Isotope Effect Studies of Chicken Liver NADP Malic Enzyme: Role of the Metal Ion and Viscosity Dependence†

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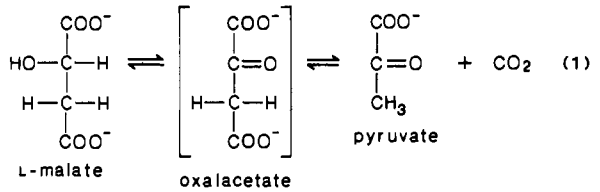
ABSTRACT: The role of the metal ion in the oxidative decarboxylation of malate by chicken liver NADP malic enzyme and details of the reaction mechanism have been investigated by ^{13}C isotope effects. With saturating NADP and the indicated metal ion at a total concentration 10-fold higher than its K_m, the following primary ^{13}C kinetic isotope effects at C_4 of malate [$^{13}(V/K_{mal})$] were observed at pH 8.0: Mg^{2+}, 1.0336; Mn^{2+}, 1.0365; Cd^{2+}, 1.0366; Zn^{2+}, 1.0337; Co^{2+}, 1.0283; Ni^{2+}, 1.025. Knowing the partitioning of the intermediate oxalacetate between decarboxylation to pyuvate and reduction to malate allows calculation of the intrinsic carbon isotope effect for decarboxylation. For Mg^{2+} as activator, this was 1.049 with NADP and 1.046 with 3-acetylpyridine adenine dinucleotide phosphate, although the intrinsic primary deuterium isotope effects on dehydrogenation were 5.6 and 4.2, and the partition ratios of the oxalacetate intermediate for decarboxylation as opposed to hydride transfer were 0.11 and 3.96 (the result of the different redox potentials of NADP and the acetylpyridine analogue). The close agreement of the intrinsic ^{13}C isotope effects with each other and with the ^{13}C isotope effect for the Mg^{2+}-catalyzed nonenzymatic decarboxylation of oxalacetate of 1.0489 [Grissom, C. B., & Cleland, W. W. (1986) *J. Am. Chem. Soc. 108*, 5582] indicates a similarity of transition states for these reactions. It was not possible to calculate reasonable intrinsic carbon isotope effects with the other metal ions by use of the partitioning ratio of oxalacetate because of decarboxylation by another mechanism. The variation of $^{13}(V/K_{mal})$ with pH was used to dissect the total forward commitment for hydride transfer into its internal (partition ratios of enzyme intermediates not affected by reaction conditions) and external (partition ratios involving substrate dissociation) components. With NADP and Mg^{2+}, the internal commitment was 0.348 and the external commitment was 0.117. When we attempted to use the variation of $^{13}(V/K_{mal})$ with solution viscosity to determine the internal and external commitments, incorrect values were obtained because of a specific effect of the viscosogen in decreasing the K_m for malate, so that V/K_{mal} actually increased with viscosity instead of decreasing, as theory predicts. This approach should prove useful, however, for enzymes where nonspecific effects of viscosity do not occur.

Chicken liver NADP malic enzyme (EC 1.1.1.38) catalyzes the oxidative decarboxylation of L-malate to pyruvate and CO_2 with concomitant reduction of NADP:



The divalent metal cation requirement is most commonly satisfied by Mg^{2+} or Mn^{2+}, although Schimerlik et al. (1977) demonstrated that Co^{2+} and Ni^{2+} could also serve as activators. The enzyme will also utilize the alternate nucleotide 3-acetylpyridine adenine dinucleotide phosphate (Acpyr-ADP).[1] Hsu (1967) showed the kinetic mechanism of pigeon liver NADP malic enzyme to be ordered sequential with NADP adding before malate, followed by release of CO_2, pyruvate, and NADPH. Pyruvate is initially produced as enzyme-bound enolpyruvate but acquires a proton before its release in the keto form (Bratcher & Hsu, 1982).

In addition to the oxidative decarboxylation of malate, the enzyme will also catalyze the NADPH-dependent reduction of oxalacetate to malate and the decarboxylation of oxalacetate to pyruvate and CO_2 (Viega Salles & Ochoa, 1950; Tang & Hsu, 1973; Hsu, 1970). Because of this, it has long been thought that dehydrogenation of malate in the central complex occurs first to yield an oxalacetate intermediate of finite lifetime, which is then decarboxylated to yield CO_2 and pyruvate. Hermes et al. (1982) used the multiple isotope effect method of examining the primary ^{13}C kinetic isotope effect with deuteriated and unlabeled substrates to show that this mechanism is correct. Grissom and Cleland (1985a) quantitated the partitioning of oxalacetate between reduction to malate and decarboxylation to pyruvate and CO_2 to provide precise estimates for the intrinsic ^{13}C and deuterium isotope effects on decarboxylation and hydride transfer, respectively, as well as the total forward commitment for malate. This commitment is the sum of the internal (partition ratios of intermediates not affected by reaction conditions) and external (partition ratios involving substrate dissociation) components.

In the present paper, we have dissected the internal and external commitments in an enzymatic reaction by examining

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[1] Abbreviations: mal, malate; pyr, pyruvate; OAA, oxalacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Acpyr-ADP, 3-acetylpyridine adenine dinucleotide phosphate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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the variation of the ^{13}C V/K isotope effect with pH and compared the result to the new method of delineating the external and internal commitments by a change in solution viscosity. Loo and Erman (1977) first used the dependence of kinetic parameters on relative viscosity to determine the extent to which an enzymatic reaction is diffusion limited. More recently, Brouwer and Kirsch (1982), Hardy and Kirsch (1984), Bazelyansky et al. (1986), and Pocker (1987) have used the variation of V/K values with relative viscosity to determine the stickiness factors for enzymatic reactions. An increase in solution viscosity should decrease the rate of substrate dissociation from the enzyme, thereby increasing the external commitment and decreasing the observed isotope effect. When the intrinsic isotope effects are known, the total commitment can be dissected into its internal and external components. This is a general technique applicable to any enzyme which is not chemically affected by viscosogen and for which the above parameters are known. We have attempted to apply this technique to NADP malic enzyme.

Because the ^{13}C kinetic isotope effect on the nonenzymatic metal-catalyzed decarboxylation of oxalacetate is now available for divalent metal cations of biological interest (Grissom & Cleland, 1986), a comparison of the transition states for enzymatic and nonenzymatic decarboxylation processes is possible. In this paper, the effect of six divalent metal cations on decarboxylation was examined and the transition state for the enzyme-catalyzed decarboxylation of oxalacetate compared to the analogous nonenzymatic reaction.

