Association-Dissociation Reactions of Mitochondrial Isocitric Dehydrogenase Induced by Protons and Various Ligands*

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SUMMARY

A nicotinamide adenine dinucleotide-specific isocitric dehydrogenase has been isolated and purified 450-fold from Blastocladiella emersonii. The enzyme has a monomeric molecular weight of 110,000 ± 10,000. It is capable of aggregating to polymers of molecular weight ranging from 200,000 to 500,000 and higher at acid pH levels. At alkaline pH values, it exists as a monomer. The aggregated enzyme is inactive and the monomer is active. Under the influence of various ligands (citrate, isocitrate, Mn++, Mg++), the aggregated enzyme can be converted to the monomer. A difference of 1 pH unit (6.5 to 7.5) changes the association-dissociation equilibrium in favor of monomer formation from the polymeric form. The enzyme was shown to exist as two distinct ionized forms with pH optima at 6.5 and 9.0. By a complex series of ligand interactions over a wide range of pH values, the two pH optima disappear and a single ionized species becomes evident with a pH optimum at 8. Although isocitrate and NAD+ display cooperative interactions, at low pH levels and high NAD+ concentrations, NAD+ is incapable of dissociating the polymer into monomer whereas isocitrate can. The same incompetence is shown by AMP but not by citrate, both of which are allosteric activators. Kinetic analyses have shown that the kinetic mechanism may be ordered binary-ternary. Attempts have been made to relate these and other findings to a system of regulation by which this enzyme is coupled to the glutamic dehydrogenase found in the mitochondria. Very tenuous correlations have been proposed for the participation of this regulatory mechanism in the germination process in this organism.

In the now "classic" paper of Monod, Wyman, and Changeux (1), a major assumption of their model of allosteric transitions was that the quaternary configuration of the oligomer depends on a symmetrical arrangement of the protomers, which symmetry is conserved during the transition from one state (R) to another (T). This being the case, noncovalent interprotomer bonds (which are of an indefinite nature) are liable to be subjected to tremendous constraints in the transition. It was further suggested that because of some inherent specificity in the association of protomers, and in the dissociation of oligomers into protomers, an equilibrium is maintained which allows for complete reassocation of the protomers when physical and other forces favor oligomer formation. Even under reasonable conditions of dilution, this reassociation process can proceed.

More and more it is being realized that oligomeric proteins are liable to further states of aggregation, and this may have important implications in the regulation of enzymes (2, 3). Sometimes these polymers are inactive and, at other times, they are active. It is a reasonable assumption that some proteins are liable to exist as polymers in vivo and may therefore be inactive unless they can be disaggregated into smaller active units. Undoubtedly, such a regulatory process must exist and the nature of the control may vary from protein to protein. Foremost in this area of research is the well known case of bovine liver glutamate dehydrogenase which can form aggregates from 350,000 to 2 million in molecular weight (2). It has been estimated that it is present in the mitochondria in concentrations as high as 1 mg of enzyme per g of mitochondrial protein. The forces that cause the oligomers to aggregate to higher orders may be similar to those that hold the protomers in a quaternary configuration. The extent of this aggregation may be influenced by a variety of factors such as pH, enzyme concentration, temperature, ionic strength, effector molecules, and cations which could also act as cofactors in the enzymic reaction.

There have been several reports which have attempted to correlate these changes in the state of aggregation with the catalytic activity. Datta, Gest, and Segal (4) reported on the inhibition and resultant aggregation of homoserine dehydrogenase by threonine. On the other hand, Vagelos, Alberts, and Martin (5) showed that the activation of acetyl-CoA carboxylase by citrate is due to the aggregation of inactive subunits. Quite recently, Iwatsuki and Okazaki (6) observed that the deoxynucleotide, dTTP, an inhibitor of the enzyme, deoxymethylidine kinase, causes aggregation of the monomer (3.5 S) to a dimer.
These are a few of the conflicting observations that prevent formulation of a unified concept on association-dissociation relationships of regulatory enzymes with respect to activation or inhibition. Although it is tempting to conclude that association-dissociation phenomenon may, therefore, not be an inherent feature in the control of allosteric enzymes, it is easy to visualize that this is not necessarily true since proteins must have different strengths of interaction between subunits in the absence of effectors. Allosteric effectors, by changing the strength of interaction, may participate either as activators or as inhibitors depending on the system. Association or dissociation could, therefore, be accompanied by either activation or inhibition.

In this paper, we report on the importance of this association-dissociation equilibrium as a probable mode of primary regulation of a NAD-specific isocitric dehydrogenase isolated from Blastocladia emersonii. The enzyme is mitochondrial and its state of aggregation is influenced by the enzyme concentration and pH. A variety of effectors, some substrates, either induce disaggregation or enhance aggregation. The enzyme aggregates into inactive polymers of over 400,000 molecular weight with decreasing pH, and exists, predominantly, as “monomers” at higher pH levels. In the monomeric state, the enzyme can exist in two well-defined conformations depending on the extent of alkalinity.

