{2+} and the protein modulator for PDE activation. Firstly, the enzyme could have binding sites for both Ca^{2+} and the protein modulator individually or, secondly, the protein modulator could bind Ca^{2+} initially and subsequently bind to the enzyme resulting in its activation. As has been previously discussed, the protein modulator is a Ca^{2+}-binding protein while no significant binding of Ca^{2+} to the modulator dependent PDE is observed

in gel filtration studies (Teo and Wang, 1973). It has thus been suggested that the second mechanism is operative in the protein modulator stimulation of PDE.

Several investigators have shown that Ca^{2+}, the protein modulator, and PDE do form a complex and indeed that the formation of this complex results in a conformational change in the enzyme. Wang et.al. (1975) showed that the enzyme is less thermostable in the presence of $Ca^{2++}$and the protein modulator than in the presence of Ca^{2+} alone, the protein modulator alone or in the absence of both agents. Similarly, Liu and Cheung (1976) also showed a decreased thermal stabilty for the enzyme upon binding Ca^{2+} and the protein modulator. This change in stability indicates that complex formation results in a conformational change in the enzyme.

The success of purification proceedures for the enzyme and modulator whereby the two are only separable when EGTA is present in the elution buffer certainly indicates that complex formation occurs (Ho et.al., 1977). Teshima and Kakiuchi (1974) demonstrated that the formation of a complex requires Ca^{2+} and they utilized this factor to isolate by gel filtration chromatography, a protein modulator-Ca^{2+}-PDE complex with a molecular weight of approximately 200,000 as compared to a molecular weight of 150,000 for the free enzyme. Lin et.al. (1975) also demonstrated, using gel filtration studies, that the formation of a protein modulator-PDE complex requires Ca^{2+} and they indicated

that complex formation increases the molecular weight of the enzyme from 170,000 to 230,000 daltons. Likewise the Ca^{2+} and modulator sensitive PDE purified 5000-fold by Ho et.al. (1977) has a molecular weight of 155,000 as determined by gel filtration studies while the enzyme in the presence of Ca^{2+} and the protein modulator has a molecular weight of 230,000. As the protein modulator exhibits anomalous behavior on gel filtration columns, it is unknown whether the molecular weights indicated by these studies are valid, however, they do indicate that complex formation does indeed occur.

The protein modulator activates the modulator free PDE from bovine heart 6 - 10 fold (Teo and Wang, 1973), however, the highly purified enzyme does not appear to be as effectively activated by the modulator and Ca^{2+} (5 - 6 fold) (Ho et.al., 1977). This may be the result of the removal during purification of a modulator binding subunit as will be discussed in section VIII.E.

Teo and Wang (1973) found that a calcium concentration of 2.3 uM, in the presence of saturating levels of bovine heart protein modulator, half-maximally stimulates PDE. Other protein modulators isolated from various sources show similar calcium requirements. For example: bovine brain modulator requires 3 uM Ca^{2+} (Lin et.al., 1974), rat testis modulator requires 1.2 uM Ca^{2+} (Dedman et.al., 1977a) and earthworm modulator requires 2 uM Ca^{2+} (Waisman et.al., 1978a) for half-maximal stimulation.

Based on all of these results, several investigators have proposed a mechanism for the activation of cyclic nucleotide PDE by the protein modulator and Ca^{2+} (Teshima and Kakiuchi, 1974; Liu et.al., 1974; Wang et.al., 1975) involving a stepwise process initiated by the binding of Ca^{2+} to the protein modulator. This binding results in the conversion of the modulator from an inactive to an active conformation allowing the modulator-Ca^{2+} complex to associate with the enzyme. This interaction then initiates a conformational change in the enzyme resulting in increased activity. A schematic representation of this model is shown below where * indicates a different conformation.

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

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

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

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

This process could be regulated by adjustments in intracellular Ca^{2+} levels, by cAMP itself, or by changes in the concentration of the protein modulator. Wang and coworkers (Wang et.al., 1972; Teo et.al., 1973) have shown that cAMP can activate PDE itself by enhancing the interaction of the enzyme with the protein modulator. Likewise Uzunov et.al. (1975) have shown by in vivo experiments,

that cAMP can stimulate its own hydrolysis, as increasing cAMP levels with carbamylcholine also results in an increased content of protein modulator in adrenal medulla. They noted that the increase in cAMP concentration preceeded the increase in modulator content. Gnegy et.al. (1976) subsequently have shown that in adrenal medulla, this increase in the protein modulator content appears to be the result of the release of membrane bound modulator, thus increasing the cytosol levels, as a result of cAMP activation of a cAMP dependent protein kinase. The protein kinase does not appear to phosphorylate the modulator but presumably phosphorylates a membrane protein associated with the modulator.

Gnegy et.al. (1977) subsequently studied the mechanism for the release of the protein modulator from its binding sites in the subcellular fractions of rat brain homogenates. The modulator was found to be present in the highest concentration in the microsomal fraction followed by mitochondrial and nuclear fractions. The release of modulator stimulated by cAMP dependent kinase phosphorylation of membrane protein occurred mainly from the mitochondrial subfractions containing synaptic membranes and synaptic vesicles. These observations infer that high concentrations of cAMP in vivo result in the phosphorylation of membrane proteins and the release of bound protein modulator which can then activate the soluble cytosol modulator dependent PDE, facilitating the hydrolysis of cAMP.

C. Protein Modulator Activation of Adenylate Cyclase

Brostrom et.al. (1975) obtained an activating factor of detergent dispersed preparations of porcine brain adenylate cyclase and demonstrated that the factor is identical to the protein modulator of cyclic nucleotide PDE. The activation process appears to be similar to the activation of PDE by the protein modulator and Ca^{2+}. The protein modulator from rat or bovine brain has also been shown to activate adenylate cyclase with Ca^{2+} being a requirement for the process (Cheung et.al., 1975). This activation lacks tissue specificity as bovine or rat protein modulators both activate the enzyme isolated from either tissue. Subsequently, Brostrom et.al. (1976) have shown that basal or norepinephrine stimulated adenylate cyclase activity of C-6 glioma cells shows a biphasic response to changes in the Ca^{2+} concentration, being stimulated by low calcium concentrations.(1uM) and inhibited by higher calcium concentrations. The enzyme is also stimulated by the protein modulator isolated from the tumor cells, however, the mechanism of activation appears to be complex as high concentrations of the modulator inhibit the enzyme (this is interesting as the protein modulator is present in great excess in bovine brain extracts). Lynch et.al. (1976) also demonstrated the presence of a modulator-adenylate cyclase complex using Sephadex G-200 chromotography. They chromotographed the enzyme in the presence and absence of Ca^{2+} and found that when Ca^{2+} is present in the eluting buffer, the

modulator is eluted with the enzyme while in the presence of EGTA the two proteins chromotographed separately. Lynch et.al. (1976) also demonstrated that formation of this complex occurs rapidly and is reversible. Thus the mechanism of the protein modulator activation of adenylate cyclase is analogous to the activation of PDE.

D. Mechanism of Cyclic Nucleotide Regulation

The role of the protein modulator in cyclic nucleotide metabolism thus appears to be a very complex one. The modulator activates the PDE resulting in the degradation of cyclic nucleotides while also possibly activating the adenylate cyclase resulting in the formation of cAMP. The picture is further complicated by the mechanism of activation as many factors become involved. Two models have been proposed which seemingly integrate some of these factors, however, the actual mechanism of regulation and the net effect on cyclic nucleotide levels is still unclear.

Model 1 (Brosham et.al., 1975; Kakiuchi et.al., 1973)

Stimulation of the cell would result in an influx of Ca^{2+} or the release of membrane bound Ca^{2+} which would in turn activate adenylate cyclase and the cGMP PDE. The net result would be increased levels of cAMP and a concommitant decrease in cGMP levels. This correlates with the theory that cAMP and cGMP act in opposition to each other and also with the evidence that the modulator dependent enzyme is predominately a cGMP PDE.

Model 2 (Cheung et.al., 1975)

Stimulation of the cell would result in an influx of Ca^{2+} or the release of membrane bound Ca^{2+} resulting in the activation of adenylate cyclase, while the membrane bound PDE is not activated by Ca^{2+} and the protein modulator. Subsequently, an increase in intracellular cAMP concentration would become evident. The increased Ca^{2+} and cAMP concentrations would then activate the cAMP PDE and thus return the cAMP levels to the prestimulated level. Thus the sequential activation of the two enzymes would allow for momentary elevations in cAMP levels.

This mechanism is supported by kinetic studies of the modulator dependent enzyme. Although the modulator dependent enzyme has a much greater Vmax for cAMP hydrolysis than other molecular forms of the enzyme (Uzunov, 1976b), it has a much lower affinity for cAMP. Activation of the enzyme by the modulator increases the affinity for cAMP by 4-fold and thus the enzyme could rapidly hydrolyze cAMP under these conditions.

V111. Other Possible Physiological Functions for the Protein Modulator

Because of its wide distribution and high concentration in many tissues (for example, the protein modulator represents approximately 1% of the soluble protein in brain) relative to either the concentrations of PDE or adenylate cyclase, many investigators (Kakiuchi et.al., 1970; Smoake et.al., 1974; Waisman et.al., 1975) have felt that the

protein modulator must have other function(s) in animal cells in addition to the activation of a specific cyclic nucleotide PDE or adenylate cyclase. The modulator has subsequently been shown to activate several other enzyme systems.

A. Stimulation of Myosin-ATPase and a Possible Role in Non-muscle Contractile Processes

The protein modulator shows a tremendous sequence homology with TNC and this led Amphlett et.al. (1976) to study the functional interchangeability of bovine brain protein modulator for TNC. They showed that the protein modulator can neutralize the inhibitory activity of TNI from skeletal muscle and can form complexes with TNT and urea stable complex with TNI. The protein modulator in the presence of TNI and tropomyosin, restores Ca^{2+} sensitivity to the Mg^{2+} stimulated ATPase of muscle actomyosin as effectively as the troponin complex and tropomyosin. Interestingly, unlike the restoration of Ca^{2+} sensitivity by TNC, the protein modulator does not require TNT. As neither TNI nor the protein modulator can bind on their own to tropomyosin, the complex of the two must be capable of binding to tropomyosin. Amphlett et.al. (1976) on the basis of these experiments suggest that the role of TNT and tropomyosin in the regulation of actomyosin ATPase activity be re-examined. Dedman et.al. (1977a) subsequently showed that rat testis protein modulator forms complexes with TNI and TNT and, likewise, that a soluble complex of these

three proteins is capable of regulating actomyosin ATPase activity although less effectively than native troponin.

These results are interesting in terms of TNC as it was originally believed that TNC might function in non-muscle contractile processes. Recently, however, it has been demonstrated by Drabikowski et.al. (1978) that TNC is not present in non-muscle cells. They demonstrated that both the protein modulator and TNC form complexes with TNI which was demonstrable with urea PAGE, however, the complexes have different mobilities and thus the proteins present in various tissues, including brain and platelets, which were originally described as TNC on the basis of their ability to sensitize ATPase and to form a complex with TNI, have been identified as the protein modulator. It is also interesting that although the protein modulator shows significant regulatory capacity in the actomyosin system, TNC shows very little, if any, ability to stimulate PDE. Several investigators (Wang et.al., 1975; Klee, 1977b) have indicated that TNC does not activate PDE. Klee (1977b) showed that while a modulator concentration of 8×10^{-9}M half-maximally stimulates PDE, TNC at a concentration of up to 10^{-5}M does not activate PDE and furthermore TNC shows no inhibitory effect on the protein modulator activation of PDE. Dedman et.al. (1977a), on the other hand, showed that TNC can substitute for the protein modulator in the stimulation of PDE, however, the affinity of the enzyme for TNC is 600-fold lower than for the modulator.

Thus it appears that the protein modulator is capable of regulating the actomyosin ATPase system and due to its ubiquitous distribution and the presence of contractile proteins in non-muscle cells, several investigators (Dedman et.al.,1977a; Vanaman et.al., 1977) have proposed that the protein modulator may have an important regulatory role in contractile processes of non-fast muscle cells. Examples of such processes are smooth muscle contraction, endo- and exocytosis, and inter- and intracellular movement.

B. Activation of Myosin Light Chain Kinase

The contraction of smooth muscle is dependent upon the interaction of actin and myosin. Most investigators now feel that this process is due to the concerted action of a protein kinase and a phosphatase. The phosphorylated form of myosin interacts with actin resulting in the hydrolysis of ATP and the generation of tension. The phosphatase then removes phosphate, resulting in the removal of the actin activation of the Mg^{2+} dependent ATPase activity and thus relaxation follows. Although smooth muscle contraction is not apparently regulated by troponin, it is a Ca^{2+}-dependent process and the Ca^{2+} regulation now appears to be affected through the protein modulator and a protein kinase. Perry and his associates (Perrie et.al., 1973; Pires et.al., 1974) first demonstrated, in rabbit skeletal muscle rather than in smooth muscle, the presence of such a kinase which they have named "myosin light chain kinase".

Perrie et.al. (1973) noted that a kinase present in

the sarcoplasm of rabbit skeletal muscle, phosphorylates the DTNB light chain of myosin transforming it into one of the other myosin light chains demonstrable by PAGE. They noted that this kinase does not phosphorylate troponin, requires Ca^{2+} for activity (Perrie et.al., 1973), is not the same as phosphorylase kinase or the traditional protein kinase, and is itself composed of a heavy and light component (Pires et.al., 1974). Yazawa and Yagi (1977) isolated myosin light chain kinase from rabbit skeletal muscle and separated the enzyme into two components by DEAE-cellulose. Both of these components, approximate molecular weights of 100,000 and 20,000, and Ca^{2+} are essential for activity. They studied the U.V. absorption difference spectra of the 20,000 dalton component in the presence and absence of Ca^{2+} and noted a great similarity with the difference spectra of TNC and the DTNB light chain of myosin. Distinct differences in the spectra were also noted, indicating that the 20,000 dalton component is not identical with either of these other two calcium binding proteins.

Yagi et.al. (1978) recently showed that this 20,000 dalton component of myosin light chain kinase (referred to as the activator component) is identical to the protein modulator. They based their identification on the chemical and physical properties of the activator component and bovine brain protein modulator, and likewise, on the functional interchangeability of the two proteins. The

activator and modulator comigrate in SDS-PAGE studies with estimated molecular weights of 16,500; have identical U.V. absorption spectra; show a typical change in U.V. absorption spectra upon the addition of Ca^{2+}, with the difference spectra induced by Ca^{2+} being identical; and have similar amino acid compositions including the presence of an unidentified basic, ninhydrin positive compound, presumably trimethyllysine. They demonstrated that in the absence of the activator component or Ca^{2+}, essentially no phosphorylation of DTNB light chain occurs. The activator component and the protein modulator are equally effective at restoring activity to myosin light chain kinase while TNC only partially restores activity. Half-maximal activation occurs at a calcium concentration of 3.5×10^{-6}M. Likewise the same amount of activator or modulator produces the same degree of activation of modulator deficient PDE. Thus the two proteins appear to be indistinguishable.

Dabrowska et.al. (1977b, 1978) studied the myosin light chain kinase in smooth muscle (chicken gizzard myosin) and also showed that the enzyme is composed of two subunits with molecular weights of approximately 105,000 and 17,000. They characterized the 17,000 dalton subunit (17K) and concluded that indeed 17K and the protein modulator are identical proteins (Dabrowska et.al., 1978). They showed that 17K and the protein modulator from adrenal medulla, brain and heart have very similar amino acid compositions. Particularly note-worthy was the lack of

tryptophan and the presence of one residue of trimethyllysine in both proteins. The proteins share similar U.V. absorption spectra; have equal mobilities on urea-polyacrylamide gels while TNC has a higher mobility; and both form urea stable complexes with TNI. Likewise, 17K exhibits a conformational change upon binding Ca^{2+} as measured by tyrosine fluorescence.

Not only did Dabrowska et.al. (1978) show that 17K and the protein modulator have similar chemical and physical properties but also that the two proteins are functionally indistinguishable. In the presence of the 105,000 dalton component, 17K or the protein modulator from adrenal medulla or brain induce identical activation of the Mg^{2+} dependent ATPase activation of actomyosin from gizzard. Likewise, brain and adrenal medulla protein modulator and 17K are equally effective in promoting phosphorylation of chicken gizzard myosin. Finally, 17K activates PDE as effectively as the protein modulator. With these activation processes, whether using the 17,000 dalton subunit of myosin or the protein modulator, Ca^{2+} is an absolute requirement for activation.

These facts prove to be very interesting and somewhat paradoxical. The discovery of a myosin light chain kinase in smooth muscle and the subsequent discovery of a protein which provides a Ca^{2+} sensitive regulatory component to this system provide evidence for a very neat theory of smooth muscle contraction involving the kinase,

the protein modulator and the phosphatase. The protein modulator would appear to function as a Ca^{2+} detector, similar to TNC for skeletal muscle contraction. The presence of myosin light chain kinase and its regulatory protein in skeletal muscle is more difficult to explain. Yagi et.al. (1978) suggest that the activity of myosin light chain kinase may be tightly coupled to the contraction-relaxation cycle of skeletal muscle, becoming fully active during contraction and inactive during relaxation. They suggest that the protein modulator and TNC are both functional in the contraction-relaxation cycle of skeletal muscle.

C. Activation of other Protein Kinases

Schulman and Greengard (1978) recently reported a calcium-dependent phosphorylation of rat cerebral cortex synaptosomal membrane fractions which require the addition of either the synaptosomal cytoplasm or the protein modulator. Purification of the synaptic membranes results in a loss of calcium dependent phosphorylation whereas this activity is recovered by reconstitution with synaptosomal cytoplasm, boiled synaptosomal cytoplasm or purified protein modulator. The boiled synaptosomal cytoplasm is incapable of independently phosphorylating substrate. This indicates that the protein modulator may also function as an activator of a protein kinase present in cerebral cortex synaptosomal membranes.

A multifunctional protein kinase has recently been

partially purified from rabbit skeletal muscle which may or may not be identical with the myosin light chain kinase previously discussed (Waisman et.al., 1978b). This kinase is also dependent on Ca^{2+} and the protein modulator for activity. The partially purified enzyme in the presence of Ca^{2+} and the protein modulator phosphorylates histone, myosin light chains and phosphorylase kinase. It is not activated by cAMP or cGMP nor is it inhibited by the heat stable inhibitor protein of cAMP dependent protein kinase and thus it appears to be a different enzyme.

D. Activation of (Mg^{2+} + Ca^{2+}) dependent ATPase and Ca^{2+} transport

Bond and Clough (1973) described a soluble protein present in membrane free hemolysates of human erythrocytes which activates a (Ca^{2+} + Mg^{2+}) dependent ATPase two-fold and this activation follows Michaelis-Menten Kinetics. This activator does not, however, activate the Mg^{2+} dependent or (Mg^{2+} + Na^{+} + K^{+}) dependent ATPase activities and thus it appears to be a specific process. Luthra et.al. (1976b) subsequently demonstrated the presence of this activator in several species and indicate that the activation is not species specific. Luthra et.al. (1977) purified the activator to apparent homogeneity utilizing carboxymethyl-Sephadex chromatography and preparative polyacrylamide gel electrophoresis. The protein was shown to have a molecular weight of approximately 16,000; a highly negative charge; and an amino acid composition consisting of 17% glutamic

acid, 14% aspartic acid, 4% lysine, and 5% arginine. These properties are similar to the properties of the protein modulator of PDE.

Jarret and Penniston (1977) purified the activator 575-fold from human erythrocyte ghosts. They demonstrated that the protein has a molecular weight of approximately 18,000 and that it exhibits properties similar to the protein modulator. The two proteins coelectrophorese and both proteins stimulate the (Ca^{2+} + Mg^{2+}) dependent ATPase and PDE. Gopinath and Vincenzi (1977) independently noted similarities between a human erythrocyte cytoplasmic activator of (Ca^{2+} + Mg^{2+}) dependent ATPase and the various Ca^{2+}-binding proteins such as the parvalbumins, TNC, and the protein modulator of PDE. They observed that high concentrations of parvalbumin or TNC stimulate (Ca^{2+}+ Mg^{2+}) dependent ATPase activity, whereas relatively low concentrations of the protein modulator activates the enzyme to the same extent as the partially purified erythrocyte cytoplasmic activator. Both the activator and the modulator increase the Vmax and the apparent affinity of (Ca^{2+}+ Mg^{2+}) dependent ATPase for Ca^{2+}.

The human erythrocyte contains very low levels of Ca^{2+} which are maintained by an ATP dependent Ca^{2+}pump. This pump is believed to be associated with the (Ca^{2+}+ Mg^{2+}) dependent ATPase present in the erythrocyte membrane. Macintyre and Green (1977) utilizing inside-out membrane vesicles prepared from human erythrocytes in the presence

of Mg^{2+}, ATP, and $^{45}Ca^{2+}$ showed that the addition of a membrane free hemolysate results in a 50% increase in $^{45}Ca^{2+}$ transport from the extravesicular space into the vesicle lumen. They indicate the possibility, and Hinds et.al. (1978) subsequently proved, that this activation is the result of the soluble activator protein of the erythrocyte membrane (Ca^{2+}+ Mg^{2+}) dependent ATPase previously discussed. Hinds et.al. (1978) also have shown that the protein modulator of PDE from bovine brain increases the rate of Ca^{2+} transport and that the Ca^{2+} transport and activation of transport by the erythrocyte soluble activator or the protein modulator is a unidirectional process.