Materials and Methods

Chemicals. The metal chloride salts used in this study were of Gold Label quality from Aldrich, and solutions were prepared with water demetaled by successive extraction with dithizone in $CHCl_3$, followed by washes with CCl_4 and aspiration to remove the organic solvent. The solutions were stored in polyethylene bottles washed with concentrated nitric acid. All other reagents except nucleotides and glutathione were demetaled in a similar manner. NADP (Boehringer-Mannheim), Acpyr-ADP (Sigma), and glutathione (Sigma) were demetaled by passage of the solutions through a 0.4 cm × 2 cm Bio-Rad Chelex 100 column in a Pasteur pipet. The reaction flasks and gas dispersion tubes were washed successively with hot Alconox and 5 M nitric acid prior to use. This procedure prevented carryover of metals between experiments.

Enzymes. Chicken liver NADP malic enzyme from Sigma contains 3 mM $MnSO_4$ which must be removed before use. The ammonium sulfate suspension was dissolved in a solution of 50 mM Pipes, pH 7, 50% v/v glycerol, 1 mM DTT, and 5 mM EDTA and dialyzed against the same solution at 4 °C for 24 h. The solution was then chromatographed on a 1 cm × 8 cm Sephadex G-10 column eluted with 10 mM Pipes, pH 7, 10% v/v glycerol, and 1 mM dithiothreitol. The coupling enzymes glutamate-pyruvate transaminase and glutathione reductase were also diluted in 50 mM Pipes, pH 7, and 50% glycerol (v/v) and chromatographed as above.

^{13}C Kinetic Isotope Effects. ^{13}C isotope effects on malic enzyme were determined by the method of O'Leary (1980). The exact procedure was patterned after Hermes et al. (1982) when possible. Since the purpose of this study was to highlight differences among the metals studied, modification of the experimental protocol for the incomplete conversions was necessary to accommodate all of the metals studied. Most notably, DTT was not included because of its high affinity for metals. Similarly, oxidized glutathione which is often used as an electron sink for NADP regeneration in conjunction with glutathione reductase was excluded from the low-conversion reactions because Ni^{2+} produced NiS_2 which led to volatile H_2S upon acidification (detectable by olfaction of the isolated CO_2). Glutamate dehydrogenase was tried as a suitable replacement for NADP regeneration, but it was inactivated by high concentrations of Ni^{2+} and Cd^{2+}. The final solution was to use a 5-fold excess of NADP and prevent reversal of the reaction by conversion of the pyruvate produced to alanine with glutamate-pyruvate transaminase (K_{eq} = 2.2 for pyruvate/glutamate production, but the equilibrium can be made favorable by the presence of 100 mM L-glutamate). Unless otherwise stated, all reactions were buffered at pH 8.0 with 100 mM Hepes. The concentrations of other reagents in a 20-mL total reaction volume were as follows: for complete conversion, 2 mM L-malate, 0.4 mM NADP (or Acpyr-ADP), 40 mM oxidized glutathione, 100 mM L-glutamate, and 5 mM $MgCl_2$; for low conversion, 20 mM L-malate, 5 mM NADP, 100 mM L-glutamate, and the specific metal concentration delivered as the chloride salt. A total of 100 units of the transaminase and 120 units of glutathione reductase (complete conversion only) were typically added to each reaction vessel. Enough NADP malic enzyme was added to obtain the desired fraction of reaction in 1–2 h. The reaction vessels were maintained at 25 °C during turnover. For each assay, the amount of CO_2 produced (measured manometrically following isolation) corresponded to the extent of reaction indicated spectrophotometrically (determined by withdrawing aliquots of the reaction solution prior to acid quenching and determining the amount of NADPH produced). The $^{12}C/^{13}C$ ratio of the CO_2 was analyzed on a Finnigan δ-E isotope ratio mass spectrometer equipped with a dual-inlet system.

Viscosity Studies. The microviscosity of the reaction mixtures was increased by the addition of ultrapure sucrose or glycerol (Sigma). Test extraction of the sucrose with dithizone showed no metal contamination; hence, it was not routinely treated with dithizone. The viscosity of the reaction mixtures was determined with a Gilmont falling-ball viscosimeter thermostated at 25 °C.

Tritium Isotope Effects. Determination of the primary tritium isotope effect for malate with Acpyr-ADP as the nucleotide was patterned after Schimerlik and Cleland (1977), but with slight modification of the chromatographic procedure. DL-Malate-*2-t* was synthesized chemically by reduction of diethyl oxalacetate with NaB^3H_4. Crude diethyl malate-*2-t* was purified on silica gel. Following acid-catalyzed deesterification, the material was chromatographed on Dowex AG-1-X2 in the formate form. DL-Malate was eluted with a gradient of 0–2 N HCOOH.

Nonreversibility of the malic enzyme reaction was ensured by coupling the pyruvate produced to L-alanine with glutamate-pyruvate transaminase. This allowed the progress of the reaction to be directly monitored spectrophotometrically at 340 (for NADP) or 363 nm (for Acpyr-ADP) to determine the fraction of reaction. Assay mixtures contained in 3 mL the following: 25 mM Hepes as buffer, pH 8.0; 25 mM NADP (or Acpyr-ADP); 5 mM $MgCl_2$; 20 mM DL-malate-*2-t*; 30 mM L-glutamate; 100 units of glutamate-pyruvate transaminase. When the desired fraction of reaction was reached, the enzymes were quenched by vigorous vortex mixing with 0.5 mL of CCl_4. Denatured protein was removed by a 0.2-μm filter, the remaining liquid adjusted to pH 10 with KOH, and sufficient salt added to bring the concentration of the sample to 0.2 M $LiCl_2$. This was loaded onto a 0.5 cm × 5 cm Bio-Rad Dowex AG-MP-1 column equilibrated with 0.2 M $LiCl_2$ adjusted to pH 10 with KOH. The column was eluted with the same buffer at 0 °C. For NADPH, those fractions

with a 260 nm/340 nm ratio of 2.5 or less were considered pure, while a ratio of 1.67 or lower for absorbance at 260 nm/363 nm was used as the criterion of purity for Acpyr-ADP. Radioactivity was determined by liquid scintillation counting of 1.000 mL of the sample in 10 mL of scintillation cocktail. Corrections for quenching were made by the subsequent addition of an internal standard containing a known amount of ^3H. Those fractions with a constant specific activity were used to determine the tritium isotope effect.

