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

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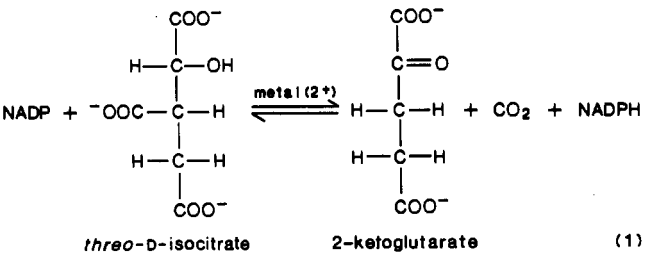
Received August 26, 1987; Revised Manuscript Received December 18, 1987

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*

†Supported by a grant from the National Institutes of Health (GM 18938). A preliminary report of this work was presented at the American Society of Biological Chemists Meeting, New Orleans, LA, April 15-23, 1982 (Grissom & Cleland, 1982), and the American Chemical Society Meeting, Philadelphia, PA, September 1982 (Grissom & Cleland, 1984).

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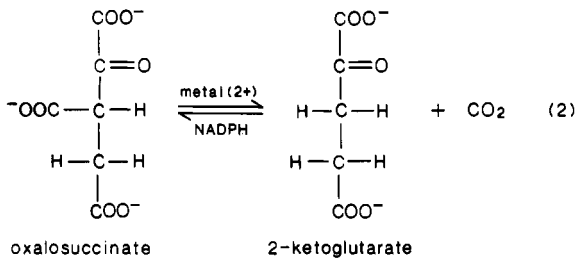
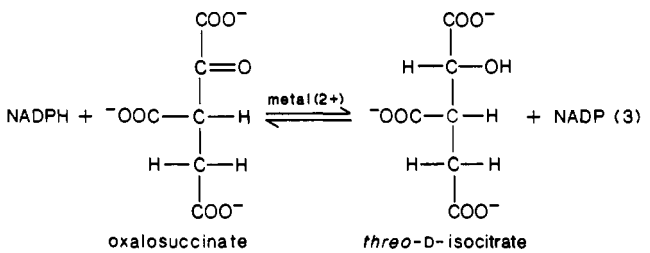
0006-2960/88/0427-2934$01.50/0

isomer of isocitrate. The divalent metal cation most often used is Mn^{2+} or Mg^{2+}, with lesser activity seen with Ni^{2+}, Zn^{2+}, or Cd^{2+}. The true substrate for reaction 1 has been postulated to be the metal–isocitrate complex (Colman & Villafranca, 1972; Colman, 1972), but this may represent highly synergistic binding of metal ion and isocitrate. The reaction is reversible with CO_2 being the oxidized carbon species used (Dalziel & Londesborough, 1968). Active enzyme centrifugation and Kurganov (1967) enzyme dilution experiments indicate the dimer is active and the monomer is inactive (Kelly & Plaut, 1981a,b). The kinetic mechanism has been thoroughly investigated by use of initial velocity, multiple inhibition, and isotope exchange studies and shown to be random sequential with catalysis more rapid than product release (Uhr et al., 1974; Northrop & Cleland, 1974). Kelly and Plaut (1981b) showed that the kinetic mechanism changes to ordered sequential with NADP binding last at low enzyme concentrations or in the absence of NADPH or EDTA.[1]

In addition to reaction 1, the enzyme will also catalyze the reduction or decarboxylation of oxalosuccinate (Siebert et al., 1957) as shown in eq 2 and 3. Because these activities reside





on the same protein, the mechanism of isocitrate oxidative decarboxylation is generally thought to be a stepwise process with hydride transfer producing enzyme-bound oxalosuccinate, which is subsequently decarboxylated. No incorporation of radioactivity into an unlabeled oxalosuccinate pool could be demonstrated when starting from isocitrate (Siebert et al., 1957). The enzyme will catalyze the exchange of the *pro-S* C_3 methylene hydrogen of 2-ketoglutarate with solvent, indicating an enzymatic base capable of stereospecifically protonating the 2-ketoglutarate enolate resulting from decarboxylation (Rose, 1960; Lienhard, 1964).

The same type of stepwise dehydrogenation, followed by metal-assisted decarboxylation, has been shown to operate with NADP and NAD malic enzymes, which are analogous oxidative decarboxylases (Hermes et al., 1982; Grissom et al., 1987). For NADP malic enzyme, an observed ^{13}C kinetic isotope effect on V/K of 1.0336 was observed at neutral pH with Mg^{2+}. For NADP isocitrate dehydrogenase, a $^{13}(V/K_{Ic})$ value of 0.9989 ± 0.0004 was observed at neutral pH (O'Leary, 1971; Limburg & O'Leary, 1977). This nearly nonexistent isotope effect is attributed to the stickiness of isocitrate (i.e., catalysis is faster than the net rate constant for dissociation of isocitrate from the central enzyme–substrate complex) such that no isotopic discrimination between ^{12}C- and ^{13}C-containing substrates occurs. The deuterium isotope effect on V/K_{Ic} varies slightly with pH: $^{D}(V/K_{Ic})$ is 1.0 at pH 7.45 and 1.08 at pH 4.5 (Cook & Cleland, 1981b). In this paper, we have used the variation of ^{13}C kinetic isotope effects with pH (Cook & Cleland, 1981a,b) and the partitioning of oxalosuccinate between reduction and decarboxylation (Grissom & Cleland, 1985) to determine the chemical mechanism and compare it to that of NADP malic enzyme. We also report the substrate activities of 3-hydroxyisocitrate and 3-fluoroisocitrate analogues.

Materials and Methods

Enzymes. Type IV porcine heart NADP isocitrate dehydrogenase (Sigma) was used in all experiments. The lyophilized powder was dissolved in a minimum amount of 50 mM Pipes, pH 7.0, 50% glycerol, 10 mM EDTA, and 1 mM dithiothreitol and dialyzed against the same buffer for 48 h at 0 °C. The solution was then chromatographed on a 1 cm × 20 cm Sephadex G-10 column eluted with 10 mM Pipes, pH 7.0, 10% glycerol, and 1 mM dithiothreitol. The coupling enzyme glutamate dehydrogenase was also diluted in 50 mM Pipes, pH 7, and 50% glycerol and chromatographed as above.

^{13}C Kinetic Isotope Effects. ^{13}C isotope effects on NADP isocitrate dehydrogenase were determined by the method of O'Leary (1980). To provide adequate buffering capacity at low pH values, reactions below pH 6.0 were run in a total volume of 280 mL rather than the standard 20-mL assay volume. The following buffers were used at a final concentration of 150 mM: pH 4.0–5.5, potassium acetate; pH 5.5–6.5, Mes; pH 6.5–7.5, Pipes. Because of the acid denaturation of isocitrate dehydrogenase, aliquots of fresh enzyme had to be periodically added to the reaction vessel to achieve the desired fraction of reaction. The enzyme was allowed to exhaust the NADP present to stop the reaction at the desired point. Because the K_m for NADP is less than 1 μM, the NADP concentration remained well-above saturation until the nucleotide was nearly exhausted. Hence, the external commitment was not significantly altered by this practice. Acid degradation of the NADPH produced and the low concentration of CO_2 prevented reversal of the reaction. Above pH 6.0, glutamate dehydrogenase was used to convert the 2-ketoglutarate produced to L-glutamate, as well as to recycle the nucleotide. The concentration of reagents was as follows: for complete conversion (20-mL volume), 8 mM *threo,erythro*-DL-isocitrate (or isocitrate analogue), 0.4 mM NADP, 30 mM NH_4Cl, and 2 mM $MgCl_2$; for low conversion (with recycling of nucleotide at neutral pH, 20-mL volume), 100 mM *threo,erythro*-DL-isocitrate (or analogue), 1 mM NADP, 30 mM NH_4Cl, and the specified $MgCl_2$ concentration; for low conversion (without recycling of nucleotide at neutral pH, 280-mL volume), 10 mM *threo,erythro*-DL-isocitrate (or analogue), 1 mM NADP, and the specified $MgCl_2$ concentration. A total of 100 units of glutamate dehydrogenase was used in those assays with the nucleotide recycle. Enough isocitrate dehydrogenase was added to obtain the desired fraction of reaction in 1–2 h. As much as 20 mL of buffer containing 3000 units of enzyme had to be added to those assays at the acid extreme of the pH profile. The vessels were maintained at 25 °C during turnover. For each assay with isocitrate or 3-fluoroisocitrate, the amount of CO_2 produced (measured manometrically following isolation) corresponded to the expected extent of reaction. The reactions were quenched with 2–5 mL of concentrated H_2SO_4. The $^{12}C/^{13}C$

[1] Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; OSA, oxalosuccinic acid; Ic, isocitrate; IcDH, isocitrate dehydrogenase; 2-KG, 2-ketoglutarate.

ratio of the CO_2 was analyzed on a Nuclides Associates RMS 6-60 isotope ratio mass spectrometer equipped with a dual-inlet system or a Finnigan δ-E isotope ratio mass spectrometer.

