Isotope Effect Studies of the Role of Metal Ions in Isocitrate Dehydrogenase†

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ABSTRACT: Pig heart $NADP^+$-dependent isocitrate dehydrogenase requires a metal ion for activity. Under optimum conditions (pH 7.5, Mg^{2+} present), the carbon isotope effect is $k^{12}/k^{13} = 0.9989 \pm 0.0004$ for the carboxyl carbon undergoing decarboxylation and the hydrogen isotope effects are $V_{max}{}^H/V_{max}{}^D = 1.09 \pm 0.04$ and $(V_{max}/K_m)^H/(V_{max}/K_m)^D = 0.76 \pm 0.12$ with *threo*-D,L-[2-^{2}H]isocitric acid. Deuterium isotope effects measured by the equilibrium perturbation technique under the same conditions are $V^H/V^D = 1.20$ for the forward reaction and 1.02 for the reverse reaction. Under these conditions the rate-determining step in the enzymatic reaction must be product release. Dissociation of isocitrate from the enzyme-isocitrate complex and the enzyme-$NADP^+$ complex must be two or more orders of magnitude slower than the chemical steps. The catalytic activity of the enzyme is about tenfold lower in the presence of Ni^{2+} than in the presence of Mg^{2+}. The carbon isotope effect in the presence of Ni^{2+} at pH 7.5 is $k^{12}/k^{13} = 1.0051 \pm 0.0012$ and the hydrogen isotope effects are $V_{max}{}^H/V_{max}{}^D = 0.98 \pm 0.07$ and $(V_{max}/K_m)^H/(V_{max}/K_m)^D = 1.11 \pm 0.14$. Thus, the rate decrease caused by substitution of Ni^{2+} for Mg^{2+} must result from the effects of metal on substrate and product binding and dissociation, rather than effects of metal on catalysis. However, a more detailed analysis of the carbon isotope effects reveals that there is also a large metal effect on the rate of the decarboxylation step, consistent with the view that the carbonyl oxygen of the oxalosuccinate intermediate is coordinated to the metal during decarboxylation.

The $NADP^+$-dependent isocitrate dehydrogenase (*threo*-D$_S$-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) catalyzes the oxidative decarboxylation of isocitric acid to α-ketoglutaric acid in the presence of a divalent metal ion. According to the accepted mechanism of action of the enzyme (eq 1),

$$\begin{array}{l} \text{isocitrate} + NADP^+ + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{E-isocitrate-}NADP^+ \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{E-oxalosuccinate-NADPH} \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad k_6 \Big\downarrow\Big\uparrow k_5 \\ E + \alpha\text{-ketoglutarate} + NADPH + CO_2 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} CO_2 + \text{E-}\alpha\text{-ketoglutarate-NADPH} \end{array} \qquad (1)$$

the reaction occurs via enzyme bound oxalosuccinic acid, but free oxalosuccinic acid is not an intermediate (Siebert et al., 1975). The addition of isocitrate and $NADP^+$ to the enzyme is random, as is the release of α-ketoglutarate and NADPH (Uhr et al., 1974; Northrop and Cleland, 1974). Not explicitly included in eq 1 is the step in which the enzyme-bound enol of α-ketoglutarate which was formed in the decarboxylation step is protonated to form α-ketoglutarate. This protonation step occurs stereospecifically with retention of configuration (Lienhard and Rose, 1964), and the enzyme catalyzes a stereospecific hydrogen exchange of α-ketoglutarate in the presence of NADPH, but in the absence of carbon dioxide. These observations are most easily understood if it is assumed that carbon dioxide release occurs prior to release of NADPH or α-ketoglutarate.

Under optimum conditions substrates and products (except carbon dioxide) bind to the enzyme quite tightly (Uhr et al., 1974). Negligible deuterium isotope effects were observed when the reaction was conducted with threo-D,L-[2-^{2}H]isocitric acid (Ramachandran et al., 1974), but these studies were limited to substrate concentrations considerably above the Michaelis constant of isocitric acid. Previous studies in our laboratory (O'Leary, 1970) showed that no carbon isotope effect is observed for the carbon atom undergoing decarboxylation. Thus, it appears that neither oxidation nor decarboxylation is rate determining in the enzymatic reaction under optimum conditions. Results of isotope exchange studies are consistent with this view (Uhr et al., 1974). The reaction is subject to a large solvent isotope effect (Colman and Chu, 1969), but the interpretation of this effect is uncertain.

Isocitrate dehydrogenase is active in the presence of a variety of metal ions. Zn^{2+} and Mn^{2+} give the highest rates, and Ni^{2+} gives the lowest of the catalytically active metal ions (Northrop and Cleland, 1970). It has been suggested (O'Leary, 1977) that the metal ion may function to coordinate the carbonyl oxygen of enzyme-bound oxalosuccinic acid and may thus serve as an electron sink in the decarboxylation step. Several lines of evidence are consistent with this proposed coordination. The dissociation constant of the enzyme-isocitrate complex is lowered by the presence of metal ions, and the dissociation constant of the enzyme-metal complex is lowered by the presence of isocitrate (Villafranca and Colman, 1972). A corresponding synergism between metal and nucleotide is not observed. Binding of isocitrate to the enzyme-metal complex appears to displace a single water molecule from the coordi-

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nation sphere of the metal (Villafranca and Colman, 1974). Nuclear magnetic resonance distance measurements for the enzyme–metal–α-ketoglutarate complex are also consistent with oxygen binding to the metal (Villafranca and Colman, 1974).

