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Diphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase from Pig Heart. Characterization of the Active Substrate and Modes of Regulation†

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ABSTRACT: This paper explores the hypothesis that only one of the ionic forms of isocitrate participates in the metal-dependent reaction catalyzed by DPN-specific isocitrate dehydrogenase and suggests that activators can function indirectly by modifying the distribution of the various forms of isocitrate. Determinations of the Michaelis constants for DPN and total isocitrate at 1.25 mM $MnSO_4$ showed that the K_m for DPN was relatively constant, but that the K_m for total isocitrate increased nearly 100-fold as the pH was raised from 6.0 to 8.0. Higher concentrations of $MnSO_4$ increased the K_m for total isocitrate at each pH. An analysis of the distribution of the various forms of isocitrate, facilitated by a computer program, revealed that the K_m for dibasic isocitrate (approximately 30.0 μM) was independent of both pH and manganese concentration. The observed variation in the K_m for total isocitrate can therefore be explained as a reflection of differential modifications of the equilibria between the ionic forms of isocitrate caused by changes in the concentrations of hydrogen and manganous ions. Free dibasic isocitrate is the actual substrate of the enzyme. Substrate inhibition, noted at pH 6, but not at 7 and 8, could be attributed to the binding of the manganous complex of dibasic isocitrate. The change of V_{max} with pH indicates the requirement for a basic form of an amino acid in the enzyme–substrate complex with a pK of 6.25. The DPN-dependent isocitrate dehydrogenase is activated by both citrate and nucleotides. "Apparent activation" results when chelating agents such as citrate, GDP, and UDP lower the K_m for total isocitrate by raising the relative proportion of the active species of isocitrate; the K_m calculated for dibasic isocitrate is unchanged. In contrast, ADP lowers the K_m for the active form of isocitrate, as well as for total isocitrate, suggesting that this nucleotide functions both as a chelating agent and as a specific allosteric modifier of enzyme activity. The demonstration that modifiers of enzyme activity can function indirectly, *via* their chelating ability, provides an alternate mechanism to that of allosteric regulation which should be considered when evaluating the kinetics of a metal-dependent enzyme.

The kinetics of the DPN-dependent isocitrate dehydrogenases [*threo*-D_s-isocitrate:NAD-oxidoreductase (decarboxylating), EC 1.1.1.41] have been extensively studied (Plaut and Aogaichi, 1968; Atkinson *et al.*, 1965; Sanwal and Cook, 1965). These enzymes, however, require divalent metal ions for activity and in the past the role of metal chelation of substrates has not generally been considered. Under physiological conditions, 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 (Grzybowski *et al.*, 1970). It seemed possible that only one of these four ionic species would specifically react with the enzyme. Any variation, therefore, in the concentrations of hydrogen and metal ions might alter the affinity of the enzyme for total isocitrate, but not for the active species of substrate. The active substrate for the pig heart TPN-dependent isocitrate dehydrogenase has previously been shown to be the metal chelate of the tribasic form of isocitrate (Colman, 1972c). A distinction in the form of isocitrate utilized by the two isocitrate dehydrogenases from the same organ and species might suggest a complementary role for the two enzymes *in vivo*.

The DPN-specific isocitrate dehydrogenases from several species have been shown to be activated by citrate and/or adenine nucleotides. In general, citrate modifies those dehydrogenases extracted from plants (Cox and Davies, 1967; Coultate and Dennis, 1969), while the adenine nucleotides modify the mammalian enzymes (Chen *et al.*, 1964; Stein *et al.*, 1967). Both citrate and adenine nucleotides, however, activate the enzymes from yeast and microorganisms (Hathaway and Atkinson, 1963; Sanwal and Cook, 1965; LéJohn *et al.*, 1969). Despite the fact that these positive effectors are all chelating agents, the possibility that they function indirectly by altering the distribution of the ionic forms of isocitrate in solution has not previously been explored. This paper reports kinetic data for the DPN-dependent isocitrate dehydrogenase from pig heart which not only allows the identification of the actual substrate of this reaction, but also analyzes the roles of different activators in terms of their effect on the Michaelis

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constant for the active substrate. Chelation is proposed as one possible mechanism for metabolic control of metal-dependent enzymes.

Experimental Section

Materials. The enzyme used for these experiments was extracted from pig heart and purified 500-fold by ammonium sulfate fractionation, chromatography on DEAE-cellulose, and gel filtration on Sepharose 6B, according to the method of Cohen and Colman (1971b). The studies were conducted with enzyme of specific activity 4–12 μmoles of DPNH/min per mg of protein. The results of kinetic experiments conducted with the cruder preparations were the same as those obtained with the purified enzyme. Substrates and coenzymes were purchased from the Sigma Chemical Co.

Assay. Isocitrate dehydrogenase activity was measured spectrophotometrically at 340 mμ using either a Cary Model 15 or a Gilford Model 240 equipped with a Model 242 recorder with an expanded scale (0.1 absorbance). The standard assay contained 0.99 mM DPN, 1.33 mM $MnSO_4$, 20.0 mM DL-isocitrate, 33.0 mM Tris-acetate buffer (pH 7.19), and enzyme in a total volume of 1.0 ml.

