Biochimica et Biophysica Acta, 370 (1974) 1–25

BBA 67323

THE KINETICS OF NAD-LINKED ISOCITRATE DEHYDROGENASE FROM CALF HEART

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(Received April 16th, 1974)

SUMMARY

From kinetic data, free manganous ion, free dibasic isocitrate and the manganese–dibasic isocitrate complex have been identified as the forms of substrate which bind to NAD-linked isocitrate dehydrogenase (EC 1.1.1.41) from calf heart. A kinetic model is proposed in which the active enzyme–substrate complex may be formed by any of three binding pathways, the predominant pathway depending upon the relative magnitude of the concentrations of substrates and the dissociation constants. Dissociation constants for the active enzyme–substrate complex and its intermediate forms have been determined at pH 6.1 and 7.1. ADP has been observed to lower significantly the Michaelis constants for manganous and isocitrate species while having no effect on maximal velocity. The pK_{es} of an essential ionizable amino acid residue has been found to be 6.51, 6.47 and 6.43 at 12, 22 and 32 °C, respectively. The heat of ionization of this residue has been determined to be $\Delta H_i = +1.56$ kcal/mole. On the basis of the above data and analogies to other enzymes, this essential amino acid residue is suggested to be either glutamyl or aspartyl. The activation energy of the enzyme catalyzed reaction was found to be $E_a = 12.6$ kcal/mole.

INTRODUCTION

NAD-linked isocitrate dehydrogenases (*threo*-D_s-isocitrate:NAD-oxidoreductase (decarboxylating), EC 1.1.1.41) have been found to require a divalent metal ion for activity [1]. The role of the divalent cation was generally not considered in earlier studies of kinetic parameters for isocitrate and NAD in the presence and absence of modifying compounds such as ADP [2–4]. Under physiological conditions, however, the substrate isocitrate exists in equilibrium with both hydrogen and metal ion and is present in both its dibasic and tribasic forms and their corresponding metal chelates [5]. More recently, Duggleby and Dennis [6] suggested that a metal–isocitrate complex is the actual substrate of the NAD-linked isocitrate dehydrogenase from

Abbreviation: TEMED, *N,N,N',N'*-tetramethylethylenediamine.

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peas, although they did not distinguish between dibasic and tribasic isocitrate chelates. Colman [7] has shown that the metal chelate of the tribasic form of isocitrate is the substrate of the pig heart NADP-linked enzyme, while both the free dibasic isocitrate ion [8] and its metal chelate [9] are actual substrates of the pig heart NAD-linked enzyme. Initial experiments indicated that in certain respects the kinetic properties of NAD-linked isocitrate dehydrogenase from calf heart are different than those of the enzyme from pig heart. Studies were, therefore, undertaken to explore the modes of regulation by manganese and hydrogen ion of the calf heart NAD-linked enzyme and to determine the species of isocitrate which bind to the enzyme to form the active enzyme–substrate complex. Results presented in this report suggest that under appropriate conditions manganous ion, free dibasic isocitrate and the manganese–isocitrate complex are all substrates of the enzyme. Kinetic parameters in the presence of ADP were obtained in order to discriminate between direct allosteric effects of ADP on the enzyme and the regulation of enzymatic activity by removal of manganese from the ligand pool by chelation with ADP.

The pH–rate profile of the pig heart NAD-linked isocitrate dehydrogenase indicated that a carboxyl or histidyl residue may be an essential group of the enzyme–substrate complex [8]. From kinetic data and chemical modification studies, Colman [10] has concluded that a glutamyl residue is essential for the catalytic function of the NADP-linked enzyme from calf heart. In order to compare the NAD-linked calf heart enzyme with the NAD and NADP-linked pig heart enzymes, pH–rate profiles were constructed at three temperatures to provide physical constants that indicate the nature of an essential amino acid residue of the enzyme–substrate complex.

METHODS

Materials

The following chemicals were purchased from the commercial sources indicated: Grade I ADP, Grade III β NAD, Type I DL-isocitric acid, Grade III *threo*-D_s-isocitric acid, Grade III imidazole, triethanolamine and reagent grade Trizma base (Tris) from Sigma; DE52 (DEAE-cellulose) from Whatman Biochemicals; Sephadex G-200 from Pharmacia; enzyme grade $(NH_2)_4SO_2$ from Schwarz/Mann; sodium barbital, $MnSO_4$ and glycerol from Fisher Scientific; and ammonium persulfate, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), (*N,N′*-methylenebisacrylamide)-acrylamide (bisacrylamide) and Column Coat from Canal Industrial Co.

Assays

NAD-linked isocitrate dehydrogenase activity was measured by the formation of NADH as monitored at 340 nm at 22 °C with a Cary Model 15 spectrophotometer. The standard assay mixture contained Tris–33 mM acetate (pH 7.2)–1.0 mM $MnSO_4$–1.0 mM NAD–10 mM D,L-isocitric acid–water and enzyme to a final volume of 1.0 ml. Measurements were made in cuvettes of 1.0 cm path length. 1 activity unit was defined as the amount of enzyme required to reduce 1.0 μmole of NAD/ml of reaction mixture per min.

Protein concentrations were determined by the method of Warburg and Christian [11] using absorbance measurements at 260 and 280 nm.

Specific activity was defined as enzyme units/mg of protein. Total enzyme units was the product of specific activity and total milligrams of protein.

Standardization of enzymatic velocities

All values for reaction velocities presented in figures and tables have been standardized with the standard assay noted above. Standardized reaction velocities are equal to the product of the measured velocity and the ratio of 0.0500 absorbance units/min and the standard assay velocity for the enzyme preparation being used.

Enzyme preparation

The NAD-linked isocitrate dehydrogenase used in these studies was purified from calf hearts obtained fresh from a local slaughterhouse. The ventricles were isolated from the hearts, placed in 0.25 M sucrose–Tris–10 mM acetate buffer, pH 7.5 at 4 °C, and homogenized with a Waring blender. The homogenate was centrifuged and the pellet discarded. The isocitrate dehydrogenase activity was precipitated with $(NH_4)_2SO_4$ between 30 and 55% saturation while maintaining the enzyme preparation at 4 °C. This fraction was then dialyzed against Tris–10 mM citrate–20% glycerol (v/v)–0.1 mM $MnSO_4$, pH 7.2 at 4 °C and applied to a DEAE-cellulose column equilibrated with the same buffer. NAD-linked isocitrate dehydrogenase activity, free of the NADP-linked activity, was eluted from the column by a stepwise increase in citrate concentration to 20 mM. Those fractions of highest specific activity collected from the DEAE-cellulose column were pooled and further purified by column chromatography with Sephadex G-200 in Tris–10 mM citrate–20% glycerol–0.1 mM $MnSO_4$–0.1 mM ADP, pH 7.2 at 4 °C. The specific activity of this preparation was 0.9 enzyme units/mg protein. Disc gel electrophoresis, followed by staining for protein and NAD-linked isocitrate dehydrogenase activity bands, revealed that the enzyme represented one of two protein bands.

Enzyme preparations used for kinetic studies were dialyzed against Tris–10 mM acetate–20% glycerol, pH 7.2 at 4 °C to remove citrate and manganese and were stored in aliquots at −90 °C. Enzyme purified by DEAE-cellulose chromatography or by DEAE-cellulose and Sephadex G-200 chromatography was used for kinetic studies. Both preparations were observed to yield the same kinetic parameters when used under the same experimental procedure. The specific activity of the enzyme preparations used varied from 0.13 to 0.84.

Determination of manganese concentrations

Concentrations of manganese were determined with a Perkin–Elmer Model 303 atomic absorption spectrophotometer equipped with a Model 165 recorder. Measurements of manganese were made at 280.3 nm using standards in the range of 0.6–80 μM $MnSO_4$.

The distilled water used in these studies had been passed through a bed of Amberlite exchange resins supplied by the Continental Water Conditioning Corp. and exhibited a specific resistance of $1.8 \cdot 10^6$ ohms.

Determination of kinetic parameters

The apparent Michaelis constants for isocitrate were determined at 22 °C at pH 6.1 and 7.1 using imidazole–36 mM HCl and triethanolamine–36 mM HCl

buffers, respectively. The total concentration of added $MnSO_4$ was maintained at a constant level while the concentration of isocitrate was varied over a range yielding zero to maximal velocities. At pH 6.1, the K_m for isocitrate was determined at 0.1, 0.5, 1.0, 5.0 and 10.0 mM $MnSO_4$; at pH 7.1, the K_m for isocitrate was determined at 0.5, 1.0, 5.0 and 10.0 mM $MnSO_4$. The concentration of NAD was always 1.0 mM, well above the K_m of 0.2 mM measured by Hruby [12] in this laboratory for the calf heart enzyme and of 0.08 mM measured by Plaut and Aogaichi [4] for the beef heart enzyme. NAD and isocitric acid solutions were adjusted with NaOH or acetic acid to pH 6 or 7 prior to assaying. Enzyme preparations in Tris–10 mM acetate–20% glycerol, pH 7.2 at 4 °C were added in a volume of 0.05 ml to each reaction mixture containing buffer–*threo*-D_s-isocitrate–NAD–$MnSO_4$; final assay volume was 1.0 ml. Initial rates of isocitrate dehydrogenase activity were measured by the appearance of NADH at 340 nm with a Cary Model 15 spectrophotometer. The pH of reacting assay mixtures were determined with a Radiometer pH Meter Type 26.