Isotope effect and isotope exchange studies indicate that under optimum conditions the rate of the oxidative decarboxylation of isocitric acid is not limited by any of the chemical steps. Oxidation and decarboxylation are much faster than product release, but no estimate has been made of how much faster they are. The nature of the effect of metal ion on the rate has not been determined, nor has the nature of the effect of pH. In this study we use carbon and hydrogen isotope effects to estimate the rates of the various chemical steps and the effects of metal and pH on these rates.

Materials and Methods

Trizma base, *threo*-D-isocitric acid, *threo*-D,L-isocitric acid lactone, $NADP^+$, NADPH, and glutamate dehydrogenase were obtained from Sigma. Celite 535 was a gift of the Johns-Manville Co. Water was deionized and doubly distilled or else purified by means of a Millipore Super Q water purification system. Other chemicals were reagent grade and were used without further purification.

Isocitrate Dehydrogenase. Pig heart $NADP^+$-dependent isocitrate dehydrogenase from Sigma (specific activity approximately 7 μmol of $NADP^+$ (mg of protein)$^{-1}$ min^{-1} in the presence of Mg^{2+} in Tris[1] buffer, pH 7.4, 25 °C) was used for all experiments except the carbon isotope effects in the presence of Ni^{2+}. For those experiments the enzyme was isolated from pig heart by an adaptation of the methods of Plaut (1962), Colman (1968), and Cleland et al. (1969) to a final specific activity of approximately 4 μmol of $NADP^+$ mg^{-1} min^{-1}. The best preparations of this enzyme to date (Ehrlich and Colman, 1976) have a specific activity of 30 μmol of $NADP^+$ (mg of protein)$^{-1}$ min^{-1}. The low purity of our enzyme preparations should have no effect on the isotope effect results as proper precautions were used to keep the amount of enzyme constant in various experiments.

D,L-[2-^{2}H]Isocitric Acid. Triethyl oxalosuccinate was prepared by the method of Friedman and Kosower (1955). The ester (1.5 g) was dissolved in 5 mL of H_2O and 3 mL of ethanol containing a trace of bromthymol blue. $NaBD_4$ (0.12 g) dissolved in 5 mL of water was added dropwise with stirring at 0 °C. The indicator color was maintained green during the addition by addition of dilute H_2SO_4. The product triethyl isocitrate was extracted with three 10-mL portions of ether and the ether was dried and removed. This product (1.2 g) was refluxed for 6 h in 10 mL of 3 M HCl. The solvent was then removed at reduced pressure and the residue was dissolved in 10 mL of hot ethyl acetate. The product isocitric acid lactone was precipitated by addition of $CHCl_3$.

Erythro and threo isomers of isocitric acid lactone were cleanly separated on Celite 535 impregnated with 0.5 N H_2SO_4 by the procedure of Gawron et al. (1958). The lactone was shown by NMR to contain 1.00 ± 0.05 deuterium atom. The lactone was hydrolyzed in 1 M NaOH at 100 °C for 10 min.

Unlabeled *threo*-D,L-isocitric acid for use in the hydrogen isotope effect studies was prepared by the same procedure, substituting $NaBH_4$ for $NaBD_4$.

threo-L-Isocitric Acid. threo-D,L-Isocitric acid lactone (2 g) was hydrolyzed with 20 mL of 1 M NaOH at 100 °C for 30 min. After cooling, 100 mL of 0.1 M Tris, 0.71 g of $Mg(OAc)_2$, and 1.32 g of NH_4OAc were added, and the pH was adjusted to 7.5. Isocitrate dehydrogenase (10 mg), 5 mg of glutamate dehydrogenase, and 10 mg of $NADP^+$ were added, and the solution was stirred at 22 °C for 48 h. A small amount of Norit was then added, and the mixture was heated briefly at 100 °C and then filtered. The filtrate was lyophilized and the residue was hydrolyzed in 1 M NaOH, neutralized, and chromatographed on a 2 × 25 cm column of Dowex-1 (Cl^-) eluted first with 500 mL of H_2O and then with 500 mL of 0.1 M HCl. Fractions having a negative optical rotation at 300 nm were pooled and lyophilized. After hydrolysis with 1 M NaOH, the product had a NMR spectrum identical with that of authentic isocitric acid and was not decarboxylated by isocitrate dehydrogenase.