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 buffer (same as specified above for different pH ranges plus Tricine, pH 7.5–8.5), 0.2 mM NADP, and the specified amount of $MgCl_2$. Because the K_m for isocitrate is submicromolar at neutral pH, 10-cm path-length quartz cuvettes were used. When it was desired to maintain the Mg^{2+} at a specific low level, the following dissociation constants were employed: Mg–NADP = 19.1 mM (Apps, 1973); Mg–isocitrate = 6.64 mM.

Synthesis of Substrates. (*A*) *threo,erythro*-DL-*Isocitrate-2-d.* Triethyl oxalosuccinate was synthesized according to the procedure of Friedman and Kosower (1955) in 97% yield. Deuteriated isocitrate was produced by reducing triethyl oxalosuccinate with $NaBD_4$ under the conditions of Limburg and O'Leary (1977). No attempt was made to influence the stereochemical course of the reduction; hence, the final product contained all four isomers of isocitrate. The isocitric lactone was hydrolyzed in 1 N KOH at 25 °C over 48 h and loaded onto a 3.5 cm × 30 cm Dowex AG 1-X2 column in the formate form. Isocitrate was eluted with a 500-mL 0–2 N HCOOH gradient. ^1H NMR showed 97% deuteriation at C_2. Overall yield was 62%. To account for the presence of three inactive isomers of isocitrate, unlabeled isocitrate was synthesized by the same procedure: ^1H NMR (270 MHz, D_2O) δ 2.00 (s), 2.5 (m), 4.05 (d).

(*B*) *threo,erythro*-DL-*3-Hydroxyisocitrate.* 3-Hydroxyisocitrate (1,2-dihydroxy-1,2,3-propanetricarboxylic acid, also called garcinia acid) was synthesized according to the procedure of Plaut et al. (1975) in which *trans*-aconitic acid is oxidized by osmium tetroxide in the presenced of potassium chlorate. The product was purified by the same chromatographic procedure described for isocitrate: ^1H NMR (D_2O) δ 2.73 (d, 2 H), 4.00 (s, 1 H). Two isomers were present in the final preparation, but only one is enzymatically active. Deuteriated 3-hydroxyisocitrate was synthesized by the same procedure, but starting from perdeuterio-*trans*-aconitic acid. This was synthesized by incubating unlabeled *trans*-aconitic acid in D_2O at 80 °C for 60 h. This exchanged all hydrogen for deuterium by a 1,3-prototrophic shift. The product, 2,4,4-perdeuteriogarcinate, was 99% deuteriated by ^1H NMR integration against an internal acetate standard.

(*C*) *3-Fluoroisocitrate.* 3-Fluoroisocitrate was synthesized according to the procedure of Gottwald and Kun (1965). The synthesis and characterization of the precursor, triethyl 3-fluorooxalosuccinate, is also reported in Grissom and Cleland (1988). A total of 2.5 g of triethyl 3-fluorooxalosuccinate was dissolved in 5 mL of ethanol and added to 8.25 mL of H_2O. The pH was adjusted to 7 with KOH and cooled to 0 °C. A total of 0.206 g of $NaBH_4$ was dissolved in 8.25 mL of H_2O at 0 °C and added dropwise over 2 h. The pH was maintained at 7–8 with dilute H_2SO_4. The workup from this point on is critical for not losing fluorine. The ether extract was dried over Na_2SO_4 and the solvent removed at 25 °C. ^1H and ^{19}F NMR showed this to be a complex mixture of at least five fluorine-containing compounds. The esters were hydrolyzed with 2:1 acetic acid:concentrated HCl for 3 days at 25 °C. Progress of the hydrolysis was followed by TLC on silica gel with $CHCl_3$ and visualized by UV and $Fe(NO_3)_3/NH_2OH$ spray. The acid was removed by the addition of 2 volumes of H_2O and rotary evaporated at 25 °C. This procedure was repeated until no acid could be detected by olfaction (ca. 6 times). The lactone of 3-fluoroisocitrate was hydrolyzed by incubation at pH 12.5 for 6 h at 25 °C (negligible F^- was lost during this time). The product was chromatographed on the same ion-exchange column described above, except the material was eluted with 2 L of 0–1 N KCl, yielding a peak with NADP isocitrate dehydrogenase activity. ^{19}F NMR showed two groups of peaks with eight lines each at −163.7 ppm and −167.9 ppm relative to $CFCl_3$ at 0 ppm. These are the two diastereomeric pairs possible with 3-fluoroisocitrate.

Determination of Enzymatic Products: 3-Hydroxyisocitrate. (*A*) ^{13}C *NMR.* A 5-mL volume of 92 mM *threo*-D-3-hydroxyisocitrate (containing equal concentrations of the three other isomers of 1,2-dihydroxy-1,2,3-propanetricarboxylic acid) was incubated with 100 mM NADP, 20 mM $MgCl_2$, and ca. 90 units of NADP isocitrate dehydrogenase at pH 7 and 25 °C. After 7 h, the solution was filtered through glass wool and transferred to a 12-mm NMR tube with a 5-mm coaxial D_2O lock containing 1,4-dioxane as a standard (67.47 ppm). ^{13}C NMR spectra at 50.3 MHz (on a Nicolet 200-MHz multinuclear spectrometer) were acquired at 8 and 12 h after the addition of enzyme. A relaxation delay of 2 s was used to ensure complete relaxation of the carbonyl and carboxyl carbons following a 90° pulse. A spectrum of the reaction components but without isocitrate dehydrogenase served as a control.

(*B*) *$NaBH_4$ Trapping Experiments.* The products of enzymatic oxidation of 3-hydroxyisocitrate in various stages of decarboxylation were trapped by reduction of the resulting carbonyl compounds with tritium-labeled $NaBH_4$ or $NaBD_4$. The resulting negatively charged species were isolated by anion-exchange chromatography, and their structure was determined by ^1H and ^{13}C NMR. The following were contained in a 200-mL reaction volume at pH 7: 100 μmol of *threo*-D-3-hydroxyisocitrate (and equal concentrations of the other three isomers of 1,2-dihydroxy-1,2,3-propanetricarboxylic acid), 100 μmol of NADP, 10 mM $MgCl_2$, and 50 units of NADP isocitrate dehydrogenase. The progress of the reaction was monitored by the appearance of NADPH at 340 nm. When ca. 65% of the *threo*-D-garcinate had been converted to products (40 min), the reaction was quenched by cooling to 0 °C; 3.0 g of Bio-Rad Chelex 100 was added to further inhibit the enzymatic reaction. The enzyme was removed by ultrafiltration with an Amicon UM-1 membrane. The resulting solution was divided into two 100-mL portions for reduction by 200 mg of $NaBH_4$ or $NaBD_4$ (each trace labeled with 10 mCi of ^3H) dissolved in 15 mL of H_2O at 0 °C. The borohydride solution was added dropwise over 20 min at 0 °C, with the pH maintained between 7.5 and 9 by the addition of dilute H_2SO_4. The solutions were further stirred for 1.5 h to ensure reaction of all materials. Several drops of formic acid were added to decompose residual borohydride. At pH 9, the solutions were loaded onto separate columns of Bio-Rad AG-MP-1 (1.5-cm diameter, total bed volume = 42 mL) in the formate form. The columns were washed with 150 mL of H_2O, and the bound material was eluted with a 500-mL 0–2 N formic acid gradient.