These observations suggest that the protein modulator of PDE and the soluble activator of erythrocyte membrane (Ca^{2+}+ Mg^{2+}) dependent ATPase are similar or identical proteins.

E. Heat Labile Modulator Binding Protein

Wang and Desai (1976) found that bovine brain PDE requires higher concentrations of the protein modulator for activation than the heart enzyme and that this is apparently due to the presence of an inhibitory factor in the brain preparations. This inhibitory factor is protein in nature, being suseptible to tryptic and chymotryptic attack, whereas it is unaffected by treatment with DNAase, RNAase, or amylase. Partial separation of the inhibitory protein and the enzyme can be obtained by Sephadex G-200 column chromatography. The inhibition of the enzyme by this protein is

reversible and appears to be modulator dependent as the inhibitory protein has little effect on the basal activity of Ca^{2+} and modulator dependent PDE or on the activity of the Ca^{2+} independent PDE. Likewise, the inhibitory protein counteracts theactivation of PDE induced by the protein modulator.

Subsequently, Wang and Desai (1977) demonstrated with gel filtration studies that the inhibitory protein associates with the protein modulator in the presence of Ca^{2+}, whereas the inhibitory protein and PDE do not interact. They concluded from these results that the inhibitory protein inhibits PDE by associating with the protein modulator, and thus prevents the protein modulator from binding to and activating PDE. The molecular weights of the free inhibitor and the inhibitor-protein modulator complex are estimated to be 95,000 and 160,000, respectively by gel filtration on a calibrated Sephadex G-200 column. Wang and Desai (1977) also showed that the inhibitor protein is heat labile and suggest that the inhibitory protein may be either another regulator of PDE or indeed may be another modulator-regulated enzyme present in bovine brain. They found that the inhibitor has no ATPase, GTPase, adenylate cyclase, or 5'-Nucleotidase activity and they designated it the "modulator binding protein".

Other investigators (Klee and Krinks, 1978; Wallace et.al., 1978) have also demonstrated an inhibitory protein of PDE in bovine brain. Wallace et.al. (1978) described a

heat labile protein which interacts with the protein modulator in the presence of Ca^{2+}, resulting in a decreased activity of adenylate cyclase or PDE. The molecular weight of the inhibitor was estimated to be approximately 80,000 using a calibrated Sephacryl S-200 column and the Stokes radius was calculated to be 3.85 nm. The inhibitor protein does not affect the basal PDE activity but increasing the amount of inhibitory protein progressively suppresses the modulator-supported PDE activity toward the basal level. Similar results were obtained for adenylate cyclase activity. This inhibitory activity of the protein is not reversed by increasing the Ca^{2+} concentration but full enzyme activity is obtained in the presence of the inhibitor if the protein modulator is added to the reaction mixture. Thus the inhibitor does not exert its effect through the chelation of Ca^{2+} but rather through interaction with the protein modulator. Wallace et.al. (1978) utilizing an affinity column composed of the protein modulator bound to Sepharose 4B, demonstrate the reversible formation of the protein modulator-inhibitor protein complex. In the presence of Ca^{2+}, the inhibitor is retained by the column while addition of EGTA to the eluting buffer causes the release of the inhibitor.

Klee and Krinks (1978) purified to apparent homogeneity an inhibitor protein of PDE from bovine brain while attempting to purify the Ca^{2+} and modulator dependent PDE on an affinity column composed of the protein modulator

bound to Sepharose 4B. They found that the inhibitor protein represents the major protein component found to bind to the immobilized modulator while PDE represents only 10% of the bound protein. Likewise, the inhibitor protein binds to the immobilized modulator more strongly than PDE. The purified inhibitor appears to be made up of two subunits, as two bands are detected on SDS-PAGE. The molecular weight of the subunits is estimated to be 61,000 and 15,000. Treatment of the protein with dimethyl suberimidate prior to electrophoresis under denaturing conditions shows only one band of approximately 80,000 molecular weight. This inhibitor protein resembles and is probably identical to the modulator binding protein of Wang and Desai and the inhibitory protein of Wallace and co-workers. It is heat labile, has the same molecular weight, and inhibits the enzyme in a similar fashion. The U.V. absorption spectrum is typical of proteins containing tryptophan although tryptophan is not detected by amino acid analysis. Klee and Krinks (1978) tested the inhibitor for enzymic activity including adenylate cyclase. guanylate cyclase, and ATPase and found that similar to the other inhibitory proteins of PDE, this inhibitory protein possesses none of these activities.

It is felt that the PDE inhibitors isolated from bovine brain in these three laboratories are identical proteins. It has been suggested (Wang and Desai, 1976; Wang and Desai, 1977; Wallace et.al., 1978) that the inhibitor

protein (modulator binding protein) is another modulator regulated enzyme which has not yet been identified. Klee and Krinks (1978), on the other hand, suggest an alternative hypothesis on the basis of data which is as yet unpublished. They indicate that the inhibitory protein co-electrophoreses with PDE in gels run under native conditions and likewise, the most highly purified PDE preparation they have obtained is contaminated with this inhibitory protein. Attempts to remove the inhibitor from the enzyme results in a partial loss of the ability of PDE to be stimulated by the modulator. On the basis of these results Klee and Krinks suggest that the inhibitory protein may be a regulatory subunit of PDE which binds the protein modulator. Indeed, this inhibitor may represent a modulator binding subunit common to all of the enzymes which are regulated by the modulator.

F. Heat Stable Inhibitory Protein of PDE

Recently, another inhibitory protein of PDE has been separated in bovine brain extracts from the heat labile modulator binding protein (Sharma et.al., 1978a). Unlike the modulator binding protein, this inhibitor retains full activity upon heating in a boiling water bath for 2 minutes. Sharma et.al. (1978b) have recently purified the heat stable inhibitor protein to apparent homogeneity utilizing standard purification techniques and affinity chromatography (Sepharose 4B bound protein modulator). The protein has a molecular weight of approximately 68,000 and appears to be mono-

meric. It is interesting that although the modulator binding protein and this heat stable inhibitor protein have very different molecular weights and heat stabilities, the larger subunit of the modulator binding protein has a molecular weight of approximately 61,000 (Klee and Krinks, 1978). The inhibitor does not inhibit cAMP dependent protein kinase or protein phosphatase and itself does not express ATPase, GTPase, cAMP PDE,cGMP PDE, 5'-nucleotidase or protein kinase activity.

The mechanism of action of this heat stable inhibitory protein is very similar to that of the modulator binding protein (Sharma et.al., 1978a) although Sharma et.al (1978b) indicate that this heat stable inhibitor has a much higher inhibitory capacity than the modulator binding protein. This protein specifically inhibits the Ca^{2+}-dependent PDE but has no effect on the basal activity of the enzyme or on the Ca^{2+} independent PDE. High concentrations of Ca^{2+} will not release the inhibition whereas addition of protein modulator to the assay mixture does release the inhibition. The higher the concentration of modulator present, the higher the concentration of inhibitor required for inhibition. Similiarly, the inhibition can be completely reversed by high concentrations of modulator. The molecular weight of the inhibitor-modulator complex as estimated by Sephadex G-100 chromatography is approximately 85,000 as compared to 70,000 for the free inhibitor protein.

The possible significance of this new inhibitor

protein is as unclear as the significance of the heat labile modulator binding protein. It may function as an inhibitor of PDE. It may function as an inhibitor of the protein modulator although as Sharma et.al. (1978a) point out, the amount of this protein in brain extracts could counteract only 5% of the total modulator present in the same extracts unless the inhibitor has a unique intracellular or tissue distribution. Likewise, the heat stable inhibitor may represent a modulator binding subunit of PDE as was previously suggested for the modulator binding protein. Indeed, it seems possible, but perhaps unlikely, that this protein is actually the large subunit of the heat labile inhibitory protein described by Klee and Krinks (1978). Finally the heat stable inhibitor may be yet another enzyme or functional protein regulated by the protein modulator and calcium.

GENERAL EXPERIMENTAL PROCEEDURES

1. Preparation of Modulator Deficient PDE

Modulator deficient PDE was prepared from bovine heart as described by Ho et.al. (1976) which is a modification of the method of Teo et.al. (1973).

A. Homogenization

The fat was removed from fresh beef heart (approximately 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, 2mM EDTA, pH 7.5 in a Waring blender at top speed for about 10 min. The homogenate was centrifuged at 11,000 x g for 30 min.

B. Ammonium Sulfate Fractionation

The pH of the homogenate was adjusted to 8.8 with 5N NaOH and the solution brought to 60% saturation with ammonium sulfate, stirred well and left for 30 min. The mixture was then centrifuged at 14,000 x g for 30 min. The pellet (which contains PDE and some modulator protein) was retained, and the supernatant (containing mostly protein modulator) was discarded. The pH 8.8 pellet was suspended in a minimum volume of 0.02M Tris, 1mM imidazole, 1mM magnesium acetate, pH 7.0, and dialyzed against 0.02M Tris, 1mM magnesium acetate, 10mM 2-mercaptoethanol, 0.01mM $CaCl_2$, pH 7.0.

C. Ultracentrifugation

The dialyzed solution was centrifuged at 160,000 x g in a Beckman Model L5-65 ultracentrifuge for 1 hr. The pellet

was discarded and the supernatant filtered with the aid of a Buchner funnel.

D. First DEAE-cellulose Column Chromatography

The filtered supernatant was applied to a DEAE-cellulose column (2.5 x 90cm) previously equilibrated with Buffer A (0.02mM Tris, 1mM imidazole, 1mM magnesium acetate 10mM 2-mercaptoethanol, 0.01mM $CaCl_2$, 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, 1mM imidazole, 1mM magnesium acetate, 10mM 2-mercaptoethanol, 0.01mM $CaCl_2$, 0.4M NaCl, pH 7.0). A flow rate of approximately 50 ml/hr was used and 6.5ml fractions were collected. Selected fractions were subjected to assay for PDE activity in the presence and absence of EGTA and the conductivity and absorbance at 280 nm measured. PDE_1 (modulator dependent PDE) is identified as the peak of activity which is inhibited by EGTA while PDE_{11} is unaffected by EGTA. The fractions corresponding to PDE_1 were pooled and dialyzed against Buffer C (0.02M Tris, 1mM imidazole, 1mM magnesium acetate, 10mM 2-mercaptoethanol, 0.1mM EGTA, 0.05M NaCl, pH 7.0).

E. Second DEAE-cellulose Column Chromatography

The dialyzed, pooled fractions from the first DEAE-cellulose column were applied to a second DEAE-cellulose column (2.5 x 60cm) previously equilibrated with Buffer C and eluted with a linear salt gradient consisting of 500ml

each of Buffer C and Buffer D (0.02M Tris, 1mM imidazole, 1mM magnesium acetate, 10mM 2-mercaptoethanol, 0.1mM EGTA, 0.4M NaCl, pH 7.0). A flow rate of approximately 50 ml/hr was used and 3.5ml fractions were collected. Again, selected fractions were subjected to assay for PDE activity in the presence and absence of EGTA and the PDE_1 activity pooled. The pooled fractions were then placed in small test tubes and stored for future use at 20°C.

11. 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 involves the conversion of 5'AMP, the product of the cAMP-PDE reaction, to adenosine and inorganic phosphate by 5'-nucleotidase. The reaction mixture, in a volume of 0.9 ml, contains in addition to PDE and the protein modulator (an amount sufficient to cause maximal stimulation of the enzyme); 1.2mM cAMP and 0.25 units of 5'-nucleotidase in 40mM Tris, 40mM imidazole, 3mM magnesium acetate, 0.11mM $Cacl_2$, pH 7.5. The reaction mixture is incubated at 30°C for 30 minutes, at which time reaction is terminated by the addition of 0.1ml of 55% (w/v) trichloroacetic acid. The resulting mixture is centrifuged at 3000 rpm in a Sorval GLC-2 general laboratory centrifuge for 10 minutes. To the supernatant (0.5ml) is added 0.5ml of 0.55% (w/v) ammonium molybdate in 1.1N sulfuric acid, followed by 0.05 ml of reducing agent (1.15M sodium bisulfite, 0.05M sodium sulfite, 0.01M 1-amino-2-naphol-4-sulfonic acid). Colour

development is allowed to proceed for at least 10 minutes and the absorbance of 660nm measured against distilled water in a Beckman model 25 spectrophotometer.

One unit of PDE activity is equivalent to the amount of enzyme which when maximally activated by the protein modulator and Ca^{2+}, hydrolyzes 1 mole of cAMP per minute at 30°C under standard conditions.

111. 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, contains in addition to the modulator and a standard amount of PDE, 1.2mM cAMP and 0.25 unit of 5'-nucleotidase in 40mM Tris, 40mM imidazole, 3mM magnesium acetate, 0.11mM $CaCl_2$, pH 7.5. The reaction mixture is incubated at 30°C for 30 minutes, at which time the reaction is terminated by the addition of 0.1 ml of 55% (w/v) trichloroacetic acid. The resulting mixture, if cloudy, is centrifuged at 3000 rpm for 10 minutes in a Sorval GLC-2 general laboratory centrifuge. To the supernatant (0.5 ml) is added 0.5 ml of 55% (w/v) ammoniom molybdate in 1.1N sulfuric acid, followed by 0.05 ml of reducing agent (1.15M sodium bisulfite, 0.05M sodium sulfite, 0.01M 1-amino-2-napthol-4-sulfonic acid). Colour development is allowed to proceed for at least 10 minutes and the absorbance is measured at 660nm against distilled water in a Beckman Model 25 spectrophotometer. Several concen-

trations of modulator sample are assayed and a standard curve constructed. This enables an accurate determination of the amount of modulator inducing half-maximal stimulation of a standard amount of PDE. One unit of modulator is defined as that amount required to give half-maximal stimulation of the standard amount of PDE.

1V. Protein Determinations

A. Lowry Protein Determination

Protein concentrations were determined by the method of Lowry et.al. (1951) unless otherwise indicated. 1 ml protein solution is mixed with 5 ml alkaline copper reagent (0.5 ml 1% $CuSO_4$ and 0.5 ml 2% sodium potassium tartrate are mixed and brought to 50 ml with 2% sodium carbonate in 0.1N sodium hydroxide). The mixture is then allowed to stand for 10 minutes at room temperature. 0.5 ml dilute Folin reagent (1 part in 2 parts distilled, deionized water) is added and the colour is developed for 30 minutes, at which time the absorbance at 660nm is measured. Bovine serum albumin (100 ug/ml to 0ug/ml) was used to develop a standard curve.

B. Direct Fluram Method of Protein Determination

The direct fluram method of Nakai et.al. (1974) was used. An appropriate sample aliquot is placed in a 13 x 100 mm disposable test tube and 1.85 ml of 0.5M sodium borate buffer, pH 8.5 added and mixed thoroughly. Fluorescamine solution (0.5 ml of 30 mg% in sequenal grade acetone) is added to the mixture with a syringe while the test tube is

vortexed and the mixture allowed to stand for 10 minutes. Rapid addition and mixing are essential. The fluorescence is measured on an Aminco-Bowman spectrophotometer using window settings (4,4,2), an excitation wavelength of 398nm and emission wavelength of 490nm. The protein content is calculated according to the following equation:

Absolute value = reading x sensitivity/scale

V. Electrophoretic Proceedures

A. 15% and 11.5% PAGE

Gel electrophoresis was performed in 15% acrylamide slab or cylindrical gels and 11.5% slab gels using the discontinuous gel system of Davis (1964). The following stock solutions were used:

Solution	Composition
A	22.2 gm acrylamide 0.6 gm MBA H_2O ——→100ml
B	0.6M Tris-HC1, pH 8.9
C	0.6M Tris-phosphate, pH 6.9
D (separating gel buffer)	40 gm sucrose 40 ml solution B H_2O ——→ 100 ml
E (spacer gel buffer)	40 gm sucrose 40 ml solution C H_2O——→ 100 ml
F (sample buffer)	70 gm sucrose 20 ml solution D 2.5 mg bromophenol blue H_2O ——→100 ml

The following solutions were prepared immediately prior to use:

Storing Buffer: 10-fold dilution of solution B

Running Buffer: (5.77 gm glycine + 1.21 gm Tris, pH 8.3) ⟶ 2000 ml with H_2O

Ammonium persulfate: 5 mg/ml in H_2O

The separating gels were prepared by mixing the following solutions:

	15% acrylamide, 0.4% MBA	11.5% acrylamide, 0.4% MBA
Solution A	20.3 ml	15.5 ml
H_2O	0.7 ml	5.5 ml
Solution D	7.5 ml	7.5 ml
Ammonium persulfate	1.5 ml	1.5 ml
TEMED	0.015 ml	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 E	3.750 ml
Ammonium persulfate	0.750 ml
TEMED	0.020 ml

Protein samples were dissolved in solution F (sample buffer) diluted 1:1 with water. Electrophoresis was performed at 100 volts (slab gels) until the tracking dye indicated the samples were focused in the separating gel, and then at 300 volts until the dye front reached the bottom of the gel. With cylindrical gels electrophoresis was performed at 3 mamps per tube. The gels were then stained in 1% Amido Black in 7% acetic acid or 0.25% Coomassie

Brilliant Blue G-250 in 7.5% acetic acid and the gels were destained electrophoretically with 7.5% acetic acid.

B. 8M urea-11.5% PAGE

Exactly the same electrophoretic system as described above was employed, with the exception that the separating gel, spacer gel, and sample buffer were made 8M in urea as follows:

Separating Gel

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

Spacer Gel

Solution A	3.275 ml
H_2O	7.125 ml
Solution E	3.75 ml
Urea	7.2 gm
Ammonium persulfate	0.75 ml
TEMED	0.020 ml

Sample Buffer

Solution F	10 ml
Urea	9.6 gm
EDTA	0.006 gm
H_2O	to 20 ml

Gels were stained in 0.25% Coomassie Brilliant Blue G-250 in 7.5% acetic acid and were destained electrophoretically with 7.5% acetic acid.

C. 8M Urea-15% PAGE

Exactly the same electrophoretic system as described in V.A. was used with the exception of the stock acrylamide solution (A), separating gel, spacer gel, and sample buffer. These solutions were prepared in the following way:

Solution A:

29.9 gm acrylamide
0.8 gm MBA
H_2O ——→ 100 ml

Separating Gel:

Solution A	20.3 ml
H_2O	0 ml
Solution D	7.5 ml
Urea	14.4 gm
EDTA	0.01 gm
Ammonium persulfate	1.5 ml
TEMED	0.015 ml

Spacer Gel

Solution A	3.275 ml
H_2O	7.125
Solution E	3.75
Urea	7.2 gm
EDTA	0.005 gm

Ammonium persulfate	0.75 ml
TEMED	0.020 ml

The gels were stained with Coomassie Brilliant Blue and destained as previously described.

D. SDS-PAGE

Gel electrophoresis was performed in 15% acrylamide and 10% acrylamide slab gels using the method described by Swank and Mankres (1971). The following stock solutions were used:

Solution	Composition
A	30 gm acrylamide 0.8 gm MBA H_2O ⟶ 100 ml
B	1.5M Tris-HCl, pH 8.8
C	10% SDS
D	0.5M Tris-HCl, pH 6.8
E (Running Buffer)	3gm Tris 14.4gm glycine 1gm SDS H_2O ⟶ 100 ml
F (Sample Buffer)	0.05M Tris 1% SDS 0.01% bromophenol blue 30% glycerol

The separating gels were prepared by mixing the following solutions:

	15% acrylamide, 0.4% MBA	10% acrylamide, 0.4% MBA
Solution A	15ml	10ml
Solution B	7.5ml	7.5ml

H_2O	5.4ml	10.4ml
Solution C	0.6ml	0.6ml
Ammonium persulfate (4mg/ml)	1.5ml	1.5ml
TEMED	0.02ml	0.02ml

The spacer gel (5% acrylamide, 0.13% MBA) was prepared by mixing the following solutions:

Solution A	5 ml
Solution B	7.5 ml
H_2O	15.7 ml
Solution C	0.3 ml
Ammonium persulfate (4mg/ml)	1.5 ml
TEMED	0.02 ml

Protein samples were dissolved in solution F and immersed in a boiling water bath for 10 minutes. Electrophoresis was performed at 25 mamps until the dye front reached the bottom of the gel. Gels were stained overnight with 0.25% Coomassie Brilliant Blue G-250 in 25% isopropyl alcohol, 10% acetic acid and subsequently destained electrophoretically with 7.5% acetic acid.