Three buffers were used for the pH studies: sodium acetate, imidazole chloride, and triethanolamine chloride. The concentration of the buffer anion was 0.036 M in all cases. For the K_m determinations at pH 6.0, 7.0, and 8.0, imidazole and triethanolamine buffers with 0.036 M chloride were selected because of their low affinity for metal ion. The substrates being tested were adjusted to the pH being studied. Solutions of DPN at pH 8.0 were used within 4 hr after being prepared. It has been shown (Cohen and Colman, 1971b) that the enzyme uses exclusively *threo*-D,-isocitrate as a substrate. The Michaelis constants reported here in terms of DL-isocitrate were found to be approximately twice those obtained for *threo*-D,-isocitrate at pH 6.0 and 7.0 and 1.25 mM $MnSO_4$. The formation of manganous chloride (K_{ass} = 3.7 M^{-1}) did not greatly influence the distribution of the ionic species of isocitrate, and was not considered in the following calculations. All studies were carried out using 0.05 ml of a stock solution of enzyme diluted with a 0.05 M Tris-acetate buffer (pH 7.19) containing 5.0 mM $MnSO_4$ and 40% glycerol. The concentrations of metal ion reported for the reaction mixtures in Results include that contributed by the enzyme solution.

Theory

The following section describes the method used to calculate the concentrations of the ionic forms of ligands present in the reaction mixture. In case I, the isocitrate present is distributed between free substrate and its metal complexes. In case II, the effect of an added activator, which is also a chelating agent, is considered. Unless indicated otherwise, manganese concentration refers to the total concentration of manganous sulfate (M_t) added and the term manganous ion indicates the free divalent cation (M^{2+}).

Case I. The standard reaction mixture for the DPN-dependent isocitrate dehydrogenase contains a buffer, isocitrate, manganous ion, and DPN. In the pH range tested, total isocitrate, (A_t), is primarily present in its dibasic (HA^{2-}), and tribasic (A^{3-}) forms and their corresponding metal complexes (MHA and MA^-). Total manganese is considered to be equal to the sum of free manganous ion and the metal chelates of isocitrate. Since the association constant for the formation of the manganese·DPN complex was found to be only 78.0 M^{-1} (Colman, 1972a), this species constituted a negligible proportion of the added metal under the conditions used and was not considered in the following equations. The mass conservation equations for total isocitrate and manganese form the basis for the calculation of the concentrations of the various ionic species present in the reaction mixture

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

$$(M_t) = M^{2+} + MHA + MA^- \quad (2)$$

The equilibrium constants of isocitrate for manganous and hydrogen ion were measured by Grzybowski *et al.* (1970), and are defined as follows: $Ka_1 = [(MHA)/(M^{2+})(HA^{2-})] = 57.6$ M^{-1}, $Ka_2 = [(MA^-)/(M^{2+})(A^{3-})] = 1150$ M^{-1}, and $Ka_3 = (H^+)(A^{3-})/(HA^{2-}) = 1.79 \times 10^{-6}$ M.

Substitution of these equilibrium constants into eq 1 and 2 results in 1a and 2a. Solving for HA^{2-} in terms of M^{2+} yields

$$(A_t) = (HA^{2-})\left[1 + \frac{Ka_3}{(H^+)} + Ka_1(M^{2+}) + \frac{Ka_2Ka_3(M^{2+})}{(H^+)}\right] \quad (1a)$$

$$(M_t) = (M^{2-})\left[1 + Ka_1(HA^{2-}) + \frac{Ka_2Ka_3(HA^{2-})}{(H^+)}\right] \quad (2a)$$

a quadratic equation in which M^{2+} is the only unknown.

$$0 = (M^{2+})^2P + (M^{2+})[(A_t)P + Q + 1 - (M_t)P] - (M_t)(Q + 1) \quad (3)$$

where the pH dependent terms, P and Q, are defined as follows: $P = Ka_1 + [Ka_2Ka_3/(H^+)]$ and $Q = Ka_3/(H^+)$. A computer program was devised to simplify the calculations of the square roots of eq 3. The real positive root was then substituted into eq 1a to calculate the concentration of dibasic isocitrate, HA^{2-}. The concentrations of the remaining ionic species of ligands were then derived from the equations defining Ka_1, Ka_2, and Ka_3.

Case II. When a chelating agent such as citrate or a nucleotide is added to the reaction mixture, a third mass conservation equation must be considered, where (C_t) represents the

$$(C_t) = HC^{2-} + C^{3-} + MHC + MC^- \quad (4)$$

total amount of added chelator which is also present in two ionic forms, (C^{3-} and HC^{2-}), and their corresponding metal complexes (MC^- and MHC). The presence of two additional metal chelates in the reaction mixture necessitates revision of the mass conservation equation for total manganese

$$(M_t) = M^{2+} + MA^- + MHA + MC^- + MHC \quad (5)$$

The equilibrium constants for metal and hydrogen ion of the chelate tested are listed in Table I and are defined in a manner analogous to those for isocitrate. Substitution of the appropriate constants into the three mass conservation equations results in three equations with three unknowns

$$(A_t) = (A^{3-})\left[\frac{(H^+)}{Ka_3} + 1 + \frac{Ka_1(M^{2+})(H^+)}{Ka_3} + Ka_2(M^{2+})\right] \quad (1b)$$

TABLE I: Equilibrium Constants for Manganous and Hydrogen Ions.

Chelator	Kc_1 (M^{-1})	Kc_2 (M^{-1})	Kc_3 (M)
Citrate	145[a]	14,100[a]	1.52×10^{-6}[a]
ADP	533[b]	20,400[b]	3.98×10^{-7}[c]
GDP	657[b]	13,600[b]	5.01×10^{-7}[c]
UDP	609[b]	8,590[b]	3.16×10^{-7}[c]

[a] Grzybowski *et al.* (1970). [b] Colman (1972a). [c] Bock (1960).

$$(C_t) = (C^{3-})\left[\frac{(H^+)}{Kc_3} + 1 + \frac{Kc_1(M^{2+})(H^+)}{Kc_3} + Kc_2(M^{2+})\right] \quad (4a)$$

$$(M_t) = (M^{2+})\left[1 + Ka_2(A^{3-}) + \frac{Ka_1(H^+)(A^{3-})}{Ka_3} + Kc_2(C^{3-}) + \frac{Kc_1(H^+)(C^{3-})}{Kc_3}\right] \quad (5a)$$

