PHYSICO-CHEMICAL AND KINETIC STUDIES OF A NICOTINAMIDE ADENINE DINUCLEOTIDE SPECIFIC GLUTAMIC DEHYDROGENASE FROM BLASTOCLADIELLA EMERSONII

by

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TO MY PARENTS

The writer wishes to thank Dr. H.B. LeJohn for his patient and generous assistance.

ABSTRACT

An NAD-specific glutamic dehydrogenase was isolated from the mitochondria of Blastocladiella emersonii and some of its kinetic properties were studied. Various purine nucleotides participated as positive (ADP, AMP) or negative (ATP, GTP, GDP) allosteric effectors. The nucleotide effect showed a strong correlation with the pH of the assay system. In the oxidative deamination of glutamate AMP (or ADP), at saturating levels, converted complex non-linear double reciprocal plots into Michaelian-type plots. For the reductive amination of α-ketoglutarate the shape of the double reciprocal (rate-concentration) plots was unchanged with saturating levels of AMP. These plots were normally linear with a sharp threshold level caused by substrate inhibition.

On the basis of product inhibition studies an Ordered Binary-Ternary kinetic mechanism (Cleland, W.W., Biochem. Biophys. Acta, 67, 104, 1963) was proposed for the oxidative deamination reaction.

Zone sedimentation analysis by sucrose density gradients revealed that protons, effectors, and some substrates protected the enzyme against hydrodynamic inacti-

vation. At alkaline pH (pH 9) the enzyme is inactivated by sedimentation. Inclusion of NAD^+, NADH, AMP, Ca^{++}, and to a lesser extent, glutamate and α-ketoglutarate stabilized the enzyme against inactivation. At pH 6, the enzyme remained partially active after sedimentation but, of all substrates, NAD^+ was peculiar in that it caused a complete inactivation of the enzyme. All other substrates and effectors failed to appreciably enhance recovery at pH 6 but did not cause inactivation.

LéJohn (Biochem. Biophys. Res. Communs., 32, 278, 1968) has been able to show a pH dependence on the regulatory action of Ca^{++} and Mn^{++} on the enzyme. Both cations inhibit the oxidative deamination reaction unidirectionally and activate the reductive amination of α-ketoglutarate. An extension of these studies led to evidence of unidirectional inhibition of the oxidative deamination reaction by the metabolites citrate, isocitrate, fumarate, succinate, fructose 1, 6-diphosphate and the non-metabolite EDTA (LéJohn, H.B., J. Biol. Chem., 243, 5126, 1968). Among the metabolites, citrate was the strongest inhibitor. This inhibition could be relieved by various cations and AMP.

From these studies a molecular model of the enzyme action has been proposed. Also a generalized physiological

concept involving the regulation of this enzyme and a "linked" mitochondrial NAD-specific isocitric dehydrogenase (McCrea, B.E., M.Sc. Thesis, Univ. of Manitoba, 1969) has been formulated.

ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine 5'monophosphate
ATP	adenosine triphosphate
CDP	cytosine diphosphate
CTP	cytosine triphosphate
DEAE-cellulose	diethylaminoethyl-cellulose
EDTA	(sodium) ethylenediamine tetra acetic acid
GDP	guanosine diphosphate
GTP	guanosine triphosphate
IDH	isocitric dehydrogenase
IMP	inosine monophosphate
ITP	inosine triphosphate
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)

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INTRODUCTION

Recently much work has been done on "allosteric regulation" of key enzymes in cell metabolism. The enzymes of the citric acid cycle and those at its branch points have received particular attention because of their central importance in aerobic metabolism. The nature of cellular differentiation may be looked upon as discrete problems of biochemical equilibria under genetic control. Because of the ease and rapidity of growth, and the fact that it is a unicellular organism which displays several of the differentiating characteristics attributed to multicellular organisms, Blastocladiella emersonii provides a near ideal system for studies of cellular differentiation. Alteration of environmental factors such as light, chemical composition of the growth medium, and temperature, can lead to a change from an ordinary colourless (O.C.) actively growing cell to a resistant sporangial (R.S.) form (by Cantino's designation, see review (5)). Because one can easily identify different morphological forms during the ontogeny of the organism, it is possible to correlate form and function, at least superficially, in this fungus. In the past two decades a large body of disconnected