Oxalacetate Partitioning with Cd, Zn, and Co. The conditions for quantitating the partitioning of oxalacetate between reduction and decarboxylation with Cd^{2+}, Zn^{2+}, and Co^{2+} were identical with those previously described (Grissom & Cleland, 1985a). By observation of NADPH at 340 nm and OAA at 281 nm (the isosbestic point for oxidation of NADPH to NADP), the relative amounts of malate and pyruvate formed can be calculated from eq 2 and 3. The observed partitioning

$$d[mal]/dt = -(dA_{340}/dt)/\epsilon_{340,NADPH} \quad (2)$$

$$\frac{d[pyr]}{dt} = \frac{(dA_{340}/dt)\epsilon_{281,OAA}/\epsilon_{340,NADPH} - dA_{281}/dt}{\epsilon_{281,OAA} - \epsilon_{281,\alpha KG}} \quad (3)$$

ratio r_H is defined in eq 4. In terms of mechanism 8 (vide

$$r_H = (d[pyr]/dt)/(d[mal]/dt) \quad (4)$$

infra) r_H is the forward commitment for decarboxylation:

$$r_H = (k_7/k_6)[1 + (k_5/k_4)(1 + k_3/k_2)] \quad (5)$$

Initial Velocity Studies. All kinetic studies were performed by monitoring changes in NADPH concentration at 340 nm with a Beckman DU monochromator equipped with an Update Instruments OD converter. The cuvette compartment was maintained at 25 °C with thermospacers. Routine assay conditions were 100 mM Hepes, pH 7.5, 0.2 mM NADP, and the specified amount of L-malate and metal. The method of Canellas and Wedding (1980) was used to determine whether free malate or the metal–malate complex was the true substrate. The following dissociation constants were employed: Mg–NADP = 19.1 mM (Apps, 1973); Mg–malate = 19.9 mM and Co–malate = 1.38 mM (Martell & Smith, 1977); Co–NADP = 8.7 mM (estimated by interpolation of dissociation constants for Co–NAD = 9.4 mM, Mg–NAD = 20.6 mM, Mg–malate, and Co–malate).

Isotope Effect Nomenclature. The nomenclature of Northrop (1977) is used in which isotope effects on a kinetic or thermodynamic parameter are indicated by a leading superscript (D, T, or 13 for deuterium, tritium, or ^{13}C). Thus, $^{D}K_{eq}$ is $K_{eq}(H)/K_{eq}(D)$ while ^{13}k is the ratio of rate constants for ^{12}C- and ^{13}C-containing substrates.

Data Analysis. Hyperbolic progress curves were fitted to eq 6 with a BASIC version of the HYPERO program of Cleland

$$v = VA/(K + A) \quad (6)$$

(1979).[2] ^{13}C isotope effects determined by the internal competition method were calculated by eq 7. In eq 7, R_f is

$$^{13}(V/K) = \log(1 - f)/\log[1 - f(R_f/R_0)] \quad (7)$$

the $^{13}C/^{12}C$ ratio in product CO_2 at fractional reaction f and R_0 is the $^{13}C/^{12}C$ ratio of the starting material (as determined by 100% decarboxylation).

[2] Compiled FORTRAN versions of these and other fitting programs for the IBM PC are available from C.B.G.

Table I: Kinetic Parameters for Activation of Malic Enzyme by Various Metals at pH 7.5[a]

metal ion	rel V_{max}	K_m (μM)
Cd^{2+}	1.07 ± 0:06	1.5 ± 0.2
Mn^{2+}	1.07 ± 0.03	2.4 ± 0.3
Zn^{2+}	0.29 ± 0.01	1.1 ± 0.2
Mg^{2+}	1.00 ± 0.02	190 ± 11
Co^{2+}	0.71 ± 0.02	20 ± 4
Ni^{2+}	0.76 ± 0.01	95 ± 5

[a] All assays contained 5 mM malate, 0.2 mM NADP, and 100 mM Hepes, pH 7.5. Low metal concentrations were chosen such that the negative cooperativity reported by Schimerlik et al. (1977) did not occur. Data were fitted to eq 6.

Theory of Viscosity Dependence of Isotope Effects. Consider the following reaction scheme for NADP malic enzyme:

$$E\cdot Mg\cdot NADP \underset{k_2}{\overset{k_1(mal)}{\rightleftharpoons}} E\cdot Mg\cdot NADP\cdot mal \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\cdot Mg\cdot NADP\cdot mal^* \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E\cdot Mg\cdot NADPH\cdot OAA \xrightarrow{k_7} E\cdot Mg\cdot NADPH\cdot pyr \xrightarrow{k_9} E\cdot Mg \quad (8)$$

where an asterisk (*) indicates the Michaelis complex geometrically poised for catalysis. k_5 and k_6 are sensitive to deuterium substitution at C_2 (hydride transfer), and k_7 is sensitive to ^{13}C substitution at C_4 (decarboxylation). The expression for the observed carbon isotope effect on V/K for malate will be[3]

$$^{13}(V/K) = \frac{^{13}k + C_{f\text{-}in} + C_{f\text{-}ex}}{1 + C_{f\text{-}in} + C_{f\text{-}ex}} \quad (9)$$

where $C_{f\text{-}in} = (k_7/k_6)(1 + k_5/k_4)$, the internal forward commitment for decarboxylation, and $C_{f\text{-}ex} = (k_7/k_6)(k_5/k_4)(k_3/k_2)$, the external forward commitment for decarboxylation.

Hardy and Kirsch (1984) have shown that an increase in solution microviscosity decreases k_1 and k_2 in a linear fashion. Since k_2 appears in the denominator of the external forward commitment, $C_{f\text{-}ex}$ increases as the viscosity increases, thereby decreasing the observed isotope effect. We can rewrite eq 9 to include this viscosity dependence:

$$^{13}(V/K) = \frac{^{13}k + C_{f\text{-}in} + C_{f\text{-}ex}\eta}{1 + C_{f\text{-}in} + C_{f\text{-}ex}\eta} \quad (10)$$

where η is the relative viscosity of the reaction medium. It should be noted that k_1, the association rate constant, never appears in the relevant equations. Similarly, k_2 only appears in the term for the external commitment. All other terms are insensitive to viscosity. The relationship between solution viscosity and observed isotope effect can be linearlized and solved for the internal and external commitments as

$$\frac{1}{^{13}(V/K)_{\eta=1} - ^{13}(V/K)_{\eta>1}} = \frac{(1 + C_f)^2}{C_{f\text{-}ex}(^{13}k - 1)}\frac{1}{\eta - 1} + \frac{1 + C_f}{^{13}k - 1} \quad (11)$$

The internal and external commitments are then given by eq

[3] The full expression for $^{13}(V/K_{mal})$ includes terms for the reverse commitment for malate and the $^{13}K_{eq}$:

$$^{13}(V/K) = \frac{^{13}k + C_{f\text{-}in} + C_{f\text{-}ex} + C_r{}^{13}K_{eq}}{1 + C_{f\text{-}in} + C_{f\text{-}ex} + C_r}$$

The reverse commitment in this mechanism is for the first product released, CO_2. Since the enzyme's affinity for CO_2 is very low, the reverse commitment is assumed to be zero. This implies CO_2 is able to leave the active site immediately after decarboxylation, thus preventing reversal of decarboxylation when the CO_2 concentration is low. This assumption removes the C_r terms from the expression.

