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Nicotinamide Adenine Dinucleotide-specific Isocitrate Dehydrogenase from a Higher Plant

THE REQUIREMENT FOR FREE AND METAL-COMPLEXED ISOCITRATE*

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RONALD G. DUGGLEBY‡ AND DAVID T. DENNIS
From the Department of Biology, Queen's University, Kingston, Ontario, Canada

SUMMARY

The stability constants of the magnesium complexes of citrate, isocitrate, and NAD^+ were measured and found to be 10.1×10^3 M^{-1}, 1.35×10^3 M^{-1}, and 0.106×10^3 M^{-1}, respectively. These data were used to calculate the concentrations of free isocitrate, free magnesium, and magnesium isocitrate in mixtures of magnesium, NAD^+, and isocitrate. The effect of these variables on the NAD^+-specific isocitrate dehydrogenase from peas (*Pisum sativum* L.) was investigated and it was concluded that (*a*) the enzyme has a substrate site specific for the magnesium isocitrate complex, (*b*) the enzyme has an activator site specific for free isocitrate, and (*c*) it is probable that the enzyme has no requirement for free magnesium.

NAD^+-specific isocitrate dehydrogenase (*threo*-D_s-isocitrate: NAD^+ oxidoreductase (decarboxylating) EC 1.1.1.41) has an absolute requirement for divalent metal ions (1–7). The effect of metal ion concentration has been described but little attention has been paid to the fact that both isocitrate and NAD^+ form complexes with metal ions, and it has not been established whether free or metal-complexed isocitrate is the substrate. Citrate, like isocitrate, activates the enzyme from plant sources (8, 9), and also forms metal complexes. It has not been shown whether the activating molecule is free or metal complexed. Cennamo, Montecuccoli, and Bonaretti (4) suggested that the magnesium isocitrate complex is not the substrate, but they ignored the activation of the enzyme by isocitrate which Atkinson, Hathaway, and Smith (10) suggested is dependent on free isocitrate. These latter workers took account of complexing effects but assumed that the stability constant for the magnesium isocitrate complex is the same as that for the magnesium citrate complex, an assumption which also seems to have been made by Plaut and Aogaichi (5). Blair (11) estimated the stability constant for the magnesium isocitrate and citrate complexes and showed that citrate forms a much more stable complex than isocitrate. The stability constants are also very dependent on ionic strength.

In this study, the same conditions were used for the determination of the stability constants of the magnesium complexes of isocitrate, citrate, and NAD^+ as were used for the assay of the enzyme. Variations due to factors such as ionic strength were, therefore, avoided.

These data were used to calculate the concentration of free isocitrate, free magnesium, and magnesium isocitrate in enzyme assays. The results indicate that magnesium isocitrate is the substrate of the enzyme, and free isocitrate (or related tricarboxylic acids) activates the enzyme. There is no requirement for free magnesium.

MATERIALS AND METHODS

Enzyme Preparation—An acetone powder from a mitochondrial fraction was prepared from etiolated pea (*Pisum sativum* L.) epicotyls by the method of Coultate and Dennis (6, 9) except for the omission of sand during homogenization. The acetone powder, at a concentration of 8 mg per ml, was suspended in 50 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonic acid-NaOH buffer (pH 7.4) in 6 M methanol[1] and centrifuged at $3000 \times g$ for 15 min at 0°. The supernatant was stable at −20° for several weeks.

Assay—The enzyme was assayed as described by Coultate and Dennis (6) except that 25 mM buffer was used, unless otherwise stated. NAD^+ was used at a saturating concentration of 1 mM and the concentrations of the other components are indicated in the text and figures. Isocitrate concentrations refer to the *threo*-D_s,L_s-isocitrate isomers (12). Biochemicals were purchased from the Sigma Chemical Company.

Determination of Stability Constants—Free magnesium concentration was measured at 25° in known mixtures of total ligand and total magnesium using a divalent cation electrode (Orion Research, Inc., Cambridge, Massachusetts), in conjunction with a Metrohm calomel reference electrode with an Orion digital pH meter. The stability constants were calculated from the equation

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[1] This concentration of methanol stabilizes the enzyme to a considerable extent. In the absence of any stabilizer the enzyme is cold labile (9). The enzyme can also be stabilized with 5 M glycerol (8), but this introduces problems of viscosity at low temperatures.