Rearrangement of eq 5a produces an equation in three unknowns

$$(M_t) = M^{2+} + (M^{2+})(A^{3-})(V) + (M^{2+})(C^{3-})(W) \quad (5b)$$

where V and W are the pH-dependent variables defined as follows: $V = Ka_2 + [Ka_1(H^+)/Ka_3]$ and $W = Kc_2 + [Kc_1(H^+)/Kc_3]$. Solving eq 1b and 4a for A^{3-} and C^{3-} allows substitution into eq 5b to produce the following equation in which M^{2+} is the only unknown

$$(M_t) = M^{2+} + \frac{(M^{2+})(A_t)V}{1 + \frac{(H^+)}{Ka_3} + (M^{2+})V} + \frac{(M^{2+})(C_t)W}{1 + \frac{(H^+)}{Kc_3} + (M^{2+})W} \quad (5c)$$

Expansion of eq 5c produces a third-order polynomial of 24 terms, the roots of which can be extracted using the scientific subroutine "DPRQD" supplied with the IBM/360 which calculates the roots of a real polynomial by means of the QD algorithm with displacement. Of the three possible roots, only one was real and positive and this value was used to obtain the concentrations of C^{3-} and A^{3-} from

$$(C^{3-}) = \frac{(M_t)}{(M^{2+})(W)} - \frac{V(A_t)}{W + \frac{W(H^+)}{Ka_3} + W(M^{2+})V} - \frac{1}{W} \quad (4b)$$

$$(A^{3-}) = \frac{(M_t)}{(M^{2+})(V)} - \frac{W(C_t)}{V + \frac{V(H^+)}{Kc_3} + V(M^{2+})W} - \frac{1}{V} \quad (1c)$$

The concentrations of all other ionic species can then be derived from eq 5a.

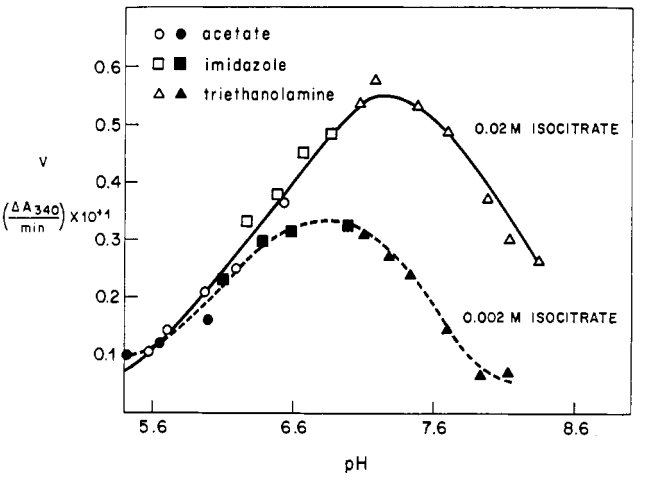

FIGURE 1: Variation of initial velocity with pH. The reaction mixtures contained 0.99 mM DPN, 1.33 mM $MnSO_4$, DL-isocitrate as indicated, buffer, and enzyme in a total volume of 1.0 ml. The three buffers used were sodium acetate, imidazole chloride, and triethenolamine chloride, 0.036 M in anion concentration.

Results

Active Form of Substrate. Figure 1 shows the curve obtained for the DPN-dependent isocitrate dehydrogenase when initial velocity is plotted *vs.* pH at two different concentrations of total isocitrate. The upper curve was obtained with 0.02 M isocitrate; the lower with 0.002 M. Between pH 5.5 and 6.5 the curves are essentially superimposable; however, as the pH is raised, the differences between the two curves become more apparent. This increasing disparity in the higher pH range suggested that the K_m for isocitrate might be pH dependent. Two possible explanations were considered. The apparent decrease in affinity for isocitrate could be either the result of the ionization of a functional group on the enzyme which is essential for substrate binding, or of a pH-dependent decrease in the concentration of the active species of the substrate isocitrate. In order to elucidate the effects of pH on the DPN-dependent isocitrate dehydrogenase, it was decided to study the affinity of the enzyme for isocitrate at pH 6.0, 7.0, and 8.0.

The first column in Table II shows the Michaelis constants for total isocitrate obtained from Lineweaver–Burk plots of the data for the K_m determinations of isocitrate at pH 6.0, 7.0, and 8.0 in the presence of 1.25 mM $MnSO_4$ and saturating concentrations of DPN (0.99 mM). It is apparent that the K_m for total isocitrate varies with pH. The fact that the K_m for

TABLE II: Michaelis Constants for Isocitrate in the Presence and Absence of Citrate.[a]

	No Citrate		1.5 mM Citrate	
pH	$K_m(A_t)$ (μM)	$K_m(HA^{2-})$ (μM)	$K_m(A_t)$ (μM)	$K_m(HA^{2-})$ (μM)
6.0	150	30	36	26
7.0	980	31	490	26
8.0	11,000	32	7200	32

[a] The Michaelis constants for DL-isocitrate were determined in the presence of 1.25 mM $MnSO_4$ as described in the Experimental Section.