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, $^DK_{eq}$ 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 saturation curves were fitted to eq 4 with a BASIC version of Cleland's HYPERO program[2] (Cleland, 1977).

$$v = VA/(K + A) \quad (4)$$

Tritium and ^{13}C isotope effects determined by the internal competition method were computed with eq 5. In eq 5, R_f

$$^{13}(V/K) = \log (1 - f)/\log [1 - f(R_f/R_0)] \quad (5)$$

is the $^{13}C/^{12}C$ ratio at fractional reaction f, and R_0 is the $^{13}C/^{12}C$ ratio of the starting material.

pH profiles which exhibited a rate plateau at both low and high pH were fitted to eq 6 with the FORTRAN program

$$\text{rate} = \log [[YL + (YH)K/H]/(1 + K/H)] \quad (6)$$

WAVL, where YL and YH are the rates at low and high pH, respectively, K is the thermodynamic dissociation constant for the titratable group, and H is the hydrogen ion concentration.

Data for deuterium isotope effects were fitted to eq 7 which

$$\text{rate} = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (7)$$

assumes isotope effects on V and V/K, where F_i is the fraction of deuterium label in the substrate, while E_V and $E_{V/K}$ are the isotope effects minus one for the respective parameters.

Partitioning of Oxalosuccinate. The partitioning of the intermediate oxalosuccinate was determined in a manner analogous to that for oxalacetate (Grissom & Cleland, 1985). The production of isocitrate and 2-ketoglutarate from oxalosuccinate was followed spectrophotometrically to determine directly the partitioning ratio R_H (also equal to C_f, the total forward commitment for decarboxylation). Oxalosuccinate reduction to isocitrate was monitored by utilization of NADPH at 340 nm. Oxalosuccinate decarboxylation to 2-ketoglutarate was followed by the disappearance of oxalosuccinate at 281 nm (the isosbestic point for conversion of NADP to NADPH). Subtracting the rate of isocitrate production from the total rate of oxalosuccinate utilization gave the amount of 2-ketoglutarate produced. The apparent oxalosuccinate extinction coefficient at 281 nm was determined at both pH values. To prevent reversal of reaction 2, 2-ketoglutarate was coupled to the production of glutamate with glutamate-pyruvate transaminase. This equilibrium was shifted in favor of glutamate production by the presence of 100 mM L-alanine in the assay. The barium salt of oxalosuccinate was obtained from Sigma and converted to the sodium salt with Dowex AG 50-Na^+. The contaminating 2-ketoglutarate was quantified with glutamate-pyruvate transaminase and lactate dehydrogenase in the presence of L-alanine and DPNH. This assay was found to give better results than glutamate dehydrogenase in the presence of oxalosuccinate. The oxalosuccinate concentration was determined by conversion of all oxalosuccinate to 2-ketoglutarate by heating to 90 °C for 15 min. The instrumentation and methods used are otherwise the same as described in Grissom and Cleland (1985). The equation for calculating the intrinsic ^{13}C isotope effect from the partitioning ratio and the observed ^{13}C isotope effect is

$$^{13}k = R_H[^{13}(V/K) - 1] + ^{13}(V/K) \quad (8)$$

where R_H is the 2-KG/Ic partitioning ratio.

RESULTS

3-Hydroxyisocitrate Product Identification: ^{13}C NMR. The progress of the enzymatic oxidation was followed by the appearance of peaks at 23 and 173 ppm (corresponding to the C_4 methylene and exocyclic amido carbons of NADPH), indicating the reduction of NADP. Resonances corresponding to the acid-catalyzed degradation product of the reduced nicotinamide ring were also observed in the 8-h spectrum and increased in intensity in the 12-h spectrum. These resonances were assigned according to the published values of Williams et al. (1976). Those resonances not corresponding to NADP, NADPH, the acid-catalyzed degradation product of NADPH, unreacted 3-hydroxyisocitrate, glycerol (added with enzyme), Pipes, or dioxane were tabulated and their intensities noted (Table I).

Table I: ^{13}C NMR Resonances Ascribable to Decarboxylation Products of 3-Hydroxyisocitrate

	8 h		12 h	
structure	chem shift (ppm)	intensity[a]	chem shift (ppm)	intensity
6	49.38	1.0	49.37	1.3
4	49.66	1.7	49.66	0.4
4	64.87	1.2	64.86	1.0
6	68.50	0.9	68.49	1.2
4	173.95	0.6	173.95	0.5
6	175.06	0.6	175.06	0.6
4	175.23	0.4		
4	206.84	0.5		
6	209.40	0.6	209.39	0.6

[a] Relative intensities of resonances.

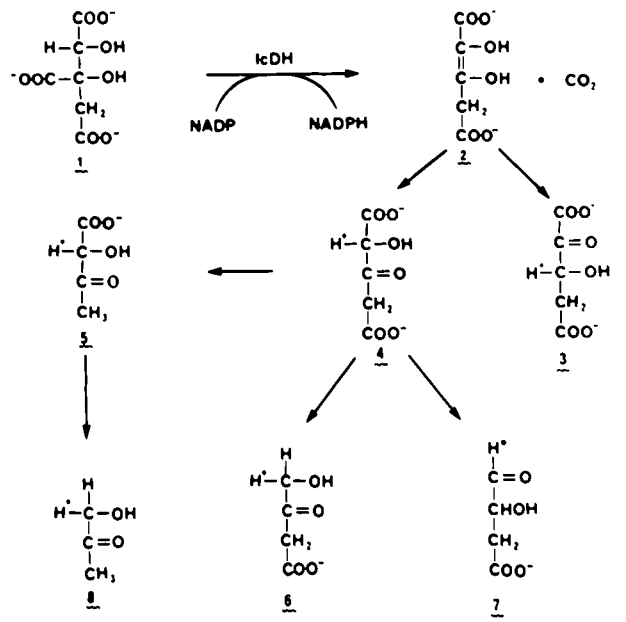


FIGURE 1: Scheme for 3-hydroxyisocitrate decarboxylation. It is thought only the conversion of **1** to **2** is catalyzed by NADP isocitrate dehydrogenase. Subsequent steps are thought to occur in solution.

Those resonances which decrease in going from the 8- to the 12-h spectrum are ascribed to an initial reaction product which decarboxylates to yield the resonances which increase in intensity. The resonances listed in Table I match those expected for structures **4** and **6** as detailed in Figure 1. Two methylene resonances in magnetically similar environments are seen at 49.38 and 49.66 ppm. C_3–C_4–C_5 of compound **4** is identical with C_3–C_4–C_5 of compound **6**. Hence, the resonance of the methylene group in **4** and **6** should be nearly identical. This is in contrast to the methylene group on **3**, which is not α to a carbonyl group and should be shifted upfield (cf. the ^{13}C resonances for the methylene group in **6** which shifts upfield by 7 ppm upon reduction of the carbonyl in the reduction trapping compound, H-I). This suggests the resonances in Table I ascribed to **4** are indeed 2-hydroxy-3-ketoglutarate and not **3** (2-keto-3-hydroxyglutarate). Structure

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

4 is seen in the spectrum taken after 8 h and diminishes in the 12-h spectrum. The resonances representing structure 6 increase in intensity in the 12-h spectrum. This suggests the carbon skeleton proceeds from 3-hydroxyisocitrate to 4 and then to 6. No evidence for acetol (8), which is the logical decarboxylation product of 6, was observed. Acetol has ^{13}C resonances at 25.9, 68.5, and 213.08 ppm under the above experimental conditions. Plaut et al. (1975), however, clearly demonstrated the presence of acetol (8) in enzymatic reaction mixtures of 3-hydroxyisocitrate by isolating the 2,4-dinitrophenylhydrazone derivative, methylglyoxal 2,4-dinitrophenylosazone.