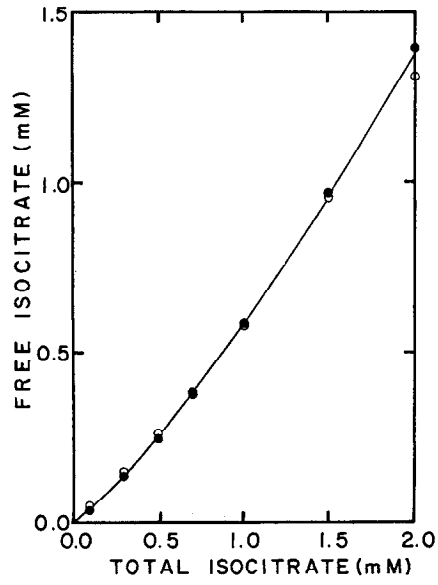

FIG. 1. Relationship between free isocitrate and total isocitrate in a typical assay mixture containing 1 mM magnesium sulfate and 1 mM NAD^+ in 25 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonate buffer (pH 7.4). The *line* represents the theoretical relationship calculated by using the stability constants of magnesium isocitrate and magnesium NAD^+ given in the text and solving between Equations 2, 3, 4, 5, and 6. The points were obtained by measuring free magnesium in the appropriate mixtures of total NAD^+, isocitrate, and magnesium and calculating free isocitrate using Equations 2 and 4 (using the determined value of K_{si}) (●) or Equations 3, 4, 5, and 6 (using the determined value of K_{sn}) (○).

$$K_{sl} = \frac{[M_t] - [M_f]}{[M_f]([L_t] - [M_t] + [M_f])} \quad (1)$$

where K_{sl} is the stability constant of the magnesium ligand complex, L_t and M_t are the total ligand and magnesium concentrations, respectively, and M_f is the free magnesium concentration.

The buffer of choice, which was compatible with the enzyme assay and the magnesium determinations, was 25 mM *N*-tris-(hydroxymethyl)methyl-2-ethane sulfonic acid (13) (pH 7.4) (ionic strength 0.015) for the following reasons. (*a*) It was at an adequate concentration to maintain pH; (*b*) it contained a low concentration of monovalent ions which interfere with the magnesium determinations at low magnesium ion concentrations; (*c*) it was nonchelating; (*d*) it was free of anions which interfere with the assay of the enzyme (6, 8). The response of the electrode did not deviate markedly from the predictions of the Nernst equation over the range 4×10^{-5} to 1×10^{-3} M magnesium, all measurements being taken in this range.

Calculation of Free Isocitrate, Free Magnesium, and Magnesium Isocitrate in Assay Mixture—The assays contained two ligands, isocitrate and NAD^+, and it was necessary to take account of both of these when calculating the concentrations of free isocitrate, free magnesium, and magnesium isocitrate. This was done using the following relationships.