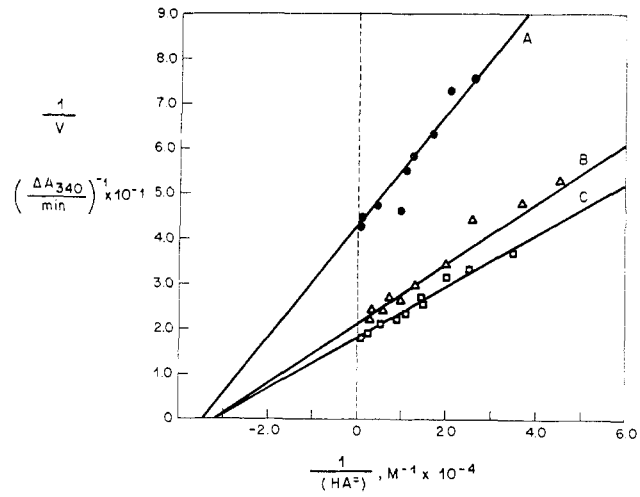

FIGURE 2: Determination of the Michaelis constants for dibasic isocitrate. This graph is a composite of the data obtained at pH 6.0 (line A), pH 8.0 (line B), and pH 7.0 (line C) plotted with dibasic isocitrate, HA^{2-} as substrate. The Michaelis constants are 30, 32, and 31 M for pH 6, 8, and 7, respectively. All experiments were performed with DL-isocitrate in the presence of 1.25 mM $MnSO_4$ as described in the Experimental Section.

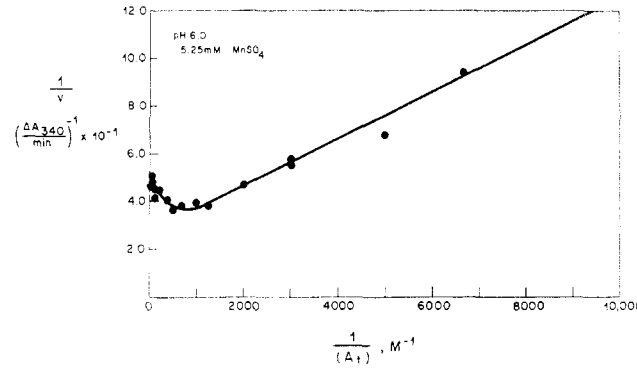

FIGURE 3: Substrate inhibition. This figure illustrates the Lineweaver–Burk plot obtained from the K_m determination for total DL-isocitrate in the presence of 5.25 mM $MnSO_4$ at pH 6.0 as described in the Experimental Section.

total isocitrate increases almost 100-fold as the pH is raised from pH 6.0 to 8.0 suggested that the active substrate might be a protonated form of isocitrate. If the concentrations of each of the four possible forms of isocitrate are calculated and the data are replotted using each of these species as substrate, the apparent variation in the K_m for isocitrate disappears only if the dibasic form of isocitrate, HA^{2-}, is considered to be substrate as recorded in column 2 of Table II. The linearity of the Lineweaver–Burk plots is illustrated in Figure 2 which is a composite of the data at all three pH's when HA^{2-} is considered as substrate. Although the V_{max} of the reaction varies with pH, the K_m in terms of HA^{2-} is relatively constant from pH 6.0 to 8.0.

An increase in the total manganese concentration might be expected to modify the distribution of the various forms of isocitrate by relatively decreasing the amounts of free dibasic and tribasic isocitrate and increasing the amounts of the chelates in solution. Therefore, if HA^{2-} is the active form of substrate, higher manganese concentrations should effectively raise the K_m in terms of total isocitrate, but have no effect on the K_m for HA^{2-}. Table III reports K_m values for total isocitrate as determined in the presence of 1.25–10.25 mM $MnSO_4$

TABLE III: Michaelis Constants for Isocitrate with Increasing Concentrations of Manganese.[a]

	pH 6.0		pH 7.0	
$MnSO_4$ (mM)	$K_m(A_t)$ (μM)	$K_m(HA^{2-})$ (μM)	$K_m(A_t)$ (μM)	$K_m(HA^{2-})$ (μM)
1.25	154	30	980	31
5.25	260	21	3100	27
10.25	400	21	9100	31

[a] The Michaelis constants for total DL-isocitrate and the dibasic form of substrate were determined as described in Experimental Section.

at pH 6 and 7. Higher metal concentrations effect a 10-fold increase in the K_m for total isocitrate at pH 7.0, but only a 2.5-fold increase at pH 6.0. This observation reflects the fact that the relative proportion of dibasic isocitrate is much smaller at pH 7.0, and thus more sensitive to changes in total metal concentration. However, at both values of pH, the K_m for HA^{2-} is little influenced by increasing manganese concentrations. Dibasic isocitrate is, therefore, the active form of the substrate isocitrate and its K_m is independent of both pH and metal concentration.

Substrate Inhibition. When the Michaelis constant for isocitrate was determined at 5.25 mM $MnSO_4$, substrate inhibition was observed with high concentrations of isocitrate at pH 6.0 as illustrated by the Lineweaver–Burk plot in Figure 3. This inhibition was not observed at pH 7 and 8, suggesting that a protonated form of isocitrate was responsible. Since this inhibition was more apparent at higher metal concentrations, it seemed likely that an isocitrate chelate was involved. The experiment in Figure 3 demonstrates the decrease in velocity observed when both the free and the metal bound forms of isocitrate are increased by raising the concentration of total isocitrate (A_t). Therefore, the two-part experiment shown in Figure 4 was devised in order to differentiate between substrate inhibition caused by a free species of isocitrate and inhibition caused by an isocitrate–metal complex. The upper graph (Figure 4A) illustrates the inhibition observed when the concentrations of the metal chelates are raised while the free forms of isocitrate are kept constant by increasing both the isocitrate and metal concentrations. This 30% decrease in velocity is well outside the experimental error of approximately 3% in the rate measurements. The lower graph (Figure 4B) indicates that no change in velocity is observed when the concentrations of the free forms of isocitrate are raised while the metal chelates are kept constant by lowering the total manganese concentration as the total isocitrate concentration is raised. This experiment demonstrates that high concentrations of a metal chelate of isocitrate can inhibit the DPN-dependent isocitrate dehydrogenase reaction.