Reduction Trapping Experiments. Three separate peaks contained ^3H were observed in the eluent. These peaks are labeled H-I and D-I for the earliest eluting peak from the $NaBH_4$ and $NaBD_4$ reductions, respectively. Similarly, the peaks which eluted second are H-II and D-II, and the third peaks are H-III and D-III. There was a fourth, less distinct radioactive peak, but this coincided with an increase in A_{260} indicating nonspecific labeling of the nucleotide or its decomposition products. Each peak was rotary evaporated to dryness and redissolved in H_2O to remove formic acid. This process was repeated until no HCOOH was detected by olfaction. Each peak was lyophilized from D_2O in preparation for ^1H and ^{13}C NMR.

Peaks H-II and D-II, although containing some radioactivity, contained no organic material with an observable ^1H or ^{13}C NMR signal and are attributed to an inorganic contaminant of the tritiated sodium borohydride. ^1H NMR in D_2O at 270 MHz of the other peaks showed the following: for H-I, δ 3.03 (dd, 1 H, J_{B-C} = 7.9 and $J_{C-C'}$ = 14.9 Hz, CH_2), 3.10 (dd, 1 H, $J_{B-C'}$ = 5.5 and $J_{C-C'}$ = 14.9 Hz, CH_2), 4.20 (dd, 1 H, J_{A-B} = 6.6 and $J_{A-A'}$ = 11.7 Hz, CH_2O), 4.33 (dd, 1 H, $J_{A'-B}$ = 3.7 and $J_{A-A'}$ = 11.7 Hz, CH_2O), 4.76 (m, 1 H, CHOH); for D-I, δ 3.01 (d, 1 H, $J_{C-C'}$ = 14.9 Hz, CH_2), 3.08 (d, 1 H, $J_{C-C'}$ = 14.9 Hz, CH_2), 4.18 (d, 1 H, $J_{A-A'}$ = 11.7 Hz, CH_2O), 4.31 (d, 1 H, $J_{A-A'}$ = 11.7 Hz, CH_2O); for H-III, δ 2.21 (dd, 1 H, J = 3.3 and 15.2 Hz, CH_2), 2.33 (dd, 1 H, J = 9.5 and 15.3 Hz, CH_2), 2.36 (dd, 1 H, J = 5.8 and 13.1 Hz, CH_2), 2.42 (dd, 1 H, J = 5.5 and 13.0 Hz, CH_2), 4.2 (m, 2 H, CHOH), 4.76 (m, 2 H, CHOHCOO); for D-III, δ 2.23 (d, 1 H, J = 15.1 Hz, CH_2), 2.35 (d, 1 H, J = 15.2 Hz, CH_2), 2.37 (d, 1 H, J = 13.0, CH_2), 2.44 (d, 1 H, J = 13.1), 4.68 (s, 1 H), 4.77 (s, 1 H).

^{13}C NMR in D_2O at 50 MHz with ^1H noise decoupling showed the following: for H-I, δ 42.04 (s, 1 C, CH_2), 66.09 (s, 1 C, CH_2O), 70.60 (s, 1 C, CHOH), 185.82 (s, 1 C, COOH); for H-III, δ 42.32 (s, 1 C, CH_2), 71.28 (s, 1 C, CHOH), 75.48 (s, 1 C, CHOCOOH). Signal to noise ratio not sufficient to observe carboxyl carbons.

Radioactive peaks H-I and D-I unambiguously correspond to the reduction product of 6. The ^1H NMR of the lactone of this compound has previously been reported (Mori et al., 1979). No evidence for the reduction products of 5 or 7 (both of which should cochromatograph with 6 by ion exchange, but be distinguishable by NMR) was observed. Fractions H-III and D-III correspond to five-carbon species with two ionized carboxyl groups. The assignment of the structure of the reduction compound of 3 or 4 is not definite. Structure 6 (and subsequent acetol formation) could not arise from 2-keto-3-hydroxyglutarate (3). Protonation of the enediol 2 at C_3 prevents further decarboxylation of 3. Hence, the major product of enzymatic decarboxylation of 3-hydroxyisocitrate is 2-hydroxy-3-ketoglutarate (4). This results from protonation of the enediol, 2, at C_2. In contrast, the enzyme protonates

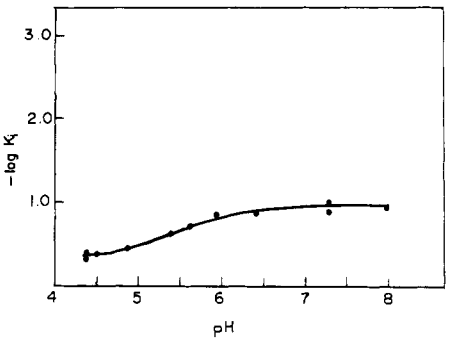


FIGURE 2: pK_i profile for oxalylglycine, a competitive inhibitor for isocitrate, versus pH. Assay conditions: 100 mM buffer, 0.1 mM dithiothreitol, 2.0 mM $MgSO_4$, and 0.5 mM NADP. Data fitted to eq 6. Calculated pK = 5.7 ± 0.1, YL = 2.0 ± 0.1, and YH = 8.8 ± 0.3.

the enolate resulting from isocitrate oxidative decarboxylation at C_3. Presumably, this enzymatic protonation is stereospecific. The stereochemistry cannot be determined from either H-I or H-III; however, since asymmetric induction during the reduction with borohydride is likely to be low, two diastereomers will result whether the enzymatic protonation at C_2 was stereospecific or not.

Isocitrate Analogue Product Identification. The stoichiometry of CO_2 release to NADPH reduction was determined for 3-fluoroisocitrate and 3-hydroxyisocitrate to ensure monodecarboxylation. For 3-fluoroisocitrate, the CO_2/NADPH ratio (NADPH measured spectrophotometrically and CO_2 measured manometrically following acid quench with H_2SO_4) was 1.0, indicating its suitability for carbon isotope effect studies. The CO_2/NADPH ratio for 3-hydroxyisocitrate was 2.8, indicating polydecarboxylation and its unsuitability for carbon isotope effect studies.

The product of 3-fluoroisocitrate oxidative decarboxylation was shown to be 2-keto-3-fluoroglutarate by ^{19}F NMR (Grissom & Cleland, 1988). The ^{19}F NMR spectrum of *threo,erythro*-3-fluoroisocitrate showed two groups of peaks with eight lines each at −163.7 ppm and −167.9 ppm (relative to $CFCl_3$ at 0 ppm). Upon incubation with isocitrate dehydrogenase, Mg^{2+}, and NADP, the peaks at −167.9 ppm diminished in intensity, and a new set of eight lines was seen to appear at −190.2 ppm. This agrees with the chemical shift of authentic 2-keto-3-fluoroglutarate (Grissom & Cleland, 1988), and this assigns the upfield peaks as *threo*-DL-3-fluoroisocitrate.

pK_i Profile. Oxalylglycine, a competitive inhibitor of isocitrate (Northrop, 1974), was used to generate the pK_i versus pH profile shown in Figure 2. The data were fitted to eq 6 with the following results: pK = 5.7 ± 0.1, YL = 2.0 ± 0.1, and YH = 8.8 ± 0.3. This likely represents the true pK of the acid/base functional group on the enzyme which deprotonates the C_2 OH to facilitate hydride transfer, although it could be another group whose protonation state is critical to catalysis. Because the rate is higher at neutral pH, the group must be ionized for catalysis.