The apparent Michaelis constants for manganese were also determined at 22 °C at pH 6.1 and 7.1 using an experimental procedure similar to that used for isocitrate K_m studies. The concentration of total isocitrate added was maintained at a constant level while the concentration of manganese was varied over a range yielding zero to maximal velocities. NAD concentration was always 1.0 mM. The enzyme preparations in Tris–10 mM acetate–20% glycerol, pH 7.2, at 4 °C contained residual manganese after dialysis. In the absence of added metal, reaction mixtures with enzyme contained 0.7–32.0 μM manganese as determined by atomic absorption spectrophotometry; the concentration of manganese in the reaction mixtures were included in the values of total manganese for the calculation of Michaelis constants. The apparent Michaelis constants for isocitrate and manganese in the presence of 0.5 mM ADP at 22 °C at pH 6.1 and 7.1 were determined using an experimental procedure identical to those for determinations in the absence of ADP.

Calculation of the concentration of ionic species

In order to elucidate the active species of isocitrate and manganese, calculations of the concentrations of all ionic species in each reaction mixture were performed. The following mass conservation equations provided the basis for these calculations:

$$I_t = HI^{2-} + I^{3-} + MHI + MI^{1-} \quad (1)$$

$$A_t = HA^{2-} + A^{3-} + MHA + MA^{1-} \quad (2)$$

$$Cl_t = Cl^{1-} + MCl^{1+} \quad (3)$$

$$M_t = M^{2+} + MHI + MI^{1-} + MHA + MA^{1-} + MCl^{1+} \quad (4)$$

Eqn 1 states that total isocitrate, I_t, is equal to the sum of the dibasic, HI^{2-}, and tribasic, I^{3-}, forms of isocitrate and their respective manganese chelates, MHI and MI^{1-}. A similar statement is made in Eqn 2 for a second chelating agent, ADP. The buffer anion, Cl^{1-}, is present in its free and manganese complexed forms (Eqn 3), and total manganese, M_t, is distributed among the free, divalent cation and all the chelated complexes found in solution (Eqn 4). The ionization constants for isocitrate [5] and ADP [13] are 1.79 μM and 0.398 μM, respectively. The dissociation

constants for MCl^{1+}, MHI and MI^{1-} are 0.27 M, 17.4 and 0.869 mM, respectively [5]. The dissociation constants of the ADP chelates of manganese [3] are 1.88 mM and 49.0 μM. A computer program [8] was used to facilitate the calculation of ionic species in each reaction mixture using the mass conservation equations and dissociation constants.

A mass conservation equation for NAD was not considered since, at concentrations present, NAD does not bind a significant proportion of the total manganese [14]. Also, the concentrations of triprotonated and monobasic isocitrate are insignificant in the experimental pH range and were not considered in the mass conservation equations [5]. Imidazole binds metals appreciably in its basic form but at pH 6.1 is present mainly in the protonated, nonchelating form; therefore, it can also be neglected in the mass conservation equations [15]. Triethanolamine does not chelate manganese to a significant extent [16].

pH–rate studies

The dependence of observed velocities on pH was determined using three buffer systems over the pH range from 5.5 to 8.6: 36 mM sodium acetate–acetic acid; imidazole–36 mM HCl and triethanolamine–36 mM HCl. Two curves were constructed using two sets of substrate concentrations: (a) 60 mM D,L-isocitric acid–3 mM NAD–1 mM $MnSO_4$ and (b) 120 mM D,L-isocitric acid–6 mM NAD–2 mM $MnSO_4$. These concentrations were chosen to be at least five times the Michaelis constants as measured at pH 6.1 and 7.1. NAD and isocitric acid solutions were adjusted prior to pH 6.5 and pH 7.5 with NaOH and HCl prior to assaying. An enzyme preparation of specific activity 0.13 in Tris–10 mM acetate–20% glycerol, pH 7.2, at 4 °C was added in a volume of 0.05 ml to bring each reaction mixture to a final volume of 1.0 ml. Spectrophotometric measurements of initial rates of NADH formation were made with a Cary Model 15 spectrophotometer. Immediately after assaying, the pH of reacting mixtures was measured with a Radiometer pH Meter Type 25.

The procedure outlined above was performed at three temperatures, 12, 22 and 32 °C, in order to permit the determination of the heat of ionization of the enzyme catalyzed reaction. The pH measurements were made at the temperature of the assay, using a pH meter which was standardized at each given temperature.

RESULTS AND DISCUSSION

Lineweaver–Burk plots

The apparent Michaelis constants and maximal velocities for various ionic species of substrates were determined by graphic analysis of Lineweaver–Burk plots.

In classical Michaelis–Menten kinetics, the concentration of one substrate is varied while the concentrations of all other components are held constant. However, in the case of isocitrate dehydrogenase, as well as other metal activated enzymes, such an experimental approach is precluded. No single ionic species can be changed in concentration without altering the concentrations of other species in the assay solution, since isocitrate is present in different states of protonation and chelation depending upon the total isocitrate, manganese and hydrogen ion concentration. In the experiments to be described, the reciprocal of the measured velocities were plotted as a function of the reciprocal of the various ionic species of substrate. The

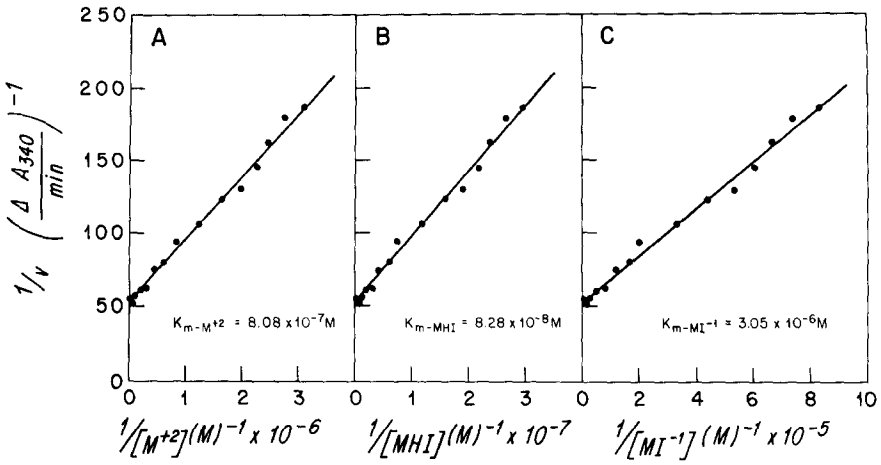


Fig. 1. K_m for manganese in the absence of ADP. Total D_s-isocitrate concentration was maintained at 5 mM while the total manganese concentration was varied over an 833-fold range. [NAD] was 1 mM and the buffer was imidazole–36 mM HCl, pH 6.1. $[HI^{2-}]$ was observed to vary only 1.07-fold over the entire range of manganese concentration. The reciprocals of the observed velocities are plotted versus the reciprocals of the concentrations of: A, free manganous ion; B, manganese–dibasic isocitrate complex and C, manganese–tribasic isocitrate complex. All plots are observed to be linear with the same maximal velocity.

K_m^{obs} values obtained are necessarily apparent values, since they are mixed constants and depend upon the stated experimental conditions. Unless otherwise indicated, all plots of $1/v$ versus $1/S$ were observed to be linear with a Hill coefficient equal to 1.0; no substrate inhibition was noted. Examples of Lineweaver–Burk plots from the data at pH 6.1 are presented in Figs 1 and 2.

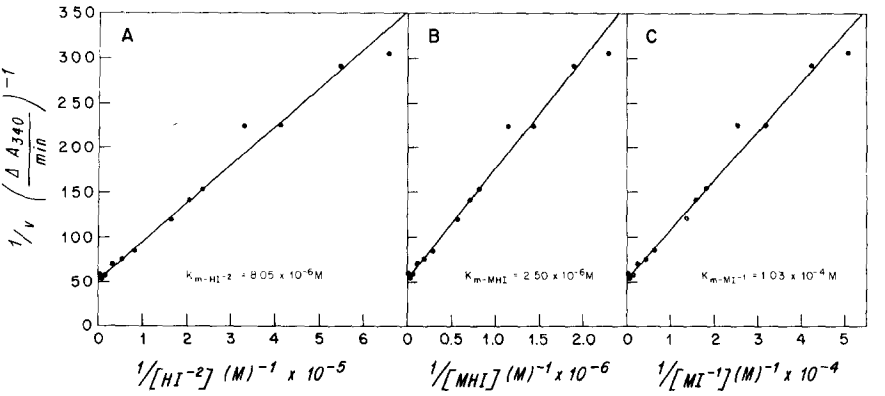


Fig. 2. K_m for isocitrate in the absence of ADP. Total manganese concentration was maintained at 5 mM while the total D_s-isocitrate concentration was varied over a 200-fold range. [NAD] was 1 mM and the buffer was imidazole–36 mM HCl, pH 6.1. $[M^{2+}]$ was observed to vary only 1.18-fold as the total isocitrate concentration was changed from 0.025 to 1 mM and 2.18-fold as the total isocitrate concentration was increased from 1 to 5 mM. The reciprocals of the observed velocities are plotted versus the reciprocals of the concentration of: A, free dibasic isocitrate; B, manganese–dibasic isocitrate complex and C, manganese–tribasic isocitrate complex. All plots are observed to be linear over the entire range of isocitrate concentration and to exhibit the same maximal velocity.