evidence has accumulated on the physiological characteristics of the organism. No attempt has been made to study the nature of regulation of enzymes, hitherto suspected to be involved, directly or indirectly, with the change in ontogeny induced by agents such as light, bicarbonate, and temperature (see review of Cantino and Lovett, (5) for an excellent account.) An NADP-specific isocitric dehydrogenase and isocitritase have been implicated in a physiological scheme of reversal of the citric acid cycle and CO_2 fixation by α-ketoglutarate dehydrogenase. It has also been inferred from labelling studies, that labelled glutamate easily entered the citric acid cycle intermediates but labelled glucose entered glutamate with great difficulty (4). The purpose of this investigation was: (i) to identify and isolate the enzyme(s) responsible for glutamate synthesis and catabolism via organic acid intermediates; (ii) to study its mode of regulation, if any; and (iii) to correlate these findings with known physiological effects of the influence of diverse cations, glutamate, and citrate on the growth of the organism. The interest in cationic effects derives from the natural habitat of the fungus. Being a freshwater aquatic organism, one can easily appreciate the importance of inorganic ions in the overall development of the mould.

HISTORICAL

I. Organism

Blastocladiella emersonii is a non-filamentous ('unicellular') water mould. The normal life cycle briefly is as follows. Motile zoospores retract their single flagellum and send out a germ tube which is destined to become the rhizoidal system of the plant. Exponential growth ensues for a period of 16 to 18 hours at 20°C. During this period, several nuclear divisions take place within each zoosporangium. The size of the sporangium increases several hundred-fold. At the termination of growth a septum delineates a sterile, subterminal rhizoidal cell which is virtually devoid of cytoplasm. The multinucleate terminal cell differentiates to form unimitochondrial zoospores which are subsequently discharged through papillae. This description represents the life cycle of the ordinary colourless (O.C.) plant (5). Variation of light, temperature, or growth medium constituents, however, can lead to an alternate form - the resistant sporangial (R.S.) form. The R.S. form builds up a thick wall and such cell constituents as soluble polysaccharide, carotenoids, melanin, chitin and it delays its zoospore release. The R.S. form has a life cycle of about 72 hours. Addition of exogenous bicarbonate

to the medium is one method of inducing this form. There exists a "point of no return" (at 36 hours) before which the R.S. form may revert to the O.C. form (e.g., by removal of bicarbonate). Beyond this point, however, the cell is irrevokably committed to the R.S. form. Thus, despite the lack of sexual reproduction this organism provides a near ideal system for studies in cell differentiation specifically with regard to enzymological study of regulation by known physiological effectors. Of particular interest is the effect of various substances on zoospore germination (LéJohn, unpublished data). Germination is inhibited by Ca^{++}, EDTA, and citrate. On the other hand, certain ions - Mn^{++}, Mg^{++}, and K^{+} enhance germination. Also, although zoospores are actively respiring, there is no evidence for nucleic acid or protein synthesis within the spores.

Enzymological studies have indicated differential rates of synthesis of certain cell constituents during exponential growth (5). An example of this phenomenon is the case of glucose-6-phosphate dehydrogenase (17). The total amount of this enzyme increases exponentially. Between 30 and 60 per cent of the generation time this rate is greater than the exponential rate of synthesis of soluble protein and is identical to that for increase in dry weight per cell. On the contrary, the total activity

of isocitritase per cell is diluted during exponential growth until about one-half generation time (40).

Labelling studies (4) indicated that glutamate could easily enter citric acid cycle intermediates but that the carbons of glucose entered glutamate with difficulty. This observation, although perhaps not convincingly substantiated, led to interest in glutamic dehydrogenase as an important branch point enzyme of the citric acid cycle. The implication of substances involved in germination inhibition as effectors of glutamic dehydrogenase (LéJohn, unpublished data) provides interesting regulatory implications of glutamic dehydrogenase in the germination process.

II. Allosteric Models

Regulation of enzymic catalysis by specific metabolites via conformational change was first proposed to explain feedback inhibition commonly found in many biosynthetic pathways (23, 42, 56). Atkinson (2) surveyed the literature and discussed how enzyme reactions may be controlled in a rather 'coarse' fashion through competitive inhibition between products and substrates. Monod, Changeux, and Jacob (42) initially proposed that direct steric hindrance between substrate and inhibitor may be impossible for stereochemically

unrelated molecules. This led to the concept that at least two stereo specifically different, non-overlapping but interacting sites are involved. The binding of the effector molecule induces a conformation change in the enzyme and this alters the binding property of the substrate site. A year earlier Gerhart and Pardee (16) had produced experimental evidence for this in their studies of aspartate transcarbamylase. Monod et al (42) coined the term allosterism to account for effectors which affect the substrate site through binding at a second (non-catalytic) site. Reiner (49) has pointed out that, if "acting at a second site" is meant, the term should be "allotropic". If on the other hand "shape changing" is meant the term should be "morphallactic" or "morphallaxic". Thus, he considers that the term allosteric, in itself, may be an improper connotation.