Kinetic Measurements. Rates of the oxidative decarboxylation of isocitric acid were measured by observing the formation of NADPH at 340 nm. Spectral measurements were made with a Gilford Model 222 spectrophotometer in 10-cm cells at 25 °C. All buffers were 0.01 M. Tris–acetate was used at pH 7.5 and 8.5, and sodium acetate at pH 5.5. All solutions contained 20 μM $NADP^+$, 10 μM EDTA, and 300 μM dithiothreitol. The total concentration of the active enantiomer of isocitric acid was measured enzymatically. Concentrations given in Figures 1 and 2 are concentrations of free *threo*-D-isocitric acid. The concentration of free *threo*-D-isocitric acid was calculated by subtracting the concentration of the Mg^{2+}–isocitrate complex from the total isocitric acid concentration (Northrop and Cleland, 1974). Initial velocities were computer fitted to the usual Michaelis–Menten equation assuming equal variance for the velocities by use of the program of Cleland (1967).

Carbon Isotope Effects. Carbon isotope effects on the oxidative decarboxylation of isocitric acid were calculated by measuring isotopic compositions of CO_2 samples isolated after 10% reaction and after 100% reaction. The system was coupled to glutamate dehydrogenase in the presence of NH_3 in order to make the reaction irreversible and in order to regenerate $NADP^+$ (O'Leary, 1970). Extent of reaction in the 100% reaction samples was measured by neutralizing the spent acidified reaction mixture and adding fresh isocitrate dehydrogenase and $NADP^+$. Correction for incomplete reaction was made by the method of Collins and Lietzke (1959).

Isotope Effects in the Presence of Ni^{2+}. Buffers and substrates were extracted repeatedly with 0.002% dithizone in CCl_4 before use. Glutamate dehydrogenase was desalted on a long column of Sephadex G-25. $NADP^+$ was chromatographed on Chelex before use. Isocitrate dehydrogenase was extensively dialyzed against buffer containing Ni^{2+}. The 100% reaction samples used in the carbon isotope effects were decarboxylated in the presence of Mg^{2+} because of the slow rate of the Ni^{2+}-dependent reaction.

Equilibrium Perturbation. To 3.0 mL of a solution containing 3.84 mM α-ketoglutarate, 39.6 mM CO_2, 275 μM NADPH, 137 μM $NADP^+$, 10 mM Tris–acetate, 3 mM dithiothreitol, and 100 μM EDTA, pH 7.5, was added a small amount of isocitrate (final concentration approximately 167 μM) such that the final solution would be at equilibrium. The amount of isocitrate was adjusted in successive solutions until addition of isocitrate dehydrogenase produced no absorbance change at 340 nm.[2] When that had been achieved, another

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

[2] Careful temperature control is required in equilibrium perturbation experiments to eliminate artifacts due to temperature changes.

TABLE I: Carbon Isotope Effects on the Enzymatic Decarboxylation of Isocitric Acid at pH 7.5, 25 °C, in 0.09 M Tris-Acetate Buffer.[a]

Metal Ion	Isotope Ratios[b] *m/e* 45/44 × 10^6		Actual % Reaction[c]		
	Low Conversion	100% Conversion	Low Conversion	100% Conversion	k^{12}/k^{13}
Mg^{2+}	13 515	13 503	10.0	99.8	0.9991
Mg^{2+}	13 479	13 468	10.0	99.8	0.9991
Mg^{2+}	13 478	13 458	10.0	99.8	0.9984
					Mean 0.9989 ±0.0004
Ni^{2+}	13 428	13 471	10.0	94.5	1.0041
Ni^{2+}	13 384	13 456	10.0	97.7	1.0069
Ni^{2+}	13 416	13 466	10.0	95.6	1.0045
Ni^{2+}	13 412	13 467	10.0	95.5	1.0049
					Mean 1.0051 ±0.0012

[a] Solutions contained, in addition to isocitrate dehydrogenase and buffer, 20 mM *threo*-D,L-isocitric acid, 20 mM ammonium acetate, 5 mM $Mg(OAc)_2$ or 18 mM $Ni(OAc)_2$, 50 μM $NADP^+$, and glutamate dehydrogenase. [b] Isotope ratios have been corrected to a constant value of the carbon dioxide standard. [c] All 100% conversions were carried out in the presence of Mg^{2+}.

solution was made using these same equilibrium amounts of reagents, except that *threo*-D,L-[2-^{2}H]isocitrate was used rather than unlabeled isocitrate. Addition of isocitrate dehydrogenase to this solution produced the absorbance change shown in Figure 3. Correction for the slow decomposition of NADPH was made by measuring the rate of the absorbance change prior to addition of enzyme and extrapolating this change over the course of the perturbation experiment. The isotope effect was calculated as described by Schimerlik et al. (1975).

Results

Inhibition of Isocitrate Dehydrogenase by threo-L-Isocitric Acid. Of the four isomers of isocitric acid, only one, *threo*-D_S-isocitrate, is a substrate for pig heart isocitrate dehydrogenase. The inhibition constant for *threo*-L-isocitric acid was determined by measuring the rate of oxidative decarboxylation of various concentrations of *threo*-D-isocitric acid in the presence of added *threo*-L-isocitrate and saturating levels of nucleotide. The double-reciprocal plots so obtained are shown in Figure 1. The L isomer is a competitive inhibitor with a K_i of 67 ± 23 μM at pH 7.5. Under these conditions the Michaelis constant for the D isomer is 0.8 μM. Thus, the rates and isotope effects observed with racemic isocitric acid are an accurate reflection of the corresponding values for the D isomer.

