Proton-Induced Association and Dissociation Effects on a Mitochondrial Isocitrate Dehydrogenase from *Blastocladiella emersonii*. Kinetics and a Regulatory Phenomenon.

by

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To my parents for their continuing aid and encouragement.

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ABSTRACT

A nicotinamide adenine dinucleotide-specific isocitrate dehydrogenase was isolated from Blastocladiella emersonii and purified 450-fold. The enzyme was demonstrated to have a requirement for a divalent metal ion (Mn^{++} or Mg^{++}) as a cofactor. Either AMP or citrate participated as allosteric activators of the reaction at low substrate concentrations. At saturating levels of all ligands, the enzyme was initially determined to have a pH optimum of 8.0 with 0.1 M Tris-Acetate buffer. It was later shown that this enzyme did in fact exhibit two ionized forms at fractional saturation of substrate ligands; one form was more prevalent at pH 6.5, the other at pH 9.0. With increasing substrate concentrations the equilibrium of these two forms shifted until, at saturation the single peak at pH 8.0 was the result.

Kinetic analysis of initial reaction rates showed that in the absence of AMP the reaction mechanism was difficult to elucidate precisely, while in the presence of AMP or citrate the oxidation of isocitrate by this enzyme became an Ordered binary-ternary (Bi-Ter) reaction as confirmed by product inhibition studies.

Zone sedimentation analysis by sucrose density gradients indicated that the enzyme could exist in several forms which were kinetically and physically different. The tendency to these forms was controlled by protons and other ligands. At pH levels greater than 7.5 an active species of molecular weight 110,000 $\pm$ 10,000 was observed. At pH 6.5, inactive aggregated forms of molecular weights ranging from 200,000 to 500,000 and larger were observed.

The substrate, NAD^{+} and the allosteric activator AMP were ineffective in causing disaggregation of the heavy molecular weight form while the alternate allosteric activator citrate, the substrate isocitrate and the cofactors, Mg^{++} and Mn^{++} could cause disaggregation.

It was concluded that the isocitrate dehydrogenase could exist in several forms and that its molecular state depended upon the concentrations of ligands, enzyme and protons. It was further concluded that these factors controlled the inter-protomer equilibrium which in turn controlled the enzyme's conformation and activity. Such a system could provide a primary control mechanism for the isocitrate dehydrogenase of Blastocladiella emersonii.

ABBREVIATIONS

These following abbreviations were used:

(NAD^+)	oxidized nicotinamide adenine dinucleotide
(NADH)	reduced nicotinamide adenine dinucleotide
(AMP)	adenosine-5'-monophosphoric acid
(EDTA)	ethylene-diaminetetraacetic acid
(Tris)	tris (hydroxymethyl)-aminoethane
(DEAE-cellulose)	diethylaminoethyl-cellulose
("TAPE"-Mg^{++}) buffer . . .	0.10 M Tris-acetate with 0.005 M K_2HPO_4, 1 mM Na_4EDTA, and 5 mM Mg^{++} $SO_4^{=}$
(PYG)	1.25 g Peptone: 1-25 g Yeast Extract: 3 g Glucose in 1 litre H_2O. Where agar is included, it is added to (2% strength).

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INTRODUCTION

Cantino and Lovett (1964) emphasized the suitability of Blastocladiella emersonii for studies of differentiation and development. In the "rounding up" process which the motile zoospore experiences prior to formation of the sporangial plant, its single giant mitochondrion apparently undergoes a related rotation process. The mitochondrion is known as the "power house" of the cell, and the citric acid cycle is localized within it. It would be reasonable to postulate that the level and activity of isocitrate dehydrogenase which affects the operation of this cycle (which in turn affects the energy level of the cell) would also affect the differentiation of these cells.