E. Isoelectric Focusing

Gel isoelectric focusing was performed according to the method of Wrigley (1968, 1971) as summarized below. The following solutions were used:

Solutions	Compositions
1) Acrylamide solution	14.8 gm acrylamide 0.33 gm MBA H_2O ——→ 50 ml

2) Sucrose	40% (w/v)
3) Ammonium Persulfate	100mg/ml (fresh each day of use)
4) Electrode buffers	a. 1% (v/v) ethanolamine b. 0.2% (v/v) phosphoric acid

Gel Preparation

The proceedure described is designed for six 0.6 x 16cm tubes. The following concentrated mixture was prepared just prior to casting the gels:

Acrylamide solution	7.0 ml
H_2O	1.62 ml
40% ampholytes (0.2ml pH3.5 - 10 0.5 ml pH2.5 - 4, 0.25 ml pH4 - 6)	0.7 ml

Each gel was mixed individually and contained the following:

concentrated mix	1.5 ml
40% sucrose	0.5 ml
sample dissolved in H_2O	2.3 ml
Ammonium persulfate	0.02 ml

The gels were overlaid with H_2O and allowed to polymerize for at least 1 hour.

Electrofocusing

The gels were fitted into the apparatus and were cooled at 5°C with a Lauda circulating water bath throughout the electrofocusing proceedure. The bottom electrode compartment was filled with 1% ethanolamine and the top chamber with 0.2% phosphoric acid. 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
50V	0.5 hour	50
100V	1 hour	50
150V	overnight	50

Staining and destaining

Gels were stained for 1 hour and 15 min. 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

Unstained gels were sliced in 2mm slices using a Gilson gel slicing apparatus. Cold, distilled, deionized water 500ul was added. The pH of each fraction was measured using a microelectrode in the cold room.

F. Periodic Acid Schiff Staining Proceedure

15% polycrylamide slab gels were produced using the proceedure described in section V.A. and stained according to the method of Fairbanks et.al.(1971). The gel was placed in a series of solutions for designated times. Most of the steps were performed in the electrophoresis apparatus to provide for constant circulation around the gel. Every solution change involved 1.5 litres. The overnight soaking of the gel in Schiff reagent was performed in a glass pan. The following solutions were used for the

designated times.

Solution	Composition	Time
1	25% isoproyl 10% acetic acid	overnight
2	10% acetic acid	2.5 hours
3	0.5% periodic acid	2 hours
4	0.5% sodium arsenite 5% acetic acid	30 - 60 min.
5	0,1% sodium arsenite 5% acetic acid	20 min.
6	repeat step 5	20 min.
7	5% acetic acid	10 - 20 min.
8	250 ml Schiff reagent*	overnight
9	0.1% sodium metabisulfite 0.01N HC1	several hours
10	repeat step 9 until the rinse solution fails to turn pink upon the addition of formaldehyde	

*Schiff Reagent

2.5 gm basic fuchsin in 500 ml H_2O
add 5 gm sodium metabisulfite and 50 ml 1N HC1
Solution is stirred for 2.5 hours and then decolourized with 2 gm activated charcoal

V1. Acid Hydrolysis and Amino Acid Analysis

Samples containing 0.02 to 0.1 moles of protein were hydrolyzed with 1 ml 6N HC1 containing 50ul 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 hrs. unless otherwise indicated. Where indicated 2 ul 2-mercaptoethanol was included in the hydrolysis solution to protect against cysteine destruction.

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.

V11. Tryptic Digestion and Peptide Mapping

1.0 mg of protein was dissolved in 100 ul of 0.1M ammonium bicarbonate, 0.1mM EGTA. The solution was saturated with nitrogen; 5ul of TPCK-trypsin (16mg/ml in 0.1M ammonium bicarbonate) was added; and the tube was covered, the sample mixed and incubated at 37°C for 2 hours. The digestion was stopped by the addition of 10 ul of 0.2N HC1. A sample (50 ul) 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, described by Tan and Stevens (1971) with 1% methyl green 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).

V111. Cyanogen Bromide Digestion

Cleavage of the protein modulator with cyanogen bromide was achieved by the method of Witkop (1961) as modified by Steers et.al.(1965). The protein (0.5 mg) was dissolved in 1.0 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

hours. Deionized water (at least 4 volumes) was then added and the mixture was lyophilized, redissolved in water, and again lyophilized.

1X. Tryptophan Determination

Tryptophan was determined spectrophotometrically according to the method of Goodwin and Morton (1946). An appropriate amount of protein is dissolved in 0.1N, NaOH, placed in a Cary 15 spectrophotometric cuvette, and the spectrum from 310nm to 240nm recorded. A spectrum of a 0.1N NaOH solution is also recorded for a blank. The concentration of tryptophan is determined by the following equation:

$$y = \frac{A_{280} - 0.6636\ A_{294.4}}{3649}$$

EXPERIMENTAL

1. Purification of Lobster Protein Modulator

Lobster protein modulator was isolated and purified by a modification of the proceedure described by Teo et.al. (1973) for bovine heart protein modulator.

A. Extraction and Heat Treatment

Live lobsters (Homarus americanus) were killed by severing the spinal cord behind the head. The muscle was then removed from the tail portion and claws, weighed, and placed in 4 volumes cold homogenizing buffer (40mM Tris, 20 uM $CaCl_2$, 1mM magnesium acetate, pH 7.5). Three lobsters give approximately 500 gm tissue. The material was then homogenized and centrifuged at 3000 x g for 30 minutes and the supernatant retained. The supernatant was then placed in two conical flasks and immersed in boiling water for 6 minutes, with occasional stirring. This material was then centrifuged at 10,000 x g for 10 minutes and the supernatant retained.

B. Concentration by Acid and Ammonium Sulfate Precipitation

In preparation for the application of the heat treated supernatant to a DEAE-cellulose column, the material was concentrated by acid and ammonium sulfate precipitation. Attemps were made to further purify the protein at this stage by ammonium sulfate fractionation as described by Teo et.al. (1973) however, this proved unsatisfactory as it resulted in very little purification and substantial losses

of the protein modulator. Adjustment of the pH to 4.1 was determined to produce optimal protein modulator precipitation.

The pH of the heat treated supernatant was adjusted to 4.1 with concentrated HCl and enough ammonium sulfate added to bring the salt concentration to 75% saturation. This solution was allowed to stand for 1 hour at $4^{o}C$, was centrifuged at 10,000 x g for 30 minutes, and the pellet suspended and dialyzed against Buffer A (0.02M imidazole, 1mM magnesium acetate, 20 uM $CaCl_2$, 0.15M NaCl, pH 6.5). The dialyzed mixture was then centrifuged at 10,000 x g for 10 minutes and the supernatant retained.

C. DEAE-cellulose Column Chromatography

The concentrated material was applied to a DEAE-cellulose cloumn (2.5 x 60 cm) previously equilibrated with buffer A and the excess protein washed off. Elution was achieved by a linear salt gradient consisting of 800 ml each of Buffer A and Buffer B (0.02M imidazole, 1mM magnesium acetate, 20 uM $CaCl_2$, 0.8M NaCl, pH 6.5). The flow rate was adjusted to approximately 100 ml/hr. The location of the protein modulator activity was determined by assaying the ability of selected fractions to activate bovine heart PDE prepared as described under general experimental proceedures. The conductivity and absorbance at 280nm of these selected fractions was also measured. A typical elution profile is seen in figure 1. The protein modulator peak was not associated with any major protein

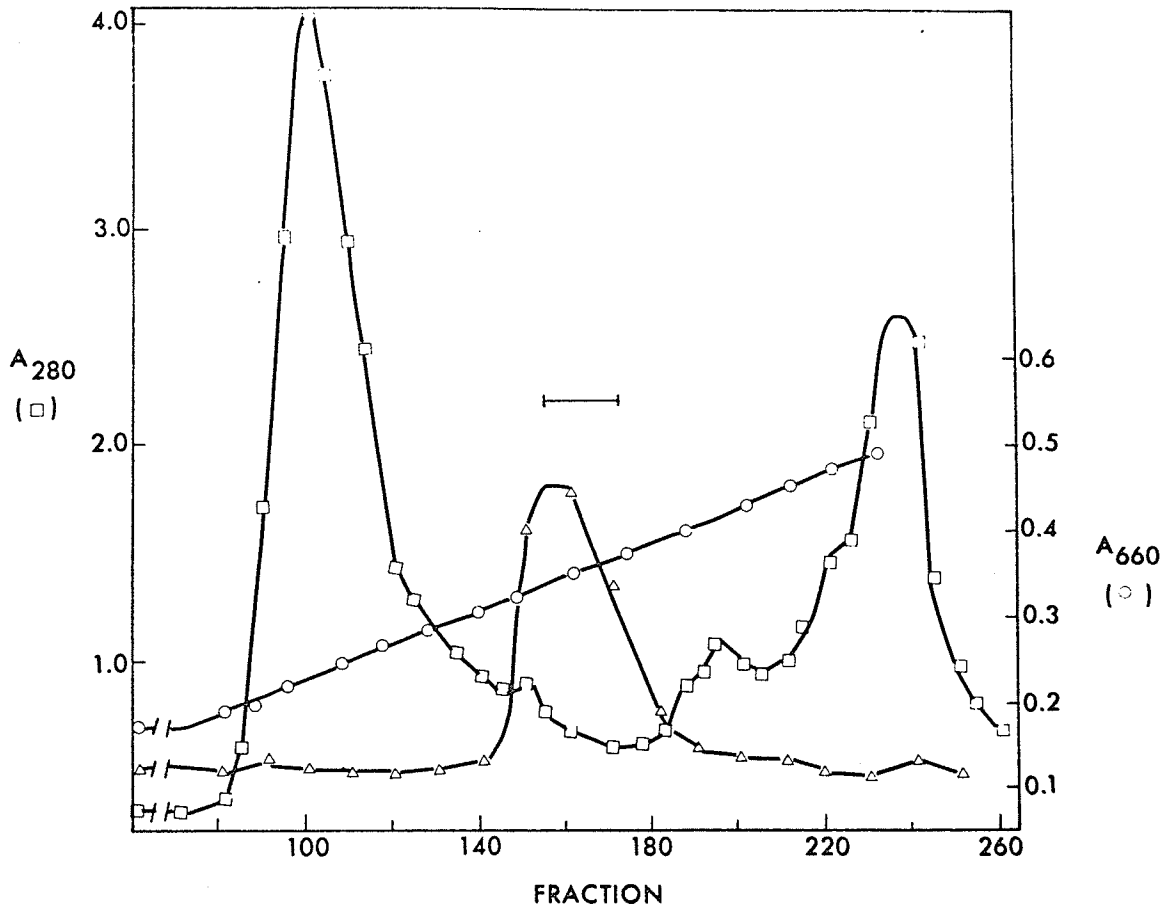


Figure 1: DEAE-cellulose chromatography of lobster protein modulator. The separation shown was performed and monitored as described in the text. 6.5 ml fractions were collected and the fractions indicated by the bar were pooled. Protein modulator activity was measured as indicated under general experimental proceedures and is shown as absorbance at 660nm(△ - △). Protein concentration was monitored by measuring the absorbance at 280nm (□ - □) . The conductivity was measured and converted to NaCl concentration by the use of a standard curve .

peaks and was typically eluted at salt concentrations between 0.32M and 0.37M. The fractions containing protein modulator activity were pooled, dialyzed against cold, distilled, deionized water; and lyophilized.

D. Sephadex G-75 Column Chromatography

The lyophilized sample from the DEAE-cellulose column was suspended in Buffer C (0.02M Tris, 1mM magnesium acetate, 1mM imidazole, 0.01mM $CaCl_2$, pH 7.5) and applied to a Sephadex G-75 column (2.5 x 90 cm) previously equilibrated with Buffer C. Protein was then eluted with Buffer C and selected fractions assayed for modulator activity and the conductivities and absorbance at 280nm were measured. A typical elution profile is seen in figure 2. Again the modulator activity peak was not associated with any major protein peak and as would be expected for a protein containing no tryptophan, the region of modulator activity shows very little absorbance at 280nm. The fractions associated with modulator activity were pooled; dialyzed against distilled, deionized water; and lyophilized for storage.

E. Preliminary Assessment of Purification

The results for a typical purification are shown in Table 1. Specific activities of modulator preparations varied between 50,000 and 86,000 units/mg protein, however, this variation is presumably due in great part to variations in the enzyme preparations used for assay.

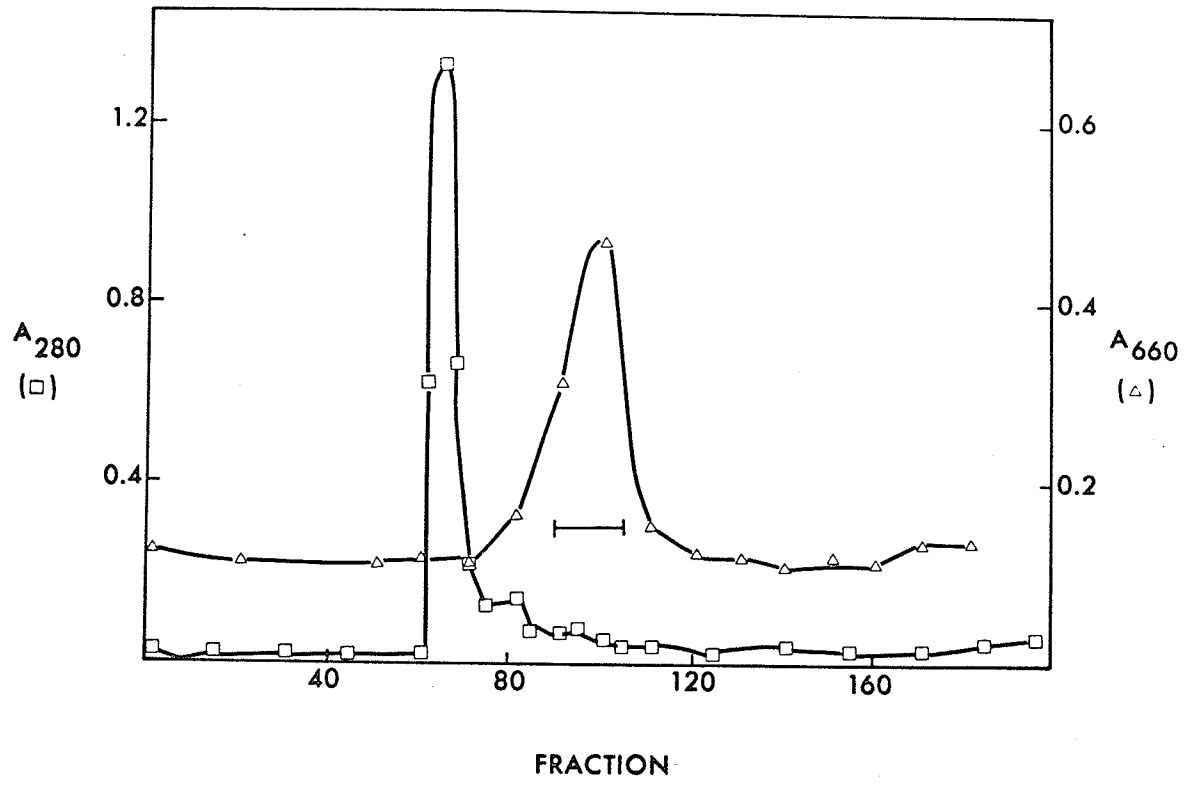


Figure 2: Sephadex G-75 chromatography of lobster protein modulator. The separation was performed and monitored as described in the text. 3.5 ml fractions were collected and the fractions indicated by the bar were pooled. Protein modulator activity is shown as absorbance at 660nm (△ - △). Protein concentration is shown as absorbance at 280nm(□ - □) .

TABLE 1. Purification of Lobster Protein Modulator

Fraction	Protein (mg)[a]	Total Activity ($\times 10^{-3}$ units)	% Yield	Specific Activity (units/mg)
Homogenate	8400	700	100	84
Homogenate after heat treatment	3300	561	80	171
DEAE-cellulose	11.4	215	31	18,900
Sephadex G-75	2.2	185	26	86,000

[a]protein concentration was determined according to the method of Lowry (1951) as described in "General Experimental Proceedures"

The purification of the modulator was also monitored by 15% polyacrylamide slab gel electrophoresis as shown in figure 3. Approximately 20 ug of material was removed at each stage in the purification proceedures; dialyzed against distilled, deionized water; and lyophilized. These samples were then dissolved in the appropriate sample buffer (see general experimental proceedures) and electrophoresed. This gel demonstrates that there is a marked purification of a very acidic protein, presumably the protein modulator. In the early stages of purification, the majority of the detectable protein runs in the upper third of the gel. A 15% acrylamide gel has a fairly small pore size and therefore these proteins presumably have higher molecular weight and/or less negative charge than the protein modulator. The protein modulator represents such a small proportion of the total protein in the early stages that it is not detectable when 20 ug is applied to the gel. DEAE-cellulose ion ex-

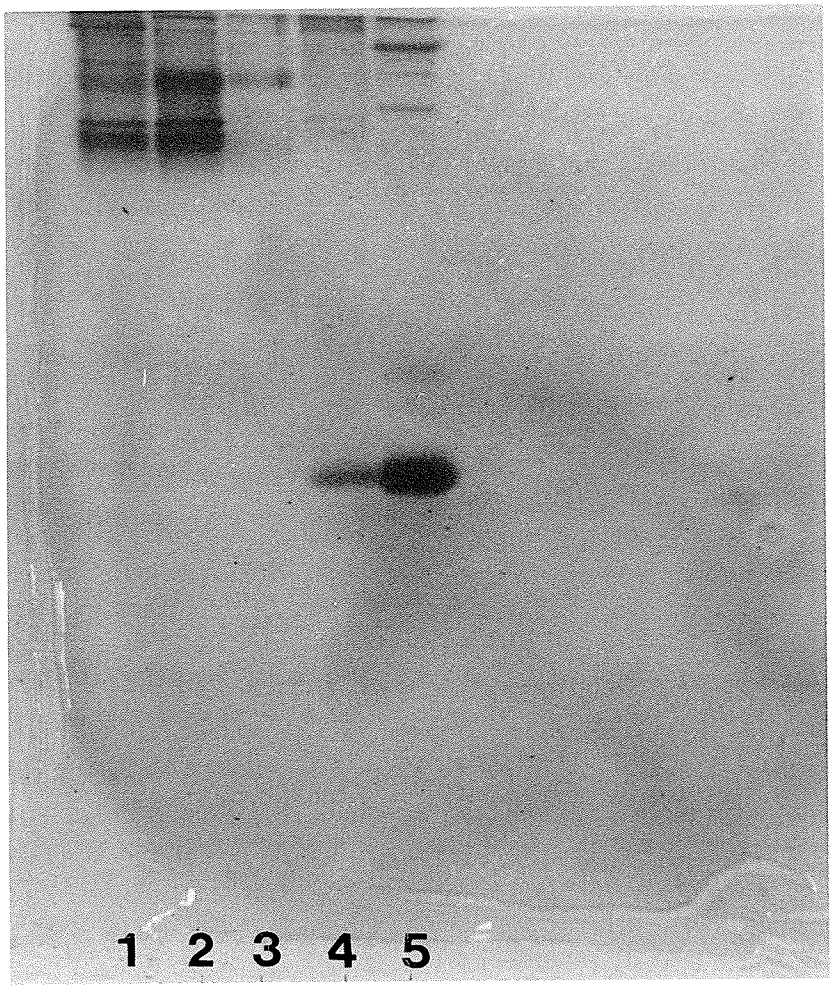


Figure 3: Purification of the lobster protein modulator monitored by 15% PAGE.
1. homogenate supernatant; 2. heat treatment supernatant; 3. dialyzed acid and ammonium sulfate concentrate; 4. DEAE-cellulose pooled fractions; 5. Sephadex G-75 pooled fractions.

-change chromatography and Sephadex G-75 gel filtration chromatography removes a large proportion of the impurities. The protein modulator is the major component at these final stages, however, the final sample after gel filtration still contains a significant amount of high molecular weight and/or less negatively charged impurities. These preparations were pooled as such, as it was assumed that the impurities would be removed by the final DEAE-cellulose chromatography carried out on the pooled sample (see page 128).