Table II: ^{13}C Isotope Effects for Malic Enzyme with Various Metal Ions at pH 8.0

metal ion	concn	high conv[a]	frac of reaction (%)	low conv	$^{13}(V/K_{mal})$
Mg^{2+}	5.0 mM	10886.1[b]	7.8	10543.7	1.033 83
		10886.1	7.5	10547.4	1.033 39
					av 1.033 6 ± 0.000 3
Mn^{2+}	70.0 μM	10886.1	26.7	10551.9	1.037 13
		10894.8	9.2	10531.8	1.036 19
		10894.8	8.5	10531.0	1.036 14
					av 1.036 5 ± 0.000 6
Ni^{2+}	18.9 mM	10886.1	11.1	10600.8	1.028 56
		10886.1	10.4	10668.3	1.021 57
		10886.1	9.8	10635.3	1.024 84
					av 1.025 ± 0.003
Cd^{2+}	5.3 μM	10886.1	6.9	10517.1	1.036 37
		10886.1	6.4	10511.1	1.036 86
		10886.1	6.7	10514.9	1.036 55
					av 1.036 6 ± 0.000 2
Zn^{2+}	9.3 μM	10886.1	7.2	10543.6	1.033 72
		10886.1	6.5	10542.2	1.033 74
		10886.1	7.2	10543.5	1.033 73
					av 1.033 73 ± 0.000 01
Co^{2+}	18.9 mM	10886.1	12.3	10608.9	1.027 92
		10886.1	14.2	10603.3	1.028 82
		10886.1	14.2	10608.7	1.028 25
					av 1.028 3 ± 0.000 5
Co^{2+}	80.0 μM	10894.8	4.9	10592.3	1.029 29
		10894.8	4.3	10595.4	1.028 89
					av 1.029 1 ± 0.000 3

[a] The high-conversion malate samples have the following standard errors associated with them: 10894.8 ± 0.8, three determinations; 10886.1 ± 2.0, five determinations. [b] Isotopic ratios of $^{13}C/^{12}C$ have been multiplied by 10^6 and corrected for ^{17}O content according to the geochemical ratio of $^{17}O/^{18}O$.

12 and 13, where m and b are the slope and vertical intercept of the plot versus $1/(\eta - 1)$ of eq 11.

$$C_{f\text{-}ex} = \frac{b^2(^{13}k - 1)}{m} \quad (12)$$

$$C_{f\text{-}in} = b(^{13}k - 1) - 1 - C_{f\text{-}ex} \quad (13)$$

Results

Kinetic Parameters for Metal Ions. In addition to Mn^{2+}, Mg^{2+}, Co^{2+}, and Ni^{2+} reported by Schimerlik et al. (1977) to serve as activators, Cd^{2+} and Zn^{2+} were found to support oxidative decarboxylation of malate. The kinetic parameters for each metal ion at saturating malate and NADP are shown in Table I.

Malate was varied at three saturating levels of metal ion to determine whether free malate or the metal ion–malate complex is the true substrate for NADP malic enzyme. Figure 1 shows reciprocal velocity plotted against reciprocal free malate and Mg–malate complex at 2.0, 8.0, and 15 mM free Mg^{2+}.

^{13}C Isotope Effects. $^{13}(V/K_{mal})$ values for malic enzyme with various metal ions are shown in Table II. All metal concentrations (except 80 μM Co^{2+} which is 4-fold the K_m) are at least 10-fold the K_m value reported by Schimerlik et al. (1977).

Oxalacetate Partitioning. Between pH 6.2 and pH 6.5 at metal ion concentrations equal to 2-fold the K_m, the pyr/mal partitioning ratios for Cd^{2+}, Zn^{2+}, and Co^{2+} as metal ion were 12, 31, and 34, respectively. These ratios, with the observed $^{13}(V/K_{mal})$ shown in Table II, gave intrinsic ^{13}k values of 1.472, 2.072, and 1.991. These values are much larger than the theoretical maximum of 1.07 calculated when the ground-state energy levels are compared for ^{13}C and ^{12}C mass differences. Concentrations of Cd, Zn, and Co greater than 2-fold the K_m gave even larger (and hence unreasonable) pyr/mal partitioning ratios.

Tritium Isotope Effects with Acpyr-ADP. The $^{T}(V/K_{mal})$ value with Acpyr-ADP as nucleotide was 3.58 ± 0.10.

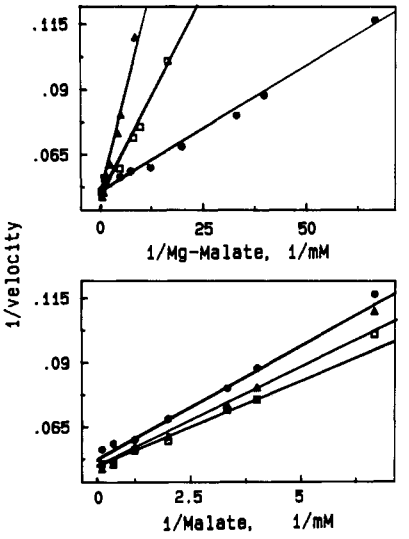

FIGURE 1: 1/velocity vs 1/[free malate(2–)] or 1/[Mg–malate complex] with saturating NADP (0.2 mM), pH 7.5, 25 °C. Saturation curves plotted against free malate are nearly superimposable, while velocities plotted versus complex are not. (▲) 2 mM Mg^{2+}; (□) 8 mM Mg^{2+}; (●) 15 mM Mg^{2+}.