Isocitrate dehydrogenases from Neurospora crassa and from yeast, as well as from other sources have been shown to be allosteric (Sanwal et al., (1965); Atkinson et al.,(1963)). Monod, Changeux and Wyman (1965) proposed a model for allosteric enzyme control in which activators which were sterically dissimilar to the substrate were bound by the enzyme and consequently, increased its affinity for substrates. In this model the importance of quarternary structure of the enzyme was emphasized. The basic subunits (protomers) were visualized as being associated into oligomers

by non-covalent bonds. This confers the quaternary configuration on the protein. Activators alter the equilibrium of the interprotomer bonds in such a way as to increase the affinity for substrates. Stable aggregates of monomers may be formed under a wide variety of conditions and harsh treatment is sometimes necessary to destroy this association. In this model activators and substrates bind at the same or overlapping sites. This would explain such phenomena as inhibition of isocitrate dehydrogenase by citrate, when present at high levels, on the basis that isocitrate and citrate were competing for the same site.

In the same year, Atkinson et al., (1965) proposed a kinetic allosteric model specifically for yeast isocitrate dehydrogenase. This concept differed significantly from that of Monod et al., (1965) but was, nevertheless, an attempt to explain the characteristic sigmoidicity of this enzyme. This will be considered more completely in the historical.

Chen, Brown and Plaut (1964) had shown an increase in the sedimentation velocity of bovine heart NAD-specific isocitrate dehydrogenase in the presence of its activator ADP and a corresponding dissociation effect in the presence of NADH and ATP. Sanwal et al., (1965) were not able to demonstrate this effect using AMP and the NAD-specific isocitrate dehydrogenase from Neurospora crassa.

The significance of Mg^{++} and Mn^{++} uptake in mitochondria and its relationship to the energy state of the cell has been considered carefully by several investigators (see, Lehninger et al., 1967). Magnesium ions had been shown to be an essential cofactor for the isocitrate dehydrogenase from yeast (Atkinson, et al., 1965) and from Neurospora (Sanwal et al., 1965) as well as for the enzyme from Blastocladiella emersonii. Metal ions were, therefore, probably involved in the control mechanism.

Valentine, Shapiro, and Stadtman (1968) working with the glutamine synthetase from E. coli were able to demonstrate a shift in the apparent pH optimum with increase in substrate concentrations. As well, they were able to show by electron microscopy that, glutamine synthetase exhibited specific aggregation-dissociation characteristics on addition of ligands.

In view of work which had been reported on control mechanisms of enzymes, it was decided that to clarify the mechanism of isocitrate dehydrogenase of B. emersonii it would be necessary to employ not only kinetic methods but also physical methods. While kinetic analyses of initial rates could indicate kinetic mechanisms they could not give adequate information about the molecular state of the enzyme.

Kinetic and zone sedimentation studies were, therefore, carried out in close correlation with one another in an attempt to develop a molecular model for this enzyme and to elucidate its possible role in differentiation.

HISTORICAL

I. General

It is essential that living things be able to control and integrate their metabolic processes so that a constant balance between biosynthesis and degradation can be maintained. Elucidation of the mechanisms of enzymes and pathways which mediate these cellular processes has led to present day investigations of metabolic regulation.

The first studies in regulation were concerned with the effects of products, substrates, and other effectors on enzyme systems. Roberts et al., (1955) decided that end products could control their biosynthesis since in Escherichia coli de novo synthesis of amino acids was not initiated if the amino acids were supplied exogenously. Yates and Pardee (1956) and Umbarger (1956), working on pyrimidine and isoleucine synthesis, respectively, reported that the end product could inhibit the first enzyme(s) unique to its own production and also curtail synthesis of the other enzymes which occurred in the pathway. Precursors of catabolic or anabolic pathways can stimulate activity of enzymes thus enhancing end product formation. Common to both these phenomena is the fact that the effector is sterically similar to the substrate, implicating some mechanism different from repression.

Monod et al., (1963) suggested that the binding sites for end products and for substrates were stereo-specifically and spatially distinct, after it was discovered that in a number of systems such as phosphoribosyl-ATP-phosphorylase, (Martin, 1963), aspartate transcarbamylase (Gerhart and Pardee, 1962) and biosynthetic threonine deaminase (Changeux, 1961), susceptibility to end product inhibition could be destroyed without concomittant loss of enzyme activity. Monod et al., (1963) coined the term "allosteric" for proteins which were regulated in this manner.