^{13}C Isotope Effects. The variation of the observed ^{13}C isotope effect on V/K_{Ic} is shown numerically in Table II and graphically in Figure 3. The data were fitted to eq 6 with the following results: pK = 4.46 ± 0.10, $^{13}(V/K_{Ic})$ plateau value at low pH is 1.040 ± 0.004, and the $^{13}(V/K_{Ic})$ plateau at neutral pH is 1.0028 ± 0.0005. The variation of the observed ^{13}C isotope effect with deuteriated isocitrate is also shown in Table II. When fitted to eq 6 (with the pK_a fixed at 4.46 because the low-pH plateau is poorly defined by lack of data points), the results are as follows: $^{13}(V/K_{Ic})_D$ plateau

Table II: ^{13}C Isotope Effects for NADP Isocitrate Dehydrogenase as a Function of pH with Mg^{2+}

substrate[a]	pH	high conv[b,c]	fraction of reaction (%)	low conv[c]	$^{13}(V/K_{Ic})$
Ic	7.0	11810.3	13.0	11768.4	1.0038
Ic	7.0	11810.3	7.3	11784.0	1.0023
Ic	7.0	11810.3	12.8	11767.7	1.0039
Ic	7.0	11810.3	9.4	11789.3	1.0019
					av 1.0030 ± 0.0010
Ic	6.5	11810.3	12.7	11768.7	1.0038
Ic	6.3	11849.8	20.2	11810.0	1.0038
Ic	5.9	11849.8	19.7	11806.9	1.0040
Ic	5.4	11849.8	17.7	11797.2	1.0049
Ic	5.3	10866.7	7.6	10777.8	1.0086
Ic	5.3	10866.7	7.6	10772.8	1.0090
Ic	5.0	11849.8	18.0	11740.1	1.0104
Ic	5.0	11849.8	17.6	11743.8	1.0100
Ic	4.85	11849.8	15.0	11724.6	1.0116
Ic	4.6	11849.8	17.8	11622.3	1.0216
Ic	4.3	11849.8	17.1	11595.3	1.0241
Ic	4.3	11849.8	17.3	11573.2	1.0263
Ic	4.1	11849.8	4.3	11537.8	1.0276
Ic-2-*d*	7.0	11899.0	10.0	11894.0	1.0005
Ic-2-*d*	7.0	11899.0	10.0	11893.0	1.0005
					av 1.0005 ± 0.0005
Ic-2-*d*	6.33	11899.0	17.0	11890.0	1.0008
Ic-2-*d*	5.82	11899.0	6.7	11887.0	1.0010
Ic-2-*d*	5.45	11899.0	6.3	11883.0	1.0014
Ic-2-*d*	5.34	10854.4	9.9	10811.1	1.0042
Ic-2-*d*	5.28	11899.0	8.1	11850.0	1.0043
Ic-2-*d*	5.03	11899.0	5.4	11849.0	1.0043
Ic-2-*d*	4.98	11899.0	6.1	11850.0	1.0043
Ic-2-*d*	4.71	11899.0	4.7	11838.0	1.0053
3-F-Ic	7.0	11022.7	13.0	10892.3	1.0129
3-F-Ic	7.0	11022.7	12.7	10893.0	1.0128
					av 1.0129 ± 0.0001
3-F-Ic	5.2	11022.7	8.4	10829.9	1.0186
3-F-Ic	5.2	11022.7	8.1	10830.7	1.0185
					av 1.0186 ± 0.0001

[a] Substrate abbreviations: Ic, unlabeled isocitrate; Ic-2-*d*, isocitrate-2-*d*; 3-F-Ic, 3-fluoroisocitrate. [b] The high-conversion isocitrate samples have the following standard deviations associated with them: 11810.3 ± 0.8, three determinations; 11849.8 ± 1.0, four determinations; 10866.7 ± 2.0, two determinations; 11899 ± 0.7, 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 $^{17}O/^{18}O$ by subtracting 740 from the observed isotopic ratio.

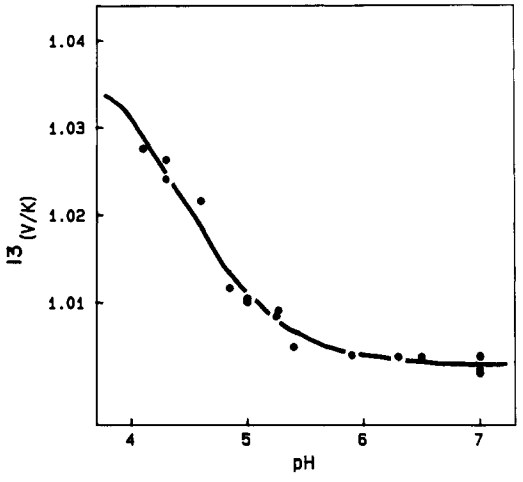


FIGURE 3: Observed ^{13}C isotope effect on V/K for isocitrate versus pH. Data fitted to eq 6 with the following results: $pK = 4.46 \pm 0.10$, $^{13}(V/K_{Ic})$ plateau at low pH = 1.040 ± 0.004, and the $^{13}(V/K_{Ic})$ plateau at neutral pH = 1.0028 ± 0.0005.

value at low pH = 1.016 ± 0.002 and $^{13}(V/K_{Ic})_D$ plateau at neutral pH = 1.0009 ± 0.0005. The ^{13}C isotope effect on V/K with 3-fluoroisocitrate is also presented in Table II. $^{13}(V/K_{3\text{-F-Ic}})$ at neutral pH is significantly above 1 and increases slightly at pH 5.2. This is the first ^{13}C or deterium isotope effect significantly greater than 1 with isocitrate dehydrogenase at neutral pH.

Viscosity Dependence of Kinetic Parameters for Isocitrate Analogues. The kinetic parameters for isocitrate and its two analogues were determined in the presence and absence of 30% sucrose as viscosogenic agent. The results are presented in Table III. The velocities varied hyperbolically with substrate concentration in each case and were fitted to eq 4. The standard errors associated with some values are large because of the low K_m values which approach the limits which can be

Table III: Viscosity Dependence of Kinetic Constants for Isocitrate Substrate Analogues, pH 7.0[a]

substrate	η^b	K_m (μM)	V_{max}	V/K
Ic	1.0	3.8 ± 0.7	0.77 ± 1.01	0.20 ± 0.03
	2.9	1.4 ± 0.4	0.090 ± 0.004	0.06 ± 0.02
3-F-Ic	1.0	5.1 ± 1.1	0.026 ± 0.006	0.005 ± 0.001
	2.9	4.3 ± 1.1	0.012 ± 0.001	0.0028 ± 0.0006
3-OH-Ic	1.0	2.4 ± 0.5	0.056 ± 0.002	0.023 ± 0.004
	2.9	2.6 ± 1.0	0.033 ± 0.003	0.013 ± 0.005

[a] Assay conditions: 0.5 mM NADP, 4 mM $MgCl_2$, and 100 mM Pipes, pH 7.0. Cuvettes with 10-cm path-length were used for all assays. [b] The viscosity was increased by the presence of 30% sucrose to give an increase in relative viscosity of approximately 2.9.

Table IV: Deuterium Isotope Effects for Isocitrate Analogues at pH 7[a]

substrate	^DV	$^D(V/K_{Ic})$
Ic	1.0[b]	1.00[b]
3-F-Ic	1.0	1.45 ± 0.15
3-OH-Ic	1.0	1.53 ± 0.13

[a] Assay conditions: 0.2 mM NADP, 2 mM MgCl, and 100 mM Pipes, pH 7.0. Cuvettes with 10-cm path-length were used for all assays. [b] Data of Cook and Cleland (1981b), but verified in the present study.