Effect of pH on K_{DPN}. Experiments were also carried out to examine the effect of the hydrogen ion concentration on the affinity of the DPN-dependent isocitrate dehydrogenase for DPN in the presence of 1.25 mM $MnSO_4$. At pH 6.0 and 7.0, when isocitrate was present at a concentration equal to 20 times the K_m for HA^{2-} at that pH, the extrapolated Michaelis constants from linear Lineweaver–Burk plots for DPN were 74 and 78 μM, respectively. At pH 8.0, a concentration of HA^{2-}

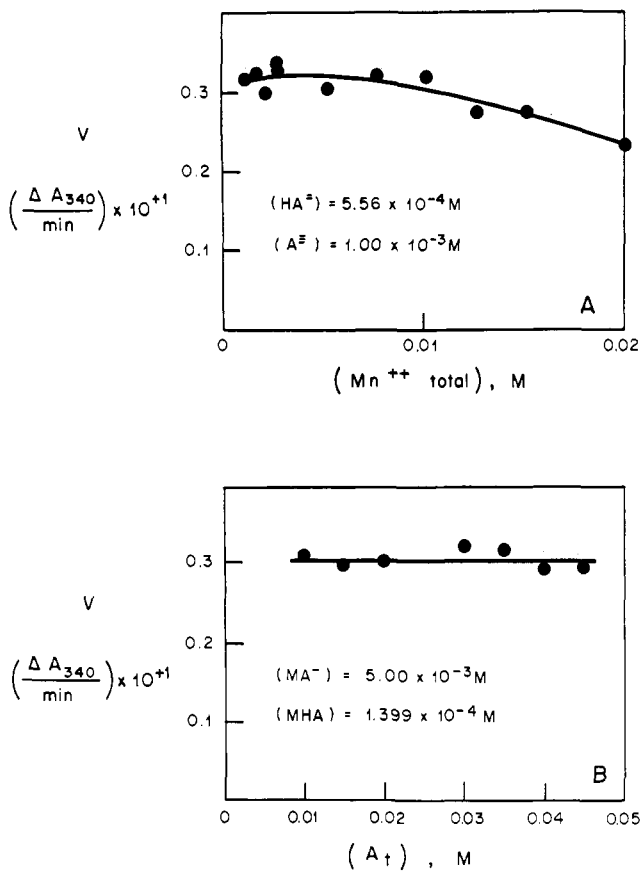

FIGURE 4: Analysis of substrate inhibition. For Figure 4A the metal chelates of isocitrate were increased and the free forms of isocitrate were kept constant as indicated. For Figure 4B the metal chelates of isocitrate were maintained constant as the concentrations of the free forms of isocitrate were raised.

as high as twenty times the K_m would have altered significantly the ionic strength of the reaction mixture. Instead, the apparent affinity for DPN was determined at two different concentrations of dibasic isocitrate (0.105 and 0.271 mM) and the intrinsic affinity for DPN was calculated to be 110 μM from the following equation (Ingraham and Makower, 1954)

$$K_{app} = \frac{K_{DPN} + \frac{K_x}{(HA^{2-})}}{1 + \frac{K_{HA^{2-}}}{(HA^{2-})}}$$

where K_{app} is the observed Michaelis constant for DPN at a particular concentration of the second substrate, HA^{2-}, whose Michaelis constant is $K_{HA^{2-}}$; K_{DPN}, the intrinsic Michaelis constant for DPN, and K_x, a complex constant, are the two unknowns in this equation. A fivefold increase in the total manganese concentration did not influence K_{DPN} at pH 7.0.

pH Dependence of Maximum Velocity. It is evident that the V_{max} of the DPN-dependent isocitrate dehydrogenase reaction does change with the concentration of hydrogen ion. By assuming the simplest model, which postulates that the V_{max} depends on the presence of the basic form of one ionizable group in the enzyme–substrate complex, it is possible to assign a value to the pK of this group from the equation (Alberty, 1956)

$$V_{max_0} = \frac{V_{max_i}}{1 + [(H^+)/K_{aes}]}$$

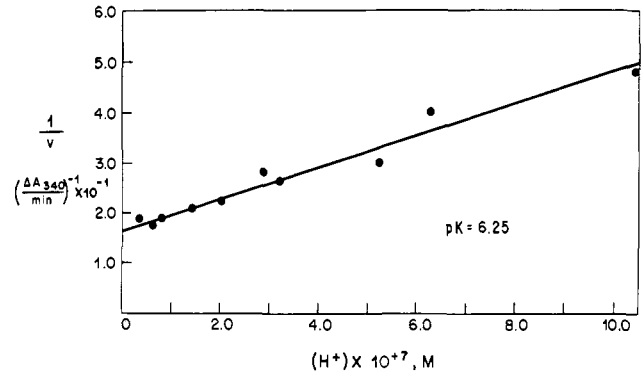

FIGURE 5: Determination of the pK of the enzyme–substrate complex. The data obtained between pH 6.0 and 7.0 in Figure 1 are plotted here in accordance with the following equation: $1/V_{max_0} = \{1/V_{max_i} + [(H^+)/(V_{max_i})(K_{aes})]\}$. The reaction mixtures contained substrates and buffer as described previously.

where V_{max_0} is the observed maximum velocity at a given hydrogen ion concentration, V_{max_i} is the intrinsic maximum velocity, and K_{aes} is the dissociation constant of an activity-dependent ionizable group in the enzyme–substrate complex. Figure 5 shows the line obtained when the data from Figure 1 are replotted to examine the pH variation of the intial velocity in the range in which the enzyme is saturated with all substrates. The slope of the line reveals the requirement for a basic form of a functional group in the enzyme–substrate complex with a pK_{aes} of 6.25.