Carbon Isotope Effects. Carboxyl carbon isotope effects on the oxidative decarboxylation of isocitric acid were measured using saturating levels of substrate and nucleotide and saturating levels of either Mg^{2+} or Ni^{2+}. Isotope effects were calculated by comparison of the isotopic composition of a carbon dioxide sample isolated after about 10% of the substrate had been decarboxylated with that of a sample isolated following complete decarboxylation. The experiments were designed to ensure that the product carbon dioxide in the 10% sample did not have an opportunity to react with enzyme-bound α-ketoglutarate. This was accomplished by choosing conditions such that carbon dioxide was always more than tenfold below its K_m and free α-ketoglutarate was removed as formed. Decarboxylations were conducted in the presence of glutamate dehydrogenase and ammonia in order to remove α-ketoglutarate as formed and in order to reoxidize the reduced nucleotide.

For the experiments in the presence of Ni^{2+}, the low rate of the enzymatic reaction necessitated careful removal of contaminating metal ions and use of a large excess of Ni^{2+}. The rate of the enzymatic reaction was, as expected (Northrop and Cleland, 1970), approximately tenfold lower in the presence of Ni^{2+} than in the presence of Mg^{2+}, and this low rate was not increased by the addition of a trace of Zn^{2+}, which has a high rate and a low K_m with isocitrate dehydrogenase.

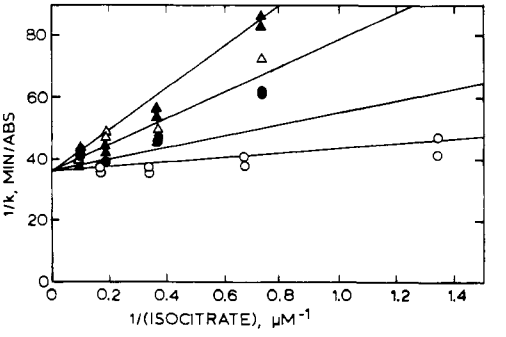

FIGURE 1: Double-reciprocal plot of the initial velocity for the oxidative decarboxylation of *threo*-D-isocitrate in the presence of various concentrations of *threo*-L-isocitrate in 0.01 M Tris-acetate, pH 7.5, 25 °C, containing 4.5 mM Mg^{2+}. Concentrations of isocitrate are those of the free (i.e., noncomplexed) material and were calculated from the known total concentrations and known dissociation constant for the Mg^{2+}-isocitrate complex. Concentrations of *threo*-L-isocitrate are 0 (○); 115 μM (●); 351 μM (△); 596 μM (▲).

Isotope ratios, percents reaction, and calculated isotope effects for the enzymatic reaction are shown in Table I. In addition to the usual correction for percent reaction in the 10% reaction sample (Bigeleisen and Wolfsberg, 1958), some of the 100% reaction samples did not react completely, and a correction was needed in that case as well (Collins and Lietzke, 1959). No significant carbon isotope effect is observed in the presence of Mg^{2+}, consistent with previous observations of O'Leary (1970). A small but significant isotope effect is observed in the presence of Ni^{2+}.

Hydrogen Isotope Effects. The kinetics of the isocitrate dehydrogenase catalyzed oxidative decarboxylation of *threo*-D,L-isocitrate and *threo*-D,L-[2-^{2}H]isocitrate were measured in the presence of saturating levels of nucleotide and metal ion. The same precautions regarding metal ions were observed as in the carbon isotope effect studies. Reciprocal

TABLE II: Summary of Hydrogen Isotope Effects on the Oxidative Decarboxylation of Isocitric Acid at 25 °C.

pH	Metal	V_{max}^H/V_{max}^D	$(V_{max}/K_M)^H/(V_{max}/K_M)^D$
5.5	4.4 mM Mg^{2+}	1.51 ± 0.11	0.95 ± 0.30
7.5	4.4 mM Mg^{2+}	1.09 ± 0.04	0.76 ± 0.12
8.5	4.4 mM Mg^{2+}	1.11 ± 0.05	1.07 ± 0.17
7.5	1.6 mM Ni^{2+}	0.98 ± 0.07	1.11 ± 0.14

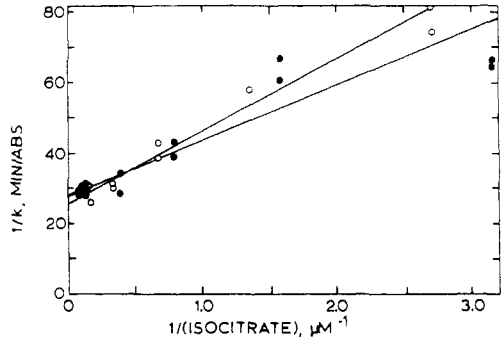

FIGURE 2: Double-reciprocal plot of the initial velocity for the oxidative decarboxylation of *threo*-D,L-isocitrate (○) and *threo*-D,L-[2-^{2}H]isocitrate (●) at pH 7.5, 25 °C, in 0.01 M Tris-acetate buffer containing 4.4 mM Mg^{2+}. Although the experiment was conducted with racemic isocitric acid, the L isomer is a very weak inhibitor (see text), and the concentrations given in the graph are those of the free D isomer.