Table V: Partitioning of Oxalosuccinate at 25 °C[a]

pH	ϵ_{OSA}[b]	initial rate (μM/min) Ic	2-KG	2-KG/Ic	$^{13}(V/K_{Ic})$[c]	^{13}k[d]
5.40	97	1.12 ± 0.06	8.2 ± 0.4	7.3 ± 0.5	1.0065 ± 0.0010	1.054 ± 0.008
5.00	113	0.325 ± 0.016	1.04 ± 0.05	3.2 ± 0.2	1.0109 ± 0.0010	1.046 ± 0.006
5.60		(Mn^{2+})		9.3 ± 0.8[e]	1.0094	1.096 ± 0.014

[a] Assay conditions: 100 mM buffer, 0.3 mM NADPH, 30 mM L-alanine, 2 mM $MgCl_2$, 5 mM OSA, and 40 units of glutamate-pyruvate transaminase in 0.5-cm cuvette. Averaged results of four determinations at each pH. [b] Apparent extinction coefficient of oxalosuccinate (M^{-1} cm^{-1}) under stated assay conditions. [c] Observed carbon isotope effect interpolated from data in Table I. [d] Calculated intrinsic carbon isotope effect with the stated 2-KG/Ic partitioning ratio and $^{13}(V/K_{Ic})$ according to Grissom and Cleland (1985). [e] 2-KG/Ic ratio calculated from Siebert et al. (1957) with 0.67 mM $MnCl_2$.

determined with 10-cm path-length cells.

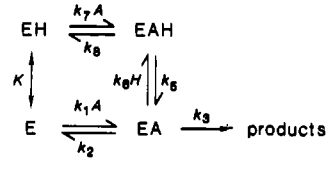
Deuterium Isotope Effects. The deuterium isotope effect was determined for isocitrate and its two analogues. The results are presented in Table IV. The data were fitted to eq 7, which assumes a deuterium isotope effect on V and V/K. The determined isotope effect on V is meaningless unless both deuteriated and unlabeled substrate preparations are free of inhibitors or contain equal concentrations of inhibitors. This condition may not have been met since three inactive isomers were present in each preparation. Hence, any isotope effect on V was ignored. Deuterium isotope effects on V/K are not affected by inhibitors. At pH 4.98 and 15 μM total Mg^{2+} ($^1/_{10}K_m$), $^D(V/K_{Ic}) = 1.06 \pm 0.03$. This is not significantly different from the value of 1.00 observed at neutral pH and saturating Mg^{2+} by Cook and Cleland (1981b).

Partitioning of Oxalosuccinate. The method of Grissom and Cleland (1985) was used to quantitate the partitioning of oxalosuccinate between decarboxylation to 2-ketoglutarate and reduction to isocitrate. The averaged results of four determinations at pH 5.00 and 5.40 are presented in Table V. The 2-ketoglutarate/isocitrate (2-KG/Ic) ratio can be used with the observed $^{13}(V/K_{Ic})$ value at the same pH to calculate a value for the intrinsic ^{13}C isotope effect (eq 8). A value abstracted from the literature (Siebert et al., 1957) for the 2-KG/Ic ratio at pH 5.6 and 0.67 mM Mn^{2+} is also presented. The values of $^{13}(V/K)$ used for the calculation of the intrinsic isotope effect at each pH are taken from the calculated parameters of eq 6. Hence, the standard errors associated with those values reflect errors distributed throughout the data in Table II. Most of the error in the determination of ^{13}k comes from the error associated with the measurement of small $^{13}(V/K_{Ic})$ values. Below pH 5.0, the isotope effect does become larger, but the stability of NADPH becomes a problem such that considerable nucleotide is degraded by acid hydrolysis, thus interfering with the accurate determination of the partitioning ratio, R_H. Above pH 6, the 2-KG/Ic partitioning ratio increases drastically, such that it becomes difficult to measure simultaneously decarboxylation and reverse hydride transfer rates, which differ by more than a factor of 15.

Discussion

3-Hydroxyisocitrate Product Identification: ^{13}C NMR. The initial product of enzymatic oxidation and decarboxylation of 3-hydroxyisocitrate is a *cis*-enediol. By analogy to the accepted enzymatic mechanism with isocitrate, an enzymatic base protonates the enol to yield the ketone, which is subsequently released into solution. Removal of carboxyls C_1 and C_5 can only result from protonation at carbon 2 of the *cis*-enediol, **2**, to give the β-keto carboxylic acid, **4**. If carbon 3 of the *cis*-enediol were protonated, the resulting 2-keto-3-hydroxyglutarate would be unable to decarboxylate further. Because isocitrate dehydrogenase can protonate either C_2 or C_3, the base must be located between C_2 and C_3. The unexpected preference for protonation of the *cis*-enediol at C_2 probably reflects the favorable thermodynamics of this molecule over 2-keto-3-hydroxyglutarate (i.e., the *cis*-enediol is electron-rich at C_2, thus favoring protonation). Rose (1960) demonstrated the ability of isocitrate dehydrogenase to detritiate 2-ketoglutarate labeled with ^3H at C_3. This indicates a base on the enzyme does exist to protonate the enolate resulting from decarboxylation and the enzyme does not release the enolate into solution unprotonated.

Carbon Isotope Effects. Figure 3 shows that the ^{13}C isotope effect increases significantly as the pH is lowered. This is the first report of an observed ^{13}C isotope effect greater than unity with Mg^{2+} for NADP isocitrate dehydrogenase. Seltzer et al. (1959) first predicted the absence of an observed ^{13}C isotope effect in those reactions where the rate of catalysis exceeded the rate of substrate dissociation from the enzyme–substrate central complex. Indeed, this is thought to be the case with isocitrate dehydrogenase. Only if catalysis can be slowed relative to substrate dissociation would the observed isotope effect be increased as the commitment to catalysis is lowered. This can be accomplished by lowering the pH of the assay such that a smaller fraction of the enzyme acid–base catalyst responsible for deprotonating the C_2 hydroxyl of isocitrate prior to hydride transfer will be in the correct protonation state for the reaction to occur. Cook and Cleland (1981a,b) fully developed the theory of the pH dependence of isotope effects. Consider the scheme



where E is enzyme–NADP–Mg^{2+} and A is isocitrate. If k_3 is greater than k_2, then isocitrate will be sticky (i.e., dissociate from the central complex much less often than it goes on to form products). This is the case with NADP isocitrate dehydrogenase at neutral pH. At low pH, a large fraction of the enzyme population will be incorrectly protonated for catalysis to occur and deplete the E and EA pools, thereby increasing the tendency of isocitrate to dissociate from the central complex relative to catalysis. The observed pK for this titration will be displaced outward (toward the acid/base extremes) from its true value. The pK_i profile for a competitive inhibitor of isocitrate will show this true pK, however, because there is no catalytic event to cause it to be sticky. Taking the true pK of 5.7 from Figure 1 and the stickiness-displaced pK of 4.46 from Figure 3, we calculate a displacement of 1.2 pH units. From eq 9, we can define a precise stickiness factor for

$$pK_{app} = pK_{true} - \log (1 + k_3/k_2) \quad (9)$$

isocitrate. Hence, catalysis is 16 times faster than dissociation of isocitrate from the central complex. It is the internal competition nature of $^{13}(V/K)$ isotope effects which allows a pK as low as 4.46 to be precisely defined. Acid lability of the

protein makes kinetics difficult even at pH 5.0. The initial velocity data of Cook and Cleland (1981b) at pH 4.5 suggested the presence of a pK about 4.5, but it is very difficult to determine such a low pK with initial velocity studies. The break in the V/K rate–pH profile seen at 6.73 (Cook & Cleland, 1981a,b) was attributed to the third pK of isocitrate. Hence, only triply ionized isocitrate is the catalytically active species.

