Role of Metal Cofactors in Enzyme Regulation. Differences in the Regulatory Properties of the *Neurospora crassa* Nicotinamide Adenine Dinucleotide Specific Isocitrate Dehydrogenase Depending on Whether Mg^{2+} or Mn^{2+} Serves as Divalent Cation†

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ABSTRACT: A number of differences in the kinetic properties of the *Neurospora crassa* nicotinamide adenine dinucleotide (NAD^+)-dependent isocitrate dehydrogenase have been found depending upon whether Mg^{2+} or Mn^{2+} served to fulfill the divalent cation requirement. With Mg^{2+} as cation, the velocity–NAD^+ saturation curves in the absence of effectors are complex at pH 7.6, indicating apparent positive and negative cooperativity. If Mn^{2+} served as the cation, however, the velocity–NAD^+ curves indicate a simple hyperbolic response. In the presence of saturating, but noninhibitory, concentrations of the activator citrate, the apparent K_m for NAD^+ is decreased, but the shapes of the velocity–NAD^+ curves are unaltered. In the presence of the activator adenosine monophosphate (AMP), negative cooperativity is observed in the presence of either Mg^{2+} or Mn^{2+}. The velocity–isocitrate curves indicate simple positive cooperativity in the presence of Mg^{2+} or Mn^{2+}, but the affinity for isocitrate is sevenfold greater in the presence of Mn^{2+}. Regardless of whether Mg^{2+} or Mn^{2+} serves as cation, citrate and AMP both activate the enzyme by reducing the apparent K_m for isocitrate. At saturating concentrations of activator, the velocity–isocitrate curves remain cooperative in the presence of Mg^{2+} but become hyperbolic in the presence of Mn^{2+}. Adjusting the pH to 6.5 appears to desensitize the enzyme, resulting in apparent hyperbolic behavior in velocity–NAD^+ curves. AMP and citrate increase the affinity of the enzyme for substrates in the presence of Mg^{2+} but not in the presence of Mn^{2+}. The kinetic data is explained by a model involving sequential ligand-induced conformational changes of the enzyme, resulting in a mixture of apparent positive and negative cooperative behavior. The metal cofactors Mg^{2+} and Mn^{2+} appear to stabilize two distinct forms of the enzyme which differ in response to varying substrate and activator concentrations. The kinetic data do not support the previously proposed model for the enzyme which did not invoke subunit interaction. An alternate model involving ligand-induced association–dissociation of the enzyme cannot be eliminated by the present results.

The NAD^+ [1]-specific isocitrate dehydrogenase from *Neurospora crassa* (*threo*-D_s-isocitrate + $NAD^+ \rightarrow$ α-ketoglutarate + CO_2 + NADH, EC 1.1.1.41) is specifically activated by citrate and AMP and requires the presence of a divalent metal cation for enzyme activity (Sanwal et al., 1963, 1965; Sanwal and Stachow, 1965). Detailed initial velocity kinetic studies of the enzyme have been reported utilizing Mg^{2+} as the divalent metal cation (Sanwal et al., 1965; Sanwal and Cook, 1966). A model for the enzyme reaction has been proposed based on such initial velocity kinetic studies. In the presence of the activator AMP, the reaction mechanism was suggested to be ordered at pH 6.5, with NAD^+ binding first followed by isocitrate (Sanwal et al., 1965). The release of products then occurred in the order: CO_2, α-ketoglutarate, and NADH. It was shown that citrate activated the enzyme by binding at an allosteric site which was also capable of accommodating isocitrate. The sigmoidal plots of velocity vs. isocitrate were interpreted as resulting from a two-site sequential binding of the substrate such that no binding occurred at the active site unless one molecule of isocitrate was bound at the allosteric site (total allosterism). The mechanism of activation of the enzyme by AMP was then examined by product inhibition and initial velocity kinetic techniques (Sanwal and Cook, 1966). It was suggested that in the absence of AMP the mechanism of the reaction became random (i.e., the steps for the addition of substrate to the enzyme became partially rate limiting). These explanations of the sigmoid response were unique at the time, as no interaction between ligand binding sites, i.e., subunit interaction, was invoked. No further data have been available to support or discard this proposed mechanism due to the difficulty in obtaining homogeneous enzyme required for direct binding studies.

It had been suggested previously that both Mg^{2+} and Mn^{2+} could fulfill the divalent metal cation requirement of the enzyme (Sanwal et al., 1964), although no detailed studies of the enzyme mechanism in the presence of Mn^{2+} have been reported. Detailed initial velocity kinetic studies have revealed a number of fundamentally different kinetic behaviors between what might be called the "Mg^{2+} enzyme" and the "Mn^{2+} enzyme", according to whether Mg^{2+} or Mn^{2+} serves as divalent cation. These properties are reported in this communication and reveal that the previously proposed kinetic model is not adequate to explain the isocitrate dehydrogenase system.