Initial observations on K_m data for manganese and isocitrate

When the Michaelis constants for total manganese at pH 6.1 and pH 7.1 were determined in the presence of 1.0 mM NAD and various fixed concentrations of isocitrate, it became apparent that higher concentrations of isocitrate facilitate the binding of manganese to the enzyme, as indicated by the decreasing values of K_m^{obs} for total manganese in Tables I and II. This observation suggested that manganese binds to the enzyme as a chelated form of isocitrate, since higher concentrations of iso-

TABLE I

OBSERVED MICHAELIS CONSTANTS FOR MANGANESE AT pH 6.1 AS A FUNCTION OF ISOCITRATE CONCENTRATION

Total isocitrate concn (mM)	$K^{obs}_{m\text{-}M_t}$ (μM)	$K^{obs}_{m\text{-}M^{+2}}$ (μM)	$K^{obs}_{m\text{-}MHI}$ (μM)	$K^{ob}_{m\text{-}MI^{-}t}$ (μM)
0.1	444	370	0.978	39.4
0.5	15.5	10.4	0.0993	3.63
1.0	5.72	2.89	0.0663	2.26
5.0	4.39	0.808	0.0828	3.05
10.0	4.30	0.448	0.0892	3.59

citrate would chelate increasing proportions of manganese and lower the apparent $K^{obs}_{m-M_t}$. Also, it was noted that $K^{obs}_{m-M_t}$ is higher at pH 7.1 than at pH 6.1. Since less of the dibasic species of isocitrate are present at pH 7.1, this observation suggested that the chelated form which binds to the enzyme might be the dibasic isocitrate–metal complex rather than the tribasic isocitrate–metal complex; a decrease in the substrate species, manganese–dibasic isocitrate, is reflected in an increase in the apparent K_m for total manganese, $K^{obs}_{m-M_t}$. Indeed, the K_m for the manganese–dibasic isocitrate

TABLE II

OBSERVED MICHAELIS CONSTANTS FOR MANGANESE AT pH 7.1 AS A FUNCTION OF ISOCITRATE CONCENTRATION

Total isocitrate concn (mM)	$K^{obs}_{m\text{-}M_t}$ (μM)	$K^{obs}_{m\text{-}M^{2+}}$ (μM)	$K^{obs}_{m\text{-}MHI}$ (μM)	$K^{obs}_{m\text{-}MI^{1-}}$ (μM)
0.5	71.9	51.8	0.0471	28.2
1.0	60.9	29.2	0.0630	34.7
5.0	41.5	8.06	0.0771	39.4
10.0	38.6	3.70	0.0866	41.6

complex, K^{obs}_{m-MHI}, appeared to be approximately constant and independent of both pH and isocitrate concentration in the range 0.5–10 mM. In contrast, the K_m for free manganous ion, $K^{obs}_{m-M^{2+}}$, was observed to decrease at least 14-fold as the isocitrate concentration was raised from 0.5 to 10 mM, and the K_m for the manganese–tribasic isocitrate complex, $K^{obs}_{m-MI^{1-}}$, was observed to increase 10-fold with the increase of 1 pH unit. Therefore, the initial hypothesesis derived from these data was that manganese–dibasic isocitrate binds directly to the enzyme, E.

TABLE III

OBSERVED MICHAELIS CONSTANTS FOR ISOCITRATE AT pH 6.1 AS A FUNCTION OF MANGANESE CONCENTRATION

Total manganese concn (mM)	$K^{obs}_{m\text{-}I_t}$ (μM)	$K^{obs}_{m\text{-}HI^{2-}}$ (μM)	$K^{obs}_{m\text{-}MHI}$ (μM)	$K^{obs}_{m\text{-}MI^{1-}}$ (μM)
0.1	191	41.5	0.339	12.7
0.5	175	35.6	1.21	64.0
1.0	218	33.6	2.06	92.5
5.0	136	8.05	2.50	103
10.0	247	6.56	6.06	231

When the Michaelis constants for total isocitrate at pH 6.1 and 7.1 (Tables III and IV) were determined in the presence of 1.0 mM NAD and various fixed concentrations of manganese, it was observed that the increase of 1 pH unit causes a 20-fold increase in $K^{obs}_{m-I_t}$, as exemplified by the rise from 218–4250 μM at a total manganese concentration of 1.0 mM. Since at a higher pH less isocitrate is in the dibasic as opposed to the tribasic form, the data strongly suggested that a dibasic form of isocitrate is the active substrate species. It was also observed that the concentration of manganese had little effect on the binding of total isocitrate at either pH. The K_m for

TABLE IV

OBSERVED MICHAELIS CONSTANTS FOR ISOCITRATE AT pH 7.1 AS A FUNCTION OF MANGANESE CONCENTRATION

Total manganese concn (mM)	$K^{obs}_{m\text{-}I_t}$ (μM)	$K^{obs}_{m\text{-}HI^{2-}}$ (μM)
0.5	3100	61.6
1.0	4250	61.7
5.0	7600	47.6
10.0	4580	14.8

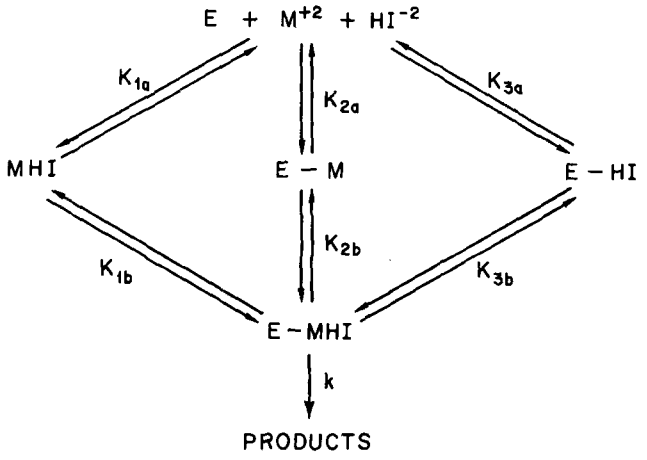
free dibasic isocitrate, $K^{obs}_{m-HI^{2-}}$, was observed to be relatively constant from 0.1–1.0 mM $MnSO_4$ at pH 6.1 and from 0.5–5.0 mM at pH 7.1, although there was a decrease in $K^{obs}_{m-HI^{2-}}$ at higher manganese concentrations. Thus, the initial hypothesis derived from the isocitrate K_m data was that free dibasic isocitrate binds to an enzyme–manganese complex. In contrast, at pH 6.1 $K^{obs}_{m-MI^{1-}}$ and K^{obs}_{m-MHI} were observed to increase 18-fold with increasing manganese concentrations and were, therefore, not considered to be active forms of substrate at the manganese concentrations present. At pH 7.1, $K^{obs}_{m-MI^{1-}}$ and K^{obs}_{m-MHI} were indeterminable from Lineweaver–Burk plots because of non-linearity. The non-linearity was not, however, interpreted as positive cooperativity for the following reason. At pH 7.1 the experimental conditions with regard to pH, the fixed concentrations of manganese, and the high concentrations of total isocitrate required for activity resulted in non-proportional variation of the concentrations of the non-chelated species, tribasic and dibasic isocitrate, with the

corresponding chelated species, as calculated with the computer program. Because all other Lineweaver–Burk plots observed in this study were linear, and because there were the indications cited above which suggested that free dibasic isocitrate was the active substrate form under these experimental conditions, it was hypothesized that the non-linear plots for the metal complexes of dibasic and tribasic isocitrate are coincidental since these forms are not the active substrate species.

The initial observations, therefore, suggested that a dibasic form of isocitrate is the active species. The dibasic form is active as either the free dibasic anion, HI^{2-}, or as the manganese–dibasic complex, MHI, with the concentration of manganese regulating the binding of these species either to an enzyme–manganese complex or to the enzyme, respectively. The isocitrate data appeared to have been collected under conditions where the former mechanism is operative, and the manganese data where the latter is operative.

A model for substrate binding

In order to more closely analyze the kinetic data by calculation of dissociation constants for binding species, a model for the possible binding pathways of substrates was devised and is presented in Fig. 3. In this model, K_{1a}, K_{1b}, K_{2a}, K_{2b}, K_{3a} and K_{3b} are defined as the dissociation constants for the reactions indicated. It is postulated



$$v = k\,[E\text{-}MHI] \qquad K_{1a}K_{1b} = K_{2a}K_{2b} = K_{3a}K_{3b}$$

$$K_{1a} = \frac{[M^{+2}][HI^{-2}]}{[MHI]} \qquad K_{2a} = \frac{[E][M^{+2}]}{[E\text{-}M]} \qquad K_{3a} = \frac{[E][HI^{-2}]}{[E\text{-}HI]}$$

$$K_{1b} = \frac{[E][MHI]}{[E\text{-}MHI]} \qquad K_{2b} = \frac{[E\text{-}M][HI^{-2}]}{[E\text{-}MHI]} \qquad K_{3b} = \frac{[E\text{-}HI][M^{+2}]}{[E\text{-}MHI]}$$

Fig. 3. Model for substrate binding by the enzyme.

that the active enzyme–manganese–dibasic isocitrate complex may be formed by any of the three pathways, and that the relative proportion of that complex formed by each of the three pathways depends upon the relative magnitude of the dissociation constants and the concentrations of substrate species. At relatively high concentrations of free dibasic isocitrate, an enzyme–isocitrate complex would be formed and Pathway 3 would be important; conversely, at high manganese concentrations, the enzyme would exist primarily in the form enzyme–manganese and Pathway 2 would predominate. At lower concentrations of both manganese and isocitrate, the preformed metal–isocitrate complex would bind directly to the enzyme as in Pathway 1.