Schimerlik et al. (1977) determined $^{T}(V/K_{mal})$ with NADP as nucleotide to be 2.02 ± 0.06. As a control on the present study, this number was redetermined and found to be 1.98 ± 0.05. Knowing $^{D}(V/K_{mal})$, $^{13}(V/K_{mal})$, and r_H, Grissom and Cleland (1985a) were able to place limits on the intrinsic deuterium isotope effect and the total forward commitment for hydride transfer with Acpyr-NADP as nucleotide. Knowing the tritium isotope effect on V/K_{mal} now allows the precise determination of ^{D}k, $C_{f\text{-}H}$, and the internal partition ratio between decarboxylation and reverse hydride transfer, k_7/k_6 [see Grissom and Cleland (1985a) for equations and details]. The results are shown in Table VII.

Viscosity Dependence of Initial Kinetic Parameters. The dependence of reaction initial velocity on solution viscosity is presented in Table III. V_{max} changes only slightly with either

Table III: Viscosity Dependence of Kinetic Parameters for Malic Enzyme with NADP at pH 5.00

rel viscosity	V (μM/min)	V/K ($\times 10^3$ min^{-1})	K_m (mM)
sucrose			
1.00	1.95 ± 0.05	10.1 ± 0.9	0.19 ± 0.02
1.17	2.22 ± 0.07	15.0 ± 1.7	0.15 ± 0.02
1.35	2.42 ± 0.05	16.5 ± 1.2	0.15 ● 0.01
1.68	2.49 ± 0.06	21.4 ± 2.0	0.12 ± 0.01
glycerol			
1.00	2.2 ± 0.1	9.5 ± 1.2	0.20 ± 0.01
1.20	2.56 ± 0.05	20.3 ± 1.4	0.130 ± 0.008
1.49	2.45 ± 0.05	36.4 ± 4.0	0.067 ± 0.008
1.94	2.55 ± 0.03	50.8 ± 3.6	0.050 ± 0.004

Table IV: Viscosity Dependence of $^{13}(V/K_{mal})$ for Malic Enzyme with NADP at pH 5.00

rel viscosity	high conv[a]	frac of reaction (%)	low conv	$^{13}(V/K_{mal})$
1.00	10880.9[b]	3.2	10505.5	1.03632
	10880.9	2.9	10511.1	1.03570
				av 1.0360 ● 0.0004
2.60	10880.9	6.3	10605.5	1.02683
	10880.9	5.4	10607.0	1.02655
				av 1.02669 ± 0.0002

[a] The high-conversion malate samples have the following standard error associated with them: 10880.9 ± 4.0, three determinations. [b] Isotopic ratios of $^{13}C/^{12}C$ have been multiplied by 10^6 and corrected for ^{17}O content according to the geochemical ratio of $^{17}O/^{18}O$.

sucrose or glycerol as viscosogen. The increase in V/K is more dramatic however, increasing 2-fold with a 1.7-fold increase in viscosity with sucrose and 5-fold with a 2-fold increase in viscosity with glycerol. An increase in either V or V/K with an increase in solution viscosity is unexpected, since theory predicts an increase in viscosity can, at most, monotonically decrease V and V/K in the absence of specific chemical interactions of the viscosogen with the enzyme.

pH Dependence of ^{13}C Isotope Effects. The pH dependence of $^{13}(V/K_{mal})$ can be used to dissect the internal and external commitment if the intrinsic isotope effect is known. Schimerlik and Cleland (1977) reported a pK of 6.0 in the log (V/K) vs pH profile with malate as substrate and Mg^{2+} as metal ion. Below this pK, V/K decreases by a factor of 10 per pH unit. If this pK is responsible for catalysis, then the ratio of catalysis to substrate release will be decreased as this group on the enzyme becomes protonated. At the pK, 50% of the external commitment will be eliminated. At pH 5.0, $^{13}(V/K_{mal})$ increases to 1.0360 ± 0.0004 (Table IV). If this increase is due to the elimination of 90% of the external commitment observed at pH 8.0 (assuming a pK of 6.0), then external and internal commitments for decarboxylation of 0.117 ± 0.009 (25% of $C_{f\text{-total}}$) and 0.348 ± 0.021 (75% of $C_{f\text{-total}}$) are calculated for pH 8.0.

Viscosity Dependence of ^{13}C Isotope Effects. The viscosity dependence of $^{13}(V/K_{mal})$ for malate with NADP and Acpyr-ADP is shown in Tables V and VI, respectively. The data from Table V were linearized according to eq 11 by fixing the intercept at 29.5.[4] The slope determined for this line is 169 ± 6. Equations 12 and 13 give external and internal commitments for decarboxylation of 0.252 ± 0.016 (57% of $C_{f\text{-total}}$) and 0.193 ± 0.012 (43% of $C_{f\text{-total}}$). With Acpyr-ADP as nucleotide, the intercept was fixed at 245, and the slope was determined to be 861 ± 126. This translates into an external and internal commitment for decarboxylation of 3.2 ± 0.5 (31% of $C_{f\text{-total}}$) and 7 ± 1 (69% of $C_{f\text{-total}}$). *As noted below, however, these values are not correct.* The difference in external and internal commitments for decarboxylation calculated by viscosity and pH variation is resolved under Discussion.

[4] The abscissa intercept in eq 11 is dependent only on the intrinsic isotope effect and the total commitment for decarboxylation, C_f. This is calculated from

$$^{13}(V/K) = \frac{^{13}k + C_f}{1 + C_f}$$

where, with NADP as cofactor, ^{13}k = 1.049 (Grissom & Cleland, 1985a). C_f is determined to be 0.445. With Acpyr-ADP as nucleotide, ^{13}k = 1.046 and C_f = 10.3.

Table V: Viscosity Dependence of $^{13}(V/K_{mal})$ for Malic Enzyme with NADP at pH 7.5

rel viscosity[a]	high conv[b]	frac of reaction (%)	low conv	$^{13}(V/K_{mal})$
1.00	10876.5[c]	7.7	10536.9	1.03355
	10876.5	6.0	10527.8	1.03416
				av 1.0339 ± 0.0004
1.59	10876.5	6.1	10562.1	1.03071
1.85	10876.5	6.9	10577.7	1.02928
2.04	10876.5	6.5	10580.7	1.02891
2.51	10876.5	9.3	10600.5	1.02735

[a] Viscosogen is sucrose. [b] The high-conversion malate samples have the following standard error associated with them: 10876.5 ± 1.6, three determinations. [c] Isotopic ratios of $^{13}C/^{12}C$ have been multiplied by 10^6 and corrected for ^{17}O content according to the geochemical ratio of $^{17}O/^{18}O$.