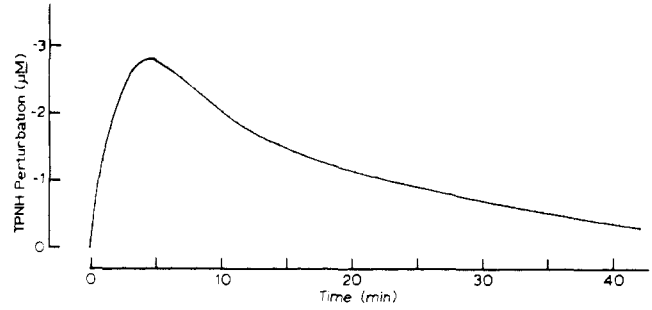

FIGURE 3: Equilibrium perturbation with isocitrate dehydrogenase using *threo*-D,L-[2-^{2}H]isocitrate at 25 °C in 0.01 M Tris-acetate buffer, pH 7.5. Concentrations of all reagents are given in the experimental section.

plots for deuterated and undeuterated isocitric acid at pH 7.5 in the presence of Mg^{2+} are shown in Figure 2. Isotope effects for this and other experiments are summarized in Table II. With the exception of the isotope effect on V_{max} at pH 5.5, none of the isotope effects differ significantly from unity. Isotope effects at pH 7.5 in the presence of Mg^{2+} are similar to those previously reported by Ramachandran et al. (1974) at a higher range of substrate concentrations in the presence of Mn^{2+}.

Equilibrium Isotope Effect. The equilibrium deuterium isotope effect on the oxidative decarboxylation of isocitric acid was measured by comparison of the equilibrium constant for the reaction of unlabeled substrate with that for *threo*-D,L-[2-^{2}H]isocitrate. Initial concentrations were adjusted to near their equilibrium values, and then isocitrate dehydrogenase was added and the reaction was allowed to come to equilibrium. The concentration of NADPH at equilibrium was measured spectrophotometrically. Other concentrations were calculated from their initial values and the final value of the NADPH concentration. The equilibrium constant is defined by eq 2.

$$K = \frac{[\alpha\text{-ketoglutarate}][CO_2][\text{NADPH}]}{[\text{isocitrate}][\text{NADP}^+]} \quad (2)$$

At pH 7.5 in 0.01 M Tris-acetate buffer at 25 °C the equilibrium constant for unlabeled isocitrate is 0.91 ± 0.02 M, in agreement with the value of 0.86 M determined under similar conditions by Londesborough and Dalziel (1968). Under these conditions the hydrogen isotope effect on the equilibrium constant[3] is $K^H/K^D = 1.18 \pm 0.03$.

Equilibrium Perturbation. Schimerlik et al. (1975) have developed a new high precision technique for measuring isotope effects in reversible enzymatic reactions. The isotope effect is similar to, though not necessarily identical with, the isotope effect on V_{max}/K_M.

An equilibrium perturbation experiment is conducted by setting all initial concentrations equal to their equilibrium values. One substrate or product is isotopically labeled in a reactive position. Upon addition of enzyme to the system, the label begins to distribute itself between reactants and products and the system is temporarily displaced from equilibrium because of the different rates of redistribution of labeled and unlabeled material. The magnitude of the isotope effect is calculated from the magnitude of the maximum displacement from equilibrium.

An equilibrium perturbation experiment for isocitrate dehydrogenase using 0.167 mM *threo*-D,L-[2-^{2}H]isocitric acid at pH 7.5, 25 °C, is shown in Figure 3. The total perturbation corresponded to 2.63 μM nucleotide. This perturbation and the equilibrium isotope effect were used to calculate kinetic isotope effects by the method of Schimerlik et al. (1975). The equilibrium perturbation hydrogen isotope effect on the forward reaction is $V^H/V^D = 1.20$, and that on the reverse reaction is 1.02.

Discussion

Carbon Isotope Effects. Carbon isotope effects have been measured for a variety of nonenzymatic and enzymatic decarboxylations. In nonenzymatic cases, carbon isotope effects in the range $k^{12}/k^{13} = 1.03$–1.06 are observed near room temperature in cases where decarboxylation is entirely rate determining (O'Leary, 1977). Isotope effects in the same range would be expected for enzymatic reactions when decarboxylation is entirely rate determining (O'Leary, 1977). The carbon isotope effects which have been observed in enzymatic decarboxylations have invariably been somewhat smaller that this, often near 1.02. These results have been interpreted to mean that in these decarboxylations the carbon-carbon bond breaking step is partially but not entirely rate determining (O'Leary, 1977).