**EXPERIMENTAL PROCEDURE**

**Organism—**B. emersonii was grown as single generation, single cell cultures as described previously (7).

**NAD-Specific Isocitric Dehydrogenase from B. emersonii—**

Freshly harvested or frozen cells were suspended with previously chilled mortar and pestle in 2 volumes of a standard buffer composed of 0.1 M Tris-acetate, 0.005 M KH2PO4, 5 mM MgSO4, \(10^{-4} \) M Na2EDTA (pH 7.5), containing \(10^{-3} \) M dithiothreitol (Cleland's reagent). A total of twice the cell weight of levigated alumina powder was added to the suspension to facilitate disruption of the cells. The slurry was rapidly ground for 5 min and 1 volume more of the standard buffer was added. The slurry was sonically oscillated in a water-cooled Raytheon ultrasonicator (10”) in 25-ml batches for 10 min to ensure complete disruption of the mitochondria. The cell debris and alumina were removed by centrifugation at 48,000 x g for 20 min. All further operations were carried out at 5°C.

The supernatant containing the enzyme was treated as follows.

1. Chilled absolute ethanol (−20°C) was added dropwise with a separatory funnel to the crude extract with continuous stirring. The solution was maintained at 0°C in an ice-salt bath. Ethanol was added to a final concentration of 30% over a period of 60 min and stirring continued for a further 2 hours. The precipitate was removed by centrifugation for 15 min at 48,000 x g. The supernatant was discarded and the precipitate was resuspended in the standard buffer. Generally, for 50 to 100 g, wet weight, of cells, 40 to 50 ml of the buffer were adequate for resuspension.

2. The suspension was recentrifuged at 48,000 x g for 1 hour and the clarified supernatant was saved. The enzyme was adsorbed onto a column of DEAE-cellulose (medium mesh), 30 x 2.5 cm, in 5-ml aliquots. A partially purified enzyme preparation was recovered from the column with a linear gradient elution system. The reservoir contained 450 ml of standard buffer with 0.5 mM EDTA adjusted to pH 7, and the mixing chamber contained 450 ml of the standard buffer only. Eight-milliliter fractions were collected automatically. The enzyme was eluted at about 0.1 mM EDTA. Fractions containing the enzyme were pooled.

3. Solid (NH4)2SO4 was added in small amounts over a period of 20 min to bring the solution to 75% saturation. The solution was stirred continuously for 1 hour. The precipitate was collected by centrifugation (48,000 x g for 20 min) and resuspended in a small volume of the standard buffer. At this point, the enzyme preparation had a specific activity between 100 and 120 times greater than that of the crude extract.

4. Further purification of the enzyme was achieved by passage through Sphadex G-200 column (30 x 2.5 cm) with the standard buffer. A highly purified preparation (single activity band in polyacrylamide gel electrophoresis) with a specific activity some 450 times greater than that of the crude extract is usually obtained. The kinetic studies reported in this paper were carried out with enzyme mainly from stocks purified some 120-fold, but cross-checks were made with the highly purified preparation in all major experiments. The zone sedimentation studies were carried out with the purified enzyme.

**Kinetic Analyses—**The reaction systems for kinetics varied and details are given in individual figure legends. In all cases, the reagents were made up fresh and at the desired pH values just prior to analyses. This was important because of the extreme sensitivity to pH shown by the enzyme. Ionic strengths were kept as near constant as possible.

**Zone Sedimentation Analysis with Sucrose Density Gradients—**Molecular weight determinations were made after Martin and Ames (8). Association-dissociation reactions of the enzyme were also detected by this method. Five milliliters of 5 to 20% continuous sucrose density gradients were prepared by the method of Britten and Roberts (9) with a Buchler Densigrad Apparatus (Buchler Instruments Company, Fort Lee, New Jersey). All gradients were buffered with 0.05 M Tris-acetate at the pH values indicated in the appropriate figure legends. When modifiers were used, these were included in the sucrose solutions before making the gradients. Centrifugations were carried out for periods varying from 16 to 20 hours at 30,000 rpm with an SW39 rotor of a Spinco Model L ultracentrifuge. For reasonable comparison, all of the data presented here are from 18-hour runs. Pig heart malate dehydrogenase (mol wt 40,000), obtained from Boehringer, was used as an internal standard in all centrifugations. Fractions were collected with a Buchler piercing unit, and 34 (10-drop) fractions were collected between the meniscus and the head of the piercing needle. Previous calibration allowed 0.25 ml of a cushion of 20% sucrose to be layered at the bottom of the tube to the head of the needle. Fractions were assayed in a standard reaction system composed of 1.25 mM NAD+ and the following (3.33 mM each): MnCl2, trisodium citrate, trisodium isocitrate, and 45 mM Tris-acetate (pH 7.8). This solution was designed to give optimal enzyme activity. The results recorded here are for 50-μl samples from approximately 140-μl fractions.