Table VI: Viscosity Dependence of $^{13}(V/K_{mal})$ for Malic Enzyme with Acpyr-ADP, pH 7.5

rel viscosity[a]	high conv[b]	frac of reaction (%)	low conv	$^{13}(V/K_{mal})$
1.00	10873.6[c]	10.1	10831.8	1.00407
	10873.6	7.6	10828.5	1.00435
				av 1.0042 ± 0.0002
1.59	10873.6	8.3	10835.9	1.00366
1.85	10873.6	8.5	10837.6	1.00349
2.04	10873.6	10.1	10844.5	1.00286
2.51	10873.6	7.0	10845.9	1.00266

[a] Sucrose is viscosogen. [b] The high-conversion malate samples have the following standard error associated with them: 10876.5 ± 1.6, three determinations. [c] Isotopic ratios of $^{13}C/^{12}C$ have been multiplied by 10^6 and corrected for ^{17}O content according to the geochemical ratio of $^{17}O/^{18}O$.

Discussion

Metal Ion Kinetic Parameters. From Table I, it is apparent the maximum velocity changes only slightly among metal ions. The variation in K_m is somewhat larger, however. Cd^{2+} and Zn^{2+} have the highest affinities, suggesting a sulfhydryl group as a metal ligand. Beyond this, no trend is apparent from Table I.

To determine whether free malate(2–) or the metal–malate complex is the true substrate for NADP malic enzyme, malate was varied at three saturating levels of Mg^{2+} or Co^{2+}. If free malate is the true substrate and the metal–malate complex is not inhibitory, then the velocities plotted versus free malate should coincide at all three levels of saturating metal ion. If the metal–malate complex is the true substrate, then enzymatic rates plotted against metal–malate complex concentration should coincide. According to the above criteria, Figure 1 suggests free malate is the true substrate with Mg^{2+}. Free malate exhibits a similar pattern with Co^{2+} as metal ion. In neither case are the velocity curves superimposable when plotted against Mg–malate or Co–malate complex. This is in agreement with the results of Canellas and Wedding (1980) for NAD malic enzyme from cauliflower and NAD malic

enzyme from *Ascaris* (P. F. Cook, personal communication).

^{13}C Isotope Effects. Although the $^{13}(V/K_{mal})$ values are clustered in a narrow range from 1.025 for Ni^{2+} to 1.0366 for Cd^{2+}, they are significantly different from each other. In each case, the observed ^{13}C isotope effect is less than the analogous isotope effect on the metal-catalyzed decarboxylation of oxalacetate (Grissom & Cleland, 1986), suggesting that a forward commitment remains in all cases examined. $^{13}(V/K_{mal})$ does not change greatly with a change in metal ion concentration as long as the activating metal is nearly saturating. The isotope effect for Co^{2+} is 1.0283 ± 0.0005 at 18.9 mM Co^{2+} ($900K_m$) and increases only to 1.0291 ± 0.0003 at 0.080 mM Co^{2+} ($4K_m$). Hence, a change in metal ion concentration changes the observed isotope effect only slightly. $^{13}(V/K_{mal})$ values with Mg^{2+} and Mn^{2+} are slightly larger than the values of 1.0302 ± 0.0005 and 1.0324 ± 0.0006 reported by Hermes and Cleland (1982). This difference may be attributable to the demetallation of reagents employed in this study.

Partitioning of Oxalacetate. The intrinsic ^{13}C isotope effect on the non-metal-catalyzed decarboxylation of dianionic oxalacetate is 1.052 ± 0.001 (Grissom & Cleland, 1986). When the values of $^{13}(V/K_{mal})$ with Mg^{2+} and Mn^{2+} determined in this study are used with the previously determined partitioning ratio for oxalacetate with these metal ions (Grissom & Cleland, 1985a), slightly larger values for the intrinsic ^{13}C isotope effect on decarboxylation are calculated. These are 1.049 ± 0.001 and 1.074 ± 0.002, respectively. The intrinsic isotope effect with Mg^{2+} as activator is identical with the ^{13}C isotope effect of 1.0489 ± 0.0001 on the nonenzymatic Mg^{2+}-catalyzed decarboxylation of oxalacetate (Grissom & Cleland, 1986), suggesting identical transition states for the enzymatic and nonenzymatic processes. Hence, catalysis by the metal ion results from stronger binding to the transition-state than to the ground-state form of oxalacetate. Although this mechanism for malic enzyme has been in vogue for at least 15 years, this is perhaps the most compelling evidence substantiating the purported role of the metal ion.

The intrinsic ^{13}C isotope effects calculated for Mn^{2+}, Cd^{2+}, Zn^{2+}, and Co^{2+} as activators of the enzymatic reaction are larger than the reasonable maximum of 1.065 for ^{13}C isotope effects and the isotope effect for the analogous nonenzymatic decarboxylation of oxalacetate. This is because the pyr/mal partitioning ratio for oxalacetate with these metals greatly favors decarboxylation over reverse hydride transfer. If the theory and technique of intermediate partitioning are assumed correct, then the prima facie conclusion is that another mechanism exists for the enzyme-catalyzed decarboxylation of oxalacetate with these metal ions. Initially, this appears to be a deus ex machina explanation but becomes more tenable when considered with Hsu's (1984) "half-of-the-sites reactivity" model for NADP malic enzyme. Hsu proposes a homotetramer which has four equivalent sites for NADP binding but two high-affinity and two low-affinity sites for metal and malate binding. Only the high-affinity sites have catalytic ability. The low-affinity sites are considered regulatory sites because binding of malate at these sites in the absence of metal causes inhibition of NADPH release. It is possible that these regulatory sites which have a low affinity for malate are capable of catalyzing the decarboxylation of oxalacetate in the presence of metals other than Mg^{2+}, all of which form much tighter complexes with organic ligands. Thus only in the presence of metal ions which form tighter stability complexes than Mg^{2+} could these low-affinity sites contribute to the rate of decarboxylation. A requisite corollary is that these sites are not capable of catalyzing reduction of oxalacetate, probably because of the more critical geometries necessary for hydride transfer.

Viscosity Dependence. The ability to dissect the internal and external commitments for any enzymatic reaction in which an intrinsic isotope effect is known is a significant advance of isotope effect theory. Previously, it has been desirable to increase observed isotope effects by decreasing the external commitment through variation in pH or cosubstrate levels. In this study, the aim was to selectively increase the external commitment by increasing viscosity, thereby decreasing the size of the observed isotope effect. As theory predicts, an increase in viscosity does indeed decrease the observed isotope effect, but to an extent greater than expected by the lack of a significant pH dependence of $^{D}(V/K_{mal})$[5] (Cook & Cleland, 1981) or the increase of $^{13}(V/K_{mal})$ at low pH.