Assuming that all equilibria are rapidly established, the following algebraic description of the model can be derived:

$$v = \frac{V}{1 + \frac{K_{1b}}{[MHI]} + \frac{K_{2b}}{[HI^{2-}]} + \frac{K_{3b}}{[M^{2+}]}} \tag{5}$$

Experimentally, the value of V used in this question was that observed in the presence of 1 mM NAD, a concentration that is at least five times the Michaelis constant for coenzyme. It was desirable to rearrange Eqn 5 into conventional Michaelis–Menten form with metal–dibasic isocitrate as substrate in order that K_m data for manganese and isocitrate could be easily related through experimental values for K^{obs}_{m-MHI}. By substituting in different ways for values in the denominator using the dissociation constant expressions, two algebraically related equations can be derived from Eqn 5 (see Appendix, *Note 1*):

$$v = \frac{V}{1 + \frac{K_{1b}}{[MHI]}\left(1 + \frac{[M^{2+}]}{K_{2a}} + \frac{[HI^{2-}]}{K_{3a}}\right)} \tag{6}$$

$$v = \frac{\frac{V}{\left[1 + \frac{K_{2b}}{[HI^{2-}]}\right]}}{1 + \frac{K_{1b}}{[MHI]}\frac{\left[1 + \frac{[HI^{2-}]}{K_{3a}}\right]}{\left[1 + \frac{K_{2b}}{[HI^{2-}]}\right]}} = \frac{V'}{1 + \frac{K_{1b}}{[MHI]}\frac{\left[1 + \frac{[HI^{2-}]}{K_{3a}}\right]}{\left[1 + \frac{K_{2b}}{[HI^{2-}]}\right]}} \tag{7}$$

where V' in Eqn 7 is the observed maximum velocity at any concentration of isocitrate and V is the intrinsic maximal velocity when $[HI^{2-}]$ is much greater than K_{2b} and NAD concentration is 1 mM. The value of K_{m-MHI} for Eqns 6 and 7 are, respectively,

$$K_{m\text{-}MHI} = K_{1b}\left(1 + \frac{[M^{2+}]}{K_{2a}} + \frac{[HI^{2-}]}{K_{3a}}\right) \tag{8}$$

$$K_{m\text{-}MHI} = K_{1b}\frac{\left(1 + \frac{[HI^{2-}]}{K_{3a}}\right)}{\left(1 + \frac{K_{2b}}{[HI^{2-}]}\right)} \tag{9}$$

In addition to the differences in the definitions of K_{m-MHI} for the Eqns 6 and 7, it is noted that Eqn 7 possesses an additional factor in the V term. Although Eqns 6 and 7 are algebraically related, they best describe the general model of Fig. 3 with different restrictive conditions as imposed by relative relationships of substrate concentrations and dissociation constants.

Eqn 6, while focusing on MHI, retains the possibility that the three pathways all function. At low concentrations of M^{2+} and HI^{2-}, K_{m-MHI} approaches the value of K_{1b}; however, when either M^{2+} or HI^{2-} becomes comparable in magnitude to K_{2a} or K_{3a}, K_{m-MHI} will increase.

Eqn 7 suggests that when $K_{2b} \ll [HI^{2-}] \ll K_{3a}$, K_{m-MHI} will approach K_{1b} and V' will approach V. If, however, $[HI^{2-}]$ becomes comparable in magnitude to K_{2b}, K_{m-MHI} and maximal velocity will decrease. These restrictions implicitly require that the concentration of M^{2+} be very much greater than MHI; this equation, therefore, can best be used to describe conditions where Pathway 2 predominates, when most of the enzyme is in the form of enzyme–manganese complex. Only when $[HI^{2-}]$ becomes comparable in magnitude to K_{2b} does the possibility arise for Pathway 1 to become proportionately significant; under these conditions, however, the isocitrate concentration must be so low that maximal velocity decreases.

From the computer program it is known that at pH 6.1 the ratio of $[M^{2+}]/[MHI]$ is approx. 10 for all experimental concentrations of total manganese and isocitrate. At pH 7.1, this ratio is approx. 100, and $[M^{2+}] \gg [MHI]$. Pathway 1 is suggested to be inherently operative at both pH values, but at pH 7.1 to be restricted by the relative availability of free manganous ion and manganese–dibasic isocitrate complex. Eqn 6 has been used to describe the kinetic data at pH 6.1 and Eqn 7 has been applied to the data at pH 7.1. The experiments which allow the calculation of the values of the dissociation constants are presented below. It is suggested that the differences between the two pH values are due to the relative concentrations of free manganous ion and manganese–dibasic isocitrate and not to inherently different mechanisms of the enzyme. Indeed, the dissociation constants at both pH values were found to be quite similar.

Detailed analysis of kinetic data for manganese and isocitrate at pH 6.1

Since both the calculation of dissociation constants and a comparison of observed and predicted values for K_{m-MHI} may be performed using Eqn 8, this equation forms the basis for the analysis of data at pH 6.1.

The dissociation constant for the enzyme–manganese complex was calculated in the following manner. From Table I, the dissociation constant for enzyme–manganese–dibasic isocitrate was selected as $K_{1b} = 6.63 \cdot 10^{-8}$ M from the data at a total isocitrate concentration of 1.0 mM; it was tentatively considered that under these conditions the concentration of free manganous ion would be low relative to K_{2a}, the concentration of free dibasic isocitrate would be low relative to K_{3a} and, therefore, K^{obs}_{m-MHI} would equal K_{1b}. At a total isocitrate concentration of 0.1 mM, the concentration of free dibasic isocitrate was assumed to be much less than K_{3a} and Pathway 3 was assumed to be negligible. Also, at a total isocitrate concentration of 0.1 mM it was found that $K^{obs}_{m-MHI} = 97.8 \cdot 10^{-8}$ M and that the free manganous ion concentration is $3.70 \cdot 10^{-4}$ M at the $[MHI]$ equal to its K^{obs}_{m-MHI}. Substituting the appropriate values into Eqn 8 rewritten in the form

$$K^{obs}_{m\text{-}MHI} = K_{1b}\left(1 + \frac{[M^{2+}]}{K_{2a}}\right) \tag{10}$$

the dissociation constant for enzyme–manganese is found to be $K_{2a} = 2.69 \cdot 10^{-5}$ M.

In order to calculate the dissociation constant for the enzyme–dibasic isocitrate complex, Eqn 8 was used. From Table I at a total isocitrate concentration of 10.0 mM, $K^{obs}_{m\text{-}MHI}$ was observed to be $8.92 \cdot 10^{-8}$ M, at which point the free metal concentration is $4.48 \cdot 10^{-7}$ M and the ratio $[M^{2+}]/K_{2a}$ is negligible compared to 1. From the computer program the concentration of free dibasic isocitrate is $3.58 \cdot 10^{-3}$M at the $[MHI]$ equal to its $K^{obs}_{m\text{-}MHI}$. Substitution of the above values into Eqn 8 resulted in the value of $K_{3a} = 1.09 \cdot 10^{-2}$ M. Now that K_{3a} was known, a check was made to test whether the value of K_{2a} was affected if the term $[HI^{2-}]/K_{3a}$ was included in the calculation of K_{2a}; indeed, at a total isocitrate concentration of 0.1 mM, $[HI^{-2}] \ll K_{3a}$ and $K_{2a} = 2.69 \cdot 10^{-5}$ M.

The dissociation constant [5] for MHI is known to be $K_{1a} = 17.4$ mM. Using the relationships $K_{1a} \cdot K_{1b} = K_{2a} \cdot K_{2b} = K_{3a} \cdot K_{3b}$, the remaining two constants may be calculated as $K_{2b} = 4.29 \cdot 10^{-5}$ M and $K_{3b} = 1.06 \cdot 10^{-7}$ M. The dissociation constants for substrate binding at pH 6.1 are summarized in Table V.