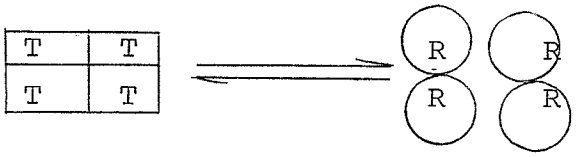
More recently Monod, Wyman, and Changeux (43) developed a statistical treatment of so-called "allosteric transitions" of regulatory enzymes. Their concept is based on the observation that the most important allosteric phenomena occur in oligomeric proteins including enzymes. A key element in the Monod-Wyman-Changeux formulation is that strict symmetry is maintained at all times during configurational changes of the enzyme. The model, outlined below, is subject to many refinements and

modifications. Most of the work reported in this thesis will provide ample evidence for one possible refinement of this theory.

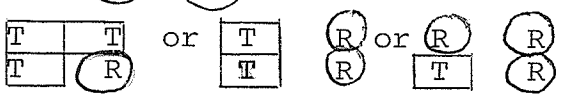
The concept of Monod et al is that oligomeric proteins are regulated via homotropic and heterotropic effectors leading to cooperative and antagonistic effects. A homotropic effector is a ligand whose binding affects the binding and possibly the catalytic behaviour of other sites for the same ligand. Heterotropic effectors alter the properties of sites reacting with dissimilar ligands. Either of these could lead to cooperative effects (increasing binding and reaction rates of other sites) and antagonistic effects (decrease in binding and reaction rates). Such complex interactions may produce K systems (substrate itself is an allosteric ligand) where binding properties are affected. It must be realized that a change in K_m is not a definition for a K system since K_m is only a function of K_s (the actual binding constant). This system is distinguished from a V system in which the reaction rates are modified (substrate does not show cooperative interaction but effectors do).

Several other models based on statistical and kinetic premises have been formulated (3, 25, 50) but the model of Monod et al, is unique because of its symmetry restriction. This model shows a best fit to a homotropic cooperative

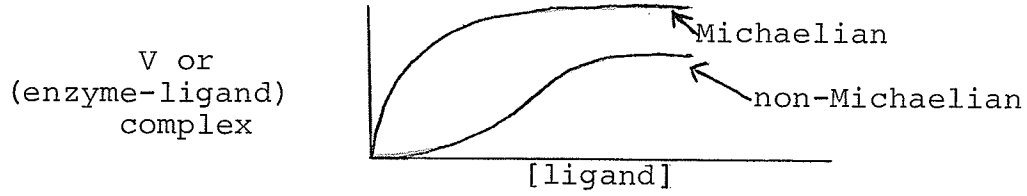
effect in a K system. As an example, the 'monomers' of a tetrameric enzyme are postulated to exist in two (at least) major conformational states designated R and T. These forms are in thermodynamic equilibrium with each other. The T form has a larger dissociation constant (K_t) for a given ligand than R does (i.e., $K_t > K_r$). The assumption is that transition of the tetramer is always complete.



i.e., there are no hybrids of the type:



Thus, a given ligand stabilizes one configuration. Unless ligands are saturating, there would be, at any instant, a mixture of R and T forms. Hence, by titrating the enzyme with a given ligand, sigmoid plots are described.



The precise form of the titration plot will depend on the ratio of K_r to K_t and the value of L ("allosteric constant").

This relationship affords facility for an extension of the Monod-Wyman-Changeux concept as observed earlier. If, in addition to conformation changes, the enzyme can be subjected to aggregation and dissociation of catalytically active forms, then the simple sigmoid plot may become further

distorted into a mixture of hyperbolic and sigmoidal patterns (30). Such complex plots have been designated 3/2 and 2/1 functions by the algebraic kinetic analysts (6, 7, 51). The fractional functions denote the power of certain constants present in algebraic formulations of the probable number of catalytically active binding sites for different substrates. These descriptions, nevertheless, fail to elucidate the molecular nature of the protein even by detailed computer analyses.