Effect of Activators on Michaelis Constant for Isocitrate. Citrate, a proposed activator of DPN-dependent isocitrate dehydrogenases from several sources, does indeed increase the velocity of the reaction catalyzed by the pig heart enzyme at all three pH values when low concentrations of isocitrate are present. In fact, a plot of initial velocity *vs.* pH in the presence of 0.002 M isocitrate and 0.0025 M citrate, analogous to Figure 1, showed that citrate addition shifted the pH optimum from pH 6.8 to 7.1. Since citrate is a chelating agent, the possibility existed that citrate could activate by competing with isocitrate

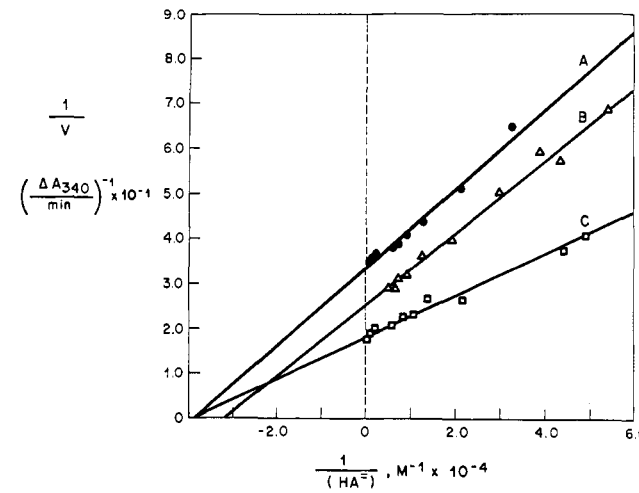

FIGURE 6: Effect of citrate on Michaelis constant for isocitrate. This graph is a composite of the data obtained at pH 6.0 (line A), 8.0 (line B), and 7.0 (line C) plotted with dibasic isocitrate, HA^{2-}, as substrate. The Michaelis constants are 26, 32, and 26 μM at pH 6.0, 8.0, and 7.0, respectively. All experiments were performed with DL-isocitrate in the presence of 1.5 mM citrate and 1.25 mM $MnSO_4$ as described in the Experimental Section.

TABLE IV: Effect of Nucleotides on the Michaelis Constant for Isocitrate.[a]

Additions	pH 6.0		pH 7.0	
	$K_m(A_t)$ (μM)	$K_m(HA^{2-})$ (μM)	$K_m(A_t)$ (μM)	$K_m(HA^{2-})$ (μM)
None	150	30	980	31
GDP	100	27		
UDP	110	27		
ADP	17	3.5	91	3.5

[a] The Michaelis constants for total DL-isocitrate and the dibasic form of substrate were determined as described in the Experimental Section in the presence of 1.25 mM $MnSO_4$. In all cases, the final concentration of added nucleotide was 1.0 mM.

for manganous ion, thereby effectively increasing the concentration of HA^{2-} in solution. Table II demonstrates that when the Michaelis constants for isocitrate is determined in the presence of 1.5 mM citrate and 1.25 mM $MnSO_4$ at pH 6.0, 7.0 and 8.0, the K_m in terms of A_t is lowered at each pH. Figure 6 illustrates the linearity of the Lineweaver–Burk plots obtained when HA^{2-} is considered to be substrate. Although the variation of V_{max} with pH remains, the K_m for HA^{2-} remains essentially constant (Table II). Similar results are obtained with 5.25 mM $MnSO_4$ and 2.5 mM citrate.

Table IV demonstrates that for the pig heart enzyme the K_m for total isocitrate is appreciably decreased by the addition of 1.0 mM ADP to the reaction mixture at both pH 6.0 and 7.0. Unlike citrate, however, ADP influences the Michaelis constants both for total isocitrate and for the active substrate, HA^{2-}. In fact, the K_m in terms of HA^{2-} is reduced about 10-fold, suggesting that ADP may interact directly with the enzyme to increase the affinity for the substrate isocitrate. Table IV also lists the results of K_m determinations for isocitrate in the presence of 1.0 mM GDP and UDP at pH 6.0. Although both nucleotides lower the K_m in terms of total isocitrate, neither directly affects the K_m for the active substrate, HA^{2-}. In all of these cases, the maximum velocity does not appear to be influenced by the addition of nucleotides.

Discussion

The three experimental observations which must be considered when determining the active form of the substrate isocitrate are as follows: (1) the K_m for A_t decreases as the pH is lowered; (2) higher manganese concentrations increase the K_m for A_t; and (3) citrate addition lowers the K_m for A_t. The 100-fold increase in the K_m for total isocitrate as the pH is raised from 6.0 to 8.0 implicates a protonated species of isocitrate, either HA^{2-} or MHA, as the active substrate. The higher values for the K_m of total isocitrate with elevated concentrations of manganese suggest that HA^{2-} is the more likely participant in the reaction. These results are consistent with the inhibition of the porcine liver enzyme by manganese above 1.5 mM which was reported by Plaut and Aogaichi (1968). The fact that these authors did not observe inhibition by magnesium may reflect in part the stronger affinity of manganese for isocitrate (Grzybowski *et al.*, 1970).

Since neither the concentrations of hydrogen or manganous ion, nor the presence of citrate influence the K_m in terms of HA^{2-}, the simplest interpretation of the data is that free dibasic isocitrate is the active substrate. The effects of higher manganese concentrations and citrate addition can then be explained as the indirect result of their ability to modify the concentration of HA^{2-} in solution.