A clue to the anomalous $^{13}(V/K_{mal})$ value is provided by the dependence of the initial velocity kinetic parameters on solution viscosity (Table III). Rather than decreasing, V/K actually increases with an increase in viscosity with either sucrose or glycerol as viscosogen. V only changes slightly; hence, a lowering of K_m is responsible for the effect on V/K. Because a similar effect is seen with sucrose and glycerol, the effect is not chemically specific to either molecule. Because V_{max} is known to be limited by release of reduced nucleotide product and V changes by a factor of less than the change in relative viscosity, the anomaly cannot come from a viscosity-induced decrease in the rate of nucleotide dissociation. Hence, the rate constants affected must be contained in the expression for K_m and not V_{max}. For mechanism 8, the expression for K_m is given by eq 14. If the only effect of viscosity were to slow down

$$K_m = \frac{k_2}{k_1}\frac{k_9}{k_3}\left(1 + \frac{k_4k_6}{k_5k_7} + \frac{k_3}{k_2} + \frac{k_4}{k_5}\right) \qquad (14)$$

diffusion, then only those rate constants involving substrate or product association or dissociation (k_1, k_2, k_7, and k_9) would be affected. Here, this is not the case. Only k_9, the rate constant for nucleotide product release which is known to be the dominant term in the expression for V, can be eliminated as being anomalously affected by viscosity. The association rate constant, k_1, is also an unlikely candidate. All other terms cannot be ruled out as being selectively affected by solvent viscosity, but the most likely rate constants to be affected are k_3 and k_4, which are for the conformational change which positions the enzyme–malate complex for hydride transfer. To determine the exact step which viscosity is affecting, r_H, $^{D}(V/K_{mal})$, and $^{T}(V/K_{mal})$ would have to be determined in solvents of varying viscosity to allow calculation of the relative free-energy profile as a function of viscosity.

Catalytic Mechanism and Free-Energy Profile. The partitioning ratios for enzyme forms which precede the first irreversible step[6] (k_7/k_6, k_5/k_4, and k_3/k_2) can be calculated from the intrinsic isotope effects and the distribution of the internal and external components of the commitment to ca-

[5] Because of the large reverse commitment for hydride transfer, a small change in the external commitment will affect $^{13}(V/K_{mal})$ more than $^{D}(V/K_{mal})$. Hence, the observed ^{13}C isotope effect will be a more sensitive reporter than will be the observed deuterium isotope effect. The increased precision possible with mass spectrometric determination of isotope ratios in product or starting materials further enhances the precision of the ^{13}C data.

[6] V/K isotope effects or kinetic parameters can only be used to probe enzymatic reactions through the first irreversible step (in this case, decarboxylation). Beyond this, the substrate must go on to product, and the commitment to catalysis is infinite. Beyond the first irreversible step, V_{max} isotope effects and kinetic parameters must be used to define the energetics of the reaction and rate of product release.

Table VII: Intrinsic Isotope Effects and Relative Rate Constants for Malic Enzyme with Mg^{2+}

parameter	NADP[a]	Acpyr-ADP[b]
$^{13}k_7$	1.049 ± 0.001	1.046 ± 0.002
$^{D}k_5$	5.6 ± 0.3	4.2 ± 0.3
$(k_5/k_4)(1 + k_3/k_2)$	3.23 ± 0.28	1.50 ± 0.10
k_7/k_6	0.11 ± 0.01	3.96 ± 0.28
k_5/k_4	2.16 ± 0.11	
k_3/k_2	0.49 ± 0.05	

[a] Observed values used for calculations with NADP: $^{13}(V/K_{mal})$ = 1.0336 ± 0.0003 (this paper); $^{13}(V/K_{mal})$ = 1.0360 at pH 5.00 and pyr/mal = 0.47 ± 0.01 (Grissom & Cleland, 1985a); $^{D}(V/K_{mal})$ = 1.47 ± 0.03 and $^{T}(V/K_{mal})$ = 2.02 ± 0.06 (Schimerlik et al., 1977). [b] Values with Acpyr-ADP: $^{13}(V/K_{mal})$ = 1.0042 ± 0.0002 (this paper); pyr/mal = 9.88 ± 0.06 (Grissom & Cleland, 1985a); $^{D}(V/K_{mal})$ = 2.18 (Hermes et al., 1982); $^{T}(V/K_{mal})$ = 3.58 (this paper).

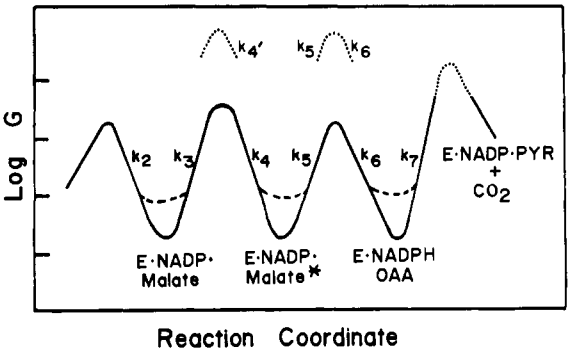

FIGURE 2: Reaction coordinate diagram for the reaction of malate and NADP catalyzed by malic enzyme. The numbers next to the activation barriers correspond to the rate constants in mechanism 8 (the reverse commitment for the decarboxylation step is assumed to be very small). Note that while the relative heights of the barriers are precisely known, the relative levels of the troughs corresponding to the intermediates are not (the dashed lines indicate this uncertainty). E·NADP-malate* and E·NADPH·OAA are shown as if $k_5 = k_6$, but there is no way to establish this without measuring the actual equilibrium constant on the enzyme. The peak heights relative to the final transition state are shown for Acpyr-ADP as dotted lines.

talysis (Table VII). From these ratios, the relative free-energy profile for the conversion of malate to enzyme-bound enolpyruvate[7] for malic enzyme can be calculated (Figure 2). The remarkable agreement of the intrinsic ^{13}C isotope effect on enzymatic carbon–carbon bond breaking with the value of ^{13}k determined for the analogous chemical process of oxalacetate decarboxylation strongly suggests a similarlity of transition states for the two processes. Hence, the enzyme's role in decarboxylation is partly to position the metal cofactor near the carbonyl of enzyme-bound oxalacetate to stabilize the enolate. (Other contributions to catalysis could involve holding C_4 in an out-of-plane position, narrowing the C_2–C_3–C_4 bond angle, and dehydrating C_4.) Other metal ions besides Mg^{2+} are assumed to have a similar role in catalysis, since the observed values of $^{13}(V/K_{mal})$ do not change greatly nor do the intrinsic ^{13}C isotope effects determined for metal-catalyzed oxalacetate decarboxylation (Grissom & Cleland, 1986). The mechanism for the enzyme-mediated metal-assisted decarboxylation of β-keto carboxylic acids originally proposed by Westheimer (Seltzer et al., 1959) thus appears correct.