The fact that allosteric proteins were susceptible to inhibition and activation by metabolites (not directly involved in their own reaction) meant that regulation of these enzymes was more rapid and more sensitive than regulation by repression and induction. Such a control system would allow a biological system to respond to compounds from related pathways thus mediating integration of biosynthesis with other metabolic functions. A combination of all of these mechanisms is necessary to control a complex biological system.

II. Regulatory Enzymes

A. Isocitrate Dehydrogenase from Other Sources

Rat liver, kidney, and brain mitochondria have been shown to have an NAD-specific and an NADP-specific isocitrate

dehydrogenase (Vignais and Vignais, 1964). The NADP-specific isocitrate dehydrogenase from B. emersonii is extramitochondrial. The NAD-specific enzyme, used in this study catalyses the reaction; D_S-isocitrate + NAD^+ $\longrightarrow$ α-KG + CO_2 + NADH + H^+

This enzyme was found to be allosteric and susceptible to effectors such as nucleotides, pH, and citrate. The enzyme from rat liver, brain and kidney mitochondria (Vignais and Vignais, 1964), yeast (Kornberg and Price, 1951; Hathaway and Atkinson, 1963), and Neurospora crassa (Sanwal et al., 1963) is activated by AMP; isocitrate dehydrogenase from locust flight muscle, (Goebell and Klingenberg, 1954) and from guinea pig heart (Plaut and Sung, 1954) by ADP. The exact mechanism of activation is not known for either effector although Plaut and Chen (1963 and 1964) were able to show with sedimentation studies that ADP caused a completely reversible aggregation of subunits in bovine heart mitochondria. This could not be demonstrated with Neurospora isocitrate dehydrogenase (Sanwal and Stachow, 1965).

Neurospora isocitrate dehydrogenase has been found to be susceptible to citrate stimulation (Sanwal et al., 1963) or as in yeast to citrate stimulation and inhibition, depending on the concentration (Atkinson, 1965). This effect was similar to the response exhibited by aspartate

transcarbamylase (Gerhart and Pardee, 1963) to its substrate analogues succinate and maleate. At low aspartate concentrations, increasing the analogue concentration over a limited low range caused increased enzyme activity. At high analogue concentration, however, inhibition occurred. Activation had occurred because the analogue mimicked the substrate causing cooperative interactions between substrate sites. The analogue effectively reduced the number of sites that had to be filled by the true substrate. A level was reached where so much analogue had been bound that only a reduced number of active sites were left available to bind the substrate. At these concentrations of analogue, competitive inhibition occurred.

In Neurospora the citrate acted only as an activator. At high concentrations it did not become a competitive inhibitor. Hathaway and Atkinson (1963) proposed a "cooperative phenomenon" with AMP and citrate. They further suggested two binding sites, one which could bind citrate and isocitrate, and an activating site which bound isocitrate, citrate, and AMP. The fact that these ligands did not inhibit at higher concentrations negated the concept that citrate was binding at unoccupied substrate sites.

Sanwal et al., (1965), using the computer-based analysis developed by Cleland (1963), proposed that the

sigmoidal relationship between isocitrate concentration and velocity resulted from sequential binding of substrate at allosteric and catalytic sites and that no binding of isocitrate occurred at the catalytic site unless it was already bound at the allosteric site.

B. pH as a Regulatory Ligand

In addition to the activation shown by nucleotides, Plaut and Sung (1964) reported a shift in pH optimum caused by ADP. At low isocitrate concentration (1.3×10^{-3} M) and at pH 6.7, maximum activity occurred in the absence of ADP. With similar isocitrate concentrations and in the presence of ADP (6.7×10^{-4} M), the pH optimum shifted to pH 7.2. A corresponding shift was observed if the isocitrate concentration was increased ten-fold with no addition of ADP.

A similar phenomenon was observed in the phosphofructokinase from skeletal muscle of mouse and frog, (Trivedi and Danforth, (1966)) where a shift in pH optimum 6.65 to 7.75 accompanied a decrease in fructose-6-phosphate concentration. In haemoglobin (Rossi Fanelli *et al.*, 1964) a change in affinity for oxygen with a change in the pH of the media has been observed.