In order to establish that the modulator is in fact the major band visualized by 15% PAGE, the following experiment was conducted. Three 15% acrylamide tube gels of equal length were prepared according to the method of Davis (1964). 40 ug of the protein modulator was applied to each of the gels and bromophenol blue was used as a tracking dye. The electrophoresis was run simultaneously and stopped when the tracking dye band reached the bottom of the gels. The position of the tracking dye marked with a small wire in two of the gels. One gel was then sliced in 2mm slices in a Gilson gel slicing apparatus. The slice containing the tracking dye was noted and 500 ul distilled water added to each slice. Aliquots from each slice were then assayed for protein modulator activity. The remaining two gels were removed from the glass holders immediately upon completion

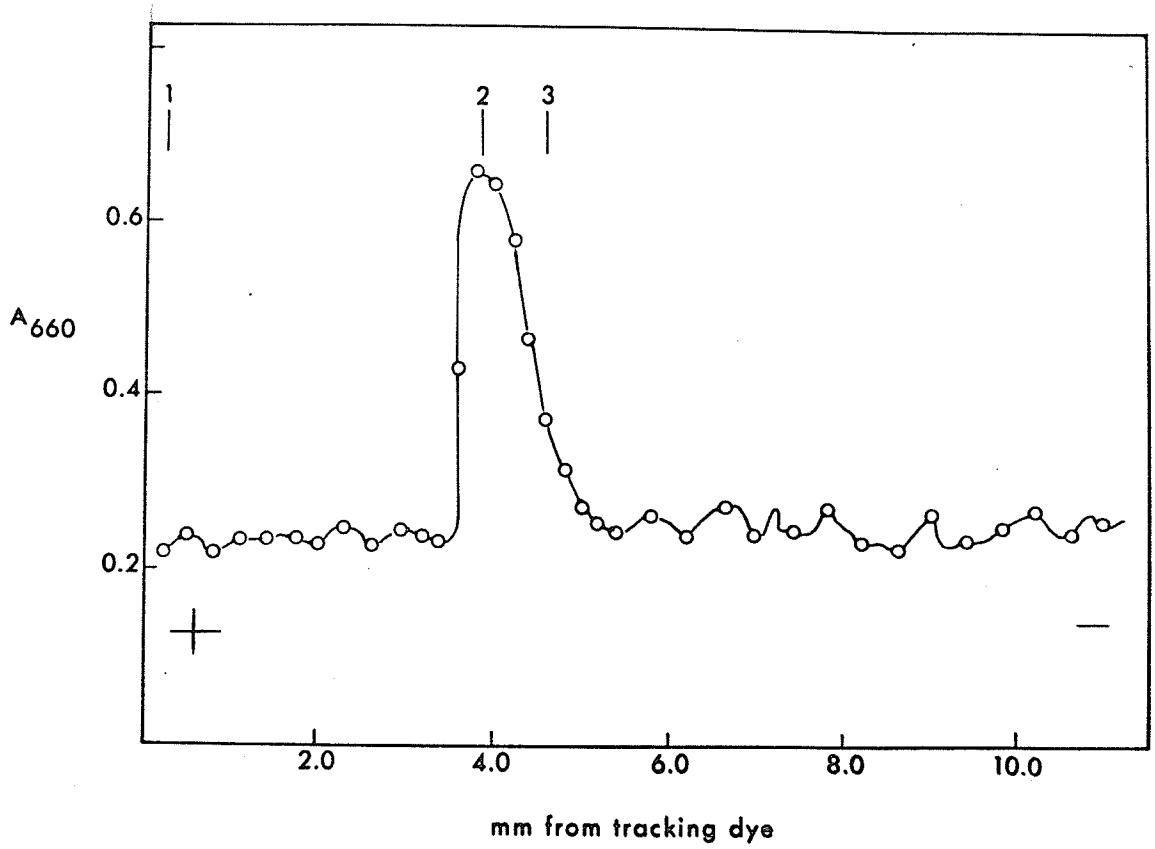


Figure 4: Identification of the major protein peak of Sephadex G-75 pooled fractions as the protein modulator.
Protein modulator activity of gel slices are shown as absorbance at 660nm. The calculated location of the three stainable protein bands are indicated.

of the run, stained with an Amido-Black solution, and destained in 7.5% acetic acid. After destaining the gel length, position of the tracking dye and position of any protein bands were recorded. In this way, the position of the protein bands after staining and destaining can be correlated with the position of modulator activity in the sliced gel. The result of this study is shown in figure 4. Three bands were clearly observed in this early preparation. Band 2 was the major protein band. Band 1 was a very faint band while band 3 is the impurity band observed throughout the modulator purification which will be discussed in section 11. Thus the major protein band observable by gel electrophoresis (band 2) is clearly the protein modulator.

11. Purity of the Protein Modulator Preparation

Attempts to determine the purity of the protein modulator isolated from lobster muscle has proven difficult. The major problem presented in the purification is the presence of a second minor protein band detectable by 15% PAGE which is stainable with either amido black or coomassie blue. All samples of lobster protein modulator were purified without the use of PMSF in the homogenizing buffer unless otherwise indicated.

A. Investigation of Impurities Detectable by 15% PAGE

The protein modulator is a highly acidic, low molecular weight protein and therefore 15% PAGE was selected for the preliminary determinations of the purity of the modulator

preparations (figure 5). One major band previously determined to exhibit modulator activity, one clearly significant minor band possessing no modulator activity and several other minor bands are observed. The gels in figure 5 were stained with amido black. 15% polyacrylamide gels of lobster protein modulator, stained with coomassie blue (figure 9; slots 9,10,11) show similar banding patterns. As was expected the major impurities were removed by the final DEAE-chromatography on pooled preparations (see page 128), however, these minor impurities were not removed by this proceedure.

The possibility that these impurities detectable by 15% PAGE are merely aggregates of the protein modulator was then assessed. Gels were prepared by the method of Davis (1964) with the addition of enough urea to the running gel, stacking gel, and sample buffer to produce a final urea concentration of 8M. Samples of lobster protein modulator electrophoresed under these denaturing conditions migrate as a single, well focused band. This could indicate that indeed protein aggregation is the cause of the impurities detectable by 15% PAGE, however, the addition of urea to the gel system described above actually produces a gel consisting of only 11.5% acrylamide. Samples of the protein modulator were then electrophoresed on a 11.5% polyacrylamide gel in the absence of urea (figure 6) to help clarify this point. As can be seen in figure 6, the modulator from lobster migrates as a single band on 11.5%

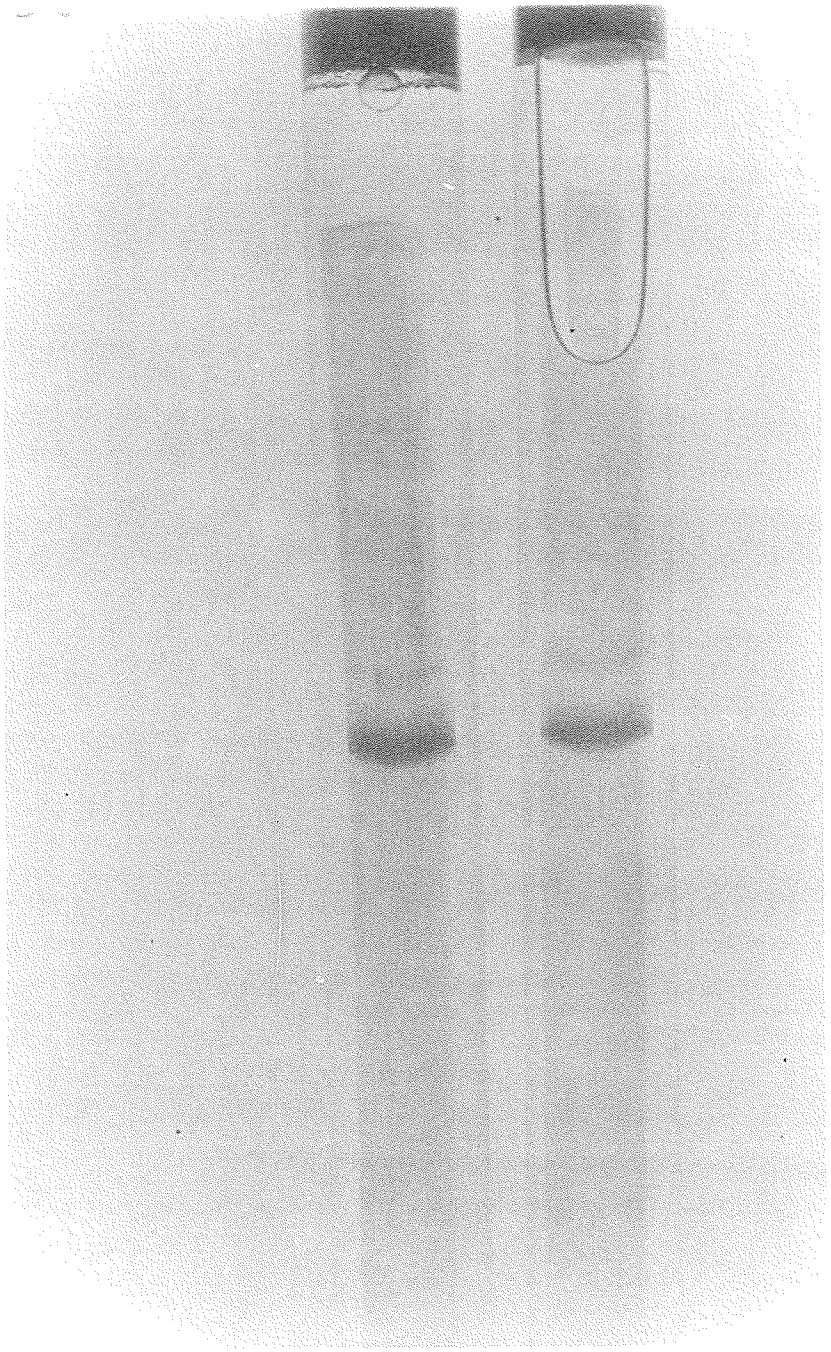
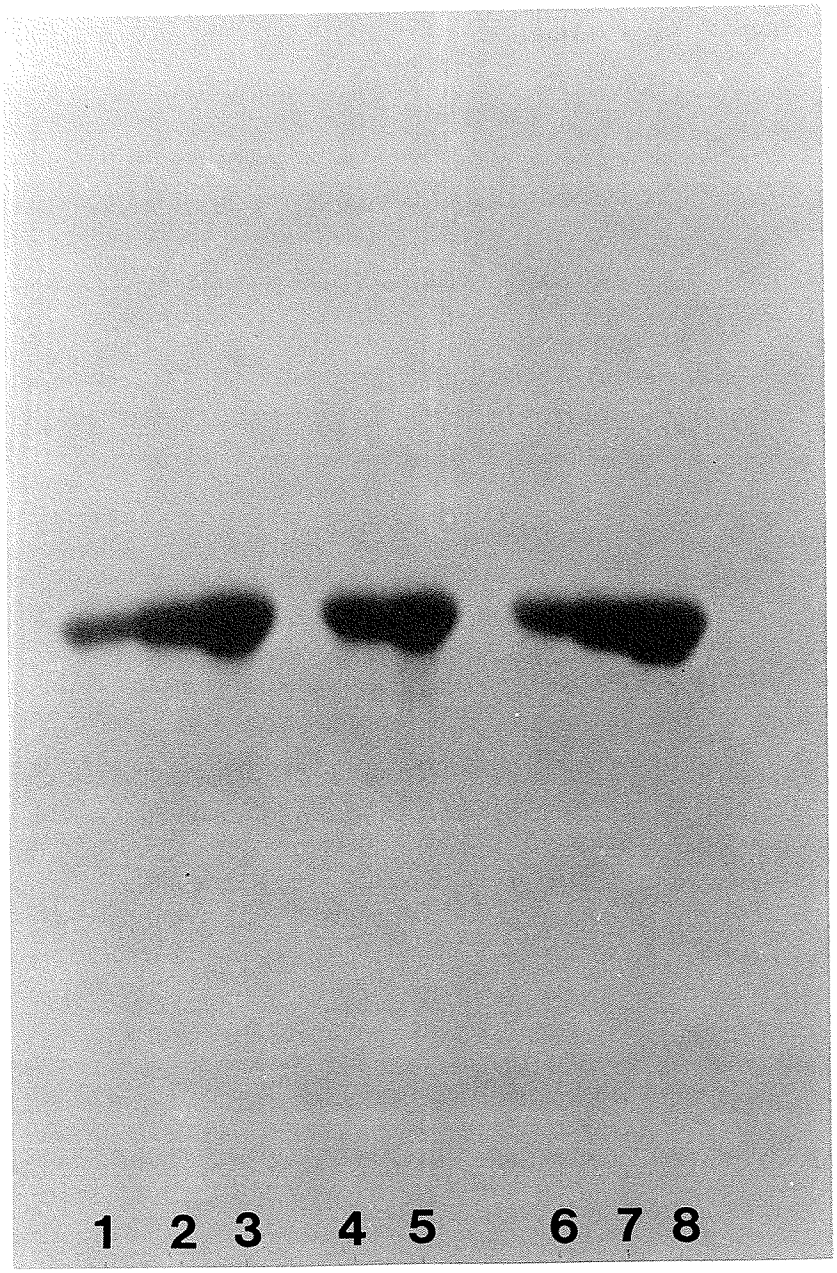


Figure 5: 15% PAGE of lobster protein modulator 15ug of protein was applied to the gels. The gels were prepared and run according to the method of Davis (1964) and stained with amido black.

Figure 6: 11.5% polycrylamide gel electrophoresis of lobster protein modulator. Three different lobster preparations were electrophoresed. Sample 3 was prepared using PMSF. 1. 10ug sample 1; 2. 20ug sample 1; 3. 40ug sample 1; 4. 15ug sample 2; 5. 25ug sample 2; 6. 10ug sample 3; 7. 20ug sample 3; 8. 40ug sample 3.



PAGE and thus would migrate as a single band on such gels whether in the presence or absence of 8M urea.

A 15% acrylamide gel containing 8M urea was prepared by modifying the method of Davis (1964) using a more concentrated stock acrylamide solution. This proceedure is described under "General Experimental Proceedures". Lobster protein modulator samples were electrophoresed in this gel system (figure 7) and the results indicate that indeed, protein aggregation is probably not the cause of the additional protein bands detectable by 15% PAGE. In fact, it appears that in this system the relative intensity of the impurity band actually increases, as the major component appears as a doublet. Figure 19 shows photographs of an electrophoretic study of the effect of Ca^{2+} on the mobility of bovine brain and lobster protein modulator in urea-PAGE. These studies indicate that there is an impurity present in the bovine brain preparation which migrates identically with the impurity of the lobster preparation, however, the lobster impurity is increased many fold by electrophoresis in urea-containing gels contrary to the results indicated for bovine brain protein modulator. Behavior such as this on urea gels is difficult to explain and the reason for this increased intensity of the impurity is unknown. This type of behavior has not been described by other investigators for the modulator isolated from other sources, and thus it is not known whether this is a unique property of lobster protein modulator.

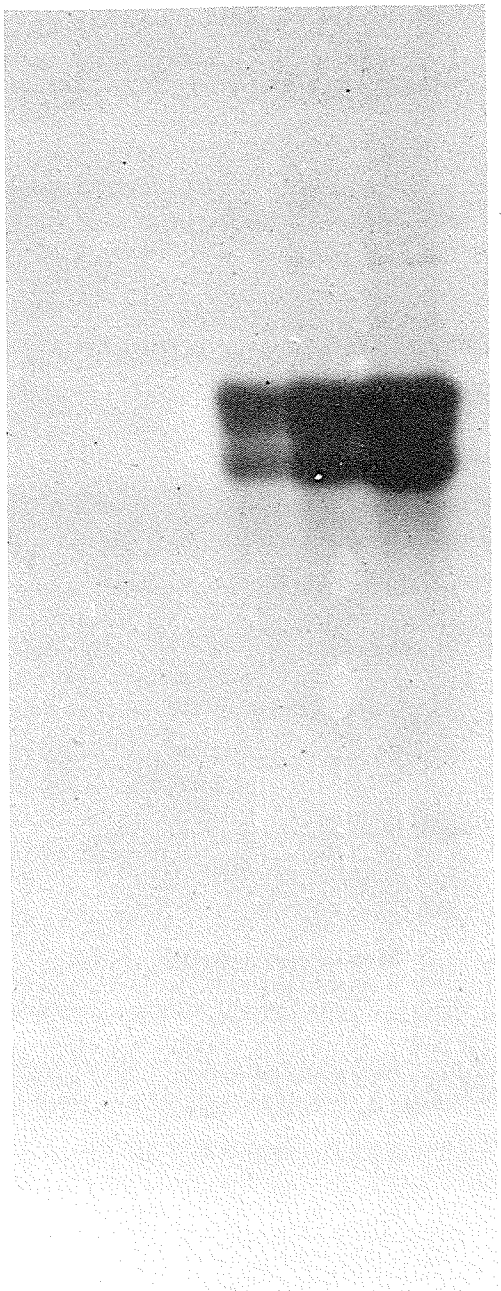


Figure 7: 15% polyacrylamide-8M urea gel electrophoresis of lobster protein modulator.

1. 10ug; 2. 20ug: 3. 40ug.

Watterson et.al. (1976a) and Walsh (1978), on the other hand, indicate that the protein modulator isolated from bovine brain does indeed show aggregation phenomena. Watterson et.al. (1976a) with bovine brain modulator using 12.5% PAGE detected impurities similar to those described for lobster protein modulator. They noted that using 6M urea-12.5% PAGE that these extra bands were no longer detectable. Walsh (1978) demonstrated an identical banding pattern on 15% polyacrylamide gels as those described by Watterson et.al. (1976a), however, 12.5% or 15% PAGE in the presence of urea was not performed. As can be seen in figure 9 (slots 7-11; slots 1-6 will be discussed on page 107) the extra bands present in lobster protein modulator samples migrate identically with extra bands present in bovine brain modulator samples. It is interesting that the impurities in both types of modulator migrate identically in 15% PAGE and yet the lobster impurity bands are apparently not eliminated by urea-gel electrophoresis. This difference in the behavior of the two proteins in urea-polyacrylamide gel electrophoretic studies may indicate that the modulator from bovine brain and lobster are significantly different.

B. Effect of a Protease Inhibitor on Modular Purification

Lobster muscle is noted for extremely high proteolytic activity and thus it is possible that the impurities observed on 15% polyacrylamide gels are due to some mild limited proteolytic digestion of the modulator itself. The protein modulator was prepared from three lobsters using a

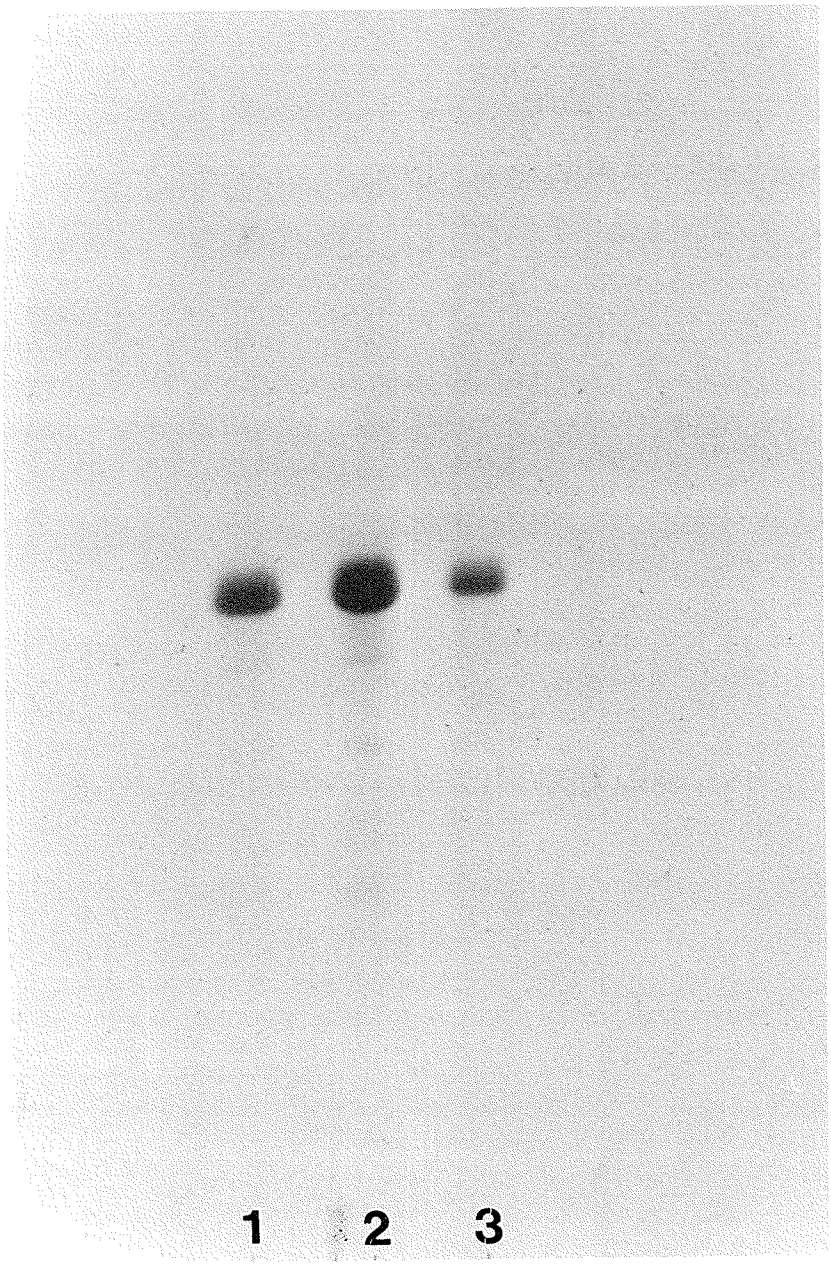


Figure 8: 15% polyacrylamide gel electrophoresis of lobster protein modulator extracted and purified in the presence of PMSF, a protease inhibitor.