$$K_{si} = \frac{[I_m]}{[M_f][I_f]} \quad (2)$$

$$K_{sn} = \frac{[N_m]}{[M_f][N_f]} \quad (3)$$

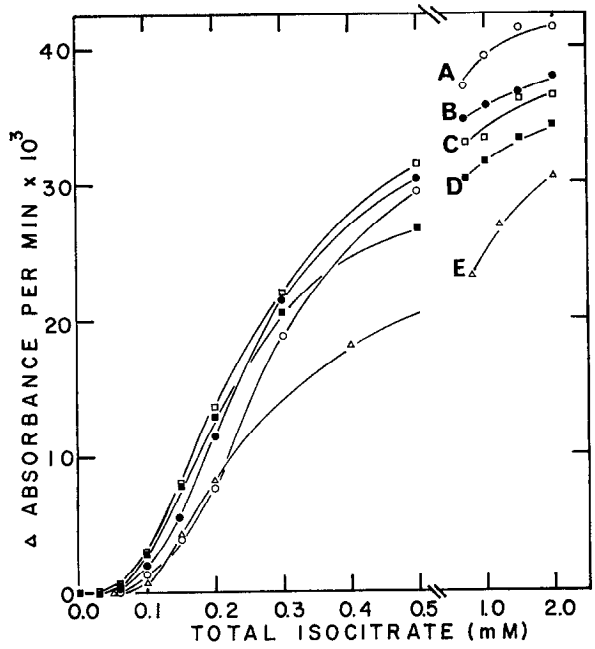

FIG. 2. The effect of isocitrate concentration on the activity of NAD^+-specific isocitrate dehydrogenase at various concentrations of magnesium. The assays contained 25 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonate buffer (pH 7.4), 1 mM NAD^+, and 0.1 mM (△), 0.5 mM (■), 1.0 mM (□), 2.0 mM (●), or 5.0 mM (○) magnesium sulfate.

$$[I_t] = [I_f] + [I_m] \quad (4)$$

$$[N_t] = [N_f] + [N_m] \quad (5)$$

$$[M_t] = [M_f] + [I_m] + [N_m] \quad (6)$$

where K_{si} and K_{sn} are the stability constants of the magnesium complexes of isocitrate and NAD^+, respectively.

I, *M*, and *N* refer to isocitrate, magnesium, and NAD^+, respectively, and subscripts *m*, *f*, and *t* refer to the magnesium complex, free ligand, and total, respectively.

RESULTS

Stability Constants of Magnesium Complexes of Citrate, Isocitrate, and NAD+—These were determined as described under "Materials and Methods," in each case making separate determinations of free magnesium in mixtures of 1 mM ligand plus 0.4 mM, 0.6 mM, 0.8 mM, and 1.0 mM magnesium. The stability constants were then calculated from Equation 1 and the four determinations expressed as a mean ± standard error. The values obtained were $10.1 \pm 0.42 \times 10^3$ M^{-1}, $1.35 \pm 0.066 \times 10^3$ M^{-1}, and $0.106 \pm 0.010 \times 10^3$ M^{-1} for the magnesium complexes of citrate, isocitrate, and NAD^+, respectively.

These values for citrate and isocitrate are considerably higher than those reported by Blair (11), but extrapolation of his values to an ionic strength of 0.015 shows that the results are in agreement. The difference in the stability constants for citrate and isocitrate is also very similar to that reported by Blair. A value for the stability constant of magnesium NAD^+ does not appear to have been published before and is much lower than that for isocitrate or citrate, indicating that NAD^+ is in a predominantly noncomplexed form in the assay. The relationship between free isocitrate and total isocitrate in the presence of 1 mM total NAD^+ and 1 mM total magnesium is shown in Fig. 1.

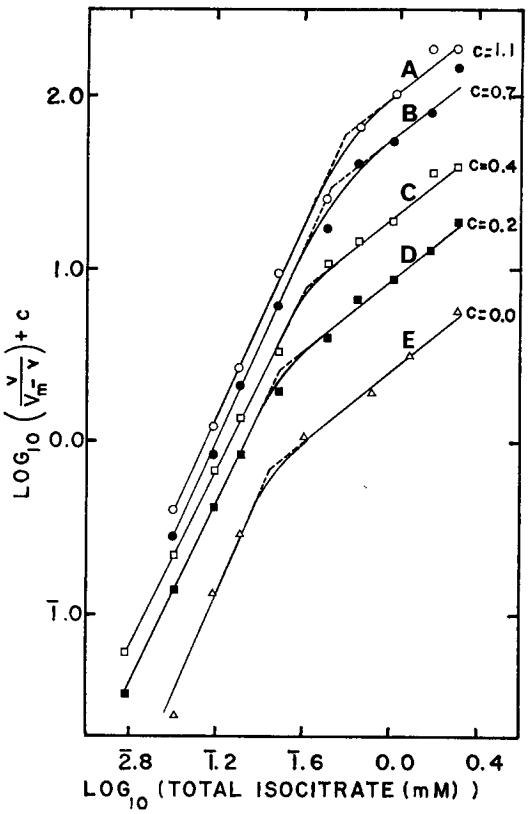

FIG. 3. The data of Fig. 2 replotted according to the Hill equation (10). Each curve was displaced vertically by a value, c, for the sake of clarity. The assay conditions are as described in Fig. 2.