The kinetic behavior of aspartate transcarbamylase of *E. coli* was shown by Gerhart and Pardee (1964) to be

influenced by proton concentration. The monomer form of this enzyme showed simple pH dependence while catalysis of the reaction by the tetramer showed a marked dependence of the pH optimum on substrate concentration. This suggested that the affinity of the tetramer for the substrate was pH dependent while the affinity of the monomer was not. The saturation curve of the tetramer is strongly dependent on pH and exhibits a sigmoid relationship reminiscent of the Bohr effect of haemoglobin. Lowering the pH of the tetramer has the same effect as increasing aspartate concentration; subunit interactions are reduced and the activity is increased.

In the glutamic dehydrogenase of B. emersonii (LeJohn and Jackson, 1968) the influence of pH on enzyme activity again resembles the Bohr effect of haemoglobin. High pH favours activation of the reductive amination of α-ketoglutarate and concomitant inhibition of the oxidative deamination of glutamate by Ca^{++} (LeJohn, 1968 a, b).

These observations strongly suggest that intracellular pH changes must be considered as a possible regulatory feature of enzymes. In the case of frog muscle phospho-fructokinase, the enzyme was observed to be sensitive to minute pH shifts within the physiological region (Trivedi and Danforth, 1966). Lowering the pH caused a decrease in

affinity of the enzyme for Fructose-6-phosphate. The enzyme was most sensitive to pH changes when it had been inhibited in the presence of ATP.

A complex system exists in intact frog muscle. A simple decrease in enzyme affinity for Fructose-6-phosphate could not reduce the rate of glycolysis by itself. If a substrate pool is small in comparison to the overall flow rate of the pathway, then a decrease in substrate affinity for any enzyme, other than the first one of the pathway, should cause a rise in substrate concentration. An elevation of the hexose phosphate concentration does not necessarily mean that the phosphofructokinase step has become rate limiting. Even a rise in substrate with an accompanying decrease in product is not absolute proof of a rate control step, because an increase in the Michaelis constant of one enzyme and a corresponding decrease in the Km of the subsequent enzyme can result in the accumulation of intermediates, (Trivedi and Danforth, 1966).

It is hard to decide which points are metabolic control points. Data based on concentration of intermediates should be tentative rather than absolute. It should also be noted that the pH-AMP curves were very steep, so that the pH control might not be widespread but a very primitive mechanism.

C. Association-Dissociation Phenomena

One of the first proteins in which association-dissociation was known to occur was haemoglobin. The most striking feature of this protein was its ability to form low molecular weight subunits when no hydrolysis or denaturation had occurred. This was attributed to the fact that normal mammalian haemoglobin was made up of four polypeptide chains which were associated by non-covalent linkages (Rossi Fanelli et al., 1964). Association-dissociation equilibrium is dependent on the characteristic structure of the protein. It is reversible, and does not involve any change in the oxygen capacity of the protein. Giodotti and Craig (1963) did experiments on the diffusion of haemoglobin through membranes which suggested that even above pH 6.5 dissociation equilibria involved tetramers, dimers and monomers.

Association-dissociation is by no means confined to haemoglobin and has also been demonstrated in enzymes. Chen, Brown, and Plaut (1964) using highly purified preparations of NAD-isocitrate dehydrogenase of bovine heart mitochondria were able to show a marked alteration in the sedimentation pattern of this enzyme on addition of the activator ADP. The enzyme was aggregated by ADP, a process which was fully reversible.

Iwatsuki and Okazaki (1967) showed by zone sedimentation on sucrose gradients that for the deoxythymidine kinase of E. coli a marked increase in the sedimentation velocity occurred on addition of activator (dCDP, dCTP, dADT) and inhibitor (dTTP) nucleotides, while no such increase occurred on addition of other nucleotides which were not effectors. There was good correlation between the extent of activation or the extent of inhibition and the increase in sedimentation rate which was exhibited. These effectors were presumed to induce dimerization; the molecular weights of the monomer and dimer were estimated to be 42,000 and 89,000-91,000 respectively. Dimerization was further interpreted to be an essential change which was involved in inhibition and activation of the protein.