TABLE V

DISSOCIATION CONSTANTS FOR SUBSTRATE BINDING AT pH 6.1 AND 7.1

pH 6.1		
$K_{1a} = 1.74 \cdot 10^{-2}$ M	$K_{2a} = 2.69 \cdot 10^{-5}$ M	$K_{3a} = 1.09 \cdot 10^{-2}$ M
$K_{1b} = 6.63 \cdot 10^{-8}$ M	$K_{2b} = 4.29 \cdot 10^{-5}$ M	$K_{3b} = 1.06 \cdot 10^{-7}$ M
pH 7.1		
$K_{1a} = 1.74 \cdot 10^{-2}$ M	$K_{2a} = 4.70 \cdot 10^{-5}$ M	
$K_{1b} = 1.34 \cdot 10^{-7}$ M	$K_{2b} = 4.92 \cdot 10^{-5}$ M	

In order to check the fit of the calculated constants to other experimental results, the following tests were performed using Eqn 8. For the manganese data, $K^{calc}_{m\text{-}MHI}$ at total isocitrate concentration of 0.5, 1.0 and 5.0 mM was calculated using the concentrations of free metal ion and free dibasic isocitrate at the $[MHI]$ equal to its $K^{obs}_{m\text{-}MHI}$. A comparison of $K^{calc}_{m\text{-}MHI}$ and $K^{obs}_{m\text{-}MHI}$ for the manganese data is presented in Table VI and shows excellent agreement.

TABLE VI

COMPARISON OF $K^{calc}_{m\text{-}MHI}$ AND $K^{obs}_{m\text{-}MHI}$ FOR MANGANESE DATA AT pH 6.1

Total isocitrate concn (mM)	$K^{calc}_{m\text{-}MHI}$ ($M \times 10^8$)	$K^{obs}_{m\text{-}MHI}$ ($M \times 10^8$)
0.5	9.30	9.93
1.0	7.55	6.63
5.0	7.94	8.28

For the isocitrate data, K^{calc}_{m-MHI} at total manganese concentrations of 0.1, 0.5, 1.0, 5.0 and 10.0 mM was calculated using the concentrations of free manganous ion and free dibasic isocitrate at the $[MHI]$ equal to its K^{obs}_{m-MHI}. A comparison of K^{calc}_{m-MHI} and K^{obs}_{m-MHI} is presented in Table VII. It was noted that the values of K^{obs}_{m-MHI} were in good agreement with K^{calc}_{m-MHI} at low concentrations of manganese but that K^{obs}_{m-MHI} was significantly lower than K^{calc}_{m-MHI} at high manganese concentrations. Values for $K^{obs}_{m-HI^{2-}}$ were also found to be lower than $K^{calc}_{m-HI^{2-}}$ at total mangansee

TABLE VII

COMPARISON OF K^{calc}_{m-MHI} AND K^{obs}_{m-MHI} FOR ISOCITRATE DATA AT pH 6.1

Total manganese concn (mM)	K^{calc}_{m-MHI} ($M \times 10^6$)	K^{obs}_{m-MHI} ($M \times 10^6$)
0.1	0.288	0.339
0.5	1.20	1.21
1.0	2.32	2.06
5.0	12.2	2.50
10.0	24.3	6.06

concentrations of 5.0 and 10.0 mM. The discrepancies between the observed and calculated values suggested that high manganese concentrations facilitate the binding to the enzyme of dibasic isocitrate and its metal chelate in a way not described by this model. The available data is too limited to provide conclusive evidence of the mode by which manganese induces this phenomenon, but two possible mechanisms are suggested. A form of manganese could bind to a regulatory site of the enzyme and lower the binding constants for free dibasic isocitrate and manganese–dibasic isocitrate at the active site. This mechanism is similar to that by which ADP is believed to modify the enzyme. Alternatively, it is possible that both dibasic isocitrate and its metal complex bind to the enzyme–manganese complex to form two reactive species, E–MHI and E–M_2HI; E–MHI may still be formed by the binding of MHI to free enzyme, E. It is possible to fit the kinetic data in the high manganese region by expanding the model to include differential binding of both free dibasic isocitrate and its metal chelate to the enzyme–manganese complex.

The mechanism of the binding of isocitrate and manganese to calf heart isocitrate dehydrogenase at pH 6.1 may be summarized in the following manner. The model presented in Fig. 3 and represented mathematically by Eqns 5 and 6 describes the binding of substrates to the enzyme. Pathway 3 is of minor importance since only under unusual conditions would free dibasic isocitrate be in the range of K_{3a}. The relative importance of Pathways 1 and 2 has been found to be dependent upon the relative concentrations of free manganous ion, free dibasic isocitrate and manganese–dibasic isocitrate. The calculated dissociation constants indicate that under the experimental conditions used for the determination of the data for the Michaelis constants for manganese (Table I), Pathways 1 and 2 are involved at $I_t = 0.1$ mM, Pathway 1 predominates at $I_t = 1.0$ mM and Pathways 1 and 3 operate at $I_t = 10.0$ mM. Under the experimental conditions used for the determination of the isocitrate K_m data (Table III), Pathway 2 predominates at all manganese concentrations.

Detailed analysis of kinetic data for manganese and isocitrate at pH 7.1

Eqn 7 appeared to best describe the trends observed in the data at pH 7.1 as noted in the initial observations. However, because Pathway 3 was of relatively small importance at pH 6.1, and because the concentration of free dibasic isocitrate is reduced by approximately 10-fold when the pH is raised to 7.1, it was assumed that Pathway 3 would be negligible at pH 7.1 with the concentrations of substrates present. Eqn 7 was, therefore, reduced to Eqn 11:

$$v = \frac{\dfrac{V}{\left(1 + \dfrac{K_{2b}}{[HI^{2-}]}\right)}}{1 + \dfrac{K_{1b}}{[MHI]} \dfrac{[I]}{\left(1 + \dfrac{K_{2b}}{[HI^{2-}]}\right)}} = \frac{V'}{1 + \dfrac{K_{1b}}{[MHI]} \dfrac{[I]}{\left(1 + \dfrac{K_{2b}}{[HI^{2-}]}\right)}} \tag{11}$$

for the detailed analysis of data at pH 7.1. It has been found that Eqn 11, which neglects Pathway 3, adequately accounts for the kinetic behavior at pH 7.1.

When V' was determined at pH 7.1 from the data for the Michaelis constants for manganese expressed in terms of metal–dibasic isocitrate, V' was observed to decrease with decreasing isocitrate concentration (Table VIII). In order to calculate

TABLE VIII

V' FROM MANGANESE DATA AT pH 7.1

Total isocitrate concn (mM)	V'
0.5	0.0135
1.0	0.0251
5.0	0.0384
10.0	0.0473

K_{2b} and the intrinsic maximum velocity at pH 7.1 (V), the following steps were performed. The numerator of Eqn 11 can be rearranged to Eqn 12:

$$\frac{1}{V'} = \frac{K_{2b}}{V} \frac{1}{[HI^{2-}]} + \frac{1}{V} \tag{12}$$

where V is the intrinsic maximal velocity when $[HI^{2-}]$ is much greater than K_{2b}. It was possible to calculate V and K_{2b} from a plot of $1/V'$ versus $1/[HI^{2-}]$, where $[HI^{2-}]$ is the concentration of dibasic isocitrate at a total manganese concentration equal to the value of $K^{obs}_{m-M_t}$. In Fig. 4, such a plot results in $V = 0.0526$ A/min and $K_{2b} = 4.92 \cdot 10^{-5}$ M.

From Eqn 11 it is noted that,

$$K^{obs}_{m\text{-}MHI} = \frac{K_{1b}}{\left(1 + \dfrac{K_{2b}}{[HI^{2-}]}\right)} \tag{13}$$

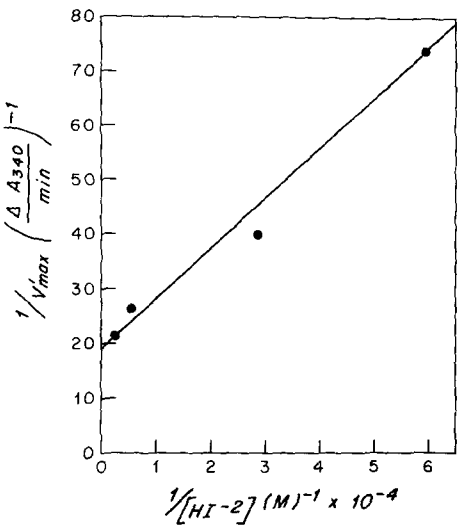


Fig. 4. Determination of K_{2b} at pH 7.1. The reciprocals of the values for V' from Table VIII are plotted versus the reciprocals of $[HI^{1-}]$, the concentrations of dibasic isocitrate at the values of $K^{obs}_{m\text{-}M^{2+}}$. Eqn 13 permits the determination of $K_{2b} = 4.92 \cdot 10^{-5}$ M from the slope of the linear function.

Substitution into Eqn 13 of the values of $K^{obs}_{m\text{-}MHI}$ from Table II and of the corresponding concentrations of free dibasic isocitrate at the $[MHI]$ equal to its $K^{obs}_{m\text{-}MHI}$ yields an average value of $1.34 \cdot 10^{-8}$ for K_{1b}. The fourth dissociation constant, K_{2a}, may be calculated as $4.70 \cdot 10^{-5}$ M from the relationship $K_{1a}K_{1b} = K_{2a}K_{2b}$, where $K_{1a} = 1.74 \cdot 10^{-2}$ M. The values of dissociation constants for substrate binding at pH 7.1 are tabulated in Table V where they may easily be compared with the corresponding values at pH 6.1.