Because great emphasis has been placed on the quaternary state of the Blastocladiella glutamic dehydrogenase in these studies, this aspect must be surveyed briefly. All quaternary forms of oligomeric proteins relate to association and dissociation of subunits. These subunits are not covalently bonded in the quaternary state. In this case monomer will be defined as that unit with one active site. Associated and dissociated forms of this refer to oligomer and subunit respectively. All protomers (subunits in an oligomer) occupy equivalent positions within the oligomer. The domain of bonding between any two protomers consists of two linked binding sets. (A binding set consists of a spatially organized collection of all protomeric groups which participate in binding to another.) Isologous associations are symmetrical and involve two identical binding sets. Consequently, there is a requirement for a finite structure with at least one axis of symmetry. Thus,

dimers and tetramers only are possible. Heterologous associations lack symmetry and can lead to greater than two protomers in association. A mixture of the two types leads to an even-numbered oligomer with a minimum of six protomers.

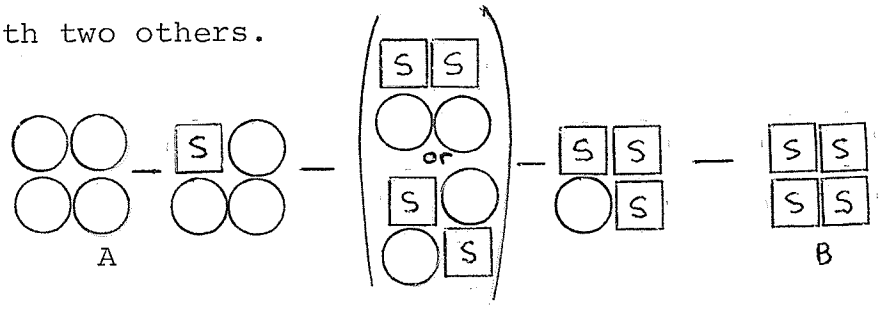
The conformation of each protomer can be constrained by its associations leading to the form T. Upon relaxation of constraints each protomer tends toward an alternate conformation (R). In such a transition the molecular symmetry, including protomeric constraints, is conserved.

Considering the extreme cases of dissociation and association, the symmetry of protomers may be destroyed upon the formation of 'super' aggregates or monomers irrespectively. The possibility exists that quaternary constraints may not induce a conformational change but only a (symmetrical) redistribution of electronic charges within the protein.

This model was based largely on studies of haemoglobin. The dissociation curve of oxyhaemoglobin as a function of oxygen tension is sigmoid showing a cooperative effect on binding sites. Haemoglobin is subject to the Bohr effect. That is, an increase of oxygen dissociation occurs as the pH is lowered. Wyman (58) showed that the Bohr effect is due to a discharge of protons provoked by the binding of oxygen.

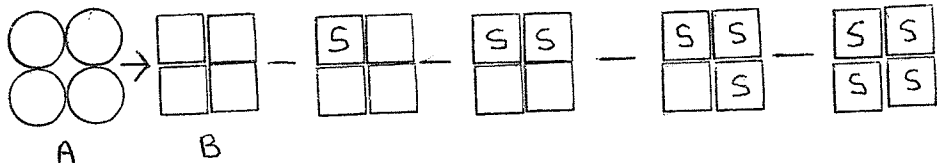
An extension of the regulatory significance of subunit interaction is contained in the molecular models of Koshland, Némethy, and Filmer (25). For simplicity identical subunits were considered primarily although this is not an essential precept.

The theory of Koshland et al considered two conformations, A and B, with the latter able to bind substrate (S). In the "square" model illustrated below diagonal interactions are considered negligible and thus each subunit interacts with two others.



i.e., non-equivalence of subunit interaction in progressive substrate binding.

The "concerted" model, unlike the rest, does not require that form B be present only in the presence of S:



This model is theoretically similar to that of Monod et al (43). The chief distinction is that the M-W-C model is restrictive by its symmetry premise whereas the K-N-F model ignores symmetry.

Two mechanisms may be considered for conformation change: catalysed transformation in which S accelerates the rate of change, and tautomerism whereby the protein itself changes to the new state which is subsequently complexed by S. The proposed "square" model of Koshland et al fits closely the binding data obtained for haemoglobin.

Certain assumptions of this theory should be considered. First, only two conformations are present. Also strong interactions exist between adjacent B conformations (similar to 'constrained" form of Monod et al). The third assumption, that A-A and A-B interactions are equivalent, seems weak. It seems implausible that a change inducing strong interaction between B forms would not alter A-B interactions. By extension of the model to include an intermediate form, this discrepancy could be resolved.