Experimental Section

Materials. The NAD^+-specific isocitrate dehydrogenase used here was purified from lyophilized cells of *Neurospora crassa* strain STA-4 essentially as described by Cook and

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[1] Abbreviations used are: AMP, adenosine 5′-monophosphate; n_H, Hill coefficient or interaction coefficient; V_{max}, maximum velocity; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; NADP, NAD^+ phosphate; NADPH, reduced NADP; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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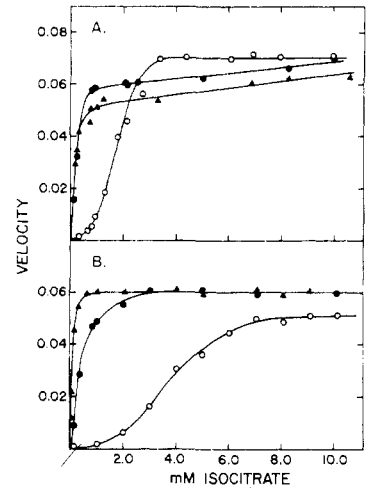

FIGURE 1: The effect of the allosteric activators AMP and citrate on initial velocity kinetic studies with varying isocitrate concentration and a fixed, saturating concentration of NAD^+ (2.0 mM) and divalent cation (3.33 mM). Conditions: 0.133 M Tris-acetate buffer, pH 7.6, containing 0.067 mM EDTA and 0.067 mM DTT, 25 °C. Velocity is expressed as the increase in absorbance at 340 nm/min: (○) no effectors, (●) saturating citrate (1.0 mM), (▲) saturating AMP (0.33 mM), (A) presence of Mn^{2+}, (B) presence of Mg^{2+}.

TABLE I: Kinetic Constants for Isocitrate Dehydrogenase.[a]

Variable substrate	Cation	Effector	K_m (mM) pH 7.6	K_m (mM) pH 6.5	n_H,[b,c] pH 7.6
Isocitrate	Mg^{2+}		3.70	0.036	2.86
		AMP	0.05	0.016	1.26
		Citrate	0.10	0.015	1.29
Isocitrate	Mn^{2+}		1.70	0.012	1.75
		AMP	0.03	0.012	0.91
		Citrate	0.03	0.014	1.09
NAD^+	Mg^{2+}		1.20	0.256	3.0/2.2
		AMP	0.42	0.051	0.87
		Citrate	0.19	0.055	2.8/1.4
NAD^+	Mn^{2+}		0.26	0.040	1.0
		AMP	0.18	0.037	0.77/1.54
		Citrate	0.16	0.040	1.1
Mg^{2+}			0.32		1.50
Mn^{2+}			0.07		1.05

[a] Kinetic constants for the variable substrate have been determined at saturating concentrations of all other fixed ligands. [b] Interaction coefficients determined from the slopes of the Hill plots. [c] An n_H value of 1.80 was observed for isocitrate at pH 6.5 with Mg^{2+} in the absence of effectors. All other interaction coefficients at pH 6.5 were approximately 1.0.

Sanwal (1969). In all experiments reported here, an enzyme preparation with a specific activity of 15–20 was used [1 unit is defined as the formation of 1 μmol of NADH min^{-1} (mg of protein)$^{-1}$]. This level of specific activity represented an approximate 400- to 500-fold purification of the enzyme over that of crude extracts. The homogeneity of the enzyme was tested by polyacrylamide gel electrophoresis following the method of Davis (1964). The enzyme was not homogeneous, exhibiting one major band (80–85% of the total protein) and five minor bands when the gels were stained with Coomassie blue or amido black 10B (50–100 μg of protein applied). A single band, corresponding to the major protein band, was observed when the gels were stained specifically for NAD^+-specific isocitrate dehydrogenase activity. The enzyme preparation was free of the following enzymes tested, which could interfere with the kinetic assay: condensing enzyme, aconitase, NADP-specific isocitrate dehydrogenase, α-ketoglutaric acid dehydrogenase complex, NADH and NADPH oxidase activity, and isocitratase. The enzyme was routinely stored in 50 mM potassium phosphate buffer (pH 6.5) containing 0.1 mM dithiothreitol (DTT) and 50 mM ammonium sulfate. Under these conditions, enzyme could be stored indefinitely at 4 °C without loss of enzyme activity. The sodium salts of NAD^+, AMP, isocitric, and citric acid were purchased from Sigma Chemical Co. The sulfate salts of Mg^{2+} and Mn^{2+} were reagent grade.