In order to check how accurately the constants calculated from the manganese kinetic data at pH 7.1 predict the experimental observations of the isocitrate data at pH 7.1, Eqn 11 was rearranged to Eqn 14 (see Appendix, *Note 2*):

$$v = \frac{V}{1 + \frac{K_{2b}}{[HI^{2-}]}\left(1 + \frac{K_{2a}}{[M^{2+}]}\right)} \tag{14}$$

where

$$K^{obs}_{m\text{-}HI}{}^{2-} = K_{2b}\left[1 + \frac{K_{2a}}{[M^{2+}]}\right] \tag{15}$$

By substituting the free manganese concentration at $[HI^{2-}]$ equal to the $K^{obs}_{m\text{-}HI^{2-}}$ into Eqn 15, $K^{calc}_{m\text{-}HI^{2-}}$ may be calculated. Table IX tabulates the calculated and observed values of $K_{m\text{-}HI^{2-}}$ at pH 7.1. Close agreement of the two sets of values is observed from 0.5 to 5.0 mM $MnSO_4$; however, $K^{obs}_{m\text{-}HI^{2-}}$ at a total manganese concentration of 10.0 mM is only one-third of the predicted value. This divergence of observed and calculated values of $K_{m\text{-}HI}{}^{2-}$ at the highest concentration of manganese is similar to the divergence of observed and calculated values at pH 6.1. The data at

TABLE IX

COMPARISON OF $K_{m\text{-}HI^{2-}}^{calc}$ AND $K_{m\text{-}HI^{2-}}^{obs}$ FOR ISOCITRATE DATA AT pH 7.1

Total manganese concn (mM)	$K_{m\text{-}HI^{2-}}^{calc}$ ($M \times 10^5$)	$K_{m\text{-}HI^{2-}}^{obs}$ ($M \times 10^5$)
0.5	6.15	6.16
1.0	5.54	6.17
5.0	5.02	4.76
10.0	4.95	1.48

pH 7.1, therefore, also suggests that high concentrations of manganese facilitate the binding of isocitrate by some means in addition to the pathways contained in the model in Fig. 3, as was discussed for data at pH 6.1.

No term except V appears in the numerator of Eqn 14, suggesting that at any concentration of manganese, an intrinsic maximal velocity is attainable if the concentration of free dibasic isocitrate is high enough. This is in contrast to the substrates M^{2+} and MHI, whose rate equations, such as Eqn 11, contain an additional factor involving HI^{2-} in the numerator. As suggested by Eqn 14, the V observed from plots of initial velocity versus HI^{2-} was found to be constant over the range 0.5–10.0 mM total manganese.

The modes of manganese and isocitrate binding to calf heart isocitrate dehydrogenase at pH 7.1 may be summarized in the following manner. The model presented in Fig. 3 is suggested to describe the possible pathways of substrate binding at pH 7.1. Pathway 3, however, is not operative at pH 7.1 because the concentration of free dibasic isocitrate in relation to K_{3a} is very low unless total isocitrate is raised to an extremely high concentration. Eqns 14 and 15 rather than Eqns 7 and 9 are, therefore, sufficient to mathematically describe substrate binding at pH 7.1. Because the concentrations of total manganese and total isocitrate used in these experiments at pH 7.1 produced a very low concentration of metal–dibasic isocitrate relative to free metal ion, Pathway 2 was observed to be predominant over Pathway 1. The dissociation constants at pH 7.1 are noted to be quite similar to the constants at pH 6.1. It is the effect of the hydrogen ion concentration on the relative proportions of I^{3-}, HI^{2-}, M^{2+}, MI^{1-} and MHI which determines the major binding pathways at the different pH values.

Observed kinetic parameters at pH 6.1 and 7.1 in the presence of ADP

The Michaelis constants for isocitrate species were determined at pH 7.1 by varying the total isocitrate concentration in the presence of 0.5 mM ADP, 1.0 mM $MnSO_4$ and 1.0 mM NAD. Fig. 5A contains representative data at pH 7.1 from which the K_m for free dibasic isocitrate in the presence of ADP, $K_{m\text{-}HI^{2-}}^{ADP}$, was determined. No substrate inhibition or non-linearity was observed in any of the Lineweaver–Burk plots. The observed constants for isocitrate species in the presence of ADP are tabulated in Table X and may be compared with the corresponding K_m values in the absence of ADP located in Table IV. ADP was found not to affect V but to lower significantly the K_m for total isocitrate, free dibasic isocitrate, metal–dibasic isocitrate and metal–tribasic isocitrate; for example, the K_m for free dibasic

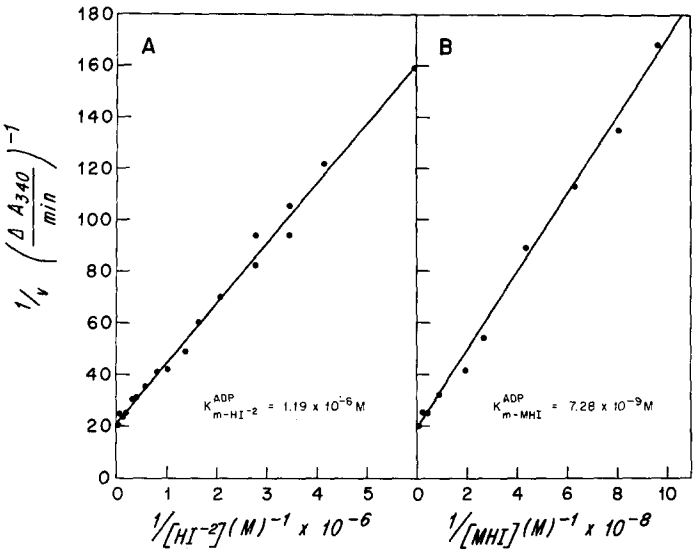


Fig. 5. K_ms for isocitrate and manganese in the presence of ADP. (A) Total D_s-isocitrate concentration was varied over a 143-fold range in the presence of 1 mM $MnSO_4$–1 mM NAD–0.5 mM ADP–triethanolamine–36 mM HCl (pH 7.1). $[M^{2+}]$ was observed to vary 1.10-fold as the total isocitrate concentration was raised from 0.007 to 0.2 mM and 1.47-fold as total isocitrate concentration was raised from 0.2 to 1 mM. The reciprocals of the observed velocities are plotted versus the reciprocals of the free dibasic isocitrate concentrations. Plots of $1/v$ versus the reciprocals of the concentrations of MHI, MI^{1-} and I_t also exhibited linearity and the same maximal velocity. (B) Total manganesee concentration was varied over a 66-fold range in the presence of 1 mM D_s-isocitrate–1 mM NAD–2 mM ADP–triethanolamine–36 mM HCl (pH 7.1). $[HI^{2-}]$ was observed to vary 1.05-fold. The reciprocals of the observed velocities are plotted versus the reciprocals of the manganese–dibasic isocitrate complex concentrations. Plots of $1/v$ versus the reciprocals of the concentrations of M^{2+}, MI^{1-} and M_t also exhibited linearity and the same maximum velocity.

isocitrate at pH 7.1 in the presence of 1 mM $MnSO_4$ is lowered from 61.7 μM in the absence of ADP to 1.19 μM in the presence of 0.5 mM ADP. It was noted in Table X that K_m values were measurable for the metal chelates of both dibasic and tribasic isocitrate at pH 7.1 in the presence of 0.5 mM ADP; the corresponding values in the absence of ADP were not measurable by Lineweaver–Burk plot analysis, as was noted above. ADP apparently lowers the K_m for total isocitrate to such an extent that with the concentrations of I_t required over the experimental range, the concentrations of MHI and MI^{1-} vary proportionately with the concentrations of total isocitrate and

TABLE X

OBSERVED MICHAELIS CONSTANTS FOR ISOCITRATE IN THE PRESENCE OF ADP
All reaction mixtures contained 1 mM $MnSO_4$, 1 mM NAD and buffer as described in Methods.

pH	[ADP]concn (mM)	$K^{ADP}_{m-I_t}$ (μM)	$K^{ADP}_{m-HI^{2-}}$ (μM)	K^{ADP}_{m-MHI} (μM)	$K^{ADP}_{m-MI^{1-}}$ (μM)
7.1	0.5	47.8	1.19	0.0363	15.9

free dibasic isocitrate; a measurement of K_m values for MHI and MI^{1-} from linear Lineweaver–Burk plots is, therefore, possible at pH 7.1 in the presence of ADP.