III. Adenylate Control

Studies on phosphofructokinase from various sources had indicated stimulation by AMP (cyclic and non-cyclic) (45, 46). Hathaway and Atkinson (19) had found AMP to be a positive modifier (i.e., activator) for yeast NAD-specific isocitric dehydrogenase. AMP produced a marked decrease in the half-saturation $(S)_{0.5}$ for isocitrate. Atkinson (2) proposed that for isocitric dehydrogenase,

assuming the aconitase reaction to be near equilibrium, the result of AMP action would be that the levels of isocitrate and citrate would vary in opposition to that of AMP.

Citrate was found to be a positive modifier of acetyl CoA carboxylase (57) which is the first enzyme unique to fatty acid synthesis. Therefore, Hathaway and Atkinson (19) proposed that one regulatory property of acetyl CoA carboxylase acting in concert with isocitric dehydrogenase is to ensure fatty acid synthesis when AMP is low and reverse this trend as the AMP level rises.

It has been suggested further that glycogen synthetase and phosphofructokinase similarly ensure polysaccharide formation when ATP is high and glycolysis as ATP drops.

From these studies an adenylate control theory was proposed. It predicted that AMP or ADP modulation would in general be found at metabolic branch points between biosynthetic and degradative pathways. On the basis of this proposal energy control of metabolism may be examined by studies on how the momentary AMP/ADP/ATP ratio affects each metabolic branch point between energy production or storage.

IV. Glutamic dehydrogenase

Glutamic dehydrogenase occupies an important position at a branch point between organic acid and amino acid metabolism. Extensive study has been carried out with this enzyme mainly from animal tissues (10-14, 53, 55). The discovery of a reversible aggregation-disaggregation reaction of the bovine liver enzyme as a function of protein concentration (44) led to intensive investigation of this phenomenon. Dilution produces disaggregation. Frieden (11) found that relatively high concentrations of NADH led to dissociation of the enzyme polymer (molecular weight 1×10^6) into monomers (molecular weight 350,000). These monomers could be further dissociated into subunits of molecular weight about 50,000. Tompkins et al (55), however, found no disaggregation with NADH alone but argued that $ZnCl_2$, GTP, diethystilbestrol (DES), and ATP in the presence of high NADH levels did cause a reduction in molecular weight. It was found that ADP and certain amino acids (leucine, isoleucine, and methionine) protected the enzyme from disaggregation and consequent inhibition of activity. Dissociation could also be produced by the aromatic chelator, o-phenanthroline.

Tompkins, Yielding, and Curran (54) found that conditions promoting disaggregation simultaneously stimulated alanine dehydrogenase activity and consequently proposed a disaggregation effect on substrate specificity. Other workers found no such relationship (9).

Recently Frieden and Colman (14) have done extensive studies on protein concentration as a determinant in aggregation-disaggregation reactions. By kinetic and binding studies it was determined that in the presence of coenzyme, GTP and GDP bind more tightly to the monomer while ADP binds preferentially to the polymer. Cooperativity in the former case is evident only at enzyme concentrations greater than 0.1 mg/ml. Further studies showed that in the absence of purine nucleotides the specific activity of the enzyme was essentially independent of enzyme concentration.

Frieden (10) had proposed that the monomer was the lowest molecular weight species to be catalytically active. He proposed that two sites, at least exist (12). The first site, the active site, could bind any of four coenzymes [NAD(H), NADP(H)]. The second site he called the purine nucleotide site. Some evidence was also found for a third site specific for NADH. The last two sites would be allosteric sites.

Frieden (12) had proposed a reversible equilibrium scheme as follows: P $\rightleftharpoons$ X $\rightleftharpoons$ Y where P is the polymer and X and Y are the active and inactive monomers respectively.

Initially it was believed that, although glutamic dehydrogenase from animal sources was responsive in varying degrees to purine nucleotides, the enzyme from microorganisms and plants showed little response to these effectors. Frieden (13) felt it was significant that the enzyme from lower and higher plants displayed specificity for one or the other coenzyme while the mammalian enzyme was nonspecific. He proposed that the purine nucleotide effect had importance in regulating the use of a particular coenzyme. Recent studies with microorganisms, however, would tend to refute this proposal. Two species of glutamic dehydrogenase, each specific for one coenzyme, have been found in certain microorganisms (27, 28, 29). In Blastocladiella emersonii a single, NAD-specific enzyme has been found (30). A definite purine nucleotide modulation has been shown for many cases (27, 29, 52). Specifically AMP activation seems a consistent property of several of the enzymes from microorganisms (27, 29, 30). An alternate reason for the existence of the two activities with distinctly different controls must be sought.