TABLE I

Effect of magnesium concentration on slope of Hill plots with respect to isocitrate, at high and at low isocitrate concentrations

The isocitrate saturation curves of Fig. 2 at various magnesium concentrations were replotted according to the Hill equation. Lines were fitted by eye and the slopes and interception point of the two straight line portions were measured.

Magnesium concentration	Slope (= n)		Inflection point	
	Low isocitrate concentration	High isocitrate concentration	Isocitrate concentration	Saturation
mM			mM	%
0.1	2.95	1.05	0.27	41
0.5	2.51	1.03	0.31	64
1.0	2.50	1.01	0.41	76
2.0	2.76	1.01	0.53	85
5.0	2.76	1.01	0.61	82

Isocitrate saturation curves for the enzyme were prepared at five concentrations of total magnesium, and a series of sigmoid curves was obtained (Fig. 2), similar to that previously reported for this enzyme (6). Increasing the magnesium concentration caused an increase in the apparent affinity of the enzyme for isocitrate, characterized by a displacement of the curve towards the ordinate. At total magnesium concentrations greater than 1 mM, the reverse occurred (Fig. 2). The data from Fig. 2 were replotted as a Hill plot (10) (Fig. 3), and gave for each magnesium concentration two straight lines with a distinct inflection point. An interaction coefficient approaching 3.0 was found at low isocitrate concentrations and of approximately 1.0 at high isocitrate concentrations. The inflection point was displaced to higher isocitrate concentrations as the magnesium concentration was raised (Table I).

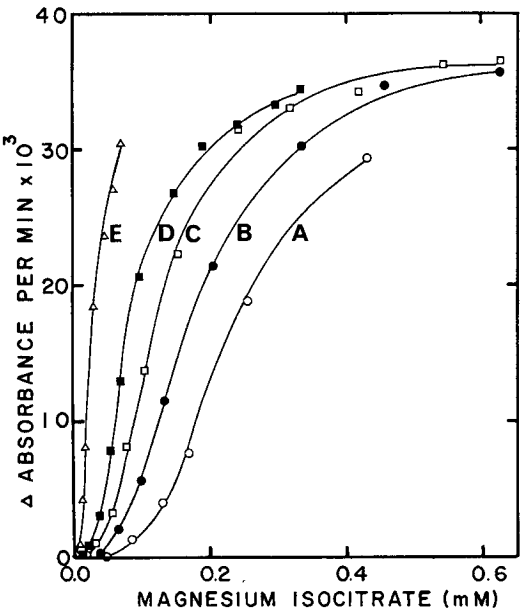

FIG. 4. The data of Fig. 2 replotted as magnesium isocitrate concentration against velocity. The magnesium isocitrate concentration was calculated from the total isocitrate concentration by the use of the stability constants for magnesium isocitrate and magnesium NAD^+. The assays are as described in Fig. 2.

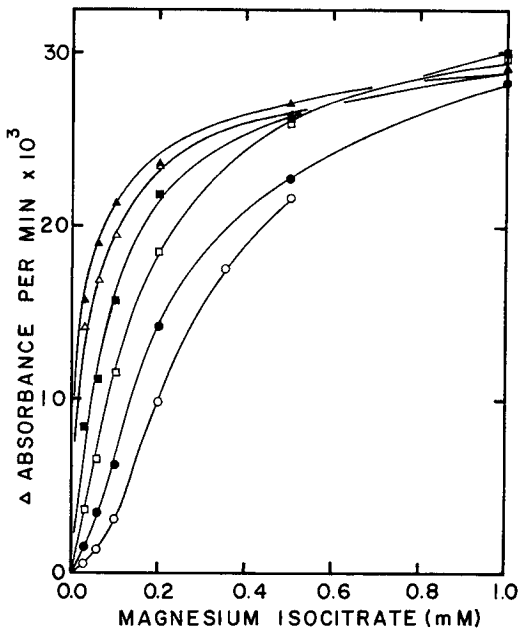

FIG. 5. The effect of magnesium isocitrate concentration on the activity of NAD^+-specific isocitrate dehydrogenase at various constant concentrations of free isocitrate. The assay contained 25 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonate buffer (pH 7.4), 1 mM NAD^+, and 0.03 mM (○), 0.06 mM (●), 0.1 mM (□), 0.2 mM (■), 0.4 mM (△), and 0.6 mM (▲) free isocitrate. The total magnesium concentration was varied to maintain constant free isocitrate concentration.