The plateau at low pH in Figure 3 represents the limiting value for the observed $^{13}(V/K_{Ic})$ for isocitrate after the external commitment has been removed. The limiting value of 1.040 for unlabeled isocitrate is about what would be expected for a decarboxylase with moderate commitments [cf. NADP malic enzyme $^{13}(V/K_{mal})$ = 1.0336]. Presumably, only internal commitments to catalysis remain to diminish the intrinsic ^{13}C isotope effect from its observed value. Since the observed value for $^{13}(V/K_{Ic})_D$ is less than $^{13}(V/K_{Ic})_H$ in all cases, the catalytic mechanism must be stepwise according to the multiple isotope effect theory of Hermes et al. (1982). The presence of deuterium increases the total commitment for decarboxylation, thus decreasing the observed $^{13}(V/K)$. The multiple isotope effect method also allows the order of the deuterium- and ^{13}C-sensitive steps to be determined when $^{D}(V/K)$ is known along with $^{13}(V/K_{Ic})$ with unlabeled and deuteriated substrates. If the deuterium-sensitive step precedes the ^{13}C-sensitive step, then eq 10 will be true. If the ^{13}C-sensitive step precedes the

$$\frac{^{13}(V/K)_H - 1}{^{13}(V/K)_D - 1} = \frac{^{D}(V/K)}{^{D}K_{eq}} \quad (10)$$

deuterium-sensitive step, then eq 11 will hold. Using the

$$\frac{^{13}(V/K)_H - ^{13}K_{eq}}{^{13}(V/K)_D - ^{13}K_{eq}} = ^{D}(V/K) \quad (11)$$

$^{13}(V/K)$ values at the low-pH plateaus, eq 10 predicts[3] a $^{D}(V/K)$ of 2.98 and eq 11 predicts a $^{D}(V/K)$ of 2.87. There is no appreciable $^{D}(V/K)$ under any condition of low pH, low metal ion concentration, EDTA, NADPH, or 1 mg/mL bovine serum albumin, however. At pH 4.5, $^{D}(V/K_{Ic})$ is only 1.08 ± 0.02 (Cook & Cleland, 1981b). We do not understand why the relationship between $^{D}(V/K)$, $^{13}(V/K)_H$, and $^{13}(V/K)_D$ appears to break down with isocitrate dehydrogenase. Besides the original application to NADP malic enzyme, it has also been successfully applied to jade plant (*Crassula*) NAD malic enzyme (Grissom et al., 1987), 6-phosphogluconate dehydrogenase (Rendina et al., 1984), and prephenate dehydrogenase (Hermes et al., 1984).

Partitioning of Oxalosuccinate. The pH dependence of the ratio of oxalosuccinate decarboxylation to reduction is illustrated in Table V. As the pH decreases, the external commitment decreases, thereby increasing the amount of isocitrate produced relative to 2-ketoglutarate. Table V shows the average intrinsic ^{13}C isotope effect on decarboxylation is 1.050 ± 0.005 with Mg^{2+} as metal ion. This is equal to the intrinsic ^{13}C isotope effect for decarboxylation of malate (1.049) in the analogous malic enzyme reaction (see preceding paper). This is not surprising, since both enzymes are thought to generate an enzyme-bound metal-coordinated β-keto carboxylic acid with the leaving carboxyl group out of the plane defined by the β-ketone carbonyl, the metal ion, and the oxygens of carboxyl C_1.

Plaut (Siebert et al., 1957) determined the ratio of oxalosuccinate decarboxylase activity to isocitrate dehydrogenase activity and the ratio of isocitrate dehydrogenase activity to oxalosuccinate reductase activity with Mn^{2+} 30 years ago. From these ratios, we can determine the ratio of oxalosuccinate decarboxylase to oxalosuccinate reductase (2-KG/Ic) activity to be 9.6. The ^{13}k calculated with this partitioning ratio (determined with Mn^{2+}) and a $^{13}(V/K_{Ic})$ value of 1.0094 (also determined at pH 5.6 with Mn^{2+}) is 1.096. This is larger than the maximum theoretical ^{13}k of 1.07 expected by comparison of ground-state energies of ^{12}C- and ^{13}C-reacting molecules.

Internal and External Commitments. The ^{13}k values of 1.05 and the pH dependence of $^{13}(V/K)$ allow the dissection of the total forward commitment for decarboxylation into internal (partition ratios of enzyme intermediates not affected by reaction conditions) and external (partition ratios involving substrate dissociation) components. The observed isotope effect is defined by the intrinsic isotope effect and the total forward commitment as given in eq 12, when the reverse

$$^{13}(V/K) = \frac{^{13}k + C_f}{1 + C_f} \quad (12)$$

commitment for CO_2 is negligible.[4] At neutral pH, C_f is 16.8. This includes internal and external contributions. At low pH, $^{13}(V/K)$ reaches a plateau value of 1.040, and according to eq 12, a forward commitment to catalysis of 0.25 remains. All external components of the commitment have been eliminated at this plateau, so the commitment is purely internal. Subtracting $C_{f\text{-int}}$ = 0.25 (1.5% of $C_{f\text{-total}}$) from $C_{f\text{-total}}$ at neutral pH, we find $C_{f\text{-ext}}$ = 16.5 (98.5% of $C_{f\text{-total}}$) at neutral pH. At neutral pH, the majority of the commitment is external in contrast to NADP malic enzyme where the internal commitment is similar in magnitude (0.348) but represents 75% of the total forward commitment for decarboxylation.

Deuterium Isotope Effects. The deuterium isotope effect on V/K never increases above 1.08, even at pH 4.5 (Cook & Cleland, 1981b). It was thought decreasing the metal concentration to a subsaturating level would decrease the external commitment for isocitrate so that a significant increase in the $^{D}(V/K_{Ic})$ value could be observed. If metal–isocitrate complex was the true substrate for the enzyme, however, decreasing the metal concentration would have no effect on the observed isotope effect. If the enzyme must bind either free metal or free malate first, then an isotope effect will be enhanced at low metal concentrations because the rate of catalysis will be less than the rate of dissociation of either substrate from the central complex. If the enzyme binds metal–isocitrate, however, the observed isotope effect will not be enhanced at low metal concentrations. The $^{D}(V/K_{Ic})$ at pH 4.98 and 15 μM total Mg^{2+} is 1.06—not significantly different from Cook and Cleland's (1981b) value of 1.08 at pH 4.5. This result agrees with other laboratories's claims that metal–isocitrate is the true substrate for NADP isocitrate dehydrogenase (Colman

[3] At the low-pH plateau, $^{13}(V/K)_H$ = 1.040 and $^{13}(V/K)_D$ = 1.016. $^{13}K_{eq}$ = 1.0027 (O'Leary & Yapp, 1978) and $^{D}K_{eq}$ = 1.17 (Cook et al., 1980).

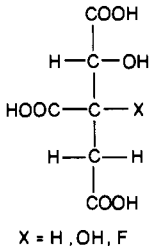
[4] The full expression for $^{13}(V/K_{Ic})$ includes terms for the reverse commitment for isocitrate and the $^{13}K_{eq}$:

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

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 effectively removes the C_r and $^{13}K_{eq}$ terms from the expression.

& Villafranca, 1972; Colman, 1972), but an alternative explanation is that the binding of metal ion and isocitrate is highly synergistic. Free isocitrate is the substrate for yeast NAD isocitrate dehydrogenase (Gabriel & Plaut, 1986).

Alternate Substrates. 3-Hydroxyisocitrate (garcinate) and 3-fluoroisocitrate are the only alternate substrates known for the oxidative decarboxylation reaction of NADP isocitrate dehydrogenase. Both isocitrate analogues are substituted at C_3. The general structure is



According to the kinetic constants in Table III, the K_m values are nearly the same for all analogues. V_{max} and V/K_{Ic} are slightly lower for 3-fluoroisocitrate and 3-hydroxyisocitrate. For each substrate, V/K decreases when the viscosity is increased. The greatest decrease in V/K should occur for the stickiest substrate. The trends are qualitative because of the large errors associated with some of the parameters. Significant deuterium isotope effects are seen for 3-fluoroisocitrate and 3-hydroxyisocitrate in Table VI. This is the first report of $^{D}(V/K_{Ic})$ greater than 1.0 at neutral pH. Similarly, $^{13}(V/K_{Ic})$ for 3-fluoroisocitrate at neutral pH is also significantly greater than unity. $^{13}(V/K_{Ic})$ for 3-hydroxyisocitrate would probably be of similar magnitude if nonenzymatic decarboxylation of the 2-hydroxy-3-ketoglutarate product did not interfere with measurement of the isotopic ratio of the enzyme-generated CO_2 product. NADP isocitrate dehydrogenase appears to tolerate substitution only at the C_3 position. Homoisocitrate and other analogues have been shown not to be substrates for the enzyme (Plaut et al., 1975).