Kinetic Measurements. All reagents (except buffer) were maintained at 0–4 °C. Solutions of AMP, NAD^+, and $MnSO_4$ were prepared just before use. All kinetic measurements were performed at 24–25 °C with a Gilford recording spectrophotometer Model 2400, equipped with dual thermoplates and a Haake thermostat. The kinetic studies at pH 7.6 were conducted in 0.2 M Tris buffer, while the studies at pH 6.5 were conducted in 0.05 M imidazole buffer. Acetic acid was used to adjust the pH of all buffers. After the addition of substrates, all cuvettes were allowed to equilibrate to 24–25 °C for 10 min prior to the initiation of the reaction by the addition of enzyme. Initiation of the reaction by the addition of any substrate gave identical reaction rates. The enzyme was checked periodically for any denaturation during the experiments by using a standard reaction mixture containing 167 mM Tris-acetate buffer, 0.667 mM AMP, 0.833 mM isocitrate, 2.0 mM NAD^+, and 3.33 mM $MgSO_4$, pH 7.6, in a final volume of 3.0 mL.

Results

The results of previous kinetic studies with isocitrate dehydrogenase of *Neurospora crassa* indicate that the enzyme activity is regulated by the activator citrate at the optimum pH 7.6 but not at pH 6.5. In this communication, initial velocity kinetic studies have been conducted at the two pH values in the presence of either Mg^{2+} or Mn^{2+} as the required cofactor. Preliminary kinetic studies indicated that maximal activation of the enzyme was attained at an AMP concentration of 0.33 mM or a citrate concentration of 1.0 mM at either pH, in the presence of either metal cofactor.

Initial Velocity Studies at pH 7.6. When the velocity of the reaction is studied as a function of isocitrate concentration in the presence of saturating Mn^{2+} (3.33 mM) and saturating NAD^+ (2.0 mM) at pH 7.6, a sigmoid response is observed in the absence of added effectors (Figure 1A). In the presence of saturating concentrations of the allosteric activators citrate (1.0 mM) or AMP (0.3 mM), the affinity of the enzyme for isocitrate is increased approximately 55-fold. No increase in the V_{max} value is observed in the presence of the activators. Hill plots of the data indicate n_H values for isocitrate of 1.75 in the absence of effectors, 0.91 in the presence of AMP, and 1.05 in the presence of citrate. The kinetic constants are summarized in Table I.

When the velocity of the reaction is studied as a function of isocitrate concentration in the presence of saturating Mg^{2+} (3.33 mM) and saturating NAD^+ (2.0 mM) at pH 7.6, a sigmoid response is again observed in the absence of effectors (Figure 1B). The affinity of the enzyme for isocitrate is approximately sevenfold lower in the presence of Mg^{2+} than in the presence of Mn^{2+}. In the presence of saturating concentrations of the allosteric activators citrate (1.0 mM) or AMP (0.33 mM), the affinity of the enzyme for isocitrate is increased approximately 40- and 80-fold, respectively. In the presence of either activator, the V_{max} is increased approximately 20%, in contrast to the results obtained in the presence of Mn^{2+}. Hill plots of the data indicated n_H values for isocitrate of 2.86 in the absence of effectors, 1.29 in the presence of citrate, and

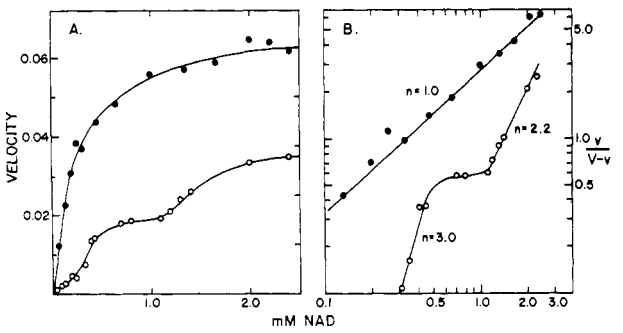

FIGURE 2: Initial velocity kinetic studies with varying NAD^+ concentration and a fixed, saturating concentration of isocitrate (10 mM) and divalent cation (3.33 mM). Conditions: 0.133 M Tris-acetate buffer, pH 7.6, containing 0.067 mM EDTA and 0.067 mM DTT, 25 °C. Velocity is expressed as the increase in absorbance at 340 nm/min: (○) saturating Mg^{2+}, (●) saturating Mn^{2+}, (A) saturation curves, (B) Hill plots.

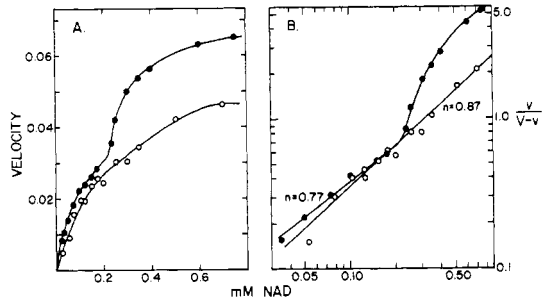

FIGURE 3: The effect of the activator AMP on initial velocity kinetic studies with varying NAD^+ concentration and a fixed, saturating concentration of isocitrate (10 mM) and divalent cation (3.33 mM). The concentration of AMP is saturating but noninhibitory at 0.33 mM. Conditions: 0.133 M Tris-acetate buffer, pH 7.6, containing 0.067 mM EDTA and 0.067 mM DTT, 25 °C; (○) saturating Mg^{2+} (3.33 mM), (●) saturating Mn^{2+} (3.33 mM), (A) saturation curves, (B) Hill plots.