The magnesium isocitrate concentration was calculated for each total isocitrate concentration and the results were replotted

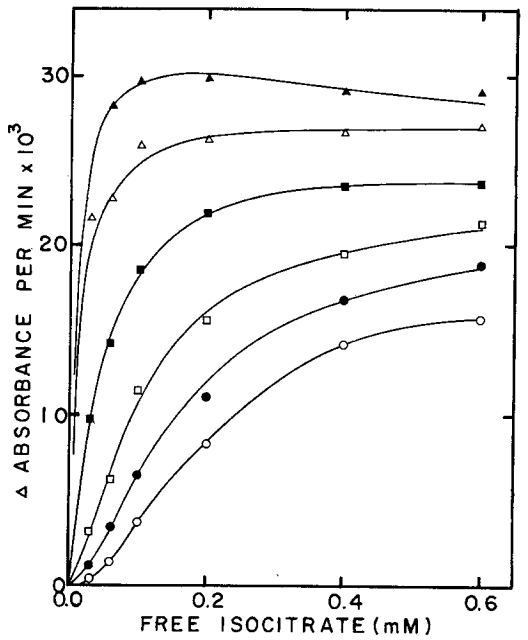

FIG. 6. The effect of free isocitrate concentration on the activity of NAD+-specific isocitrate dehydrogenase at various constant concentrations of magnesium isocitrate. The assay contained 25 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonate buffer (pH 7.4), 1 mM NAD^+, and 0.03 mM (○), 0.6 mM (●), 0.1 mM (□), 0.2 mM (■), 0.5 mM (△), and 1.0 mM (▲) magnesium isocitrate. The magnesium concentration was varied to maintain constant magnesium isocitrate concentration.

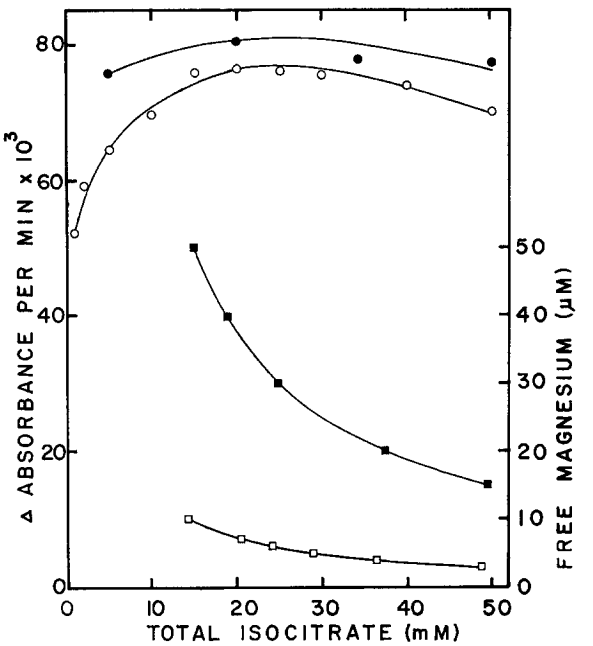

FIG. 7. The effect of high isocitrate concentrations on the activity of NAD+-specific isocitrate dehydrogenase. The assays contained 25 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonate buffer (pH 7.4), 1 mM NAD^+, and 0.2 mM (○) or 1.0 mM (●) total magnesium. Free magnesium concentration at the various total isocitrate concentrations was calculated from the stability constants of magnesium isocitrate and magnesium NAD^+ when total magnesium was 0.2 mM (□) or 1.0 mM (■).

using these values (Fig. 4). If the enzyme only required magnesium isocitrate it would be expected that the curves would be superimposable, which is not the case, and magnesium isocitrate concentration cannot be the only factor determining the rate of the enzyme reaction. Similar plots using calculated values for free isocitrate indicated that free isocitrate is not the substrate. The series of curves in Fig. 4 is similar to the saturation curves of many allosteric enzymes over a range of effector concentrations and it is noteworthy that at any particular magnesium isocitrate concentration, the free isocitrate concentration in the assay increases from *Curve A* to *E*, *i.e.* as the total magnesium concentration is lowered. However, the free isocitrate concentration also varies along each curve, making interpretation difficult.