Concluding Remarks. The usefulness of the pH variation of isotope effects to dissect internal and external commitments has been illustrated. NADP isocitrate dehydrogenase, which shows a nearly nonexistent ^{13}C isotope effect on V/K at neutral pH, exhibits a ^{13}C isotope effect on V/K which is only moderately diminished by commitments at low pH. An examination of the ^{13}C isotope effect with deuteriated and unlabeled isocitrate has determined the catalytic mechanism to be stepwise with hydride transfer likely preceding decarboxylation. This generates an enzyme-bound β-keto carboxylic acid intermediate which undergoes facile decarboxylation. The breakdown of eq 10 and 11 to describe the order of hydride transfer and decarboxylation is unexpected. The decrease in $^{13}(V/K_{Ic})$ is larger than predicted by the size of $^{D}(V/K_{Ic})$. One possible explanation for the discrepancy is a change in the kinetic mechanism between the conditions of an internal competition experiment to determine $^{13}(V/K_{Ic})$ (high levels of enzyme, high concentration of Mg^{2+} and isocitrate) and a direct comparison experiment to determine $^{D}(V/K_{Ic})$ (low levels of enzyme, low concentrations of isocitrate, and high levels of Mg^{2+}). Kelly and Plaut (1981b) showed that the kinetic mechanism changes from random to ordered sequential with NADP binding last at low enzyme concentrations or in the absence of NADPH or EDTA. The experimental protocol to determine $^{D}(V/K_{Ic})$ precisely matches these conditions. If the kinetic mechanism were ordered with isocitrate binding first, then at saturating NADP the commitment for isocitrate would be infinite, and no isotope effect would be seen. This is not supported by our experimental data, however. $^{D}(V/K_{Ic})$ does not increase in the presence of EDTA or NADPH. Perhaps a change to an ordered sequential kinetic mechanism under the conditions used to determine $^{D}(V/K_{Ic})$ is not overcome by the presence of NADPH or EDTA. It is prudent to refrain from further speculation without additional experimental data and supporting theory.

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Two-Step Internalization of Ca^{2+} from a Single E~P·Ca_2 Species by the Ca^{2+}-ATPase†

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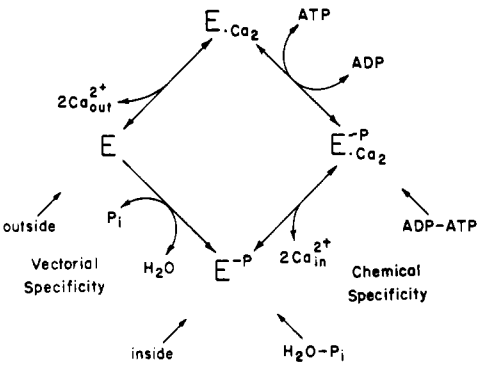
ABSTRACT: Phosphorylation by ATP of E·*Ca_2 (sarcoplasmic reticulum vesicles (SRV) with bound $^{45}Ca^{2+}$) during 5–10 ms leads to the occlusion of 2 *Ca^{2+}/EP_{tot} [quench by ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) alone] in both "empty" (10 μM free Ca^{2+}_{in}) or "loaded" SRV (20–40 mM free Ca^{2+}_{in}). The rate of Ca^{2+} "internalization" from the occluded E~P·*Ca_2 was measured by using an ADP + EGTA quench; a *Ca^{2+} ion that is not removed by this quench is defined as internalized. In the presence of 20–40 mM unlabeled Ca^{2+} inside SRV, 1 *Ca^{2+}/EP_{tot} is internalized from ^{45}Ca-labeled E~P·*Ca_2 with a first-order rate constant of $k_1 = 34\ s^{-1}$. Empty SRV take up 2 *Ca^{2+}/EP_{tot} with the same initial rate, but the overall rate constant is $k_{obsd} = 17\ s^{-1}$. The apparent rate constant ($k_b = 17\ s^{-1}$) for internalization of the second *Ca^{2+} is inhibited by $[Ca]_{in}$, with $K_{0.5} \sim 1.3$ mM and a Hill coefficient of $n = 1.1$. These data show that the two Ca^{2+} ions are internalized sequentially, presumably from separate sequential sites in the channel. [^{32}P]EP·Ca_2 obtained by rapid mixing of E·Ca_2 with [γ-^{32}P]ATP and EGTA disappears in a biphasic time course with a lag corresponding to $\sim 34\ s^{-1}$, followed by EP* decay with a rate constant of $\sim 17\ s^{-1}$. This shows that both Ca^{2+} ions must be internalized before the enzyme changes its specificity for catalysis of phosphoryl transfer to water instead of to ADP. Increasing the concentration of ATP from 0.25 to 3 mM accelerates the rate of $^{45}Ca^{2+}$ internalization from 34 to 69 s^{-1} for the first Ca^{2+} and from 17 to 34 s^{-1} for the second Ca^{2+}. High [ATP] also accelerates both phases of [^{32}P]EP·Ca_2 disappearance by the same factor. The data are consistent with a single form of ADP-sensitive E~P·Ca_2 that sequentially internalizes two ions. The intravesicular volume was estimated to be 2.0 μL/mg, so that one turnover of the enzyme gives 4 mM internal $[Ca^{2+}]$.

The mechanism of Ca^{2+} transport by the Ca^{2+}-ATPase[1] of sarcoplasmic reticulum may be described by a set of rules that define vectorial and chemical specificity in the coupled process (Jencks, 1980, 1983; Pickart & Jencks, 1984). The rule for vectorial specificity is that phosphorylation of the Asp-COO^- of the Ca^{2+}-ATPase changes the direction of the Ca^{2+} dissociation–binding to the enzyme (Verjovsky-Almeida et al., 1978; Dupont, 1980; Takisawa & Makinose, 1981, 1983), acting as a vectorial switch that tells the enzyme whether to react with calcium outside or inside (Scheme I). Before phosphorylation Ca^{2+} dissociates outside but not inside (Ikemoto, 1975; Inesi et al., 1980), whereas after phosphorylation it dissociates inside from the "occluded" EP·Ca_2 but not outside (Dupont, 1980; Takisawa & Makinose, 1981, 1983; Champeil, et al., 1986). The rule for chemical specificity is that dissociation–binding of Ca^{2+} acts as a chemical "switch" that tells the enzyme whether to react with ATP or P_i: In the absence of Ca^{2+} the enzyme can be phosphorylated by P_i but not by ATP, and E·Ca_2 can be phosphorylated by ATP but not by P_i (Sumida et al., 1978; Scofano et al., 1979; de Meis, 1981; Martonosi & Beeler, 1985). After phosphorylation of E·Ca_2 by ATP,

Scheme I



all of the EP·Ca_2 is ADP sensitive but not H_2O sensitive (Pickart & Jencks, 1982; Stahl & Jencks, 1984, 1987; Fernandez-Belda & Inesi, 1986). After calcium comes off EP·Ca_2, the covalently bound phosphate can be transferred

† Publication No. 1645. This research was supported in part by grants from the National Institutes of Health (GM20888) and the National Science Foundation (PCM-8117816).
‡ Supported by a Chaim Weizmann postdoctoral fellowship.

[1] Abbreviations: E or Ca^{2+}-ATPase, calcium adenosinetriphosphatase; SRV, sarcoplasmic reticulum vesicles; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; TNP, trinitrophenyl.

0006-2960/88/0427-2943$01.50/0