1.26 in the presence of AMP. The kinetic constants obtained are summarized in Table I.

When the velocity of the reaction is studied as a function of NAD^+ concentration at saturating isocitrate (10 mM) at pH 7.6, the saturation curves obtained in the presence of Mg^{2+} and Mn^{2+} are strikingly different (Figure 2A). In the presence of Mg^{2+} (3.33 mM) a complex saturation curve is obtained containing an intermediary plateau region, while in the presence of Mn^{2+} (3.33 mM) an apparent hyperbolic response is observed. When the data were replotted in the Hill plot (Figure 2B), the n_H value for NAD^+ in the presence of Mn^{2+} was 1.0, while in the presence of Mg^{2+} a biphasic curve was obtained with maximum n_H values of 3.0 and 2.2.

In the presence of the activator AMP (0.33 mM), the saturation curves for NAD^+ are significantly altered (Figure 3A). In the presence of Mg^{2+}, the saturation curve fails to reach a maximal velocity, even at high NAD^+ concentrations (5.0 mM), indicative of simple negative cooperativity. In the presence of Mn^{2+}, however, a biphasic curve is now observed in contrast to the simple hyperbolic curve observed in the absence of effectors. When the data are presented in the form of the Hill plot (Figure 3B), weak negative cooperative-like behavior is observed for NAD^+ in the presence of Mg^{2+} (n_H = 0.87), while a biphasic curve for NAD^+ in the presence of Mn^{2+} is observed, with maximum n_H values of 0.77 and 1.5.

In the presence of the activator citrate (1.0 mM), the saturation curves for NAD^+ (Figure 4A) are similar to those obtained in the absence of effectors (see Figure 2A). A complex

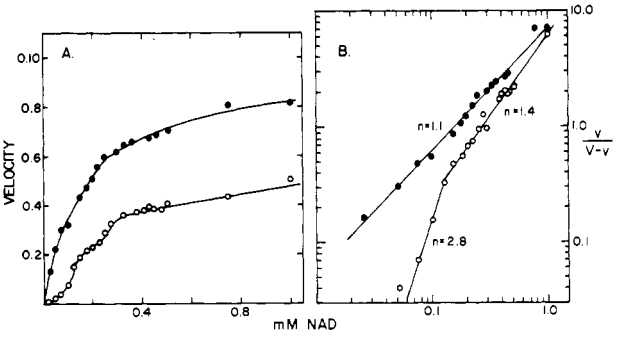

FIGURE 4: The effect of the activator citrate on initial velocity kinetic studies with varying NAD^+ concentration and a fixed, saturating concentration of isocitrate (10 mM) and cation (3.33 mM). The concentration of citrate is saturating but noninhibitory at 1.0 mM. Conditions: 0.133 M Tris-acetate buffer, pH 7.6, containing 0.067 mM EDTA and 0.067 mM DTT, 25°C; (○) saturating Mg^{2+} (3.33 mM), (●) saturating Mn^{2+} (3.33 mM), (A) saturation curves, (B) Hill plots.

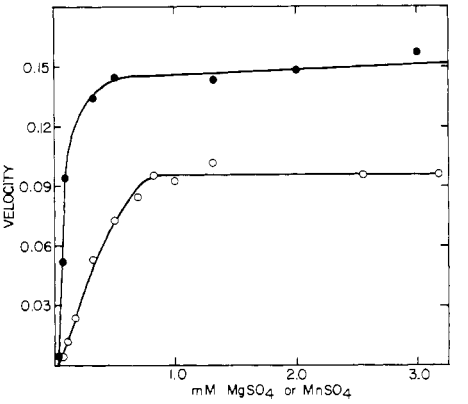

FIGURE 5: Initial velocity kinetic studies with varying cation concentration at a fixed, saturating concentration of isocitrate (10 mM) and NAD^+ (2 mM). Conditions: 0.133 M Tris-acetate buffer, pH 7.6, containing 0.067 mM EDTA and 0.067 mM DTT, 25 °C. Velocity is expressed as the increase in absorbance at 340 nm/min: (○) Mg^{2+} concentration, (●) Mn^{2+} concentration.