1. 20ug; 2. 40ug; 3. 10ug.

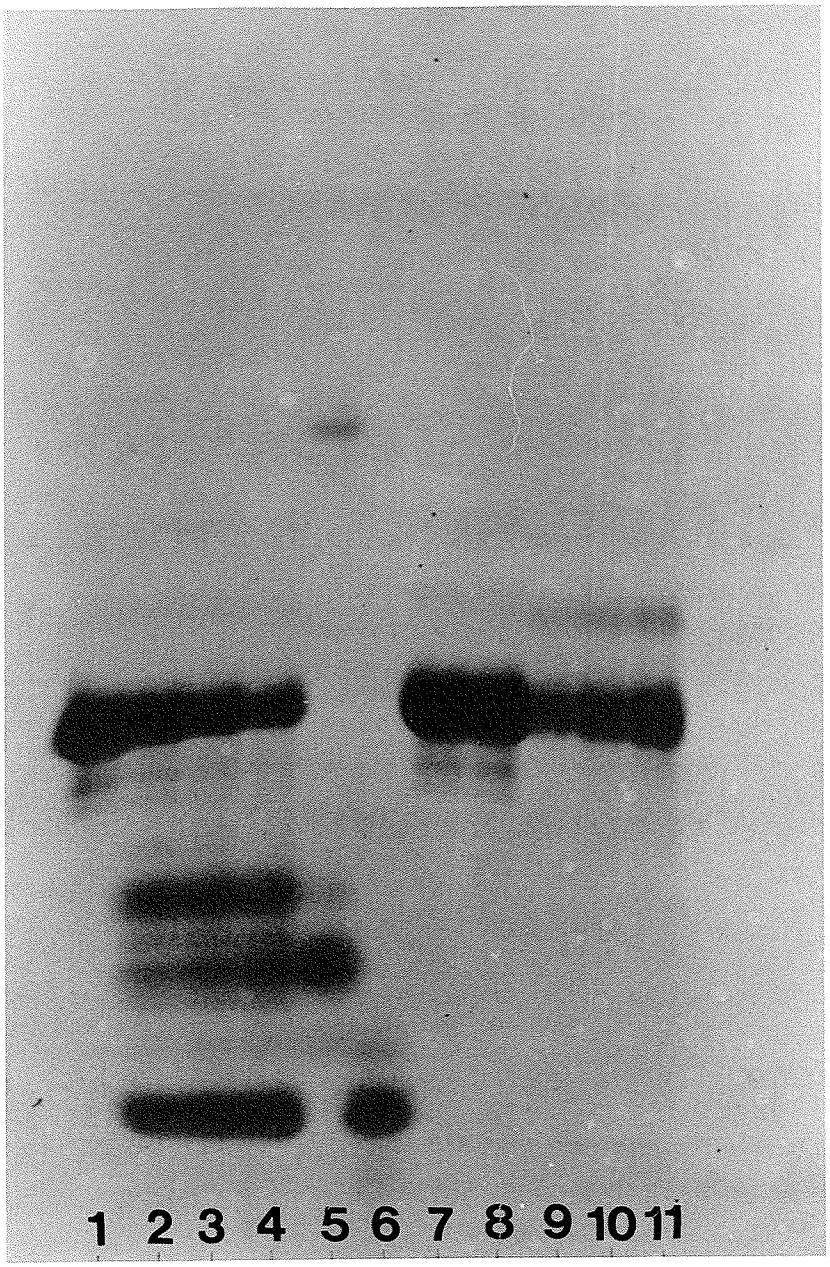


Figure 9: 15% PAGE of modulator proteins and tryptic digestion products.

1. bovine brain modulator; 2-4. tryptic digestion products of bovine brain protein modulator; 5-6 purified tryptic digestion products of bovine brain modulator; 7-8. bovine brain modulator; 9. 12ug lobster modulator; 10. 20ug lobster modulator; 11. 30ug lobster modulator

purification proceedure identical to the one previously described with the addition of PMSF (phenyl methyl sulfonyl fluoride) to the homogenizing buffer. PMSF is a protease inhibitor and a stock solution of 6mg/ml PMSF in 95% ethanol was prepared and the homogenizing buffer made 5% v/v with this solution. The elution profiles of the DEAE-cellulose and Sephadex G-75 chromatography of this preparation were virtually identical to those obtained in other preparations. The protein modulator, purified in this manner, migrated as a single band on 15% polyacrylamide gels (figure 8). Thus it appears that the impurity band is the result of proteolytic digestion occurring prior to heat treatment. This impurity may be a digestion product of the protein modulator or of another unrelated protein. Whatever its source, it is difficult to remove from the modulator preparation.

As mentioned in the literature review, the protein modulator is fairly stable to tryptic attack in the presence of Ca^{2+} and limited proteolysis has been shown to occur (Walsh et.al., 1977). Such a limited proteolysis might occur with the lobster modulator prior to heat treatment if PMSF is not present in the homogenizing buffer. Lobster protein modulator and bovine brain protein modulator have identical mobilities on 15% polyacrylamide gels, while products of controlled tryptic digestion of bovine brain protein modulator migrate much closer to the anode (figure 9) (Walsh et.al., 1977). This indicates that the

impurities present in the lobster modulator preparations are probably not tryptic digestion products of the protein unless the modulator contains a lysine or arginine residue in an unusual position. Indeed, as will be shown in Experimental Section 1V, lobster protein modulator appears to have two trypsin susceptible lysine residues which are not susceptible to trypsin in bovine brain protein modulator. They may also be due to the activity of some other proteolytic enzyme resulting in the production of smaller peptides which appear closer to the cathode in this gel system.

C. SDS-PAGE

Many attempts were made to visualize lobster protein modulator using SDS-PAGE. When methanol was used as the protein fixing agent, the modulator was barely visible as a diffuse smear. Using isopropyl alcohol as fixing agent, according to the method of Fairbanks et.al. (1971), the detection of the protein modulator is improved. 10% polyacrylamide SDS gels (figure 10) and 15% polyacrylamide SDS gels (figure 11) of the protein modulator fixed with isopropyl alcohol show one relatively well focused band and a trailing, diffuse region while the marker proteins appear as distinct, well focused bands. The banding pattern observed is identical whether the modulator preparations were prepared in the presence or absence of PMSF.

It is well known that glycoproteins often show abnormal behavior, including smearing, on SDS gels. Although

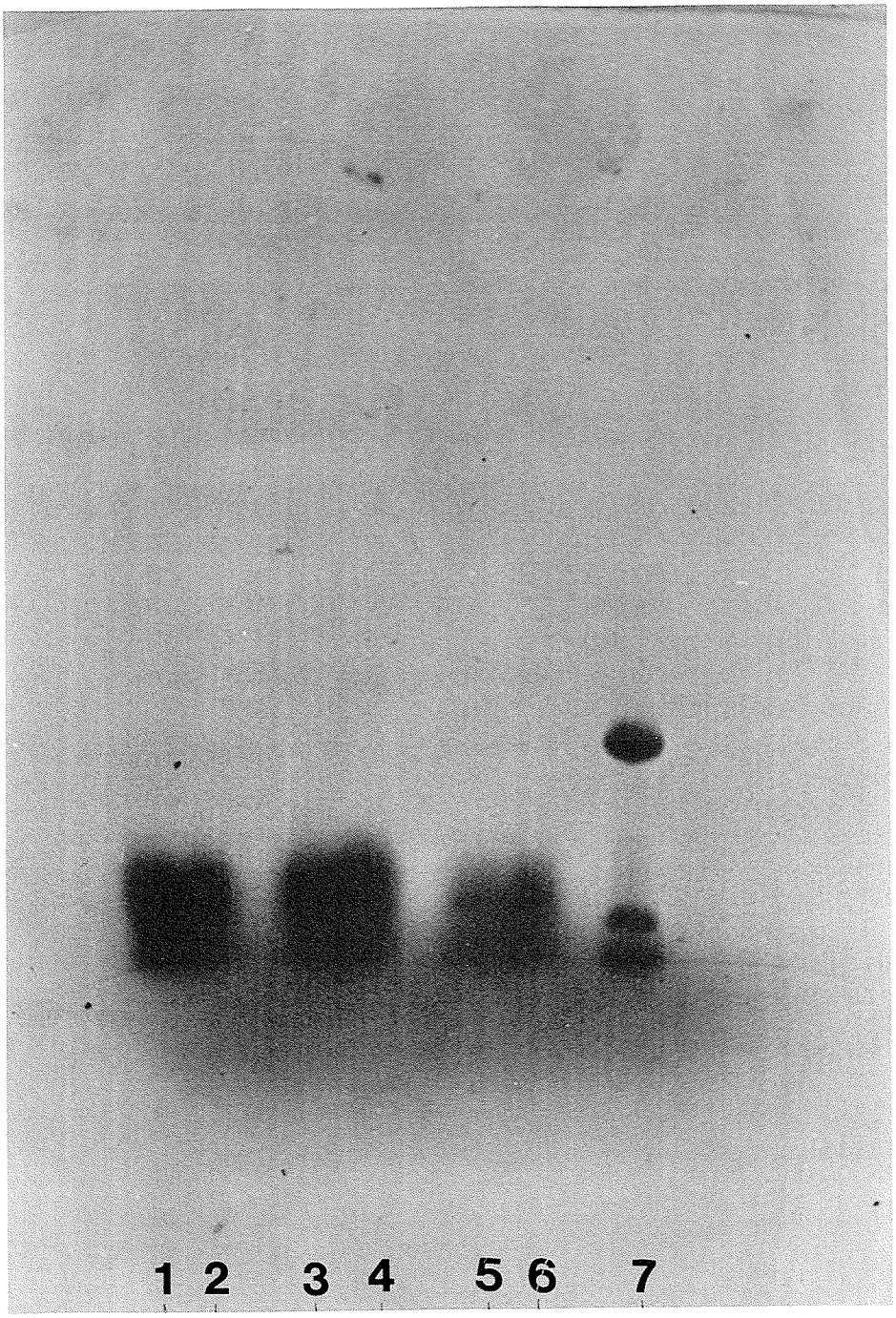


Figure 10: 10% polyacrylamide-SDS gel electrophoresis of lobster protein modulator. Three different lobster preparations were analyzed. Sample 3 was prepared using PMSF.

1. 30ug sample 1
2. 50ug sample 1
3. 30ug sample 2
4. 50ug sample 2
5. 30ug sample 3
6. 50ug sample 3
7. marker proteins (myoglobin, chymotrypsinogen, cytochrome c)

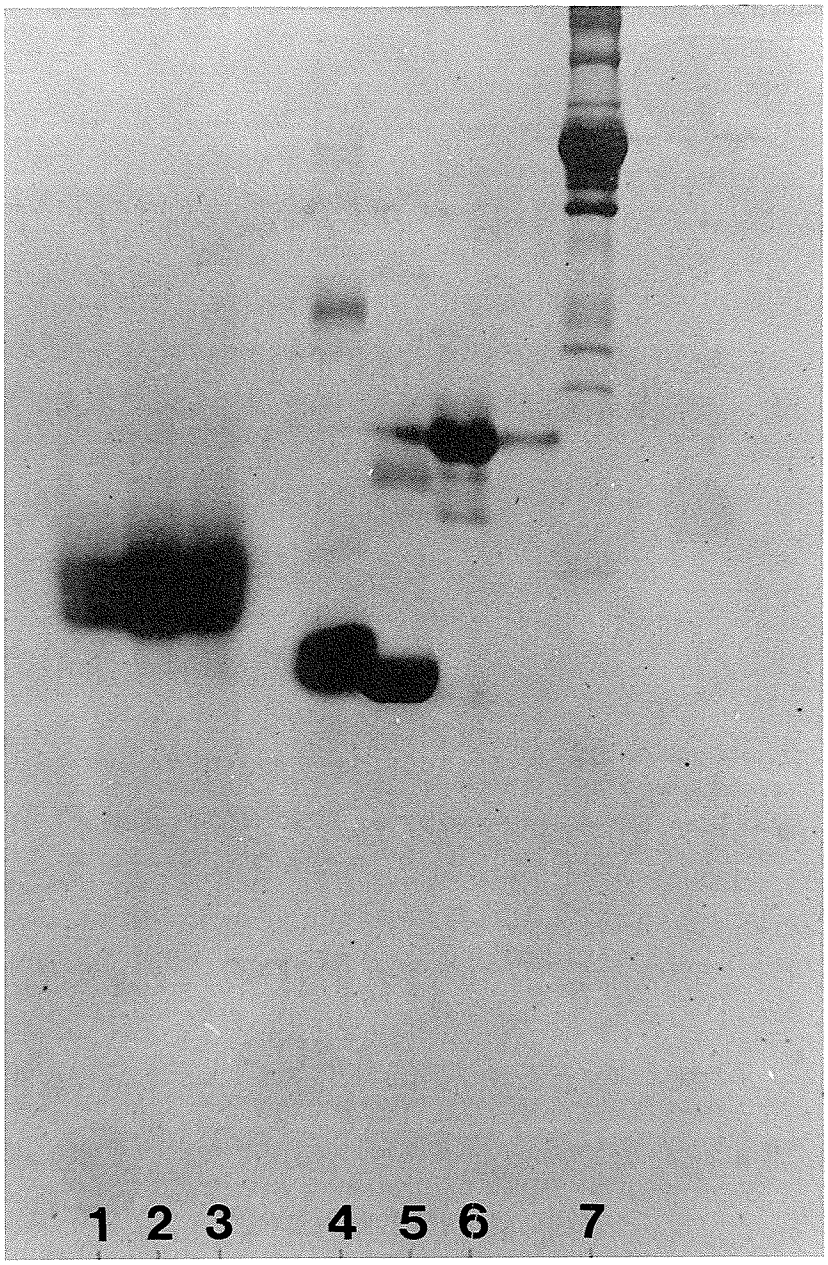


Figure 11: 15% polyacrylamide-SDS gel electrophoresis on lobster protein modulator prepared using PMSF.

1. 10ug modulator
2. 20ug modulator
3. 40ug modulator
4. 10ug myoglobin
5. 10ug cytrochrome c
6. 10ug chymotrypsinogen
7. 10ug albumin

many investigators have indicated that the protein modulator has no carbohydrate component (see Section 1VB in the literature review), the lobster protein modulator was examined for carbohydrate content. 15% polyacrylamide gels were prepared by the method of Davis (1964) and stained by the periodic acid Schiff proceedure (Fairbanks et.al., 1971) also described in "General Experimental Proceedures". Samples of ovomucoid, lobster protein modulator prepared with PMSF, bovine brain protein modulator, and earth-worm protein modulator were electrophoresed and stained (figure 12) and all the protein modulator samples appeared to be Schiff positive although not nearly as strongly positive as ovomucoid. The experiment was then repeated using ovomucoid (17-33% carbohydrate), ovalbumin (3% carbohydrate), and chymotrypsinogen (0% carbohydrate) as standards (figure 13). Lobster protein modulator was again shown to be Schiff positive with an intensity of staining approximately equal to ovalbumin while chymotrypsinogen was schiff negative as would be expected. This carbohydrate may represent an impurity present in the lobster protein modulator samples, however, it also would have to be an impurity present in bovine brain and earthworm modulator preparations.

The presence of approximately 3% carbohydrate as a component of the protein modulator might explain its anomalous behavior on SDS gels, however, bovine brain modulator preparations exhibit normal behavior on SDS gels despite the apparent presence of this carbohydrate.

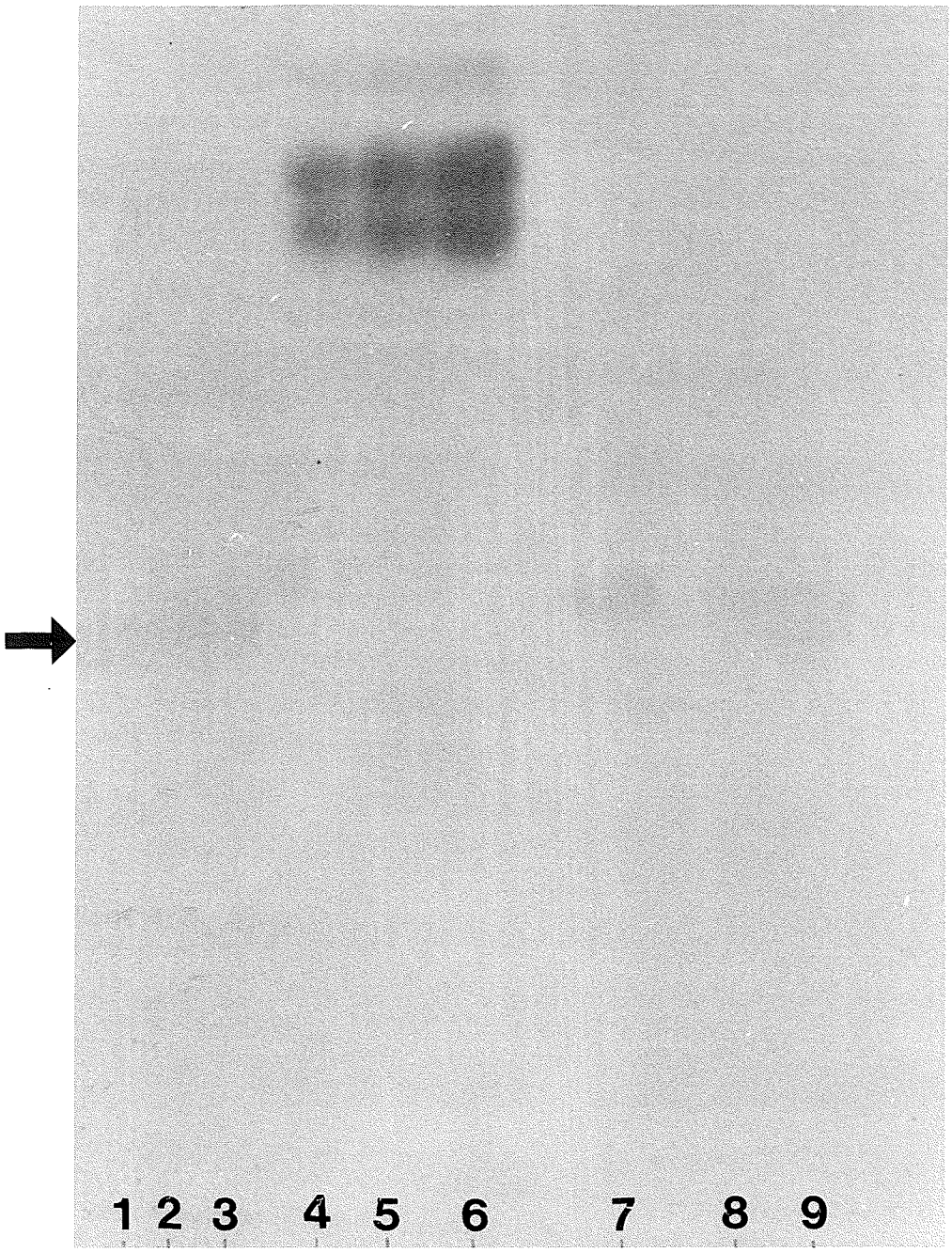


Figure 12: 15% PAGE of protein modulators stained with the PAS proceedure. The lobster preparations were purified in the presence of PMSF

1. 20ug lobster modulator
2. 30ug lobster modulator
3. 50ug lobster modulator
4. 20ug ovomucoid
5. 30ug ovomucoid
6. 50ug ovomucoid
7. 15ug bovine brain modulator
8. 15ug earthworm modulator
9. 60ug earthworm modulator

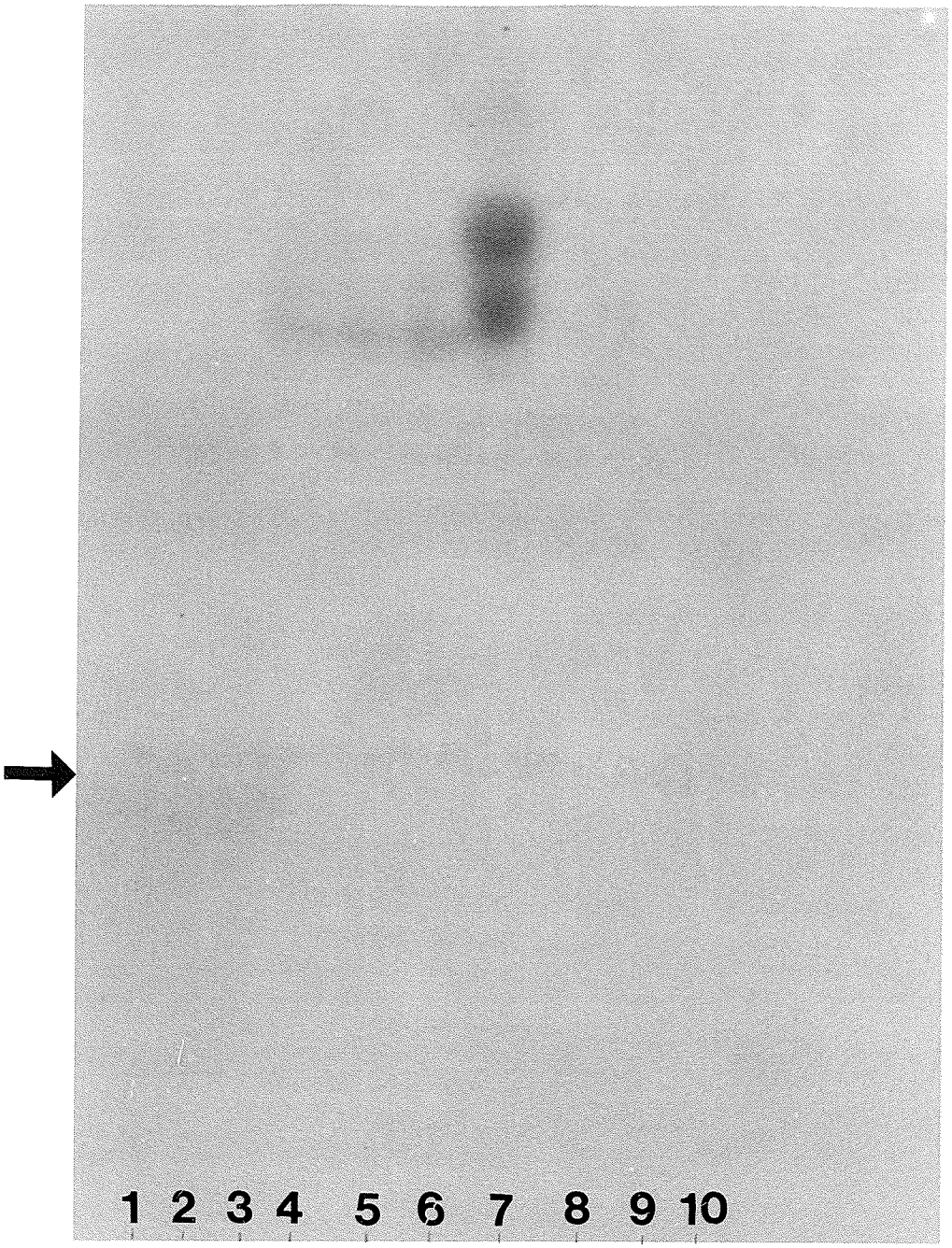


Figure 13: 15% PAGE if PMSF lobster protein modulator stained by the PAS proceedure.