**Enzyme Assays—**All assays were carried out in 10-mm light path cuvettes in 3-ml volumes. Reaction rates were measured as ΔA340 μm min-1 by means of a Gilford model 2000 recording spectrophotometer. The method of Lowry et al. (10) was used for protein determinations.

**Reagents and Chemicals—**These were purchased from Sigma.
2486 Proton as Regulatory Ligand for Isocitric Dehydrogenase

Vol. 244, No. 9

Fig. 1. Cooperative effects of NAD\(^+\) and isocitrate on the reaction catalyzed by \(B.\ emersonii\) isocitric dehydrogenase. \(a\), NAD\(^+\) as the variable substrate and isocitrate held at several fixed levels. \(b\), isocitrate as the variable substrate and NAD\(^+\) held at several fixed concentrations. The concentrations of reactants are shown. The reaction system contained 150 mM Tris-acetate buffer (pH 8), 3.33 mM Mg\(^++\), and 30 \(\mu\)g of enzyme protein.

Fig. 2. Cooperative effects of NAD\(^+\) and AMP (O) and of NAD\(^+\) and citrate (■) on the reaction catalyzed by \(B.\ emersonii\) isocitric dehydrogenase. The concentrations of AMP and citrate are as indicated. The reaction system contained 150 mM Tris-acetate buffer (pH 8), 3.33 mM Mg\(^++\), 0.8 mM isocitrate, and 10 \(\mu\)g of enzyme protein.

Fig. 3. \(a\), rate concentration plots of the effect of divalent metals (Me\(^++\)) on the oxidation of isocitrate catalyzed by \(B.\ emersonii\) isocitric dehydrogenase. The substrates, NAD\(^+\) and isocitrate, were held at fixed concentrations of 1.33 mM and 3.33 mM, respectively. The reaction system also contained 100 mM Tris-acetate buffer (pH 8) and 10 \(\mu\)g of enzyme protein. \(b\), cooperative effects of Mg\(^++\) and isocitrate on the reaction catalyzed by \(B.\ emersonii\) isocitric dehydrogenase. The reaction system contained 1.33 mM NAD\(^+\); 1 mM AMP, 100 mM Tris-acetate buffer (pH 8), and 10 \(\mu\)g of enzyme protein. Isocitrate concentrations were as indicated.

RESULTS

When NAD\(^+\) is the variable substrate and isocitrate is fixed at several different levels, the rate concentration plots of Fig. 1\(a\) indicate that the \(V_{\max}\) increases as the half-saturation \((S_{0.5})^2\) decreases. The same pattern is evident with isocitrate as the variable substrate and NAD\(^+\) fixed (Fig. 1\(b\)). These are now familiar non-Michaelian kinetics usually shown by allosteric enzymes.

In keeping with the isocitric dehydrogenases from other sources (11-19), the \(B.\ stadtmanii\) NAD-specific isocitric dehydrogenase...
requires a cation (Mn$^{++}$ or Mg$^{++}$ specifically) as a cofactor with either ADP or AMP as an allosteric effector which activates the enzyme at low concentrations of substrates. As for the Neurospora isocitric dehydrogenase (14, 15), citrate is a very strong allosteric activator. The results of Fig. 2 illustrate the cooperative interactions between AMP and NAD$^+$. A similar relationship holds for isocitrate and AMP. For brevity, this is not shown.

Although most of the kinetic studies reported here were carried out with Mg$^{++}$ as the cofactor, Mn$^{++}$ was more effective than Mg$^{++}$ when the allosteric activators (citrate and AMP) were absent (Fig. 3a). The $S_{0.5}$ values for Mn$^{++}$ and Mg$^{++}$, at the same substrate levels, were 60 nM and 600 nM, respectively. In the presence of AMP, the $S_{0.5}$ values for these two cations were identical at 2 nM (see Table I for the kinetic constants). This relatively high Mg$^{++}$ requirement, quite likely, is due to the formation of Mg$^{++}$-isocitrate complexes which effectively reduces the concentration of ligands available for the reaction. This interpretation is supported by the observation that, whereas higher levels of Mg$^{++}$ cause strong inhibition, increasing Mn$^{++}$ was without any effect on the reaction. However, the extremely large decrease in $S_{0.5}$ for Mg$^{++}$ in the presence of AMP is not understood. The fact that AMP or citrate at comparatively low concentrations (100 nM) can change the affinity of the enzyme for Mg$^{++}$ from low to high suggests that a configuration change has occurred on the enzyme—a change that Mn$^{++}$ is capable of causing in the absence of effectors. This conclusion is also supported by sedimentation studies which are reported later. Our decision to use Mg$^{++}$ rather than Mn$^{++}$ was based on the observation that the enzyme is very sensitive to pH and a large part of the work is on pH effects. Mn$^{++}$ is not a stable cation outside the pH range of 6 to 7.5.