NAD^+ saturation curve is again observed in the presence of Mg^{2+} but an apparent hyperbolic curve in the presence of Mn^{2+}. When the data are presented in the form of the Hill plot (Figure 4B), little cooperative behavior is observed for NAD^+ in the presence of Mn^{2+} (n_H = 1.1), but a biphasic curve is observed in the presence of Mg^{2+} with n_H values of 2.8 and 1.4.

When the velocity of the reaction is studied as a function of total cation concentration at saturating isocitrate (10 mM) and NAD^+ (2.0 mM), it is obvious that Mn^{2+} is the preferred cofactor in vitro. The affinity of the enzyme for Mn^{2+} is four- to fivefold greater than for Mg^{2+} and the maximum velocity is greater in the presence of Mn^{2+} (Figure 5). When the data are presented in the form of the Hill plot, positive cooperativity is observed with Mg^{2+} (n_H = 1.5) but no cooperativity with Mn^{2+} (n_H = 1.05). The resultant kinetic constants obtained at pH 7.6 are summarized in Table I.

Initial Velocity Studies at pH 6.5. When the velocity of the reaction is studied at pH 6.5 as a function of isocitrate concentration in the presence of saturating NAD^+ (2.0 mM) and Mg^{2+} (3.33 mM), sigmoid behavior is observed (Figure 6). In contrast, in the presence of saturating Mn^{2+}, a hyperbolic response with isocitrate is observed. Both the affinity of the enzyme for isocitrate and the V_{max} are doubled when Mn^{2+} is substituted for Mg^{2+}. In the presence of saturating concentrations of AMP or citrate, the velocity–isocitrate curve

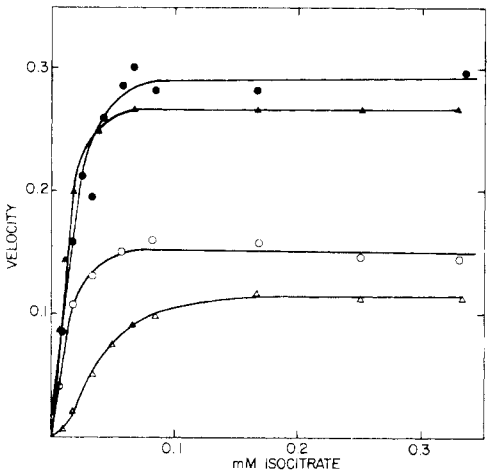

FIGURE 6: The effect of the activator citrate (or AMP) on initial velocity kinetic studies at pH 6.5 with varying isocitrate concentration and a fixed, saturating concentration of NAD^+ (2 mM) and cation (3.33 mM). The concentration of citrate is saturating but noninhibitory at 1.0 mM. In the presence of saturating AMP (0.33 mM), the saturation curves for isocitrate are identical to those obtained in the presence of citrate. Conditions: 33 mM imidazole buffer, pH 6.5, containing 0.067 mM EDTA and 0.067 mM DTT, 25 °C; (△) presence of Mg^{2+}, no effectors; (○) presence of Mg^{2+} and 1.0 mM citrate; (▲) presence of Mn^{2+}, no effectors; (●) presence of Mn^{2+} and 1.0 mM citrate.

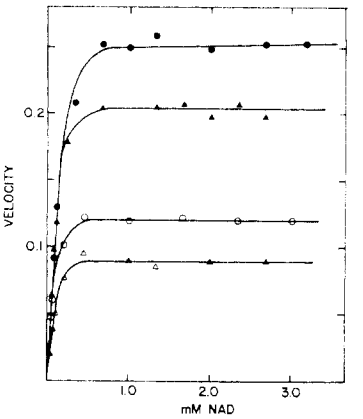

FIGURE 7: The effect of the activator AMP (or citrate) on initial velocity kinetic studies at pH 6.5 with varying NAD^+ concentration and a fixed, saturating concentration of isocitrate (10 mM) and cation (3.33 mM). The concentration of AMP is saturating but noninhibitory at 0.33 mM. In the presence of saturating citrate (1.0 mM), the saturation curves for NAD^+ are identical with those obtained in the presence of AMP. Conditions: 33 mM imidazole-acetate buffer, pH 6.5, containing 0.067 mM EDTA and 0.067 mM DTT, 25 °C; (△) presence of Mg^{2+}, no effectors; (○) presence of Mg^{2+} and 0.33 mM AMP; (▲) presence of Mn^{2+}, no effectors; (●) presence of Mn^{2+} and 0.33 mM AMP.

becomes hyperbolic in the presence of Mg^{2+} (Figure 6) and the affinity of the enzyme for isocitrate is approximately doubled. A slight increase in V_{max} is observed in the presence of either activator. In the presence of Mn^{2+}, AMP and citrate have no effect on the K_m for isocitrate but slightly increase the V_{max}. The resultant Hill plots of the data of Figure 6 indicated an n_H value of 1.8 for isocitrate in the presence of Mg^{2+} in the absence of allosteric effectors but n_H values of approximately 1 for all other cases.