Michaelis constants for manganese species were determined by varying the total manganese concentration in the presence of 1.0 mM isocitrate, 1.0 mM NAD and ADP. At pH 6.1, one experiment was performed with 2 mM ADP; at pH 7.1, two experiments were performed with 0.5 and 2 mM ADP. Fig. 5B contains representative data at pH 7.1 from which K^{ADP}_{m-MHI} was obtained. As was observed for the K_m data for isocitrate in the presence of ADP, no substrate inhibition or non-linearity was noted in any of the Lineweaver–Burk plots for the manganese K^{ADP}_m data. The observed Michaelis constants in the presence of ADP are tabulated in Table XI and may be compared with the corresponding constants in the absence of ADP located

TABLE XI

OBSERVED MICHAELIS CONSTANTS FOR MANGANESE IN THE PRESENCE OF ADP
All reaction mixtures contained 1 mM *threo*-D_s-isocitrate, 1 mM NAD and buffer as described in Methods.

pH	[ADP]concn (mM)	$K^{ADP}_{m-M_t}$ (μM)	$K^{ADP}_{m-M^{2+}}$ (μM)	K^{ADP}_{m-MHI} (μM)	$K^{AD}_{m-MI^{1-}}$ (μM)
6.1	2.0	9.01	0.592	0.0106	0.475
7.1	0.5	41.1	3.77	0.0101	4.43
7.1	2.0	113	2.98	0.00728	3.39

in Tables I and II. At both pH values it is observed that $K^{ADP}_{m-M_t}$ in the presence of 2 mM ADP is actually higher than the corresponding K_m in the absence of ADP; however, the K_m values for the species free manganous ion, manganese–dibasic isocitrate and manganese–tribasic isocitrate are 5–10-fold lower in the presence of 2 mM ADP than in the absence of ADP. These opposite effects may be explained by the chelation of manganese by ADP [14] and by direct modification of the enzyme by the binding of ADP. In the presence of a relatively high concentration of ADP, much of the manganese in solution is bound by ADP, thereby reducing the actual amount of manganese available as substrate; the effect of chelation by ADP is therefore to raise the apparent K_m for total manganese. ADP is also suggested to bind to a regulatory site of the enzyme thereby lowering the Michaelis constants for the ionic species of substrate. Further support for the above model for the modification of kinetic parameters by ADP is derived from the observation in Table XI that at pH 7.1 lowering the concentration of ADP from 2 to 0.5 mM results in almost identical values for the K_m values of manganous species but in a value for $K^{ADP}_{m-M_t}$ which is lower than even $K^{obs}_{m-M_t}$ in the absence of ADP. An ADP concentration of 0.5 mM under the experimental conditions, therefore, appears to saturate the regulatory binding site on the enzyme, and raising the ADP concentration to 2 mM only functions to remove manganese from the ligand pool by chelation.

pH–rate profiles

The simplest and most common interpretation of pH–rate profiles constructed under saturating substrate conditions is that they represent the titration curve of an

amino acid residue of the enzyme–substrate complex. This same interpretation has been made in the present case although it is noted that in certain cases more complex explanations have been proposed and required.

In the present study, pH–rate profiles were constructed at three temperatures, 12, 22 and 32 °C, in order to permit the calculation of both the pK_{es} and the heat of ionization of an essential amino acid residue. With these two values, a more reliable proposal for the nature of an essential residue can be made than if only one is available. In addition, at each temperature, pH–rate profiles were determined with two sets of substrate concentrations in order to assess the state of saturation of the enzyme over the entire pH range. If the two curves obtained with the two sets of substrate concentrations diverged, then a mixture of effects of pH on apparent K_m and apparent V was implied; such portions of the pH–rate profile could, therefore, not be relied upon to exhibit true maximal velocities.

The dependence of observed "maximal" velocities on pH at 12, 22 and 32 °C is shown in Fig. 6. It has been concluded that there is only one essential residue ionizable in the pH range 5.5–8.6. Two observations support this conclusion: (1) at

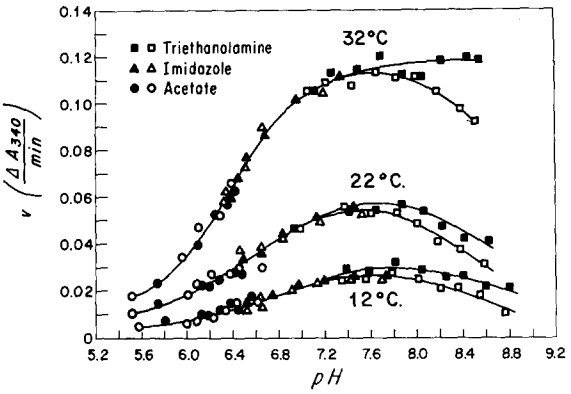


Fig. 6. pH–rate profiles. The dependence of observed enzymatic velocity is plotted as a function of pH at 12, 22 and 32°C. ■ and □, triethanolamine–36 mM HCl; ▲ and △, imidazole–36 mM HCl; ● and ○, 36 mM sodium acetate–acetic acid. Solid symbols, 120 mM DL-isocitric acid–6 mM NAD–2 mM $MnSO_4$; open symbols, 60 mM DL-isocitric acid–3 mM NAD–1 mM $MnSO_4$.

all temperatures the observed maximal velocities between pH 5.5 and 7.5 are almost identical for the two sets of substrate concentrations used. However, above pH 7.5 the observed velocities with the two sets of substrate concentrations diverge, suggesting that with the set of lower substrate concentrations saturation of the enzyme was not achieved. A question, therefore, concerning the saturation of the enzyme with the higher set of concentrations is also raised. It is possible that the higher set of substrate concentrations also does not permit the observation of true maximal velocities above pH 7.5 and that the decrease in velocities above pH 7.5 are due solely to K_m effects. It is observed that at 32 °C with the higher concentrations of substrates a constant maximum velocity is attained above pH 7.5, indicating that K_m values may decrease as temperature is raised. The possibility that there is a second essential ionizable group on the enzyme–substrate with a pK between pH 7.5 and 8.6 thus

seems unlikely. (2) The phenomena noted above are consistent with the K_m studies performed at pH 6.1 and 7.1. It was observed that the K_m for total isocitrate at pH 7.1 is approximately 20-fold higher than the corresponding K_m at pH 6.1; however, the K_m value for the dibasic and chelated dibasic forms of isocitrate are quite similar at both pH values. The explanation appears to be that as pH is raised a decreasing proportion of isocitrate is found in the active dibasic forms and that the apparent K_m for total isocitrate, therefore, rises. The concentrations of isocitrate (0.03 and 0.06 M in D$_s$-isocitrate) used in the pH–rate studies were chosen to be 7.5 and 15 times greater than the K_m for total isocitrate at pH 7.1. If, therefore, the K_m for total isocitrate were to rise by as much as 5-fold from pH 7.1 to 8, non-saturating conditions would result with those concentrations of isocitrate used in these studies. Observed velocities would, therefore, not be maximal.

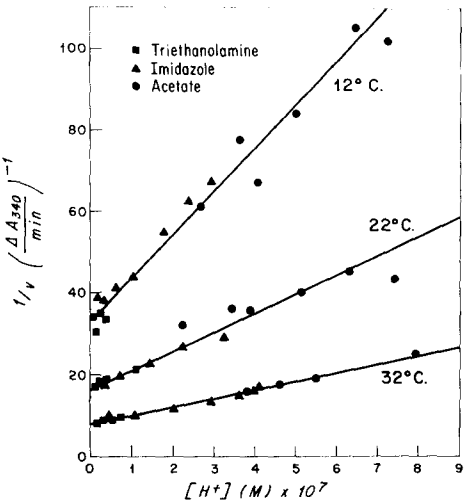


Fig. 7. Determination of pK_{es} at 12, 22 and 32 °C. The reciprocals of observed enzymatic velocities from the high substrate concentration curves of Fig. 6 are plotted versus hydrogen ion concentration. Eqn 16 permits the calculation of pK_{es} to be 6.51, 6.47 and 6.43 at 12, 22 and 32 °C, respectively. ■, triethanolamine–36 mM HCl; ▲, imidazole–36 mM HCl; ●, 36 mM sodium acetate–acetic acid. All velocities were measured in the presence of 120 mM D,L-isocitric acid–6 mM NAD–2 mM $MnSO_4$.

The values of pK_{es} at 12, 22 and 32 °C have been determined from Fig. 7 to be 6.51, 6.47 and 6.43, respectively, by using the relationship

$$v = \frac{V}{1 + \frac{[H^+]}{K_{es}}} \tag{16}$$

where K_{es} is the dissociation constant for an essential ionizable group in the enzyme–substrate complex. As noted by Cohn and Edsall [18], a pK_a of 6.5 is in the range of an imidazolium ring, is high for a carboxyl group, and is low for an α-ammonium group.

Heat of ionization of an essential amino acid residue

Van 't Hoff's law [19] may be written in the form:

$$d(pK_{es}) = \frac{\Delta H_i}{2.303\,R}\, d\left(\frac{1}{T}\right) \tag{17}$$

where T is expressed in °K and $R = 1.986$ cal/°K per mole. In Fig. 8, Eqn 17 has been applied to a plot of pK_{es} versus 1/T to calculate the heat of ionization of an essential amino acid residue to be $\Delta H_i = +1.56$ kcal/mole. Values of $\Delta H_i = +1.56$ kcal/mole and $pK_{es} = 6.47$ at 22 °C have been calculated for the ionization of an essential residue in calf heart isocitrate dehydrogenase. The ΔH_i value falls approximately in the range $\Delta H_i = \pm 1.5$ kcal/mole common for carboxyl residues as listed by Cohn and Edsall [18]. No other amino acid residues have ΔH_i values in this range, including the imidazolium ring of histidine ($\Delta H_i = +6.9$–7.5 kcal/mole, $pK = 5.6$–7.0 at 25 °C) or the α-ammonium group ($\Delta H_i = +10$ kcal/mole; $pK = 7.6$ –8.4 at 25 °C).