The relationship of the carbon isotope effect observed in the enzymatic decarboxylation of isocitric acid to the mechanism

[3] This experiment was performed by Dr. Paul Cook.

of eq 1 is given[4] by eq 3 and 4. Again, we assume that $k_5^{12}/k_5^{13} = 1.03$–1.06 and that only this step shows a significant carbon isotope effect.

$$\frac{k^{12}}{k^{13}}(\text{obsd}) = \frac{k_5^{12}/k_5^{13} + R_1}{1 + R_1} \quad (3)$$

$$R_1 = \frac{k_5}{k_4}\left[1 + \frac{k_3}{k_2}\right] \quad (4)$$

We also assume that reaction of the enzyme–nucleotide–α-ketoglutarate complex with carbon dioxide does not occur; i.e., $k_6 = 0$ under the conditions of measurement. The validity of this assumption will be discussed in more detail subsequently.

The partitioning factor R_1 is equal to the ratio of the rate of decarboxylation to that of the reverse reaction for the enzyme–oxalosuccinate–NADPH complex. Because of the nature of the isotope effect experiment, this reverse reaction must proceed all the way through dissociation of isocitrate from the enzyme (step k_2 in eq 1). However, binding of substrates to the enzyme is random (Uhr et al., 1974), and this dissociation may occur either from the enzyme–isocitrate–$NADP^+$ complex or from the enzyme–isocitrate complex. Qualitatively, the lack of a significant carbon isotope effect indicates that decarboxylation is much faster than this dissociation. Quantitatively, the lack of a significant carbon isotope effect indicates that R_1, and therefore either k_5/k_4 or $(k_5/k_4)(k_3/k_2)$ or both, is much greater than unity.[5]

One other possible explanation for the small size of the carbon isotope effect must be considered. Decarboxylation and release of carbon dioxide probably do not occur in a single step. Isocitrate dehydrogenase also catalyzes the carboxylation of α-ketoglutarate. The substrate for the carboxylation is carbon dioxide, rather than carbonate or bicarbonate (Dalziel and Londesborough, 1968), and carbon dioxide binds to the enzyme–NADPH–α-ketoglutarate complex with a K_m of 2.2 mM (Uhr et al., 1974). Thus, it is possible that in the forward reaction decarboxylation of the enzyme–oxalosuccinate–NADPH complex might produce a complex of sufficient lifetime that carboxylation (k_6 in eq 1) might occur at a rate which is sufficiently high to compete with carbon dioxide release. Such a reverse reaction, if it were to occur under the conditions of our carbon isotope effect experiments, would have the effect of decreasing any carbon isotope effect which would otherwise be observed. Neither the rate constant for carbon dioxide release nor the rate constant for carboxylation can be estimated with any certainty. If the K_m value for carbon dioxide equals the dissociation constant for this species, then the rate constant for dissociation of carbon dioxide should be sufficiently high that carboxylation could not compete. Little is known about the rates of carboxylation reactions of enolates or metal-bound enolates in model systems. These reactions are endergonic by a considerable factor and are not easily studied. A number of such carboxylations are known in enzymatic reactions but the thermodynamics of the carboxylation/decarboxylation step has not been worked out. The contribution of entropy may be crucial; it is possible that the rate of carboxylation of an enzyme-bound enolate intermediate by a properly poised carbon dioxide molecule might be quite high.

Thus, carbon isotope effects on the oxidative decarboxylation of isocitric acid in the presence of Mg^{2+} at pH 7.5 indicate that decarboxylation is much faster than dissociation of isocitrate from the enzyme–isocitrate complex, provided that dissociation of carbon dioxide from the enzyme–ketoglutarate–NADPH–carbon dioxide complex is sufficiently fast that carboxylation does not occur under these conditions.

Hydrogen Isotope Effects. The hydrogen isotope effect on V_{max}/K_m for the enzymatic decarboxylation of isocitric acid under optimum conditions is slightly inverse, but the magnitude of the experimental error makes it questionable whether this inverse effect is real. The important point is that the isotope effect is approximately unity.

Hydrogen isotope effects in the range $k^H/k^D = 4$–6 are often observed for nonenzymatic reactions in which the hydrogen is being transferred in the rate-determining step (Melander, 1960). In enzymatic reactions hydrogen isotope effects for transfer of a hydride ion between a substrate and a pyridine nucleotide are often small because the hydrogen transfer step is much faster than other steps in the overall reaction (Simon and Palm, 1966; Klinman, 1977). Hydrogen isotope effects have been measured specifically for the hydride transfer step, and they fall in the range $k^H/k^D = 4$–6 (Brooks and Shore, 1971).

The relationship between the observed hydrogen isotope effect on V_{max}/K_m and the mechanism of eq 1 for isocitrate dehydrogenase is given by eq 5.

$$\frac{(V_{max}/K_m)^H}{(V_{max}/K_m)^D} = \frac{k_3^H/k_3^D + k_3/k_2 + (K^H/K^D)(k_4/k_5)}{1 + k_3/k_2 + k_4/k_5} \quad (5)$$

In the derivation of eq 5, only k_3 and k_4 are assumed to show significant isotope effects. Decarboxylation has been assumed to be irreversible under the conditions used for measurement of the isotope effect on V_{max}/K_m; thus, k_6 and subsequent rate constants do not enter. The occurrence of the isotope effect on the overall equilibrium constant in eq 5 is an algebraic device used to avoid the presence of k_4^H/k_4^D in the equation. The hydrogen isotope effect on the overall equilibrium constant ($K^H/K^D = 1.18$) is sufficiently close to unity that for our purposes it is adequate to rewrite eq 5 as eq 6

$$\frac{(V_{max}/K_m)^H}{(V_{max}/K_m)^D} = \frac{k_3^H/k_3^D + R_2}{1 + R_2} \quad (6)$$

in which case the partitioning factor R_2 (eq 7) is similar to partitioning factor R_1.

$$R_2 = k_3/k_2 + k_4/k_5 \quad (7)$$

We expect that k_3^H/k_3^D is in the range 4–6. Thus, R_2, and therefore either k_3/k_2 or k_4/k_5 or both, must be considerably larger than unity.