The cooperative interactions described above are very similar.
Proton as Regulatory Ligand for Isocitric Dehydrogenase

FIG. 6. Product inhibition by HCO$_3^-$ of the reduction of NAD$^+$ catalyzed by B. emersonii isocitric dehydrogenase. The reaction system contained 150 mM Tris-acetate (pH 8), 2 mM AMP, 16.66 mM isocitrate, 3.33 mM Mg$^{++}$, and 20 µg of enzyme protein. Inset, replot of intercepts against inhibitor concentration.

to those reported by Atkinson et al. for the enzyme from yeast (11, 12) and by Sanwal et al. for the enzyme from Neurospora (14, 15). As is evident below, the kinetic mechanism that could be formulated from standard product inhibition patterns and modifier influences on the kinetic behavior of the Blastocladiella isocitric dehydrogenase is not very different from the mechanism proposed for the enzyme from Neurospora (15). The Michaelis constants for NAD$^+$ and isocitrate were determined from the double reciprocal plots of Fig. 4. Since normal Michaelian plots could only be obtained for low substrate levels in the presence of saturating concentrations of AMP or citrate, these Michaelis constants were evaluated under these conditions.

Product Inhibition Patterns—Cleland (20) has discussed rather ample the immense value of studying product inhibition patterns to elucidate enzymic reaction mechanisms, but the procedure is of little use when double reciprocals of rate concentration plots are nonlinear. To achieve linearity in these studies, AMP or citrate had to be used as allosteric activators as in the case of proper $K_m$ determinations.

During the oxidation of isocitrate by isocitric dehydrogenase, the products are NADH, CO$_2$, and α-ketoglutarate. In these studies, we followed the suggestions of Cleland in terms of concentrations of the reagents. One substrate (isocitrate) was held saturating in each experiment, and NAD$^+$ was the varied substrate interacting with each of the three products, individually. In Fig. 5, the results of an experiment with NADH as a product inhibitor, at pH 8, in the presence of AMP, are shown. The inhibition is competitive. The replot of the slopes is linear. With HCO$_3^-$ as product inhibitor (Fig. 6), the inhibition pattern is noncompetitive and the replot is linear. When α-ketoglutarate was used as inhibitor, the inhibition pattern was uncompetitive except that at high α-ketoglutarate levels the pattern became noncompetitive (Fig. 7a). The replot of intercepts was linear. However, the slope replot was nonlinear. We suspected that complex formation of the cofactor (Mg$^{++}$) by α-ketoglutarate may, in part, be responsible for this response. Indeed, when citrate was used as an activator in the product inhibition studies (Fig. 7b) we had to use 10 times as much Mg$^{++}$ (33.33 mM) before we could obtain product inhibition patterns that were similar to those cases of which AMP was used as the activator. It was impossible to use much higher concentrations of Mg$^{++}$ because of precipitation difficulties. Even with large increases in the concentration of isocitrate at these high levels of Mg$^{++}$, the product inhibition patterns remained the same. Since the inhibition by α-ketoglutarate was largely uncompetitive, it seemed reasonable to suggest that the products are probably released in an ordered sequence: CO$_2$ first, followed by α-ketoglutarate, and finally NADH. This kinetic approach

FIG. 7. Product inhibition by α-ketoglutarate of the reduction of NAD$^+$ catalyzed by B. emersonii isocitric dehydrogenase. The reaction system contained 150 mM Tris-acetate (pH 8), 16.66 mM isocitrate, 3.33 mM Mg$^{++}$, and 40 µg of enzyme protein. a, AMP as activator; b, citrate as activator and Mg$^{++}$ at 33.33 mM. Inset, replot of intercepts against inhibitor concentration.
tells us very little about the molecular state of the enzyme, an important and probably primary aspect of its regulation. Physical techniques were therefore used in attempts to elucidate a molecular mechanism.