When the velocity of the reaction is studied at pH 6.5 as a function of NAD^+ concentration in the presence of saturating isocitrate (10 mM), normal hyperbolic responses are obtained under all conditions (Figure 7). The affinity of the enzyme for NAD^+ is approximately sixfold greater in the presence of Mn^{2+} than in the presence of Mg^{2+} in the absence of added effectors. In the presence of saturating AMP or citrate, the affinity of the enzyme for NAD^+ is increased approximately fivefold in the presence of Mg^{2+}. In the presence of Mn^{2+}, however, AMP and citrate have no effect on the affinity of the enzyme for NAD^+. An increase in the maximum velocity is observed in the presence of citrate or AMP whether Mg^{2+} or Mn^{2+} is used as cofactor.

In Figures 6 and 7, the saturation curves in the presence of AMP or citrate are essentially identical and therefore the experimental data for only one effector has been presented. The resultant kinetic constants at pH 6.5 for all cases are summarized in Table I.

Discussion

It has been realized for some time that the NAD^+-dependent isocitrate dehydrogenase from a variety of sources bears at least three types of site: a substrate site specific for *threo*-D_S-isocitrate, NAD^+, and a metal cofactor and two regulatory sites, one specific for isocitrate, citrate, and structurally related molecules and a second specific for adenine nucleotides (Sanwal and Stachow, 1965; Atkinson et al., 1965; Coultate and Dennis, 1969). In general, in the absence of allosteric effectors, initial velocity kinetic studies have revealed positive cooperative behavior between isocitrate binding sites but no cooperativity between NAD^+ binding sites. The interpretation of the cooperative response has been complicated by the fact that isocitrate is capable of binding to both the active and allosteric sites of the enzyme, resulting in models for the enzyme involving no subunit interaction. The interpretation is further complicated by the ability of isocitrate (and several allosteric ligands) to bind the essential metal cofactor, resulting in several possible chelated and unchelated forms of the substrate. The role of the metal cofactor in the enzyme mechanism has been examined in detail with the enzyme from pig heart (Cohen and Colman, 1972, 1974) and from pea epicotyls (Duggleby and Dennis, 1970). The cooperative response of isocitrate observed with the pig heart enzyme has been explained in terms of a random kinetic mechanism involving binding of unchelated isocitrate to an enzyme-Mn^{2+} complex or the binding of a Mn^{2+}-isocitrate complex directly to the enzyme (Cohen and Colman, 1974). In comparison, the cooperativity observed in the pea enzyme has been explained by the binding of unchelated isocitrate to the allosteric site but the binding of a Mg^{2+}-isocitrate complex to the active site of the enzyme. Neither of these explanations involved an interaction between the active sites on neighboring subunits, i.e., subunit interaction.

In the present study, we have compared the effect of substituting Mn^{2+} for Mg^{2+} on the catalytic and regulatory properties of the NAD^+-dependent isocitrate dehydrogenase of *Neurospora crassa*. The most striking feature of the kinetic curves is the differences observed in the velocity-NAD^+ curves in the presence of the two cations presented in Figures 2-4. The simplest explanation of these results is that Mn^{2+} (or a Mn^{2+}-isocitrate complex) induces a change in the conformation or structure of the enzyme significantly different from that induced in the presence of Mg^{2+}. No cooperativity between NAD^+-binding sites is observed in the absence of allosteric effectors or in the presence of citrate (Figures 2 and 4), indicating that the binding sites for NAD^+ are most likely equivalent and independent in the "Mn^{2+} enzyme". In contrast, the NAD^+ saturation curves observed in the presence of Mg^{2+} are complex and can be explained on the basis of a model involving NAD^+-induced sequential changes in conformation (Koshland et al., 1966). The shape of the Hill plots

presented in Figures 2B and 4B can be interpreted as original positive cooperativity between NAD^+-binding sites, followed by negative cooperativity and finally positive cooperativity (Cornish-Bowden and Koshland, 1975). A model for the isocitrate dehydrogenase system involving ligand-induced sequential changes in enzyme conformation is further supported by the occurrence of apparent negative cooperative behavior between NAD^+-binding sites (Figure 3) and isocitrate-binding sites (with Mn^{2+}, Figure 1A) in the presence of the activator AMP. This conclusion is based on the interaction coefficients of less than unity and the biphasic Hill plots obtained with NAD^+. The interaction coefficient greater than one observed for the second "step" of the biphasic response (see Figures 2–4) is typical of negative cooperative interactions as previously reported in kinetic studies with yeast glyceraldehyde-3-phosphate dehydrogenase (Rock and Cook, 1974) and human erythrocyte phosphofructokinase (Lee et al., 1973). The complex NAD^+ saturation curves support a subunit interaction model for isocitrate dehydrogenase, indicating that the kinetic model proposed previously for this enzyme is most likely incorrect (Sanwal et al., 1965; Sanwal and Cook, 1966).