The information provided by the equilibrium perturbation hydrogen isotope effect is similar to, though not identical with, that provided by the hydrogen isotope effect on V_{max}/K_m. The relationship between the equilibrium perturbation isotope effect and the mechanism of eq 1 is given by eq 8.

$$\frac{V^H}{V^D} = \frac{\frac{k_3^H}{k_3^D} + \frac{k_3}{k_2} + \frac{K^H}{K^D}\frac{k_4}{k_5}\left[1 + \frac{k_6}{k_7}\right]}{1 + \frac{k_3}{k_2} + \frac{k_4}{k_5}\left[1 + \frac{k_6}{k_7}\right]} \quad (8)$$

[4] Throughout this discussion rate constants lacking superscripts are those for the normal isotopic species, i.e., ^1H and ^{12}C. Rate constants for other isotopic species, when different from those for the normal species, are always indicated by specific superscripts.

[5] Based on the results of equilibrium binding studies, Ehrlich and Colman (1975) have argued that isocitrate dehydrogenase does not operate by a random mechanism, but rather that isocitrate binds prior to the binding of nucleotide. Although we find these data less compelling than the kinetic data (Uhr et al., 1974), our conclusion would be qualitatively unchanged if the mechanism were ordered; that is, dissociation of isocitrate from the enzyme *and* from the enzyme–NAD^+ must be slow compared with decarboxylation.

In this case it should be noted that the isotope effect was measured under equilibrium conditions, and it is not correct to assume that $k_6 = 0$, as we have done previously. Instead, the equation must include rate constants through k_7, the product release step. Rate constant k_7 must be the rate constant for release of the product which contains the isotopic label, in this case, NADPH.

For present purposes we will again assume that $K^H/K^D = 1$, and thus reduce eq 8 to eq 9 and 10.

$$\frac{V^H}{V^D} = \frac{k_3{}^H/k_3{}^D + R_3}{1 + R_3} \tag{9}$$

$$R_3 = k_3/k_2 + k_4/k_5 + (k_4/k_5)(k_6/k_7) \tag{10}$$

Partitioning factor R_3 is equal to R_2 plus an additional term which is related to product release $[(k_4/k_5)(k_6/k_7)]$. The small magnitude of the observed equilibrium perturbation isotope effect indicates that R_3 is very large.

Carbon and hydrogen isotope effects thus indicate that R_1, R_2, and R_3 are larger than unity. Because of the uncertainties in the observed isotope effects and in the predicted values of $k_5{}^{12}/k_5{}^{13}$ and $k_3{}^H/k_3{}^D$, it is not possible to obtain actual values for these partitioning factors, but it is possible to estimate a range of possible values. In the most pessimistic case (small isotope effects on k_3 and k_5, large errors in the observed isotope effects), the values of R_1, R_2, and R_3 are in the vicinity of 100. For more realistic assumptions, they are considerably larger than this.

The difference between the hydrogen isotope effect on V_{max}/K_m and that obtained by the equilibrium perturbation technique must be due to the presence of the additional term in eq 8 not present in eq 5. Chemically, this differences arises because the decarboxylation step must be considered reversible in the equilibrium perturbation experiment, whereas it is effectively irreversible in the steady-state measurements. The equilibrium perturbation isotope effect obtained for the oxidative decarboxylation of isocitrate is equal to the equilibrium isotope effect for the overall reaction, whereas the isotope effect in the reverse reaction is unity. The equality of equilibrium and equilibrium perturbation isotope effects probably arises because the last term in eq 8 dominates. Chemically, this means that release of NADPH from the final complex is much slower than release of isocitrate from the initial complex. Both steps are slower than any of the chemical transformations.

The small magnitude of the hydrogen isotope effect on V_{max} arises in a different way. The relationship of the observed isotope effect to the mechanism of eq 1 is given by eq 11.

$$\frac{V_{max}{}^A}{V_{max}{}^D} = \frac{\frac{k_4{}^H}{k_4{}^D} + \frac{k_4}{k_5}\left(1 + \frac{K^H}{K^D}\frac{k_3}{k_4}\left[1 + \frac{k_5}{k_7}\right]\right)}{1 + \frac{k_4}{k_5}\left(1 + \frac{k_3}{k_4}\left[1 + \frac{k_5}{k_7}\right]\right)} \tag{11}$$

Assumption that $K^H/K^D \approx 1$ results in eq 12 and 13.