**pH Effects**—In preliminary studies, we had observed that the pH optimum of the reaction changed considerably depending on the concentration of substrates. At high substrate levels (16.66 mM isocitrate and 6.66 mM NAD$^+$), the pH optimum was 8. However, when the substrates were reduced to about $K_m$ values (see Fig. 1, a and b), the pH optimum was found to be 6.5. From Fig. 2, it was already apparent that the Michaelis constants of NAD$^+$ and isocitrate were reduced about 10-fold when AMP was present in the reaction. Therefore, we rechecked the pH optimum again in the presence of AMP, with the same concentrations of substrates as before. It was found that the pH optimum was fixed at about pH 8 as long as AMP was saturating. At low levels of AMP, and limiting amounts of substrates, the pH optimum dropped to about 7. Holding the substrate levels low and fixed, but varying AMP concentration over several different pH values, the results shown in Fig. 8 were obtained. The pH optimum for the reaction increased progressively from 6.5 to 8 with different concentrations of AMP. This suggested that high substrate concentrations and AMP were capable of altering the ionization properties of the enzyme. Similar findings have been reported by Trevedi and Danforth (21), Gerhart and Pardee (22); LehJohn and Jackson (20), Tompkins et al. (23), and Kovac, Bednarova, and Groksak (24).

Our first concern was to examine the rate concentration plots at pH 6.5 in the absence of AMP. The results of an experiment in which NAD$^+$ was the varied substrate and isocitrate was held fixed at several different levels are shown in Fig. 9. Surprisingly, NAD$^+$ was found to be strongly inhibitory. The plots were sigmoidal at relatively low concentrations of substrates, but hyperbolic when substrate levels were high. Since we had observed that high substrate concentrations changed the pH optimum to 8, we looked at the rate concentration profiles within

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**Fig. 8.** Rate of the reaction catalyzed by *B. emersonii* isocitric dehydrogenase as a function of pH over a wide range of AMP concentrations. The reaction system contained 150 mM Tris-acetate (pH as indicated), 16.66 mM isocitrate, 1 mM NAD$^+$, 3.33 mM Mg$^{++}$, and 25 $\mu$g of enzyme protein.

**Fig. 9.** Rate concentration patterns of the reduction of NAD$^+$ catalyzed by *B. emersonii* isocitric dehydrogenase at different pH values. The reaction system contained 100 mM Tris-acetate (pH as indicated), 3.33 mM Mg$^{++}$, isocitrate as indicated, and 20 $\mu$g of enzyme protein.

**Fig. 10.** Rate of the reaction catalyzed by *B. emersonii* isocitric dehydrogenase as a function of pH over a wide range of NAD$^+$ concentrations. The reaction system contained 100 mM Tris-acetate (pH as indicated), NAD$^+$ as indicated, 3.33 mM Mg$^{++}$, 3.33 mM isocitrate, and 20 $\mu$g of enzyme protein.
the pH range 7.5 to 9.0. For adequate comparison, the findings are incorporated into Fig. 9. Careful analysis immediately revealed that the pH effect was very complex. At very high NAD⁺ concentrations, and at alkaline pH values, substrate inhibition was no longer apparent. If anything, NAD⁺ served as an activator. On the other hand, at low NAD⁺ concentrations, with isocitrate limiting, the enzyme was more reactive at pH 6.5 than at 7.5 and 8.0. Clearly, the pH profile observed in Fig. 8, when AMP was absent, must be incomplete. A second pH optimum should be present at alkaline pH values. To confirm this, we repeated the study over a wider range of pH values at low substrate concentrations. In the absence of AMP, with both substrates held low, two pH optima at pH 6.5 and 9.0 were obtained (Fig. 10). With increasing NAD⁺ levels and all other parameters unchanged, both pH optima were altered. The enzyme became increasingly reactive between pH 7.0 to pH 8.0. At saturating concentrations of NAD⁺ (10.66 mM), there was a complete inhibition of the reaction at acid pH values and the enzyme became optimally active between pH 7.5 and 8.0 at which it was previously minimally reactive when low levels of substrate ligands were used.

It was necessary to show that isocitrate was also capable of causing the same changes in pH optimum as NAD⁺. In Fig. 11, the result of an experiment in which isocitrate was the varied substrate and NAD⁺ was held fixed at a low concentration (0.66 mM) over a wide range of pH is shown. Two pH optima are clearly discernible. Relatively lower concentrations of isocitrate were required to bring about a change from two to one pH optimum. We have shown that citrate can replace AMP as an allosteric activator (Fig. 2). We interpreted the effectiveness of isocitrate in these experiments to be the result of isocitrate mimicking citrate. As becomes clear in the sedimentation studies, isocitrate served as a better dissociating ligand than NAD⁺ and these transitions from two to one pH optimum are correlated with association and dissociation of the protein.

Because of the instability of Mn⁺⁺ at pH values below 6 and above 8, we could not obtain a clear pattern on the ionization process of the enzyme when Mn⁺⁺ replaced Mg⁺⁺. In the experiments in which Mn⁺⁺ was used (the results of which are not illustrated here) there were two pH optima, one at pH 7.5 and another around pH 9. The latter was not clearly defined because of pH interference with Mn⁺⁺. A single optimum appeared at pH 7.8 as substrate concentrations were increased. Since the effect of the 2 metal ions appears to be similar, we restricted our use of Mn⁺⁺ in subsequent studies to routine assays of enzyme activity.