If MHA were the active form of isocitrate, competition with free manganous ion (M^{2+}) would have to be postulated in order to account for the lower affinity for total isocitrate at higher concentrations of manganese. In this case, increasing concentrations of manganese would necessarily raise the concentrations of both the substrate (MHA) and the inhibitor (M^{2+}), and citrate would activate by chelating the inhibitory manganous ion. Because Lineweaver–Burk plots using either HA^{2-} or MHA as substrate appear linear, the experimental data fit the following equation, where K_{obsd} is a constant. If

$$v = \frac{V}{1 + \frac{K_{obsd}}{(S)}}$$

HA^{2-} is the active form of substrate, K_{obsd} would be expected to be constant under all experimental conditions. That this is indeed true is illustrated by the kinetic data. However, if MHA is the active substrate, the equation must be modified to include competitive inhibition with free manganous ion

$$v = \frac{V}{1 + \frac{K_{MHA}}{(MHA)}\left(1 + \frac{(M^{2+})}{K_i}\right)}$$

where K_{MHA} and K_i are the dissociation constants for the enzyme·MHA and the enzyme·M^{2+} complexes, respectively. Substitution of the appropriate equilibrium constants allows the following rearrangement of the equation in which K_{obsd} is

$$v = \frac{V}{1 + \frac{K_{MHA}}{56.7(HA^{2-})}\left(\frac{1}{(M^{2+})} + \frac{1}{K_i}\right)}$$

now equal to $(K_{MHA}/57.6)[(1/M^{2+}) + (1/K_i)]$. It is apparent that a constant value for both K_{MHA} and K_i cannot be calculated from this relationship. Indeed, the values for these constants calculated directly from the data are dependent on the concentration of manganous ion in every case. Since a relatively constant value can be calculated for the Michaelis constant of HA^{2-} under all experimental conditions, free dibasic isocitrate must be the active form of isocitrate which combines with the DPN-dependent isocitrate dehydrogenase from pig heart. It should be pointed out that this analysis does not imply that manganous ions are unnecessary for catalysis of the isocitrate dehydrogenase reaction: addition of a chelating agent, such as EDTA, to an assay mixture totally abolishes activity. The results of this paper bear only on the active species of isocitrate and do not yield any information regarding the form in which the essential metal is bound by the enzyme; this question is currently under investigation.

The apparent decrease in the affinity for total isocitrate as the pH is increased could be the result of either the ionization of a functional group on the enzyme which is essential for the binding of substrates, or of a pH-dependent decrease in the concentration of the active species of substrate. Since the pH variation of the apparent Michaelis constants for total isoci-

trate can be entirely explained by the change in the concentration of HA^{2-} as the pH is increased from 6.0 to 8.0, it is unnecessary to postulate that an ionizable enzymic group is involved in substrate binding. Examination of the data reveal, however, that at saturating concentrations of all substrates a basic form of an amino acid residue with a pK of 6.25 is essential for proper functioning of the enzyme–substrate complex. Although this pK is within the range considered typical for the ionization of histidyl residues (Cohn and Edsall, 1943), it may also reflect the dissociation of a carboxyl group with an unusually high pK or another functional group whose pK has been drastically altered by the environment of the protein (Schmidt and Westheimer, 1971). The pK of the enzyme–substrate complex of the TPN-dependent isocitrate dehydrogenase from the same source has been shown to be 5.7 and is thought to be due to the ionization of a carboxyl group (Colman, 1968; Colman and Chu, 1969).

That a broad pH optimum is seen when initial velocity is plotted *vs.* pH at low concentrations of isocitrate demonstrates the fact that the increasing affinity of enzyme for substrate is partially compensating for the decrease in V_{max} as the pH is lowered. This observation suggests that the opposing consequences of pH change may provide a mechanism for maintaining a relatively constant level of DPN-dependent isocitrate dehydrogenase activity *in vivo* during small fluctuations in pH.

Extensive kinetic studies to determine the active substrate species for the TPN-dependent isocitrate dehydrogenase from pig heart have also been carried out in this laboratory (Colman, 1972b,c). In contrast to the DPN enzyme, the TPN enzyme uses the metal chelate of the tribasic form of isocitrate as substrate and is inhibited by both free manganous ion and free tribasic isocitrate. The difference in the substrate specificities of these two proteins suggests that the concentration of hydrogen ion and manganous ion *in vivo* may be involved in determining the DPNH:TPNH ratio in the cell by regulating the availability of substrate for these two isocitrate dehydrogenases.

Although various estimates have been made of the concentrations of total metals in animal tissues (Vallee and Altschule, 1949; Comar and Bronner, 1964), more precise information regarding the subcellular metal concentrations is required to permit a meaningful assessment of the role of the metal ions in the regulation of this enzyme. Unlike the mammalian enzyme, the DPN-dependent isocitrate dehydrogenase from the pea uses the metal–isocitrate complex as the active substrate, indicating that the substrate specificity of this enzyme is source dependent (Duggleby and Dennis, 1970).

The substrate inhibition observed at pH 6.0 in this study was more apparent if the concentration of either isocitrate or manganese was increased in the reaction mixture. High concentrations of *threo*-D$_s$-isocitrate also inhibited the reaction, thereby eliminating the possibility that either an impurity in the DL-isocitrate solutions or the L enantiomer itself was responsible for the inhibition. Free manganous ion was discounted as an inhibitor because inhibition was observed at high concentrations of isocitrate when the concentration of free metal was low. Because the inhibition was apparent only at pH 6.0 and because higher concentrations of manganese increased the extent of inhibition, it seemed likely that the metal chelate of the dibasic isocitrate (MHA) was responsible. Examination of the distribution of the various forms of isocitrate present at all three pH's revealed that only the concentration of MHA was appreciably increased at pH 6.0 and 5.25 mM $MnSO_4$. The 30% decrease in velocity exhibited in Figure 4A when MHA is raised from 0.1 to 0.3 mM agrees with the amount of inhibition seen in the K_m determinations of isocitrate at pH 6.0 and 5.25 mM $MnSO_4$. The data shown in Figure 4, together with an analysis of the distribution of the forms of isocitrate, show that the metal chelate of the active form of isocitrate can inhibit at high concentrations and that this inhibition is not competitive in nature since increasing concentrations of HA^{2-} do not overcome the inhibition. It was not possible to distinguish between noncompetitive and uncompetitive inhibition from these data; both models yield a K_i of approximately 0.29 mM. No inhibition was observed at pH 7.0 or pH 8.0 because the concentrations of MHA were not sufficiently elevated to produce inhibition.