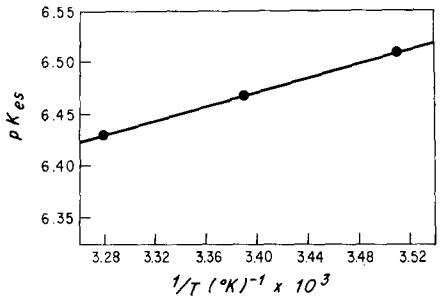


Fig. 8. Determination of the heat of ionization of an essential residue of the enzyme–substrate complex. pK_{es} is plotted as a function of the reciprocal of temperature. Eqn 17 permits the calculation of $\Delta H_i = +1.56$ kcal/mole from the slope of the linear function.

Although a pK of 3.0–4.7 is commonly found for carboxyl residues [18], the pK may be significantly higher if the carboxyl group is located in a hydrophobic environment. An upward shift of the pK for the enzyme–substrate complex might also be accomplished by a negatively charged environment, which in this case could be provided by the dibasic isocitrate species or by nearby negatively charged amino acid residues. The correlation of chemical data and structural analysis for lysozyme has demonstrated that in this enzyme the residue Glu-35 has a pK_a of about 6.3 while functioning as a general acid catalyst [20, 21]. By chemical modification and kinetic studies, Colman [10] has identified a glutamyl residue in the pig heart NADP-linked isocitrate dehydrogenase which is essential for catalytic function. The pK_{es} of this residue is 5.7 and is independent of temperature in the range 10–30 °C. The serine proteases, in particular the α-lytic proteases of *Myxobacter* 495, appear to have aspartic acid residues with pK values of approx. 6.7 [22]. Well documented precedents do exist, therefore, for the pK of a carboxyl residue being shifted well out of the range generally considered typical for that class of amino acids.

On the basis of the above observations it is suggested that calf heart isocitrate dehydrogenase contains an essential residue, either aspartyl or glutamyl, with $pK_{es} =$

6.47 at 22 °C and ΔH_i = 1.56 kcal/mole. At present, the mechanistic role of such a residue is unclear without further knowledge of the structure and function of the active site of the enzyme.

Activation energy of the enzyme catalyzed reaction

The empirical Arrhenius equation:

$$d(\log k) = -\frac{E_a}{2.303\,R}\,d\left(\frac{1}{T}\right) \tag{18}$$

may be applied to a plot of log V versus 1/T to determine E_a from the slope of the linear function. V may be substituted in the Arrhenius equation for k (the reaction velocity constant) in order to determine E_a, since V will alter the position of the function without changing the slope. Values for V at 12, 22 and 32 °C were obtained from Fig. 7. Values for V at 17, 27 and 37 °C were calculated from values of K_{es} read from Fig. 8 and from individual velocities measured at known pH and temperature. The activation energy of the oxidative decarboxylation reaction catalyzed by isocitrate dehydrogenase from calf heart has been calculated as E_a = 12.6 kcal/mole in Fig. 9.

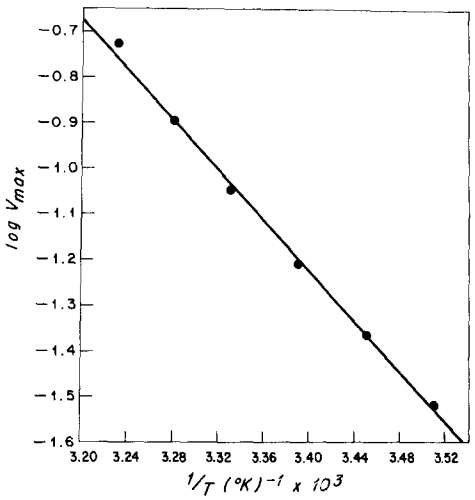


Fig. 9. Determination of the activation energy of the enzyme-catalyzed reaction. Log V is plotted versus the reciprocal of temperature. Values of V at 12, 22 and 32 °C were obtained from Fig. 7; values of V at 17, 27 and 37 °C were calculated from values of K_{es} from Fig. 8 and individual velocities measured at known pH and temperature. Eqn 18 permits the activation energy of the enzyme catalyzed reaction to be calculated as E_a = 12.6 kcal/mole.

That the activation energy of the overall reaction catalyzed by calf heart isocitrate dehydrogenase is constant over the range of 12–37 °C indicates that several phenomena do not occur with this enzyme. The oxidative decarboxylation of isocitric acid must be a complex reaction with a rate constant for each individual reaction step. It is possible for different reaction steps to be rate limiting at different temperatures for an overall reaction and hence for E_a to change sharply at a specific temper-

ature, as Stern [23] has proposed for pancreatic lipase, invertase and trypsin. Kistiakowsky and Lumry [24] and Sizer [25] have proposed two alternate explanations for the temperature dependent nature of the activation energy of urease. According to these respective investigators, the activation energy for urease changes at 22 °C due to reversible inactivation or to the existance of two conformational forms of differing activities. Over the temperature range investigated for calf heart isocitrate dehydrogenase, it appears that both the activity-dependent conformation and the rate-determining steps remain invariant. The observation justifies the implicit assumption that in studying the pK of enzyme–substrate complex at several temperatures the rate-determining step and the essential ionizable group involved remained the same.

APPENDIX

Note 1. By substitution into Eqn 5 with the dissociation constant expressions, two algebraically related equations (Eqns 6 and 7) may be derived:

$$\frac{K_{2b}}{[HI^{2-}]} = \frac{[E\text{–}M]}{[E\text{–}MHI]} = \frac{\dfrac{[E]\,[M^{2+}]}{K_{2a}}}{[E\text{–}MHI]} = \frac{[E]}{[E\text{–}MHI]} \cdot \frac{[M^{2+}]}{K_{2a}} = \frac{K_{1b}}{[MHI]} \cdot \frac{[M^{2+}]}{K_{2a}}.$$

Also:

$$\frac{K_{3b}}{[M^{2+}]} = \frac{[E\text{–}HI]}{[E\text{–}MHI]} = \frac{\dfrac{[E]\,[HI^{2-}]}{K_{3a}}}{[E\text{–}MHI]} = \frac{[E]}{[E\text{–}MHI]} \cdot \frac{[HI^{2-}]}{K_{3a}} = \frac{K_{1b}}{[MHI]} \cdot \frac{[HI^{2-}]}{K_{3a}}.$$

By substituting for $K_{3b}/[M^{2+}]$ and $K_{2b}/[HI^{2-}]$ in Eqn 5 and factoring $K_{1b}/[MHI]$ in the denominator, Eqn 6 may be obtained.

$$v = \frac{V}{1 + \dfrac{K_{1b}}{[MHI]}\left(1 + \dfrac{[M^{2+}]}{K_{2a}} + \dfrac{[HI^{2-}]}{K_{3a}}\right)} \tag{6}$$

Alternatively, by dividing both the numerator and denominator of Eqn 5 by $(1 + K_{2b}/[HI^{2-}])$,

$$v = \frac{\dfrac{V}{\left(1 + \dfrac{K_{2b}}{[HI^{2-}]}\right)}}{1 + \dfrac{\dfrac{K_{1b}}{[MHI]} + \dfrac{K_{3b}}{[M^{2+}]}}{\left(1 + \dfrac{K_{2b}}{[HI^{2-}]}\right)}}$$

is obtained. By substituting for $K_{3b}/[M^{2+}]$ in the above equation and then factoring out $K_{1b}/[MHI]$, Eqn 7 may be derived.

$$v = \frac{\dfrac{V}{\left(1 + \dfrac{K_{2b}}{[HI^{2-}]}\right)}}{1 + \dfrac{K_{1b}}{[MHI]} \dfrac{\left(1 + \dfrac{[HI^{2-}]}{K_{3a}}\right)}{\left(1 + \dfrac{K_{2b}}{[HI^{2-}]}\right)}} = \frac{V'}{1 + \dfrac{K_{1b}}{[MHI]} \dfrac{\left(1 + \dfrac{[HI^{2-}]}{K_{3a}}\right)}{\left(1 + \dfrac{K_{2b}}{[HI^{2-}]}\right)}} \tag{7}$$

Note 2. Eqn 14 may be derived from Eqn 11 by substitution of the dissociation constant expressions and by rearrangement. From *Note 1* of the Appendix:

$$\frac{K_{1b}}{[MHI]} = \frac{K_{2a}}{[M^{2+}]} \frac{K_{2b}}{[HI^{2-}]}$$

and, therefore,

$$v = \frac{\dfrac{V}{\left(1 + \dfrac{K_{2b}}{[HI^{2-}]}\right)}}{1 + \dfrac{K_{2a}}{[M^{2+}]} \dfrac{K_{2b}}{[HI^{2-}]} \dfrac{1}{\left(1 + \dfrac{K_{2b}}{[HI^{2-}]}\right)}}.$$

By rearrangement,

$$v = \frac{V}{1 + \dfrac{K_{2b}}{[HI^{2-}]} \left(1 + \dfrac{K_{2a}}{[M^{2+}]}\right)} \tag{14}$$

which is in conventional Michaelis–Menten form for HI^{2-}.

ACKNOWLEDGMENT

This work was supported in part by U.S.P.H.S. grants 5 R01-AM 17552-02 and 5 R01-AM-14635 and by a grant from the Delaware Heart Association.

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