**Sedimentation Studies**—We reasoned that, if the two conformations of the enzyme are sufficiently different to elucidate in simple pH kinetic analyses, they may also have molecular differences which could be resolved by zone sedimentation analysis. For this investigation, we studied the sedimentation behavior of the enzyme at different pH values. The findings are summarized in Figs. 12 to 14. Using the commercially built Buchler Densigrad apparatus (see “Experimental Procedure”), the centrifugations were reproducible with 5% error. All of the centrifugation data given here represent a minimum of four and in some cases six runs. In Fig. 12, typical profiles of the sedimentation studies are shown.

**Fig. 11.** Rate of the reaction catalyzed by *B. emersonii* isocitric dehydrogenase as a function of pH over a wide range of isocitrate concentrations. Conditions as for Fig. 9 with NAD⁺ fixed at 0.66 mM and isocitrate held constant at different levels as indicated.

**Fig. 12.** Sedimentation behavior of *B. emersonii* isocitric dehydrogenase as a function of pH. Enzyme protein, 250 μg, was used in each gradient. For details of preparing the gradients and assay of the fractions, see “Experimental Procedure.” The molecular weight of the enzyme sedimenting at pH 7.5 and at pH 9 was estimated as 120,000 in each case. Molecular weight of the enzyme sedimenting at pH 6.5 varied from 180,000 to 200,000 or more. Malate dehydrogenase peaks at tube 26. Centrifugations were carried out for 18 hours at 30,000 rpm.
Fig. 13. Influence of various ligands—NAD⁺ plus Mn²⁺; NAD⁺, Mn²⁺, and citrate; citrate, isocitrate, and Mn²⁺—on the sedimentation behavior of B. emersonii isocitric dehydrogenase at pH 6.5. Concentrations of ligands were as follows: NAD⁺, 4 mM; Mn²⁺, isocitrate, and citrate each at 3.33 mM. Centrifugations were carried out for 18 hours at 30,000 rpm. Enzyme protein, 250 μg, was used.

Fig. 14. Influence of various ligands—Mn²⁺, Mn²⁺ and citrate, Mn²⁺ and isocitrate—on the sedimentation behavior of B. emersonii isocitric dehydrogenase at pH 6.5. The concentrations of ligands are as for Fig. 13. Centrifugations were carried out for 18 hours at 30,000 rpm. Enzyme protein, 250 μg, was used.

The sedimentation behavior of the enzyme at three pH values are shown. At pH 6.5, in the absence of substrates or modifiers, the enzyme existed as a heterogeneous aggregate that is probably inactive. The molecular weight of this aggregate varied from 200,000 to 500,000 and higher because some of the enzyme was present in the cushion of 20% sucrose. A shift by 1 pH unit to 7.5 caused a dissociation of the enzyme to a nearly homogenous preparation with a molecular weight of about 110,000 ± 30,000. The existence of the two closely set peaks was consistent and also depended on the concentration of enzyme used. If the enzyme is an aggregate at pH 6.5, and it appears to be an inactive form, then the rate concentration curves for pH 6.5 in Fig. 9 should represent changes that occur during partial dissociation of the enzyme in the course of assays. This implies that substrate ligands can induce a dissociation of the aggregated enzyme.

In the next series of experiments, the influence of substrates and effectors on the dissociation-aggregation equilibrium was studied. At pH 6.5 the enzyme exists as an aggregate. If any ligand is capable of causing dissociation, we should be able to observe this when the ligand is included in the sucrose density gradients at this pH. The results of Figs. 13 and 14 summarize the findings. The centrifugations were performed at pH 6.5 in the presence of different ligands and in various combinations. Only citrate (activator), Mn²⁺, Mg²⁺ (weakly effectively), and the substrate isocitrate could cause dissociation of the enzyme into monomers. Both NAD⁺ and AMP (an activator) failed to antagonize the association forces at pH 6.5. Even in the presence of Mn²⁺, NAD⁺ was ineffective. Apparently, NAD⁺ at high concentrations enhanced aggregation. This interpretation has been confirmed because low (0.33 to 0.67 mM) NAD⁺ concentrations in the gradients did not cause significant aggregation. This plot is not recorded here.