1. 20ug lobster modulator
2. 30ug lobster modulator
3. 50ug lobster modulator
4. 20ug ovalbumin
5. 30ug ovalbumin
6. 50ug ovalbumin
7. 50ug ovomucoid
8. 20ug chymotrypsinogen
9. 30ug chmotrypsinogen
10. 50ug chymotrypsinogen

D. Isoelectricfocusing

Analytical isoelectric focusing was performed on the lobster protein modulator as described in "General Experimental Proceedures". It was hoped that preparative isoelectric focusing might be used to remove the impurity present in the sample, however, the analytical gel (results not shown) showed the presence of only one major protein band with an isoelectric pH of 4.1 and an extremely small impurity band with an isoelectric pH of 4.7. This proceedure would not be suitable for purification.

E. Conclusions about the Impurity Material

The ideal solution to this problem would be to prepare adequate amounts of modulator using PMSF in the purification proceedure. The required amounts of modulator had already been prepared, however, by the original purification proceedure and time did not allow for the production of a new modulator. The impurity is present in relatively small proportion and as it may be simply a digestion product of the modulator, the material already prepared was used for the final experimental proceedure involving tryptic digestion of the modulator and the isolation of the peptides by Dowex ion exchange Chromatography.

111. Characterization of Lobster Protein Modulator

A. U.V. Absorption Spectrum

The U.V. absorption spectrum of the protein modulator isolated from lobster was recorded between wavelengths of

310nm and 240nm by means of a Cary spectrophotometer model 15 (figure 14). The protein concentration was 1.4mg/ml as determined by the method of Lowry (1951) and the pH of the solution 7.5. At this pH, the U.V. absorption spectrum shows absorption peaks at 253,259,266,269, and 276nm. These values correlate well with the absorption peaks present in the spectra of the protein modulator isolated from other tissues and species and the spectra of other members of this group of homologous calcium binding proteins. These unique optical properties are indicative of proteins containing no tryptophan and a high phenylalanine to tyrosine ratio.

B. Amino Acid Composition

The amino acid composition of lobster protein modulator was determined as indicated in "General Experimental Proceedures" (Table 11). The molecular weight of lobster protein modulator is not known and therefore the amino acid composition was calculated assuming the protein contains 148 residues as does bovine brain protein modulator (Vanaman et.al., 1977). The lobster modulator shows the general properties previously described for all of the calcium binding proteins (literature review Section 1V.A.) namely: (1) the protein contains no trypyophan; (2) the protein has a high phenylalanine to tyrosine ratio; and (3) the protein contains a high proportion of acidic residues.

Table 11 also shows a comparison between the compositions of lobster protein modulator, bovine brain protein

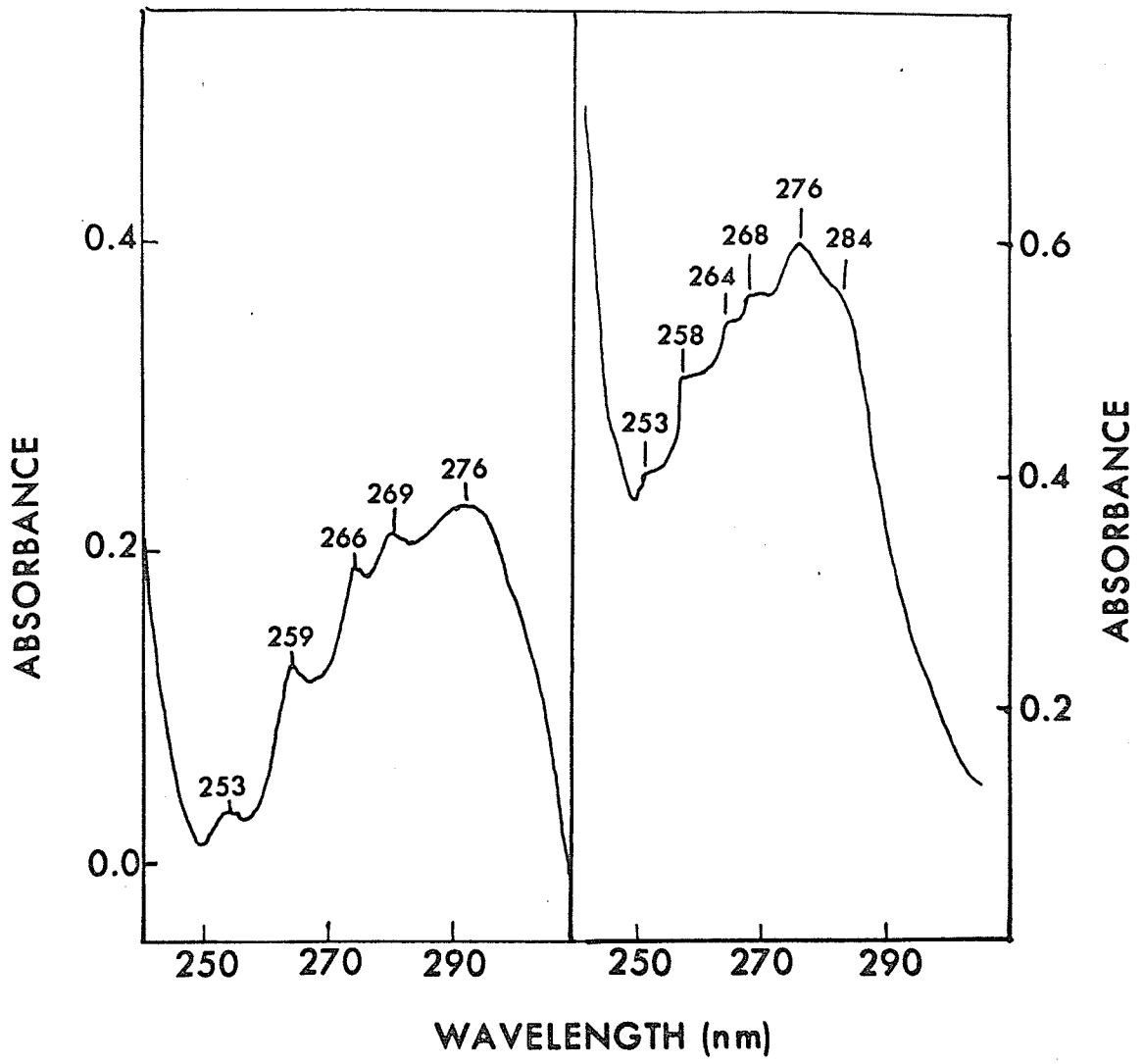


Figure 14:

UV absorption spectrum of lobster protein modulator.

A. lobster protein modulator (1.4mg/ml) dissolved in 20mM Tris, 1mMg acetate, pH 7.5

B. bovine brain protein modulator (5.0mg/ml) dissolved in 20mM Tris, 1mM magnesium acetate, pH 7.5

Table 11. Amino Acid Composition of Calcium-binding Proteins

AMINO ACID	AMINO ACID COMPOSITION			
	Lobster Protein Modulator[a]	Bovine Brain Modulator[b]	Rabbit Skeletal Muscle TNC[c]	Lobster Muscle TNC[d]
Lys	8.37 (8)	8	9	15
His	1.84 (2)	1	1	1.92
Arg	5.67 (6)	6	7	6.72
Asp	20.59 (21)	23	22	18
Thr	11.5[f] (12)	12	6	6
Ser	7.10[f] (7)	4	7	5.12
Glu	31.16 (31)	27	31	28.9
Pro	4.96 (5)	2	1	7.8
Gly	12.31 (12)	11	13	24.8
Ala	9.36 (9)	11	13	12.3
Cys	-	0	1	-
Val	7.92 (8)	7	7	9.4
Met	6.11 (6)	9	9	-
Ileu	6.19 (6)	8	10	6.9
Leu	7.73 (8)	9	9	11.8
Tyr	1.40 (1)	2	2	-
Phe	7.02 (7)	8	10	5.44
Tryp	0[e]	0	0	-

[a]The lobster sample used for this analysis was purified using PMSF and the values calculated assuming the protein contains 148 residues.

[b]Taken from its known primary sequence (Vanaman et.al., 1977)

[c]Taken from its known primary sequence (Collins et.al., 1973)

[d]Calculated from Regenstein and Szent-Gyorgyi (1975)

[e]determined spectrophotometrically

[f]extrapolated to 0 time using 22hr, 48hr and 72 hr hydrolysis time figures

modulator, rabbit skeletal muscle TNC, and lobster muscle TNC. As can be seen, the lobster modulator and bovine brain modulator compositions are very similar with only major differences being observed in the values obtained for histidine, serine, proline, and methionine. Comparison of the compositions of the two proteins with their mammalian counterparts provide some interesting results. Both the lobster modulator and TNC have two histidine residues whereas bovine brain protein modulator and rabbit muscle TNC have only one histidine residue. Likewise, both the lobster proteins have significantly higher values for proline whereas mammalian TNC has only two proline residues and mammalian modulator has only one proline residue. The difference in the number of methionine residues is also interesting. Bovine brain protein modulator contains 9 methionine residues whereas the protein modulator isolated from lobster contains only 6 methionine residues. Walsh and Stevens (1977a, 1978b) and Walsh (1978) have implicated three methionine residues in the PDE binding site of the protein modulator. Presumably these three residues which are conserved in the protein modulator from bovine brain and in TNC are also present in lobster protein modulator whereas three other methionine residues have been replaced.

C. Tryptophan Determination

Tryptophan was determined by the method of Goodwin and Morton (1946) as described in "General Experimental

Proceedures". 2.55 mg lobster protein modulator was dissolved in 2 ml 0.1N NaOH (1.275 mg/ml). The spectrum obtained on a Cary 15 spectrophotometer was recorded and the results obtained are shown in Table 111. The tryptophan concentration (y) was calculated and found to be 0.0749 umoles/ml. Assuming that lobster protein modulator has a molecular weight of 18,000, the sample contained 70.8 umoles modulator/ml and thus 0.001 umole tryptophan was detected per umole protein modulator.

Table 111. Tryptophan Determination of Lobster Protein Modulator

A	Sample + Blank	Blank	Sample
280 nm	0.752	0.014	0.738
294.4 nm	0.712	0.012	0.700

D. Tryptic Peptide Map

Peptide maps of many tryptic digests of lobster protein modulator preparations were produced as described in "General Experimental Proceedures". These maps show many similarities to the peptide maps obtained from tryptic digests of bovine brain protein modulator (figure 15). The two proteins are, however, certainly not identical.

E. Cyanogen Bromide Digestion

Digestion of proteins with cyanogen bromide results in cleavage at methionine residues. The amino acid analysis of lobster protein modulator shows that the protein contains six methionine residues and therefore cyanogen bromide digests of lobster protein modulator would be

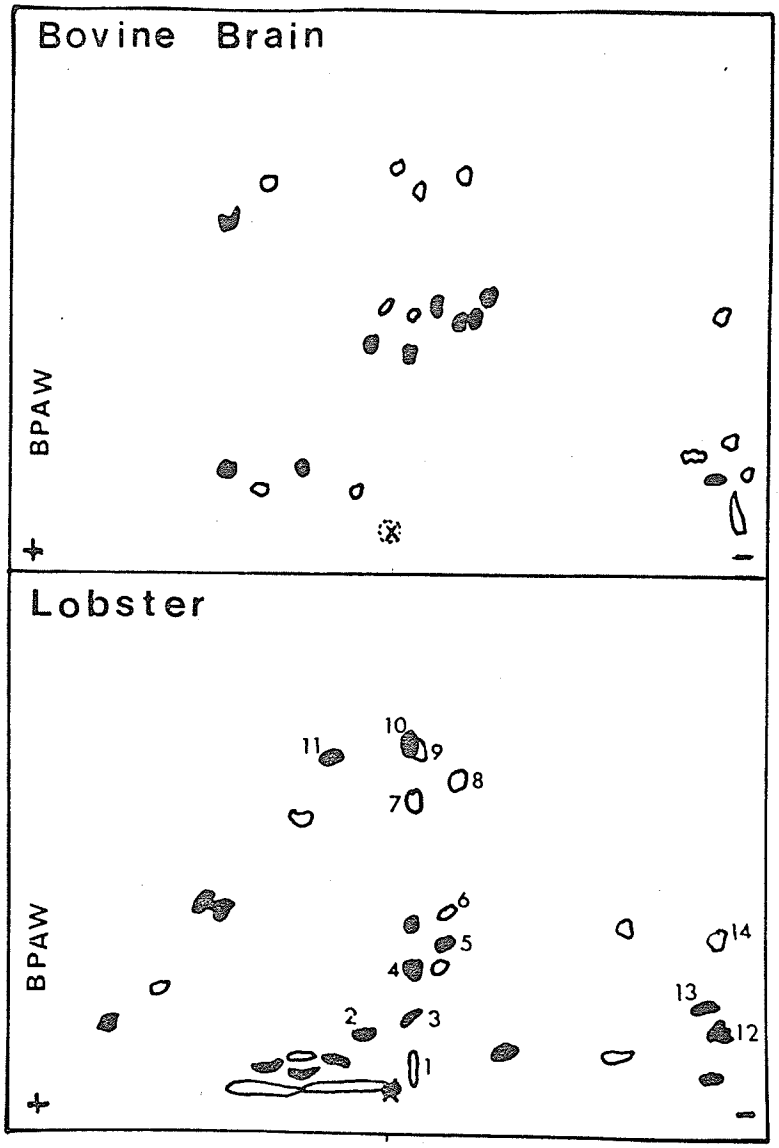


Figure 15: Tryptic peptide maps of protein modulator obtained from bovine brain and lobster

Major Spots (●)

Minor Spots (o)

expected to produce 7 peptides. The protein modulator purified using PMSF in the purification proceedure was digested with cyanogen bromide as described in "General Experimental Proceedures" and aliquots of the digest were subjected to 15% PAGE according to the method of Davis (1964)(see figure 16). As can be seen in figure 16, 7 peptides were produced by this digestion proceedure; clearly confirming the presence of 6 methionine residues in the protein.

F. Role of Ca^{2+} in the Lobster Protein Modulator Activation of PDE

The protein modulator isolated from other tissues and species requires Ca^{2+} for its ability to activate PDE. To determine the role of Ca^{2+} in the activation of PDE by lobster protein modulator, the following experiment was conducted and the results are shown graphically in figure 17.

Equivalent amounts of bovine heart PDE was present in each of 18 assay tubes. Curve A demonstrates the effect of Ca^{2+} on the basal PDE activity, that is, in the absence of protein modulator. Curve B demonstrates the increasing activity of PDE in the presence of saturating levels of protein modulator (16 units/assay tube) upon the addition of Ca^{2+}. It can therefore be concluded that as with the protein modulator isolated from other tissues and species, lobster protein modulator requires Ca^{2+} for its ability to activate PDE and furthermore Ca^{2+} has a maximum activation effect which is not increased by the addition of further

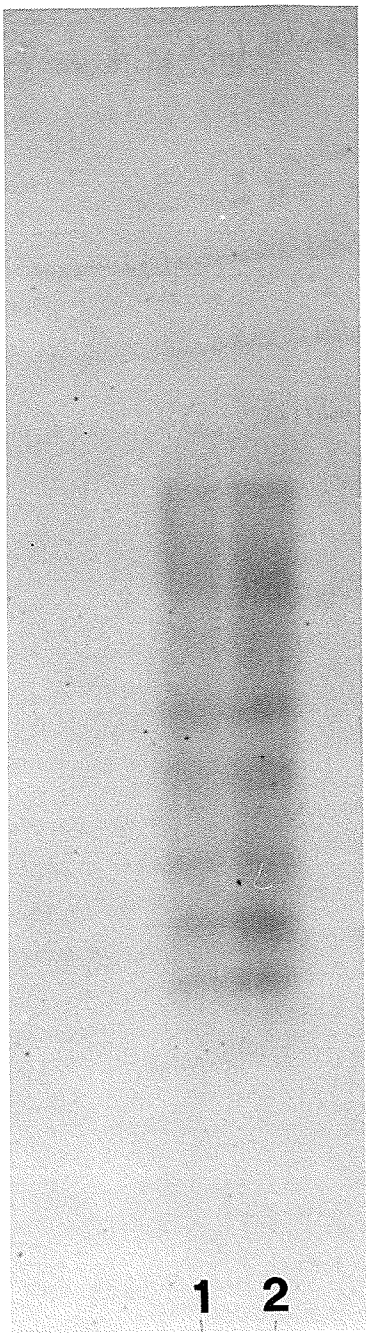


Figure 16:

15% PAGE of cyanogen bromide digestion of lobster protein modulator.

1: 100ug digest

2: 150ug digest

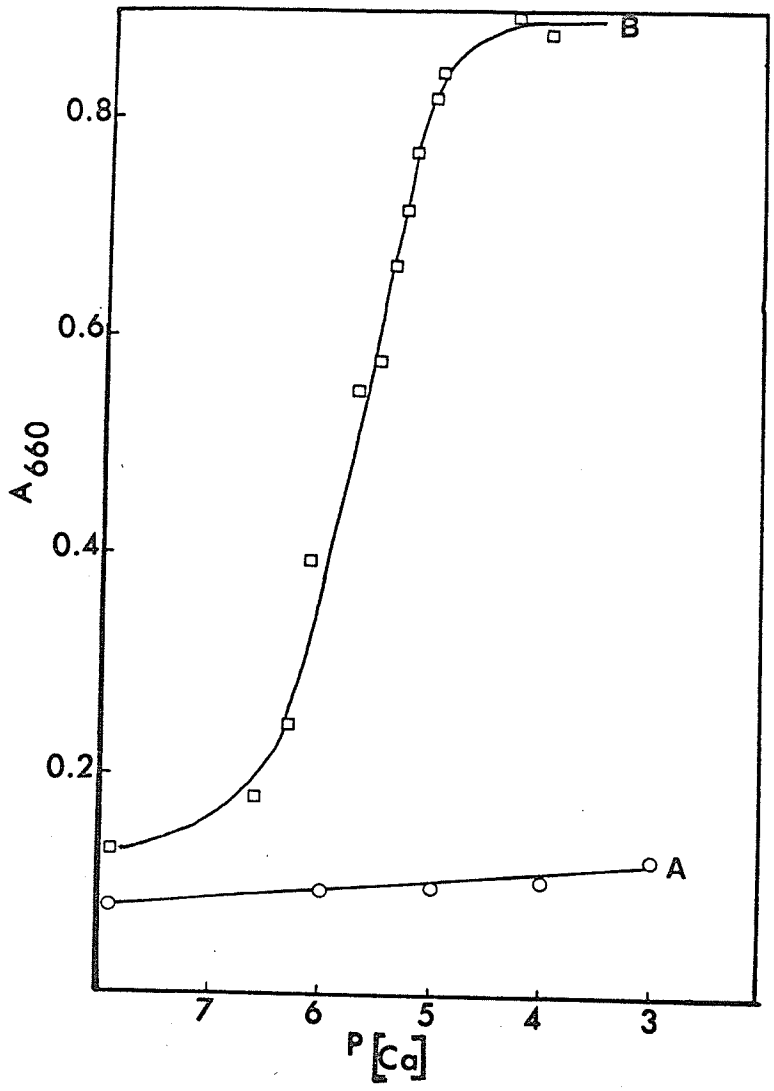


Figure 17: Role of Ca^{++} in lobster protein modulator activation of PDE.