With Equations 2 through 6, it is possible to calculate total isocitrate and total magnesium concentrations that will allow magnesium isocitrate to be varied while free isocitrate is kept constant. Enzyme assays were performed under such conditions and the results shown in Fig. 5. The maximum velocity was unaffected by free isocitrate concentration, but free isocitrate caused a considerable stimulation at low magnesium isocitrate concentrations. This result would be expected if magnesium isocitrate is the substrate of the enzyme and free isocitrate is an activator.

Similarly, when free isocitrate is varied and magnesium isocitrate is kept constant, the series of curves in Fig. 6 were obtained. In this case maximum velocity is dependent on magnesium isocitrate which further indicates its role as substrate for the enzyme.

It has so far been tacitly assumed that free magnesium is not required for enzyme activity. To demonstrate the absence of a

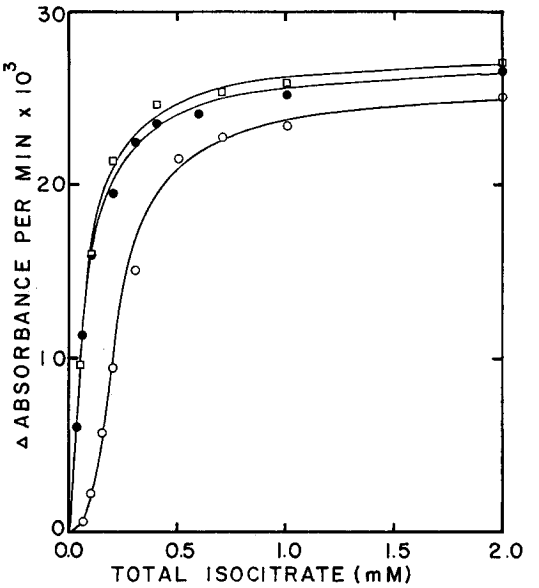

FIG. 8. The activation of NAD+-specific isocitrate dehydrogenase by citrate or propane-1,2,3-tricarboxylic acid. The assays contained 50 mM *N*-tris(hydroxymethyl)methyl-2-ethane sulfonate buffer (pH 7.4), 1 mM magnesium sulfate, 1 mM NAD^+, and 1.0 mM propane-1,2,3-tricarboxylic acid (●), 1.0 mM citrate (□), or no activator (○).

requirement for free magnesium, advantage was taken of the fact that isocitrate complexes magnesium, so that increasing the total isocitrate concentration reduces free magnesium to very low levels. Under these conditions, a powerful inhibition should be seen if free magnesium is required by the enzyme. It was

found that concentrations of isocitrate up to 50 mM produce only slight inhibition at 0.2 mM total magnesium (Fig. 7). Increasing total magnesium 5-fold (which causes an approximately 5-fold increase in free magnesium) does not reverse this inhibition. The free magnesium concentration shown in Fig. 7 will be somewhat in error because of the increasing ionic strength contributed by the high isocitrate concentrations, but this does not detract from the conclusion that free magnesium is probably not required by the enzyme or that, if it is required, the apparent affinity of the enzyme for it is very high.