The effectiveness of the various ligands can be seen in the magnitude of enzyme activity recovered. The larger the number of different ligands that are interacting, the more homogeneous the enzyme became. A reasonable schematization would be, at low pH levels,

\[
\begin{align*}
\text{Low [NAD⁺]} & \\
\text{Citrate} & \\
\text{Isocitrate} & \\
\text{Mn²⁺ or Mg²⁺} & \\
\text{OH⁻} & \\
\text{Polymer \textit{vs} monomer} & \\
\text{High [NAD⁺]} & \\
\text{H⁺} &
\end{align*}
\]

Results not recorded here indicate that the molecular weight of the enzyme at pH 7.5 differs slightly from that at 9.0. The enzyme sedimenting at pH 7.5 is consistently heavier than that sedimenting at pH 8.5 to 9.0. The latter is fairly homogeneous, and the monomeric molecular weight that we have established in
these studies is a representation of this fraction. All of the ligands that dissociate the polymer into monomer convert the monomeric form into the pH 8.5 fraction, particularly when multiple ligands are present. The equilibrium equation presented above is therefore a representation of the extreme configurations that are induced by the diverse ligands.

**DISCUSSION**

Before we deal with the probable involvement of isocitric dehydrogenase in the respiratory and germination processes of this organism, it is necessary to show that the kinetic mechanism falls short of providing the information about the molecular state and consequent regulation of the isocitric dehydrogenases.

**Kinetics**—From product inhibition patterns, elucidation of a reasonable kinetic mechanism is complicated by the fact that the enzyme is, as far as we know, irreversible in vitro. Extremely high concentrations of α-ketoglutarate and HCO₃⁻ are required to elicit some measure of inhibition. The levels of products used in the assays certainly affected the ionic strength and pH of the reaction. In view of the extreme sensitivity of the enzyme to ionic strength and pH, we are reluctant to interpret the kinetic data beyond the fact that the kinetic mechanism appears to be an ordered binary-ternary reaction (see Cleland (20), for a description of this mechanism) when saturation kinetics are carried out.

Although the kinetic analyses might have led us to conclude that the substrates, NAD⁺ and isocitrate, are completely cooperative ligands, sedimentation studies refute this. It appears that the kinetic behavior of this enzyme depends on a complex of conformation changes involving association and dissociation equilibrium. The peculiar response of the enzyme to NAD⁺ at pH 6.5 (see Figs. 9 and 13) in kinetics and sedimentation behavior appears to be due to some special mode of interaction between the enzyme and this ligand. High NAD⁺ concentration, at pH 6.5, inhibits the enzyme and also enhances aggregation. Only citrate can overcome the inhibitory effects of NAD⁺ (Fig. 13). Isocitrate cannot because inhibition is still evident at high isocitrate concentrations (Fig. 9).

When the pH of the reaction system is increased from 6.5, the enzyme displays normal cooperative kinetics. A reasonable suggestion would be that NAD⁺ and isocitrate have two distinct interrelations depending on the pH. In the polymer form, they are cooperative only when NAD⁺ concentration is low, and in the monomer form they are completely cooperative at all NAD⁺ levels. It is pertinent to mention that we have now found that the homotropic cooperative responses of NAD⁺ and isocitrate differ. When Mn²⁺ is used as the cofactor in kinetic analyses, the cooperative response for NAD⁺ is negative, whereas that for isocitrate is positive.⁴ In reactions in which Mg²⁺ is used as cofactor, a similar cooperative pattern is observed but it is not as pronounced as the Mn²⁺ assays, presumably because the latter is a better cofactor. A complete report is being prepared,⁴ and attempts will be made to correlate these opposing cooperative effects to the action of NAD⁺ that is elucidated in this paper.

**Association-Dissociation Reactions**—Using the same sedimentation analysis technique, we were able to observe that the kinetic patterns obtained are influenced by association-dissociation equilibrium reactions. The presence of various ligands can pull the equilibrium in favor of monomer formation. The concentration of these ligands must affect the heterogeneous population of enzyme forms that may be present at any instant. Therefore, the nature of the rate concentration plots in the absence of activating ligands must reflect two things at least: (a) allosteric transitions involving monomeric enzyme species; (b) the dissociation of the aggregate into monomers. The rate concentration plots of Fig. 1, a and b, are therefore misleading.

Frieden (26) has formulated a useful kinetic equation which may be used to evaluate the extent to which association-dissociation equilibrium influences regulatory enzymes. This method is based on conducting kinetic analysis with varied enzyme concentrations at fixed ligand levels. The technique is simple for dimer-monomer transitions, but, when higher aggregates prevail as in these studies, it becomes very difficult to utilize that approach. The fact that the enzyme that we selected is mitochondrial and is strongly affected by pH makes it possible to study the association-dissociation effects quite simply.