A. activity of PDE in the absence of protein modulator and with increasing concentration of Ca^{++}.

B. activity of PDE in the presence of 16 units protein modulator and with increasing concentration of Ca^{++}.

Ca^{2+}.

It was also determined that a calcium concentration of 2×10^{-6}M was required for half maximal stimulation of PDE at saturating levels of protein modulator; a value comparable to the values reported for bovine brain modulator (Lin et.al., 1974), rat testis modulator (Dedman et.al., 1977a), and earthworm modulator (Waisman et.al., 1978a). These proteins require calcium concentrations of 3×10^{-6}M, 1.2×10^{-6}M, and 2×10^{-6}M respectively for half maximal stimulation of bovine heart PDE,

G. Calcium Effected Change in Modulator Mobility on Urea-PAGE

The protein modulator exhibits a marked change in conformation upon binding calcium (see literature review, Section 1V.F.) and several investigators have indicated that this conformational change is expressed as a change in the mobility of the modulator in urea-PAGE (Head and Mader, 1975a; Amphlett et.al., 1976 ; Kobayashi and Field, 1978). Urea- 15% PAGE of lobster protein modulator shows that at least one component of the lobster modulator preparation exhibits this change in mobility when Ca^{2+} is removed from the sample (figure 18). The bewildering behavior of lobster protein modulator on 8M urea- 15% PAGE was previously discussed (see 11.B.) and it appears that the impurity protein band present in lobster modulator preparations which is increased in intensity by urea-PAGE does not exhibit calcium dependent mobility changes in this

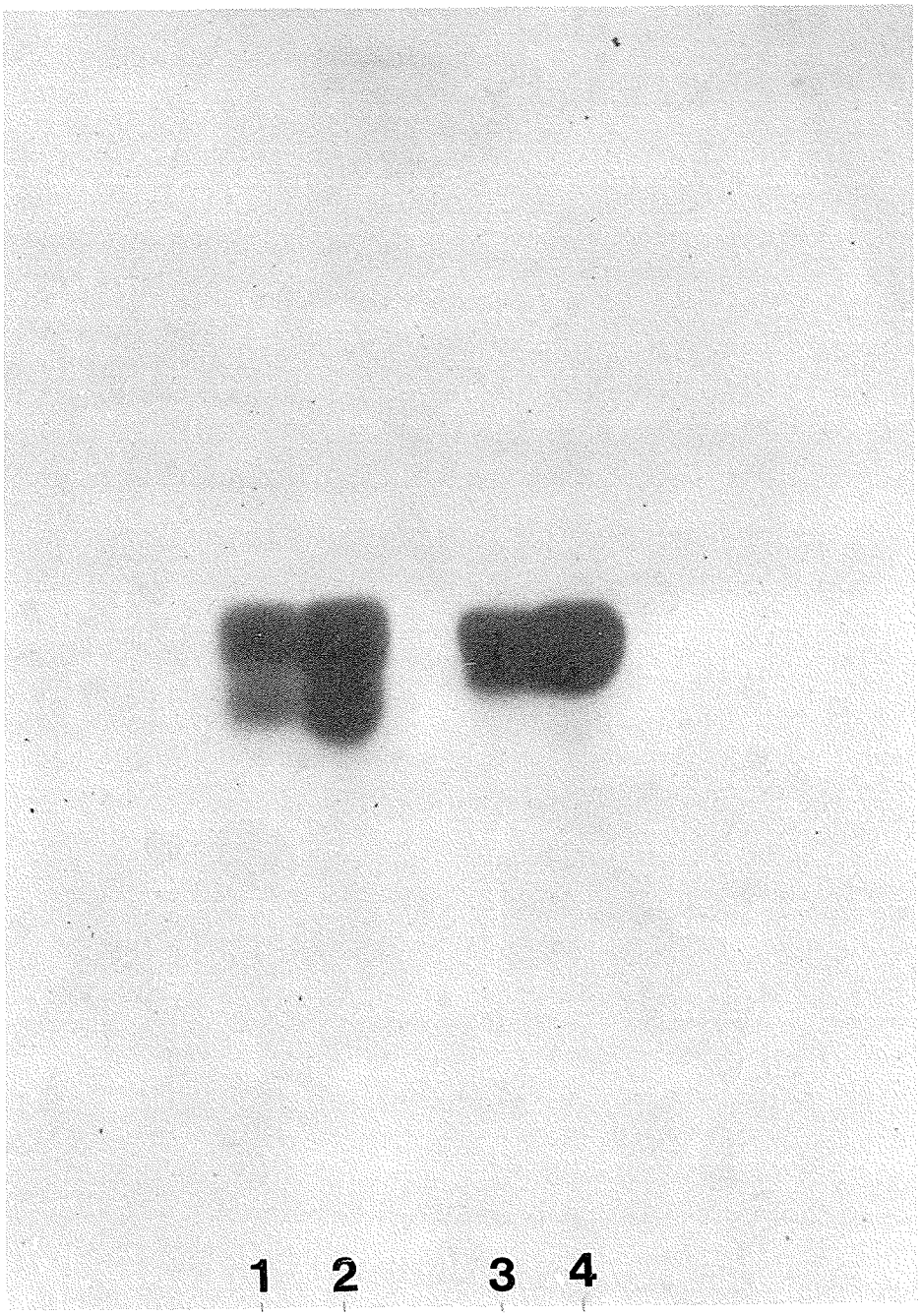


Figure 18: 8M urea-15% PAGE of lobster protein modulator.
1: 20ug lobster protein modulator ($4mMCa^{++}$)
2: 40ug lobster protein modulator ($4mMCa^{++}$)
3: 20ug lobster protein modulator (4mMEGTA)
4: 40ug lobster protein modulator (4mMEGTA)

gel system.

A gel study (figure 19) comparing the behavior of lobster protein modulator and bovine brain protein modulator on 8M urea- 15% PAGE, demonstrates that the lobster modulator protein band which has different mobility in urea-PAGE in the presence or absence of Ca^{2+} does indeed correspond to the major protein band present in the bovine brain modulator sample which also exhibits a different mobility in the presence or absence of calcium. In the bovine brain modulator sample, a second protein band was also identified, which exhibited equal mobility to the second protein band present in the lobster modulator sample. This second protein band present in both modulator samples appears to be present in much greater concentration in the lobster preparation.

H. Molecular Weight

The molecular weight of the protein modulator isolated from lobster was not determined. The protein modulator isolated from several tissues (Cheung, 1971; Lin et.al., 1975) behaves anomalously on Sephadex gel filtration columns and as a result values derived from gel filtration studies are approximately double values obtained from other techniques. Likewise, the lobster protein modulator behaves anomalously on SDS-PAGE and therefore the molecular weight could not be determined in this way. Ultracentrifugation studies were not carried out.

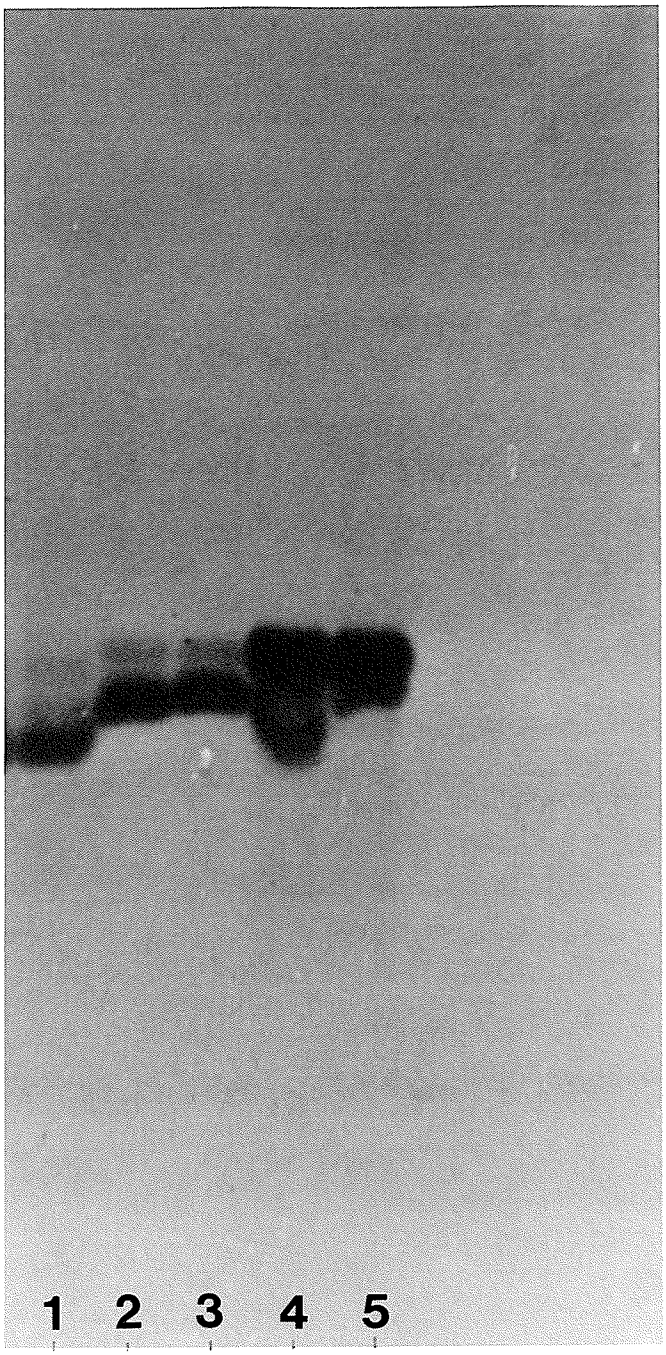


Figure 19: 8M urea-15% PAGE of lobster and bovine protein modulator.

1. bovine brain protein modulator (Ca^{2+})
2. bovine brain protein modulator (4mMEGTA)
3. bovine brain protein modulator (no addition)
4. lobster protein modulator (4mMCa^{2+})
5. lobster protein modulator (4mMEGTA)

IV. Isolation and Composition of Lobster Protein Modulator Tryptic Peptides

A. Sample Preparation

The accumulated modulator material was subjected to a final DEAE-cellulose chromatography purification step and the amino acid composition and tryptic peptide map of this final preparation were found to be virtually identical to the composition and peptide map of the modulator preparation purified in the presence of PMSF. The minor impurity previously discussed was, however, still present. Despite this fact, this material was used for this final analysis.

As small a volume as possible is desirable for application on to a Dowex 50 column as this will provide for optimal peptide separation. The digest of the modulator from bovine brain forms a gel-like material when too concentrated in buffers suitable for Dowex chromatography and therefore it was decided to digest only 30 mg of lobster protein modulator.

30 mg of modulator was weighed, dissolved in 1.0 ml of 45 mM calcium chloride and 1.0 ml distilled, deionized water and the pH adjusted to 7.8 with 0.05N NaOH (approximately 200 ul). The pH of the digestion mixture was maintained at 7.8 utilizing a radiometer pH stat; the 0.5 ml burette being filled with 0.05N NaOH. This also provided a mechanism to monitor the process and determine when the digestion is complete. TPCK-trypsin (100 ul of 10 mg/ml in 0.04M acetic acid and 0.02M $CaCl_2$) was added to the modulator mixture with a microsyringe and the digestion was

allowed to continue until an obvious slowdown occurred. At this point, another 100 ul TPCK-trypsin was added. The entire digestion required approximately 100 ul of 0.05N NaOH to neutralize the acid produced by peptide bond hydrolysis. The reaction was terminated by adjusting the pH to 2.0 with concentrated HCl. The total volume after digestion was approximately 2.5 ml and the resultant solution was not completely clear. A sample (40 ul) was removed at this stage for analysis by peptide mapping.

B. Dowex 50 Chromatography Separation

The digest was fractionated using the cation exchanger Dowex 50 according to the method of Schroeder (1972). The Dowex was suspended in pH 3.1 buffer (64.5 ml pyridine + 1114 ml glacial acetic acid $\longrightarrow$ 4000 ml with H_2O) and packed and equilbrated under pressure in a 0.6 x 100 cm column. The tryptic digest was applied to the top of the column under pressure, however, this application did prove difficult as the sample gelled on the top of the column, reducing the flow rate dramatically. This problem was solved only by stirring a very small portion of the top mixture after which the application proceeded much faster. It is not known, however, whether the gelling of the sample on the top of the column resulted in the loss of significant amounts of the sample or not. It is assumed, however, that as low yields of peptides were obtained for amino acid analysis, substantial losses did occur.

The column was developed with a nonlinear gradient

composed of 355 ml of pH 3.1 buffer and 650 ml pH 5.0 buffer (645 pyridine + 573 ml glacial acetic acid ⟶ 4000ml with H_2O) Elution was completed using approximately 25 ml pH 5.0 buffer followed by pH 5.6 buffer (684 ml pyridine + 180 ml glacial acetic acid ⟶ 1000 ml with H_2O). 1.8 ml fractions were collected and a flow rate of 18.0 ml/hr was used. The elution was monitored using the direct fluram method of Nakai et.al. (1974) as described in "General Experimental Proceedures". Aliquots (45 ul) were removed from alternate tubes and analyzed in this manner. Figure 20 shows the elution profile of the first 250 fractions obtained from the column while the remaining 350 fractions showed no major protein peaks. The peptide peaks were pooled as indicated in figure 20 and Table IV and the remaining material up to tube #600 was pooled into 17 arbitary fractions. The pooled fractions were taken to dryness on a rotatory evaporator at 36°C and redissolved in 0.5 - 0.75 ml of a suitable solvent (Table IV).

C. Isolation of Peptides from Dowex Fractions

Approximately 50 ul of each redissolved Dowex 50 fractions was mapped and stained with ninhydrin as described in "General Experimental Proceedures". Fractions Ia, VIIa, VIII - XXIV were found to contain no peptides. The remaining sample of fractions I,II,IIa,III,IV,IVa,V,VI, and VII was subjected to peptide mapping and stained with 10 x dilute ninhydrin (0.01% in 95% ethanol). As soon as spots appeared at room temperature, they were cut out and

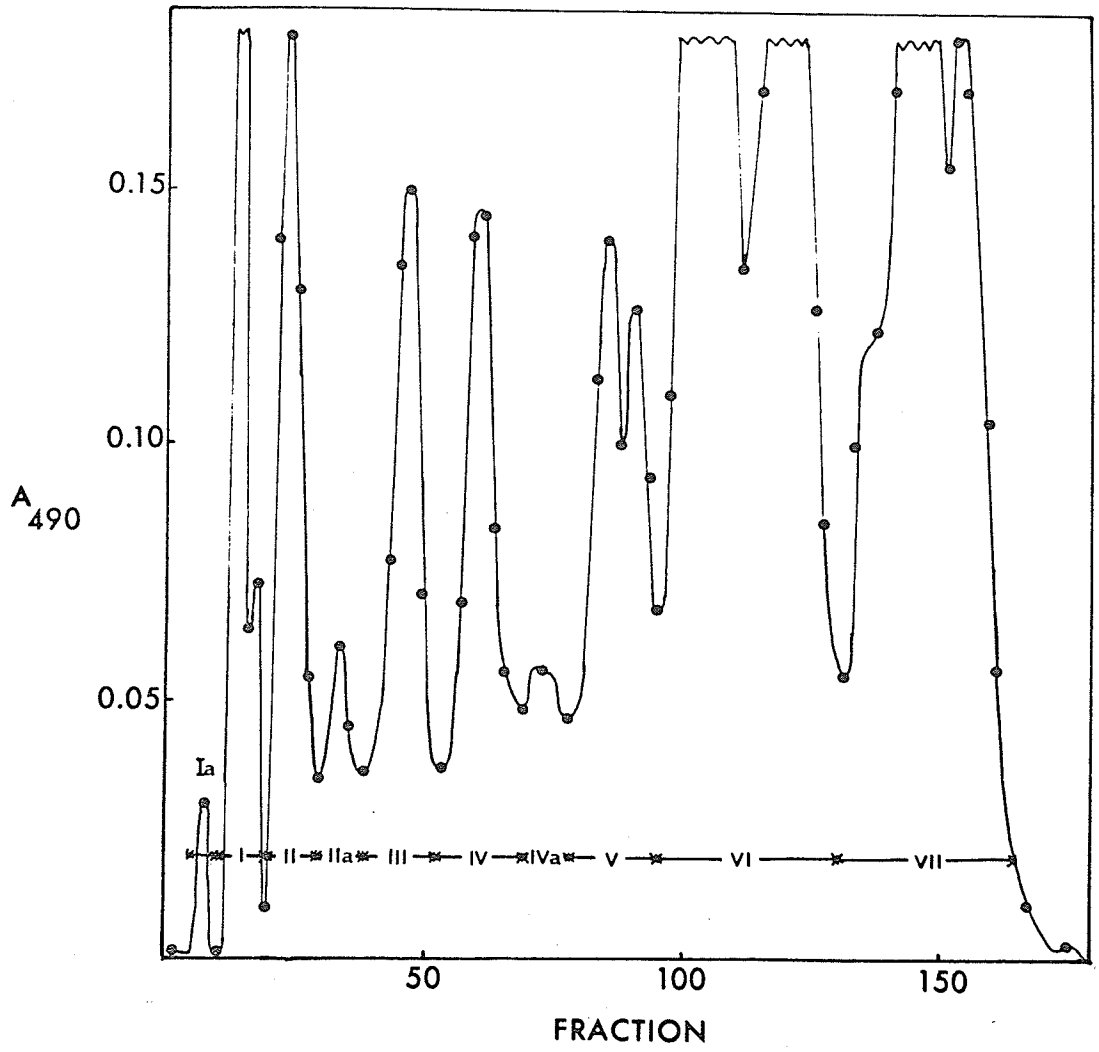


Figure 20: Dowex 50 chromatographic separation of peptides obtained from the tryptic digest of lobster protein modulator. The separation was performed and monitored as indicated in the text and the fractions indicated by bars were pooled.

and rinsed with ether to remove the excess ninhydrin. The strips were then eluted with 10% acetic acid followed by 50% pyridine and the eluant taken to dryness in a vacuum dessicator. The dried samples were hydrolyzed in 6N HCl at 110°C for 22 hours and subjected to amino acid analysis. It must be remembered in the analyses of peptides obtained in this manner, that ninhydrin reacts with lysine residues and the N-terminus of peptides unless the N-terminus is acetylated. Consequently, these residues may be partially or even totally destroyed.

TABLE IV. Preparation of Peptide Fractions Obtained from Dowex 50 Column Chromatology

Fraction Number	Tubes Pooled	Solvent	+/- Peptides
Ia	4-9	50% pyridine	-
I	10-18	50% pyridine	+
II	19-29	10% acetic acid	+
IIa	30-38	10% acetic acid	+
III	39-52	10% acetic acid	+
IV	53-69	10% acetic acid	+
IVa	70-78	10% acetic acid	+
V	79-95	50% pyridine + formic acid + 10% acetic acid	+
VI	96-130	50% pyridine + formic acid + 10% acetic acid	+
VII	131-165	50% pyridine + formic acid	+
VIIa-XXIV	166-600	50% pyridine	-

D. Analysis of Lobster Protein Modulator Tryptic Peptides

All peptides were isolated in the above manner and the concentration of the amino acids determined for each peptide.* The amino acid composition of those peptides recovered in sufficient quantity for analysis (16 peptides) was estimated by designating one of the stable amino acids as unity and calculating the remaining amino acid composition with this as reference. Many of the isolated peptides were not present in sufficient quantity for analysis and presumably represent contaminants from other fractions, but may also represent minor protein contaminants of the purified material.

A roman numeral is used to designate the Dowex 50 fraction from which each peptide was obtained and is followed by an arabic numeral indicating the specific peptide from that fraction.

Table V correlates the isolated Dowex 50 peptides with peptides identified in the tryptic peptide map (see figure 15).

The amino acid composition of the lobster peptides could then be compared with the composition of bovine brain protein modulator tryptic peptides taken from the known primary sequence (Appendix 1). It was hoped that in this way the degree of homology between lobster and bovine brain protein modulator tryptic peptides could be determined and thus give an indication of the degree of homology between

the two proteins.