While Figs. 5 and 6 strongly indicate that magnesium isocitrate is the substrate and free isocitrate is the activator, their roles could be reversed. Citrate and propane-1,2,3-tricarboxylic acid are known to activate the enzyme (6) and presumably they do so in a manner analogous to isocitrate. The stability constants of the metal complexes of citrate and propane-1,2,3-tricarboxylic acid differ considerably (14) and very little magnesium propane-1,2,3-tricarboxylic acid will be present in the assay. Nevertheless it was found that citrate and propane-1,2,3-tricarboxylic acid activate to a similar extent (Fig. 8), suggesting that it is the free form which activates. However, citrate and propane-1,2,3-tricarboxylic acid, because of their different complexing abilities, will differentially affect the concentrations of free magnesium, magnesium isocitrate, and free isocitrate. In the presence of propane-1,2,3-tricarboxylic acid the concentrations of free isocitrate and magnesium isocitrate will not be significantly different from the control. It is probable that there is a direct effect of free propane-1,2,3-tricarboxylic acid on the enzyme rather than an indirect effect due to alterations of the concentration of free isocitrate and magnesium isocitrate. A logical extension of this is that free isocitrate and free citrate also activate the enzyme. When citrate is the activator much of it will be present as magnesium citrate so that free magnesium and magnesium isocitrate will be lower than in the control and free isocitrate will be at a higher concentration. It is assumed in this case that the enzyme is activated by free citrate and free isocitrate. Finally, if free citrate (or propane-1,2,3-tricarboxylic acid) could activate and free isocitrate was also the substrate, the enzyme would be active in the absence of magnesium and this is not observed. Thus the magnesium isocitrate complex is probably the substrate and free isocitrate the activator.

DISCUSSION

The problem of metal complexing by the substrates, activators, and inhibitors of metal-activated enzymes has always presented a problem in the interpretation of results. The difficulty of obtaining exact and reliable estimates of the stability constants of these complexes has discouraged quantitative descriptions. Specific ion electrodes offer a simple method for the estimation of these constants. NAD^+-isocitrate dehydrogenase offered a good system for the study of metal chelation effects.

It has been realized for some time that NAD^+-isocitrate dehydrogenase bears two types of site; a substrate site specific for *threo*-D_s-isocitrate and a regulator site capable of binding the substrate and closely related molecules (6, 10, 15). In the light of the present work, it appears that there is a further difference in the binding properties of these sites. The substrate site specifically requires magnesium isocitrate complex, whereas the activator site binds free isocitrate. This is also consistent with observations on the enzyme from yeast which has four binding sites for isocitrate but only two sites for magnesium (10). The demonstration that the substrate site requires the magnesium complex provides an explanation for this observation. Free magnesium itself is probably not required (Fig. 7).

The model which has been proposed for the yeast enzyme (10) assumes that the enzyme molecule has two substrate binding sites and two activator sites. If a similar situation occurs in the plant enzyme, and there is evidence that the enzyme may be a dimer (16), it is not surprising to find that in the presence of nonsaturating concentrations of free isocitrate, the magnesium isocitrate concentration curve is sigmoid (Fig. 5). Similarly there are two activator sites and the free isocitrate saturation curve is sigmoid (Fig. 6) at low substrate concentrations.

In view of this, the fact that the Hill plots of activity against total isocitrate concentration had slopes greater than 1 was anticipated. The sharp inflection points in the plots are unusual but this is probably a result of the two types of isocitrate site, one binding free isocitrate and the other magnesium-complexed isocitrate. At low isocitrate concentrations, four binding sites may be available per enzyme molecule. Since the two types of isocitrate site probably have different affinities for their respective ligands, one type will become saturated first and the number of available binding sites will be reduced to two. At this point an inflection will be observed in the Hill plots. The interaction coefficient before the inflection approaches 3.0 and after the inflection is approximately 1.0. It must be remembered that this value is only an indication of the degree of interaction between the binding sites but not their actual number. It is suggested that when two sites are occupied there is little interaction between the two remaining sites. This would account for the interaction coefficients of 3 and 1 in the Hill plots and also the tendency towards hyperbolic kinetics in the plots of velocity against either magnesium isocitrate or free isocitrate as the concentration of free or magnesium isocitrate is increased respectively (Figs. 5 and 6). If free isocitrate is the activator it would be expected that at low total isocitrate and high total magnesium concentrations the free isocitrate would fall to sufficiently low concentrations that an inhibition would be seen. This is in fact observed in Fig. 2.

The importance of the different ligand requirements of the two types of site in the regulation of the tricarboxylic acid cycle is difficult to evaluate. The effect of magnesium concentration on the activity of the enzyme is accentuated in the presence of both citrate and isocitrate (17) and may be a factor in the regulation of the cycle.

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