**Multiple pH Optima**—Reiner (27) mentioned that multiple pH optima may be possible if more than one charge distribution in the enzyme can permit the same catalytic activity to occur. Schwimmer (28) extended this concept and derived steady state rate equations which defined three conditions under which it is possible to observe two pH optima. These are as follows: (a) the existence of two isoenzyme species with distinctly different pH optima; (b) the participation of an ampholyte inhibitor that combines with only one ionic form of the enzyme and has no effect on another; (c) the formation of two enzyme-substrate complexes that are different by at least 2 protons in their ionic forms. The latter is a special case of the concept of Reiner (27). The enzyme that we used in these studies showed no evidence, in polyacrylamide gels, of being heterogeneous. If an inhibitor is responsible for the two pH optima, then it must be endogenous to the enzyme or it might be a contaminant of reagents used. We could eliminate the possibility of the substrates acting as ampholyte inhibitors because they were used at low concentrations. A more convincing interpretation is that two enzyme-substrate complexes are formed. If that is the case, there are two ionized forms of the enzyme.

**Correlations with Glutamic Dehydrogenase**—The results reported in this communication show that protons, as ligands, can play an important regulatory role in the activity of enzymes. In our work, two enzymes that are closely linked in mitochondrial metabolism (NAD-specific glutamic and isocitric dehydrogenases) have been studied. The influence of pH on the reaction kinetics of the glutamic dehydrogenase has been well documented elsewhere (7, 29, 30). Whereas the NAD-specific isocitric dehydrogenase is activated by citrate, the glutamic dehydrogenase is inhibited unidirectionally by this metabolite, permitting the reductive amination of α-ketoglutarate to proceed but inhibiting the oxidative deamination of glutamate (30). Mn²⁺ and Mg²⁺, the cofactors of isocitric dehydrogenase, inhibit the oxidation of glutamate but strongly activate the amination of α-ketoglutarate by the glutamic dehydrogenase (29). In the presence of AMP (ADP), this unidirectional control is lost. In addition, at acidic pH levels, the glutamic dehydrogenase becomes insensitive
to a diversity of regulatory ligands (30). Both enzymes have been found to be associated with the mitochondria of the organism (31). The isocitric dehydrogenase (as reported here) is very sensitive to pH; it exists as an aggregate and is inactive at acid pH levels, but dissociates into active monomers at slightly alkaline pH values. In addition, citrate is a strong activator of acid pH levels, but dissociates into active monomers at slightly alkaline pH values. In addition, citrate is a strong activator of the enzyme, and either Mg++ or Mn++ is a cofactor for the reaction.

Biology—To appreciate the physiological implications, a brief summary on the biology of the organism is pertinent. The reader is referred to up to date reviews on this aspect (32, 33).

B. emersonii goes through a simple alternation of generations in its life cycle from a motile, actively respiring, uninucleate, unicellular zoospore which contains a single large mitochondrion to a nonmotile multinucleate, fertile single cell sporangial plant. The latter contains equivalent numbers of nuclei and mitochondria which may vary from 100 to 500 depending on the size of the organism at maturity. The life cycle is completed within 12 to 20 hours, depending on the growth condition. At the end of the life cycle, between 100 and 500 new zoospores are released. Since it is known that these zoospores do not synthesize any amino acids, glutamate must be a required nutrient for growth in a defined medium composed of mineral salts, glucose, thiamine, and methionine. It is not necessary for germination.5

Mitochondrial Control—Chance (34) has shown that mitochondria are capable of a change of 1 pH unit across the mitochondrial membrane. During the translocation of Ca++ (and Mn++ by a similar mechanism (35)), the internal pH rises as the external pH drops. For the isocitric dehydrogenase in Bistocladiella, this would amount to a unit change in pH, from 6.5 to 7.5. This pH change can cause dissociation of the aggregated enzyme into active monomers. The kinetic and sedimentation studies had shown that only citrate is capable of dissociating the enzyme when NAD+ concentration is high at pH 6.5. It is well agreed that a consequence of the rise of intramitochondrial pH is ATP hydrolysis, hence continued operation of the citric acid cycle (see Lehninger (35), Mitchell (36), Pullman and Schartz (37) for a comprehensive coverage). It is possible then that the actively respiring motile zoospore has a highly energized mitochondrion which is triggered into active biosynthetic processes by cations and protons. This theory is not unrealistic since it is known that these zoospores do not synthesize any macromolecule until they germinate (38) and Lehner (39), at which time biosynthesis is rapid and unhindered until just prior to sporulation.

The regulatory mechanisms that we have elicited for these two enzymes certainly support this interpretation. The glutamic dehydrogenase which is easily reversible and connects amino acid metabolism with organic acid metabolism has a unique regulatory mechanism whereby it is unidirectionally inhibited and activated. Most of the inhibitors are also activators of the reductive reaction of the same glutamic dehydrogenase; others have no noticeable effect on it. A regulatory mechanism, whereby the two enzymes act in concert, therefore, becomes extremely plausible.

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