A comparison of the effects of various activators on the DPN-specific isocitrate dehydrogenase from pig heart demonstrates the existence of two possible mechanisms for the regulation of a metal requiring enzyme. Apparent activation by a chelating agent, such as citrate, is the indirect result of its ability to compete with isocitrate for manganous ion and effectively increase the concentration of the active species of isocitrate. Citrate appears to activate only in terms of A_t; inspection of the distribution of the various forms of isocitrate at each concentration of total isocitrate reveals that the K_m for the active substrate, HA^{2-}, does not change. In contrast, ADP activation affects the K_m for both HA^{2-} and A_t, suggesting that ADP may act not only by chelation but also by direct interaction with the enzyme. The specificity of allosteric activation by ADP is illustrated by the fact that GDP, another purine nucleotide, functions only as an apparent activator and does not lower the K_m for the active substrate. That modifiers of enzyme activity can function indirectly, *via* their chelating ability, presents an alternate or supplementary model to that of allosteric regulation which should be considered when studying the metabolic control of metal-dependent enzymes.

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Active-Site Studies on Rabbit Liver Nicotinamide Deamidase†

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ABSTRACT: The possibility that control of the Preiss–Handler pathway of NAD biosynthesis is at the nicotinamide deamidase catalyzed reaction has stimulated investigations into the kinetic, physical, and structural properties of this enzyme. This work describes the number of, and relation between, the active centers for amidase and esterase activity of rabbit liver nicotinamide deamidase. The presence of three active sites was determined by reaction of the site-specific reagents diisopropyl fluorophosphate (DFP) and carbobenzoxyamido-2 phenylethyl chloromethyl ketone (ZPCK) with the enzyme and from binding studies. DFP treatment inhibited both esterase and amidase activities, whereas ZPCK inhibited amidase activity but did not affect esterase activity or amide binding. The relation between the catalytic sites was further investigated by kinetic and binding studies using competitive inhibitors (nicotinic acid, *p*-nitrophenol, benzoic acid, hydrocinnamic acid, and 3-indolepropionic acid). The pH dependence of the amidase and esterase activities established that the esterase activity has a higher pH optimum than the amidase activity. Ionizable groups on the ES complex with pK_a values of 5.6–5.8 affect both activities. Chemical characterization of the enzyme included COOH-terminal studies (0.9 mole of leucine/mole of protein as measured by carboxypeptidase action) and sulfhydryl group determination (0.8 mole of SH/mole of enzyme). It was concluded that: (1) the ester and amide substrates share three binding sites on the enzyme, one of which is not equivalent to the other two; (2) both the esterase and amidase activities involve seryl residues; (3) the amidase activity requires histidyl residues which are not involved in ester hydrolysis or amide binding; and (4) a nonhistidine nucleophilic group may be involved with the seryl residues in the esterase activity.

The widespread occurrence of the enzyme nicotinamide deamidase (Preiss and Handler, 1958a,b; Imsande, 1961; Joshi and Handler, 1962; Bernheim, 1967; Pallini *et al.*, 1965; Petrack *et al.*, 1963, 1965; Kirchner *et al.*, 1966; Marki and Greengard, 1966) and the possibility that control of the Preiss–Handler pathway of NAD biosynthesis may occur *in vivo* at this reaction (Greengard *et al.*, 1963, 1965, 1969) have stimulated investigations into the physical, kinetic, structural, and control properties of the enzyme (Su *et al.*, 1969). Of particular interest was the finding that nicotinamide deamidase from rabbit liver also catalyzes the hydrolysis of a variety of ester substrates (Su *et al.*, 1969). This dual activity of the enzyme is analogous to that of a number of other enzymes (*e.g.*, chymotrypsin, trypsin, carboxypeptidase-A, elastase, thrombin) which also hydrolyze amide or peptide bonds and ester bonds. Furthermore, nicotinamide deamidase activity was affected by the site-specific reagents DFP and ZPCK[1] (Su and Chaykin, 1971; Gillam *et al.*, 1972), thus implicating seryl and histidyl residues in the active center of the enzyme. These amino acid residues have been well established as part of the active centers of other amidase and esterase enzymes such as trypsin, chymotrypsin (Schoellmann and Shaw, 1963), elastase (Naughton *et al.*, 1960; Smillie and Hartley, 1964), plasmin (Groskopf *et al.*, 1969), and others (Dixon, 1966) using the same or related site-specific inhibitors.

This work was undertaken to elucidate the nature of the active center(s) of rabbit liver nicotinamide deamidase, determine the number of active sites and substrate binding sites, and ascertain if the active center for amidase activity is identical with the active center for esterase activity.

Materials and Methods

Chemicals. [7-^{14}C]Nicotinamide (specific activity 42 mCi/mmole) was purchased from New England Nuclear. It was

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[1] The abbreviations used are: ZPCK, carbobenzoxyamido-2-phenylethyl chloromethyl ketone; DIP, diisopropylphosphoryl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DFP, diisopropyl fluorophosphate.