*Note that the same peptide can be isolated from more than one Dowex 50 fraction.

TABLE V. Correlation between Dowex 50 Peptides and the Tryptic Peptides identified in Figure 15

Dowex 50 Peptides	Identical Peptides Figure 15
I-7b	10
I-8	11
II-10	5
II-11	6
IIa-14	4
III-17	3
III-18	1
III-23a	2
IV-2	8
VI-34	7
VI-37	9
VI-38	10
VI-45	14
VII-27	not identified
VII-30	13
VII-31	12

1. Lobster Tryptic Peptides I-7b and I-8

Analysis of lobster peptides I-7b and I-8 (Table VI) indicates that both of these peptides have amino acid compositions which are similar if not identical to that of bovine brain modulator residues 1-13. It can be seen in figure 15 that these two peptides (I-7b and I-8) are widely separated on the peptide map and therefore it would not appear to be possible that they are identical to each other, however, if lobster peptide was partially deamidated, the deamidated peptide and the original peptide would possess different charges and therefore would separate upon peptide mapping. Indeed, the apparently homologous region in the bovine brain protein modulator (residues 1-13) does possess 2 amidated acidic residues.

The lysine residue of lobster peptide I-7b appears to have been largely destroyed in this proceedure, presumably during the ninhydrin staining of the peptide map before elution.

TABLE VI. Amino Acid Composition of Lobster Peptides I-7a and I-8

	Lobster Tryptic Peptide		Bovine Brain
Amino Acid	I-7a	I-8	Residues 1 to 13[a]
Lys	0.24 (1)	0.61 (1)	1
Asp	1.05 (1)	1.45 (1)	1
Thr	1.02	1.0 (1)	1
Ser	-	0.17 (0)	0
Glu	4.36 (4)	4.68 (5)	5
Gly	0.41 (0)	0.46 (0)	0

Ala	1.79 (2)	1.73 (2)	2
Val	0.15 (0)	0.18 (0)	0
Meth	0.34 (0)	0.49 (0)	0
Ileu	1.0 (1)	1.04 (1)	1
Leu	1.06 (1)	1.15 (1)	1
Phe	0.79 (1)	0.90 (1)	1

[a]Vanaman et.al., 1977

2. Lobster Tryptic Peptides VI-38 and III-17

In the bovine brain modulator sequence lysine 21, situated between aspartic acid 20 and aspartic acid 22, is apparently not susceptible to tryptic attack for reasons as yet unknown. A single lobster peptide with a composition corresponding to residues 14-30 of bovine brain protein modulator was not identified, however, the sum of the compositions of lobster peptide VI-38 (Table VII) and III-17 (Table VIII) corresponds identically with the composition of residues 14-30. Lobster peptide VI-38 has a composition identical to bovine brain residues 14-21 while lobster peptide III-17 has a composition identical with bovine brain residues 22-30. It would appear that the lysine residue in the lobster protein modulator comparable to lysine 21 in bovine brain protein modulator is susceptible to tryptic attack.

TABLE VII. Amino Acid Composition of Lobster Peptide VI-38

Amino Acid	Lobster Peptide	Bovine Brain Residues 14 to 21[a]
Lys	1.0 (1)	1
Arg	0.3 (0)	0
Asp	1.2 (1)	1
Thr	0.3 (0)	0
Ser	0.9 (1)	1
Glu	1.2 (1)	1
Gly	0.5 (0)	0
Ala	0.9 (1)	1
Val	0.4 (0)	0
Met	0.3 (0)	0
Ileu	0.1 (0)	0
Leu	1.4 (1)	1
Phe	1.8 (2)	2

[a]Vanaman et.al., 1977

TABLE VIII. Amino Acid Composition of Lobster Protein III-17

Amino Acid	Lobster Peptide	Bovine Brain Residues 22 to 30[a]
Lys	0.87 (1)	1
Arg	0.07 (0)	0
Asp	1.83 (2)	2
Thr	3.0 (3)	3
Glu	0.58 (0)	0
Gly	1.89 (2)	2
Ala	0.08 (0)	0
Val	0.08 (0)	0
Met	0.04 (0)	0
Ileu	1.0 (1)	1
Leu	0.07 (0)	0
Tyr	0.03 (0)	0
Phe	0.04 (0)	0

[a]VANAMAN et.al., 1977

3. Lobster Tryptic Peptides VI-37 and VI-34

Lobster peptides VI-37 and VI-34 appear to be identical in amino acid composition to each other and to bovine brain tryptic peptide, residues 31 to 37 (Table IX). Interestingly, both these lobster peptides also migrate very differently on peptide maps but the reason for this difference is not known. There are no amidated acidic residues present in bovine brain modulator residues 31-37, however, the comparable lobster peptide may contain one or more amidated residues and therefore partial deamidation might explain the different migration properties of the two peptides.

TABLE IX. Amino Acid Composition of Lobster Peptide VI-37 and VI-34

Amino Acid	Lobster Tryptic Peptide VI-37	Lobster Tryptic Peptide VI-34	Bovine Brain Residues 31 to 37[a]
Lys	0.25 (0)	0.15 (0)	0
Arg	1.0 (1)	0.93 (1)	1
Asp	0.39 (0)	0.14 (0)	0
Thr	1.0 (1)	0.92 (1)	1
Ser	0.35 (0)	0.14 (0)	0
Glu	0.8 (1)	0.7 (1)	1
Gly	1.4 (1)	1.10 (1)	1
Ala	-	0.10 (0)	0
Val	1.2 (1)	1.01 (1)	1
Met	1.1 (1)	0.95 (1)	1
Leu	1.3 (1)	1.0 (1)	1

[a]Vanaman et.al., 1977

4. Lobster Tryptic Peptide II-11

Lobster tryptic peptide II-11 appears to be identical in composition to residues 38 to 74 of bovine brain protein modulator (Table X).

5. Lobster Tryptic Peptide VII-31 and VI-45

The lysine residue at position 75 in the bovine brain protein modulator sequence is not susceptible to tryptic cleavage and as a result a peptide, Lys-Met-Lys, residue (75 to 77) is produced. No peptide of this composition was identified in the lobster digest, however, lobster peptide VII-31 has a composition containing one lysine and one methionine residue (Table XI). It is possible that lobster peptide VII-31 has 2 lysine residues but that a large proportion of these residues were destroyed in the staining proceedure and thus the results could show the presence of only one lysine residue. It is assumed, however, that the spot would be detected with relatively little destruction of lysine due to the presence of 2 lysine residues in the peptide.

Lobster peptide VI-45 is composed of a sole arginine residue (Table XII). In the bovine brain sequence, residue 74 is an arginine residue. It seems possible that in the lobster modulator sequence, lysine 75 has been replaced by arginine, allowing for cleavage at arginine 75 and arginine 74; thus forming two peptides, Lys-Met and Arg alone.

This is an interesting proposal as lysine 75 has been

implicated in the activity of the protein modulator as an activator of PDE (Walsh, 1978).

TABLE X. Amino Acid Composition of Lobster Peptide II-11

Amino Acid	Lobster Peptide	Bovine Brain Residues 38 to 74
Lys	0.3 (0)	-
Arg	1.0 (1)	1
Asp	6.8 (7)	7
Thr	2.9 (3)	3
Ser	1.0 (1)	1
Glu	6.5 (6)	6
Pro	2.4 (2)	2
Gly	3.4 (3)	3
Ala	3.0 (3)	3
Val	1.2 (1)	1
Met	3.1 (3)	3
Ileu	2.2 (2)	2
Leu	3.3 (3)	3
Phe	2.0 (2)	2

[a]Vanaman et.al., 1977

TABLE XI. Amino Acid Composition of Lobster Peptide VII-31

Amino Acid	Lobster Peptide	Bovine Brain Residues 75 to 77[a]
Lys	1.15 (1)	2
Gly	0.13 (0)	0
Ala	0.13 (0)	0
Met	1.0 (1)	1

[a]Vanaman et.al., 1977

TABLE XII. Amino Acid Composition of Lobster Peptide VI-45

Amino Acid	Lobster Peptide
Arg	1.0

6. Lobster Tryptic Peptide VII-27

The amino acid composition of lobster peptide VII-27 closely resembles residues 87 to 90 in the bovine brain protein modulator sequence, differing only in the absence of one glutamic acid residue (Table XIII).

TABLE XIII. Amino Acid Composition of Lobster Peptide VII-27

Amino Acid	Lobster Peptide	Bovine Brain Residues 87 to 90[a]
Arg	1.0 (1)	1
Asp	0.14 (0)	0
Glu	0.4 (0)	0
Gly	0.26 (0)	0
Ala	1.06 (1)	1
Phe	1.04 (1)	1

[a]Vanaman et.al., 1977

7. Lobster Tryptic Peptide IV-2

Similar to lysine 21, lysine 94 of bovine brain protein modulator is situated between two aspartic acid residues in the primary sequence and also is not susceptible to tryptic attack; thus a peptide is produced (residues 91-106) which contains two lysine residues. A single peptide with a similar composition was not isolated from the lobster protein modulator tryptic digest, however, lobster tryptic peptide IV-2 (Table XIV) closely resembles bovine brain residues

95-106 in amino acid composition. This would indicate that a second lysine residue stable to tryptic attack in the bovine brain protein modulator is susceptible to tryptic attack in the lobster protein modulator. A peptide was not isolated, however, from the lobster modulator digest which is comparable in amino acid composition to bovine brain residues 91-94.

8. Lobster Tryptic Peptide II-10

Lobster peptide II-10 was the only lobster peptide analyzed which contained histidine (Table XV) despite the fact that the amino acid analysis of the undigested lobster protein modulator indicates that the molecule contains a total of 2 histidine residues. Bovine brain protein modulator contains only one histidine residue (residue 107), however, lobster peptide II-10 bears little resemblance to bovine brain tryptic peptide 107 to 126.

The unusual amino acid, trimethyllysine (residue 115) is also present in bovine brain tryptic peptide 107-126. The routine amino acid analyses which were carried out in these experiments do not distinguish between lysine and trimethyllysine but there is qualitative evidence indicating that the lobster protein modulator does contain trimethyllysine. Lobster peptide II-10 appears to contain one residue each of lysine and arginine and thus the value for lysine might represent trimethyllysine.

TABLE XIV. Amino Acid Composition of Lobster Peptide IV-2

Amino Acid	Lobster Peptide	Bovine Brain Residues 95 to 106[a]
Lys	0.3 (0)	1
His	0.2 (0)	0
Arg	0.9 (1)	0
Asp	1.3 (1)	2
Thr	0.2 (0)	0
Ser	0.9 (1)	1
Glu	1.3 (1)	1
Gly	1.9 (2)	2
Ala	2.0 (2)	2
Val	0.2 (0)	0
Ileu	0.9 (1)	1
Leu	1.1 (1)	1
Tyr	0	1
Phe	1.0 (1)	0

[a]Vanaman et.al., 1977

TABLE XV. Amino Acid Composition of Lobster Peptide II-10

Amino Acid	Lobster Peptide	Bovine Brain Residues 107 to 126[a]
Lys	1.0 (1)	TML 1
His	0.7 (1)	1
Arg	0.5 (1)	1
Asp	2.4 (2)	4
Thr	1.2 (1)	2
Ser	2.5 (3)	0
Glu	4.1 (4)	3
Pro	2.4 (2)	0
Gly	3.2 (3)	1
Ala	1.4 (1)	0
Val	1.3 (1)	2
Met	0.6 (1)	2

Ileu	o.9 (1)	1
Leu	1.9 (2)	0
Tyr	0.6 (1)	2
Phe	1.0 (1)	0

[a]Vanaman et.al., 1977

9. Lobster Tryptic Peptides IIa-14 and III-23a

The amino acid composition of two other large peptides (IIa-14, III-23a) isolated from the lobster protein modulator digest were not found to be similar to any of the tryptic peptides isolated from bovine brain protein modulator (Table XVI and Table XVII).

Lobster peptide III-23a was not identified as a distinct spot on the peptide map but rather was present as a diffuse smear. This may indicate that it is a mixture of peptides and thus the data would be unreliable.

TABLE XVI. Amino Acid Composition of Lobster Peptide IIa-14

Amino Acid	Lobster Peptide
Lys	1.0 (1)
His	0.19 (0)
Asp	2.76 (3)
Thr	1.16 (1)
Ser	0.77 (1)
Glu	3.11 (3)
Gly	1.76 (2)
Ala	0.82 (1)
Val	1.52 (2)
Met	1.18 (1)
Ileu	0.75 (1)
Leu	0.46 (0)
Tyr	0.71 (1)
Phe	0.66 (1)

TABLE XVII. Amino Acid Composition of Lobster Peptide III-23[a]

Amino Acid	Lobster Peptide
Lys	1.0 (1)
His	1.29 (0)
Arg	0.61 (1)
Asp	3.6 (4)
Thr	2.04 (2)
Ser	1.18 (1)
Glu	3.82 (4)
Pro	1.35 (1)
Gly	2.06 (2)
Ala	0.78 (1)
Val	0.98 (1)
Ileu	1.27 (1)
Leu	0.90 (1)
Tyr	0.63 (1)
Phe	0.55 (1)

10. Lobster Tryptic Peptide VII-30

Lobster peptide VII-30 is a small peptide identified in the tryptic digest of lobster protein modulator which is not similar to any peptide identified in the tryptic digest if bovine brain protein modulator. Peptide VII-30 appears to contain only 2 amino acid residues, arginine and alanine (Table XVIII).

TABLE XVIII. Amino Acid Composition of Lobster Peptide VII-30

Amino Acid	Lobster Peptide
Arg	1.0 (1)
Gly	0.13 (0)
Ala	0.94 (1)

11. Lobster Tryptic Peptide III-18

Despite the efforts to insure that the tryptic digestion of 30 mg of lobster protein modulator was complete, a large peptide (III-18; data not shown) was isolated which appears to represent a major portion of the protein modulator molecule indicating that incomplete digestion occurred. It is interesting to note that this peptide spot is consistently obtained in tryptic digests of lobster protein modulator. The reason for a major portion of the modulator sample remaining undigested is not known. This peptide may be extremely resistant to tryptic digestion for some as yet unknown reason (chemical modification, etc.) and may likewise represent the impurity band previously discussed which is so difficult to remove from the lobster protein modulator.

12. SUMMARY

Tryptic digestion of bovine brain protein modulator results in the production of 10 peptides (Vanaman et.al., 1977) while a total of 16 peptides were recovered from the tryptic digest of lobster muscle protein modulator in sufficient quantity for analysis (Table VI-XVIII). Of these lobster peptides, 11 possess similar or identical amino acid composition to bovine brain peptides representing approximately 76% of the bovine brain protein modulator primary sequence (Table XIX). An additional 5 peptides were recovered which could not be related with certainty to the remaining bovine brain protein modulator sequence. Lobster tryptic peptides similar to the regions from residue 78-86,

90-94, and 127-148 of the bovine brain protein modulator primary sequence were not identified. It can be estimated from the amino acid composition of the apparently homologous peptides that the two proteins are probably more than 68% homologous.

TABLE XIX. Comparison of Lobster Muscle and Bovine Brain Modulator Tryptic Peptides

Lobster Peptide	Corresponding Region Bovine Brain Modulator Sequence[a]	% homologous Composition
I-7b, I-8	1-13	100
VI-38	14-21	100
III-17	22-30	100
VI-37, VI-34	31-37	100
II-11	38-74	100
VII-31	75-77	67
VII-27	87-89	100
IV-2	95-106	75
II-10	107-126	70
VI-45	-	?
VII-30	-	?
IIa-14	-	?
III-23a	-	?
III-18	-	?

[a]Vanaman et.al., 1977 (see appendix I)

Table XX compares the total amino acids recovered from the isolated tryptic peptides as compared to the total amino acid compositions of lobster muscle protein modulator. It is obvious from the results shown in Table XIX that the entire sequence of the lobster protein modulator is not re-

presented by the peptides which were analyzed. Furthermore, the recovery of 10 arginine residues from isolated peptides (only 6 arginine residues were present in the amino acid composition of the intact modulator) indicates that there is probably duplication or contamination within the peptide population analyzed. For example, peptide VI-45 (one arginine residue) and peptide VII-30 (one arginine and one alanine residue) may merely represent fragments of other peptides. Similarly, lobster peptide III-23a was not isolated as a distinct peptide and indeed the amino acid composition may not represent a single tryptic peptide.

It was obvious after peptide mapping was performed on the fractions obtained from the Dowex 50 fractionation of the tryptic digest of the lobster protein modulator sample that this is not an ideal proceedure for the isolation and analysis of these peptides. The peptides present within a given fraction were located in close proximity on the peptide maps and thus no real benefit was obtained by the technique. Furthermore, with the difficulty found in sample application onto the Dowex 50 column and the fact that losses of material always occur in purification proceedures, it is likely that a large amount of material was lost throughout the proceedure.

TABLE XX. Amino Acid Recovery from Lobster Peptide Isolation

Amino Acid	Amino Acids Recovered from Peptides	Amino Acid Composition of Lobster Protein Modulator
Lys	7	8
His	1	2
Arg	10	6
Asp	14	21
Thr	10	12
Ser	7	7
Glu	21	31
Pro	4	5
Gly	13	12
Ala	12	9
Val	5	8
Meth	7	6
Ileu	8	6
Leu	7	8
Tyr	2	1
Phe	9	7

CONCLUDING REMARKS

In the past, the study of the function of various proteins has been greatly aided by the analysis and comparison of proteins isolated from different tissues and species. It now appears that this may be a valuable techinque in the study of the protein modulator. The protein modulator isolated from bovine brain has been extensively studied and shown to be essentially identical in amino acid sequence to bovine heart protein modulator (Stevens et.al., 1976) and rat testis protein modulator (Dedman et.al., 1978). The experiments presented in this thesis indicate, however, that although the protein modulator isolated from lobster is similar to bovine brain protein modulator, it is certainly not an identical protein. As the two proteins appear to be equally effective in the activation of PDE, they may represent ideal examples for the study of the calcium binding domain, the nature of protein-protein interaction which occurs between PDE and the protein modulator, and the regulation of these processes.

As was described in the experimental section, the lobster protein modulator is similar to bovine brain protein modulator with respect to heat stability, isoelectric point, UV absorption spectrum, mobility in various PAGE systems, amino acid composition, the presence of small amounts of carbohydrate, tryptic peptide map patterns and the composition of many of the isolated peptides, the ability to stimulate PDE, and the dependence on Ca^{2+} for

activity. Indeed, the two proteins appear to be more than 68% homologous.

Differences which were noted for the two proteins include the behavior in SDS-PAGE systems; the increased concentration of an "impurity band" in urea-PAGE of lobster protein modulator in the presence of Ca^{2+}, a characteristic not observed with bovine brain protein modulator; the susceptibility of certain lysine residues (residue 21 and 94) to tryptic attack; the possible absence of lysine residue 75 in lobster protein modulator, a residue which has been implicated in the activity of bovine brain protein modulator; the inability to identify homology between 5 lobster protein modulator tryptic peptides and bovine brain protein modulator tryptic peptides; and the presence of a large peptide in the tryptic digest of lobster protein modulator which represents undigested protein.

Future studies into these differences in structure and the possible effect on the physical and chemical properties of the modulator and, indeed, on its function may yield considerable information about the mechanism of action of the protein modulator and perhaps proteins in general.

APPENDIX 1: Amino Acid Sequence of Bovine Brain Protein Modulator

Ac - Ala - Asp - Gln - Leu - Thr - Glu - Glu - Ile - Ala - Glu - Phe -
(1) (10)

- Lys - Glu - Phe - Ser - Leu - Phe - Asp - Lys - Asp - Gly - Asx - Gly -
(20)

Thr - Ile - Thr - Thr - Lys - Glu - Leu - Gly - Thr - Val - Met - Arg - Ser -
(30)

Leu - Gly - Glx - Asx - Pro - Thr - Glx - Ala - Glx - Leu - Glx - Asx - Met -
(40) (50)

Ile - Asn - Gln - Val - Asp - Ala - Asp - Gly - Asx - Gly - Thr - Ile - Asp -
(60)

Phe - Glu - Pro - Phe - Leu - Thr - Met - Met - Ala - Arg - Lys - Met -
(70)

Lys - Asp - Thr - Asp - Ser - Glu - Glu - Glu - Ile - Arg - Glu - Ala -
(80)

Phe - Arg - Val - Phe - Asp - Lys - Asp - Gly - Asx - Gly - Tyr - Ile -
(90) (100)

Ser - Ala - Ala - Glu - Leu - Arg - His - Val - Met - Thr - Asx - Leu -
(110)

Gly - Glu - Tml - Leu - Thr - Asp - Glu - Asp - Val - Asp - Glu -
(120)

Met - Ile - Arg - Glu - Ala - Agn - Ile - Asp - Gly - Asp - Gly -
(130)

Glx - Val - Asx - Tyr - Glx - Glx - Phe - Val - Gln - Met -
(140)

- Met - Thr - Ala - Lys - COOH
(148)

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