In contrast to the results obtained with NAD^+, the substitution of Mn^{2+} for Mg^{2+} has little effect on the velocity–isocitrate response except to lower the degree of cooperativity between isocitrate binding sites (Figure 1). The interpretation of the isocitrate saturation curves is complicated, as previously discussed, by the ability of isocitrate to chelate the metal cofactor and to bind to the regulatory site. We have routinely analyzed the data in terms of the different species of ligand both with regard to ionic form (at pH 7.6 and 6.5) and the various free and metal-chelated forms of ligand following the procedure of Cohen and Colman (1972). It is obvious from such analyses that NAD^+ and AMP, at the concentrations used in the present study, do not bind an appreciable quantity of metal cofactor. Thus, the data obtained with varying NAD^+ concentrations provides the strongest evidence that the differences in the kinetic curves obtained with Mn^{2+} and Mg^{2+} are not due to differential chelating effects of the various ligands. An explanation of the data solely on the basis of chelating effects is further ruled out by the observation that the addition of citrate or lowering the pH to 6.5, increases the affinity of the enzyme for isocitrate *and* NAD^+ in the presence of Mg^{2+}. These two factors, which alter the distribution of the various chelated and unchelated forms of isocitrate, have been shown to have no effect on the affinity of the enzyme for NAD^+ in the enzyme from other sources (Cohen and Colman, 1972, 1974; Duggleby and Dennis, 1970). We are thus left with the conclusion that Mg^{2+} and Mn^{2+} must have a direct effect on the structure of the enzyme.

Although the present experiments were not designed to determine the mode of binding of the metal cofactor, analysis of the results presented in Figures 1 and 5 would indicate two possible mechanisms for metal binding. The results of Figure 5 are consistent with the conclusion that interaction occurs between substrate binding sites in the presence of Mg^{2+} but not in the presence of Mn^{2+}. Analysis of the data indicates that the Me^{2+}–isocitrate^{3-} complex curves are superimposable on the curves presented in terms of total Me^{2+} concentration, suggesting that the Me^{2+}–isocitrate^{3-} complex may be the actual form by which the metal binds. Plotting the results in terms of *free* metal concentration again yields a hyperbolic response with Mn^{2+} ($K_m = 6\ \mu M$) and a cooperative response with Mg^{2+} ($K_m = 63\ \mu M$). In comparison, similar analysis of the data in Figure 1 indicates that the velocity–concentration curves for Mn^{2+}–isocitrate^{3-}, Mn^{2+}–isocitrate^{2-}, isocitrate^{3-}, and H–isocitrate^{2-} all remain sigmoid. This result indicates that the enzyme may be capable of binding either Mn^{2+} directly followed by isocitrate^{3-} or the Mn^{2+}–isocitrate^{3-} complex, depending upon total Mn^{2+} concentration. A similar conclusion is drawn from the data with Mg^{2+} as the cofactor. These results are in qualitative agreement with the results of Cohen and Colman (1974).

It is obvious that the two activators citrate and AMP cause different structural changes in the enzyme. Thus, citrate has little effect on the NAD^+ saturation curves but does eliminate the positive cooperativity observed in the isocitrate saturation curves. The action of citrate can be explained by assuming that citrate occupies the allosteric site of the enzyme, resulting in isocitrate binding only to the active sites. In contrast, AMP results in increased negative cooperative interaction between substrate binding sites, indicating a more gross structural alteration in the enzyme. Lowering the pH to 6.5 must also cause a structural change in the enzyme, eliminating the complex interactions observed at pH 7.6. The activators AMP and citrate have essentially no effect on the affinity of the enzyme for NAD^+ and isocitrate in the presence of Mn^{2+}, indicating again that the Mn^{2+} enzyme is structurally distinct from the Mg^{2+} enzyme.

In view of the lack of definitive data on the structure of the enzyme, a model involving ligand-induced association–dissociation cannot be eliminated by the present results. Thus, Mg^{2+} and Mn^{2+} may stabilize different molecular weight forms of the enzyme with different enzymatic properties. Similarly, the substrates and effectors may induce changes in the size of the enzyme. Detailed molecular weight studies in the presence of various ligands are required to substantiate or eliminate an association–dissociation model for the enzyme. Such studies are presently under investigation.