$$\frac{V_{max}{}^H}{V_{max}{}^D} = \frac{k_4{}^H/k_4{}^D + R_4}{1 + R_4} \tag{12}$$

$$R_4 = k_4/k_5 + k_3/k_5 + k_3/k_7 \tag{13}$$

The small value of the observed isotope effect indicates that one or more of the terms k_4/k_5, k_3/k_5, and k_3/k_7 is large. No statements can be made with certainty about the first two of these terms, but k_3/k_7 is likely to be quite large. Rate constants k_2 and k_7 are likely to be of similar magnitudes since both represent rates of dissociation of rather sticky substrates or products from the enzyme. In fact, k_7 is likely to be the smaller of the two, because as used here k_7 includes the dissociation of both α-ketoglutarate and NADPH from the enzyme, whereas k_2 only includes dissociation of isocitrate. Since k_3/k_2 is quite large, k_3/k_7 will be quite large also. This conclusion is consistent with previous studies of enzyme-catalyzed isotope exchange, which indicated that the rate-determining step in the overall reaction is product release (Uhr et al., 1974).

Thus, the following picture of the mechanism of action of isocitrate dehydrogenase emerges: Under optimum conditions, the binding of isocitrate to the enzyme is virtually irreversible and is followed rapidly by oxidation and decarboxylation. The oxidation and decarboxylation steps must be at least two orders of magnitude faster than the substrate and product release steps. Dissociation of NADPH from the enzyme is slow.

The magnitudes of the carbon and hydrogen isotope effects indicate that dissociation of isocitrate from the enzyme is slow. From this it follows that dissociation of $NADP^+$ from the enzyme must also be slow; were that not so, dissociation of $NADP^+$ would always occur prior to dissociation of isocitrate, contrary to the observed random mechanism for this enzyme.

Effects of Metals. When Ni^{2+} is substituted for Mg^{2+} in isocitrate dehydrogenase, the value of V_{max} decreases by approximately a factor of ten (Northrop and Cleland, 1970) and the value of V_{max}/K_m decreases by somewhat more than this amount. We shall examine the reason for this rate decrease in terms of the mechanism of eq 1.

The simplest explanation of the effect of metal ions would be that Ni^{2+} functions less efficiently than Mg^{2+} in either the oxidation step or the decarboxylation step. This would require that, although the binding and dissociation steps are the kinetically significant steps in the presence of Mg^{2+}, substitution of Ni^{2+} has slowed either hydride transfer or decarboxylation to the point where the observed rate is limited by one of these steps. Under such conditions a large hydrogen isotope effect will be observed if hydride transfer is rate determining and a large carbon isotope effect will be observed if decarboxylation is rate determining. Hydrogen isotope effects in the presence of Ni^{2+} are still essentially unity, so hydride transfer clearly has not become rate determining. The carbon isotope effect observed in the presence of Ni^{2+} is significantly different from unity but much smaller than that expected for rate-determining decarboxylation. An isotope effect of this magnitude indicates that partitioning factor R_1 is of the order of 10. Thus, the change in rate of the enzymatic reaction is not a result of a change in the rate of oxidation or decarboxylation.

However, hidden from view in this argument is the fact that the change in metal ion does have a large effect on the rate of the decarboxylation step. This arises in the following way: We estimated previously that in the case of Mg^{2+} the value of R_1 is at least 100, and perhaps several times larger than this. In the case of Ni^{2+} the value is approximately 10. It appears that V_{max} is very nearly equal to k_7 for both Ni^{2+} and Mg^{2+}. If we assume that the effect of metal on k_2 is similar to that on k_7, then consideration of eq 4 reveals that k_5 is decreased by at least a factor of 100 on changing from Mg^{2+} to Ni^{2+}. Thus, the metal has a large effect on the rate of the decarboxylation step, even though decarboxylation is never rate limiting. This conclusion is consistent with the suggestion based on magnetic resonance studies (Villafranca and Colman, 1972) that the metal coordinates the carbonyl oxygen of α-ketoglutarate. By this means the metal can function as an electron sink during decarboxylation as it does in nonenzymatic decarboxylations of β-keto acids (O'Leary, 1977).

The effect of metal ions on the overall rate of the enzymatic reaction appears not to result from metal ion effects on the chemical steps at all. Instead, it appears that the metal is in some way affecting rates of substrate and product binding and dissociation. This conclusion is consistent with NMR studies of the binding of isocitrate to the enzyme (Villafranca and Colman, 1972, 1974).

Conclusion

Isocitrate dehydrogenase operates at maximum efficiency at pH values near 7 in the presence of saturating levels of Mg^{2+}. Isotope exchange studies (Uhr et al., 1974) have shown that under these conditions the rate-determining step in the overall reaction is product release. Carbon and hydrogen isotope effects are consistent with this conclusion and indicate further that the chemical transformations are at least two orders of magnitude faster than the substrate and product dissociation steps.

The essential metal ion appears to function in at least two different ways. It functions in the decarboxylation step, and large differences in decarboxylation rate can be obtained by changing metal ion. The metal ion also functions in the substrate and product binding and dissociation steps, particularly those involving isocitrate and α-ketoglutarate, although the mechanism of this metal ion effect is not clear.

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