Frieden (1967) showed that for bovine liver glutamate dehydrogenase the polymer bound the inhibitor less tightly than did the monomer form of the enzyme, and that for this glutamate dehydrogenase the association-dissociation was rapid in relation to the overall reaction. He postulated that this phenomenon was analogous to the allosteric concept of Monod except that in his model the two states of the enzyme are monomer and polymer form rather than a relaxed or tight enzyme configuration (Monod et al., 1963).

One of the latest reports of association-dissociation is that of Valentine, Shapiro, and Stadtman (1968) who worked with glutamine synthetase from E. coli. Here the phenomenon was controlled by addition of divalent cations. Substrates and inhibitors were incapable of altering the molecular structure in any way as demonstrated by electron microscopy.

D. The Role of Divalent Cations

It has also been shown that divalent cations such as Mn^{++}, Mg^{++}, and Co^{++} are necessary for the activity of NAD-isocitrate dehydrogenase of bovine heart mitochondria (Chen et al., 1964). Hathaway and Atkinson (1963) indicated a requirement for Mg^{++} or Mn^{++} ions for the isocitrate dehydrogenase of yeast. Mg^{++} ions were necessary for activity of the isocitrate dehydrogenase of Neurospora crassa (Sanwal et al., 1965).

Mitochondria are able to translocate and accumulate divalent ions such as Ca^{++}, Mg^{++}, Mn^{++}, and Sr^{++}. Two hypotheses have been developed to account for the ion transport process and for its relationship to the energy processes of the cell. Some features of translocation are, however, common to any hypothesis on ion translocation.

1. The cation intake stimulates ATP hydrolysis, i.e, electron transport.

2. There is a stoichiometric relationship between cation uptake and stimulation of respiration.

3. During uptake of cations, H^+ ions are ejected with concomitant rise in intramitochondrial pH.

Two mechanisms of mitochondrial ion transport exist at present. The more traditional is the "Chemical Coupling" hypothesis which proposes that high energy intermediates, formed during electron transport, are available either to be used for ATP or to transport ions by coupled enzymic reactions which have common intermediates.

The second mechanism, the H^+-pump theory, does not support the hypothesis of high energy intermediates but makes an alternate proposal of separation of H^+ from OH^- which results in a pH gradient and a transmembrane potential. The gradient provides the energy for transport and oxidative phosphorylation (Lehninger et al., 1967).

While the mitochondrion is accumulating cations it is in a highly "energized" state and the citric acid cycle is operating. The electron transport chain is also operating at this time. There is a stoichiometric relationship between Ca^{++} uptake and electron transport activity (ATP hydrolysis).

Ca^{++} uptake has an absolute requirement for operation of an electron transport chain; it also requires ATP or ADP, Mg^{++}, and phosphate in rat liver mitochondria. The mechanisms for uptake of Mn^{++}, Ca^{++}, and Sr^{++} seem to be identical, while, accumulation of Mg^{++}, at least in beef heart, apparently requires an inducer, (Lehninger et al., 1967). During ATP synthesis extrusion of these cations will occur with concomitant uptake of H^{+} ions.

III. Blastocladiella emersonii

Blastocladiella emersonii was first described as a species in 1953 by Cantino and Hyatt. This Phycomycete offers a model system for experiments in differentiation because large, synchronized, single generation cultures are easily obtained by controlled growth on defined media. The organism is a useful subject for experiments on regulation because of its life cycle. When grown in synchronous culture it was possible to keep B. emersonii in the O.C. state for an indefinite period.

A change from O.C. to R.S. cells could be induced by additions of exogenous bicarbonate. The change from O.C. to R.S. development could be arrested if the bicarbonate were removed before two-thirds of the regular generation time (16 hr) had elapsed. After this time, the cells were irreversibly

O.C. - ordinary colorless
R.S. - resistant sporangial