It is obvious from the results presented in this study that the regulatory properties of isocitrate dehydrogenase are significantly altered by the choice of metal cofactor. Several enzymes have been shown to have different regulatory properties in the presence of Mg^{2+} and Mn^{2+}, including ADP-glucose pyrophosphorylase (Gentner and Preiss, 1968), several gluconogenic enzymes (Wimhurst and Manchester, 1970), and pyruvate kinase (Gabrielli and Baldi, 1973). The conclusion can be drawn from such studies that the role of metal ions in enzyme reactions is not simply as a necessary cofactor but rather as a distinct regulatory entity. It is difficult to assess the physiological significance of the observed effect. We have estimated the internal concentrations of Mg^{2+} and Mn^{2+} by atomic absorption spectrophotometry with the tentative conclusion that the maximum concentration of Mg^{2+} and Mn^{2+} in the soluble fraction of the cell is approximately 10 and 0.1 mM, respectively. These concentrations are tenuous due to our lack of knowledge regarding local concentration effects, the extent of binding of metal ions by internal metabolites, and the known variability of the internal volume of *Neurospora crassa* with growth conditions. The results are in good agreement with the concentrations of Mn^{2+} (0.1–0.15 mM) and Mg^{2+} (5–10 mM) estimated in liver (Gabrielli and Baldi, 1973; Long, 1967; Wimhurst and Manchester, 1970).

In conclusion, our results would indicate that the activity of isocitrate dehydrogenase is much more sensitive to regulation by citrate and AMP when Mg^{2+} serves as the required cation. If Mn^{2+} replaces Mg^{2+} as the required cation, the enzyme has a greater affinity for substrates and is partially desensitized to the effect of the allosteric activators. Since both cations may be available to the enzyme in vivo, the enzyme may be distributed normally between the Mn^{2+} enzyme and Mg^{2+} enzyme forms. Since these two forms of enzyme vary in regulatory properties, control of the Mg^{2+}/Mn^{2+} ratio of the cell

may be an important mechanism of regulation of the Krebs cycle in *Neurospora crassa*.

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Cyclic Adenosine 3′,5′-Monophosphate Dependent Protein Kinase of Rat Leydig Cells: Physical Characteristics of Two Holoenzymes and Their Subunits†

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ABSTRACT: The interstitial cells of the rat testis contain two forms of cAMP-dependent protein kinase which are activated in vitro by low concentrations (10^{-11} M) of human chorionic gonadotropin. The two cAMP-dependent holoenzymes of purified Leydig cells have been further characterized by cAMP binding and phosphokinase assay during ion-exchange chromatography, gel filtration, and sucrose density gradient centrifugation. Equilibrium binding studies performed with interstitial cell extracts and [^{3}H]cAMP revealed a single order of binding sites with high affinity for cAMP ($K_a = 10^9$ M^{-1}). After chromatography on DEAE-Sephadex and DEAE-cellulose, the two holoenzymes were identified by density gradient centrifugation as discrete peaks with sedimentation constants of 4.0 S and 6.2 S. From these values and the Stokes radii of 37.9 Å and 47.7 Å derived from gel filtration on Sephadex G-200, the estimated molecular weights of the holoenzymes were 59 600 and 116 400, respectively. The regulatory subunits of the protein kinase holoenzymes were also identified by cAMP binding analysis after gel filtration and sucrose density centrifugation, as 3.0S and 4.2S components with estimated molecular weights of 35 500 and 66 300. The molecular weight of the 2.9S catalytic subunit was estimated to be 33 000. The contributions of the 6.2S and 4.0S forms of the holoenzyme to the total cAMP-dependent protein kinase activity in Leydig cell extracts were approximately 70% and 30%, respectively. The main major 6.2S holoenzyme was eluted by 120 mM NaCl during chromatography on DEAE-cellulose at pH 7.4 and exhibited properties comparable with those of the type I protein kinase present in other tissues. However, the 4.0S holoenzyme eluted by 220 mM NaCl did not correspond to the type II holoenzyme of other tissues and appeared to be derived from the 6.2S holoenzyme. Selective or sequential activation of the individual forms of protein kinase during hormone stimulation could provide a mechanism for the expression of discrete biological responses in the Leydig cell.

Activation of cAMP[1]-dependent protein kinase by gonadotropic hormones has been recently demonstrated in collagenase-dispersed interstitial cells of the rat testis (Podesta et al., 1976a,b). Enzyme activity was stimulated by incubation of the isolated cells with low concentrations of luteinizing hormone (LH) and human chorionic gonadotropin (hCG). The extent of enzyme activation was correlated with the production of cAMP by the hormone-stimulated cells (Podesta et al., 1976a,b; Cooke & van der Kemp, 1976). The interstitial cell has been shown to contain two forms of cAMP-dependent protein phosphokinase, with sedimentation constants of about 6.5 S and 3.8 S. During incubation of isolated interstitial cells with trophic hormones, total protein kinase activity was stimulated in a dose-dependent manner, leading to the formation of a single 2.9S catalytic subunit. Conversion of the

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[1] Abbreviations used: cAMP, cyclic adenosine 3′,5′-monophosphate; LH, luteinizing hormone; hCG, human chorionic gonadotropin; BSA, bovine serum albumin.