Other malic enzymes are thought to catalyze hydride transfer and decarboxylation by a similar mechanism. Jade plant NAD malic enzyme, which is morphologically different from mammalian malic enzymes, still catalyzes hydride transfer before decarboxylation, even though it does not utilize exogenous oxalacetate (Grissom et al., 1987).

Acpyr-ADP as Nucleotide. When Acpyr-ADP replaces NADP as nucleotide, the intrinsic ^{13}C isotope effect on decarboxylation is not significantly different than the value obtained with NADP (Table VII). The intrinsic deuterium isotope effect on hydride transfer decreases markedly to 4.2, however. This argues for an earlier transition state for hydride transfer with the more favorable equilibrium constant afforded by the more positive redox potential of Acpyr-ADP. This is most apparent in the ratio of decarboxylation to reverse hydride transfer, k_7/k_6. With Acpyr-ADP as nucleotide, this ratio increases 36-fold. The greater energy barrier to reverse hydride transfer (and hence increased propensity to undergo decarboxylation, thus lowering the observed carbon isotope effect) is graphically depicted by the dotted lines in Figure 2.

Concluding Remarks. The role of the metal ion in the catalytic mechanism of NADP malic enzyme seems well-defined and in accord with the accepted theory for metal-assisted decarboxylation of β-keto carboxylic acids (in this case, generated by first oxidizing malate to oxalacetate). Altering the redox potential of the nucleotide does not change the intrinsic ^{13}C isotope effect on decarboxylation significantly. The intrinsic isotope effect on hydride transfer decreases, however, indicating the transition state occurs earlier with the more redox-positive nucleotide Acpyr-ADP. Seventy-five percent of the total commitment to catalysis is internal, presumably representing a conformational change necessary to poise the central complex for catalysis. The remaining 25% is external and represents steps involved in substrate dissociation.

The variation of initial velocity kinetic parameters with sucrose and glycerol as viscosogens shows an anomalous change in one of the terms which contributes to K_m. The net effect is to *increase* V/K_m with an increase in solution viscosity. The variation of $^{13}(V/K_{mal})$ with solution viscosity provides further evidence for a specific chemical effect of the viscosogen. The theory for the variation of observed isotope effect with solution microviscosity clearly shows the utility of the technique with enzymes which respond normally to the presence of viscosogen.

[7] Only the heights of the free-energy barriers are precisely defined by V/K isotope effects. The "depths of the troughs" must be defined with V_{max} isotope effects, or by measurement of equilibrium constants on the enzyme.

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Isotope Effect Studies of the Chemical Mechanism of Pig Heart NADP Isocitrate Dehydrogenase†

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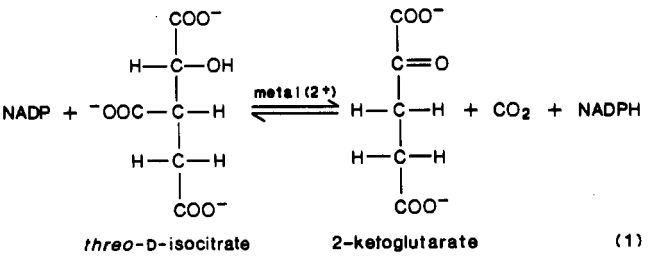
ABSTRACT: The catalytic mechanism of porcine heart NADP isocitrate dehydrogenase has been investigated by use of the variation of deuterium and ^{13}C kinetic isotope effects with pH. The observed ^{13}C isotope effect on V/K for isocitrate increases from 1.0028 at neutral pH to a limiting value of 1.040 at low pH. The limiting ^{13}C isotope effect with deuteriated isocitrate at low pH is 1.016. This decrease in $^{13}(V/K_{Ic})$ upon deuteriation indicates a stepwise mechanism for the oxidation and decarboxylation of isocitrate. This predicts a deuterium isotope effect on V/K of 2.9, but $^{D}(V/K)$ at low pH only increases to a maximum of 1.08. It is not known why $^{13}(V/K_{Ic})$ with deuteriated isocitrate decreases more than predicted. The pK seen in the $^{13}(V/K_{Ic})$ pH profile for isocitrate is 4.5. This pK is displaced 1.2 pH units from the true pK of the acid/base functionality of 5.7 seen in the pK_i profile for oxalylglycine, a competitive inhibitor for isocitrate. From this displacement, catalysis is estimated to be 16 times faster than substrate dissociation. By use of the pH-dependent partitioning ratio of the reaction intermediate oxalosuccinate between decarboxylation to 2-ketoglutarate and reduction to isocitrate, the forward commitment to catalysis for decarboxylation was determined to be 7.3 at pH 5.4 and 3.2 at pH 5.0. This gives an intrinsic ^{13}C isotope effect for decarboxylation of 1.050. 3-Fluoroisocitrate is a new substrate oxidatively decarboxylated by NADP isocitrate dehydrogenase. At neutral pH, $^{D}(V/K_{3\text{-F-Ic}}) = 1.45$ and $^{13}(V/K_{3\text{-F-Ic}}) = 1.0129$. At pH 5.2, $^{13}(V/K_{3\text{-F-Ic}})$ increases to 1.0186, indicating that a finite, but diminished, external commitment remains at neutral pH. The product of oxidative decarboxylation of 3-hydroxyisocitrate by NADP isocitrate dehydrogenase is 2-hydroxy-3-ketoglutarate. This results from enzymatic protonation of the *cis*-enediol intermediate at C_2 rather than C_3 (as seen with isocitrate and 3-fluoroisocitrate). 2-Hydroxy-3-ketoglutarate further decarboxylates in solution to 2-hydroxy-3-ketobutyrate, which further decarboxylates to acetol. This makes 3-hydroxyisocitrate unsuitable for ^{13}C isotope effect studies.

Porcine heart NADP isocitrate dehydrogenase (EC 1.1.1.42) catalyzes the oxidative decarboxylation of *threo*-D_s-isocitrate to 2-ketoglutarate and CO_2 with reduction of NADP:



(1)

It is entirely specific for $NADP^+$ and the fully ionized 2*R*,3*S*

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