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## pH Profiles and Isotope Effects for Aconitases from *Saccharomyces lipolytica*, Beef Heart, and Beef Liver. $\alpha$ -Methyl-*cis*-aconitate and *threo*-D<sub>5</sub>- $\alpha$ -Methylisocitrate as Substrates<sup>†</sup>

John V. Schloss,<sup>†</sup> Mark H. Emptage,<sup>†</sup> and W. W. Cleland\*

**ABSTRACT:**  $\alpha$ -Methyl-*cis*-aconitate (*cis*-2-butene-1,2,3-tricarboxylate) was converted only to  $\alpha$ -methylisocitrate (3-hydroxybutane-1,2,3-tricarboxylate) by aconitases from beef liver or *S. lipolytica*. While the kinetic parameters of beef liver (cytoplasmic) or heart (mitochondrial) aconitases did not vary over the pH range 4.9-9 with the natural substrates, and only slightly with the  $\alpha$ -methyl substrates, the yeast aconitase exhibited a bell-shaped pH profile with all substrates and for binding of the competitive inhibitor, tricarballic acid, with pK values around 7 and 9. The third pK of the substrates does not affect  $V/K$ , showing that these pK's are for catalytic groups on the enzyme. One of these catalytic groups presumably removes a proton to give the carbanion intermediate in the reaction, and the other protonates the hydroxyl group when it is eliminated to give water, possibly with the assistance of

the Fe-S center. Beef liver aconitase showed a primary deuterium isotope effect of 1.12 (measured by equilibrium perturbation with deuterated  $\alpha$ -methylisocitrate) which was pH independent and only slightly greater than the equilibrium isotope effect. Isotope effects with the yeast enzyme were also pH independent but about 1.22 on  $V/K$  (or when measured by equilibrium perturbation) and 1.7 on  $V$ . These data suggest a kinetic mechanism for beef aconitases in which product release occurs only by displacement by the substrate in a step independent of pH or of the protonation state of the substrate. With the yeast enzyme, product displacement either depends on the protonation state of the catalytic groups on the enzyme or can occur spontaneously at a finite rate. For all enzymes, binary complexes with reactants cannot have the catalytic groups incorrectly protonated.

Aconitase (EC 4.2.1.3), the second enzyme of the citric acid cycle, catalyzes the reversible hydration of *cis*-aconitate to either citrate or isocitrate. Because this reaction involves no net oxidation or reduction, the revelation that aconitase is an Fe-S protein (Kennedy et al., 1972; Suzuki et al., 1975b; Ruzicka & Beinert, 1978) came as a surprise. Further, aconitase loses activity on purification but can be activated with a reductant and Fe<sup>2+</sup> (Dickman & Cloutier, 1950; Morrison, 1954; Villafranca & Mildvan, 1971; Gawron et al., 1974).

These observations can now be explained by recent experiments which indicate that inactive aconitase contains a [3Fe-4S] cluster (Beinert et al., 1983) and that activation with iron and reductant converts this cluster to a [4Fe-4S] cluster (Kent et al., 1982; Kennedy et al., 1983). In addition, an intact [4Fe-4S] cluster in aconitase is required for full enzymatic activity (Emptage et al., 1983a).

Some insight into the potential role of the iron-sulfur cluster during catalysis has come from Mössbauer spectroscopy and perturbations in the EPR<sup>1</sup> spectra of the Fe-S cluster by

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<sup>1</sup> Abbreviations: EPR, electron paramagnetic resonance; Mes, 2-(*N*-morpholino)ethanesulfonate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonate; Tris, tris(hydroxymethyl)aminomethane; Caps, (cyclohexylamino)propanesulfonate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonate; Taps, *N*-[tris(hydroxymethyl)methyl]glycine; SDS, sodium dodecyl sulfate; kDa, kilodaltons. <sup>2</sup> $K_{eq}$ , <sup>3</sup> $V$ , <sup>4</sup> $V/K$ , and <sup>5</sup>(Eq.P.) represent deuterium isotope effects on  $K_{eq}$ ,  $V$ ,  $V/K$ , or one measured by equilibrium perturbation (that is,  $K_{eq,H}/K_{eq,D}$ ,  $V_H/V_D$ , etc.).

$^{17}\text{O}$ -labeled substrates (Emptage et al., 1983b), which suggest that the hydroxyl group of the substrate may form an inner-sphere complex with the most labile iron atom during catalysis. It is also known from the work of Rose & O'Connell (1967) that the hydrogen removed from substrate is not rapidly exchanged with solvent and can be recovered in product, while the oxygen is exchanged and not found in product. Thus, the elimination reaction appears to be stepwise and involve a carbanion intermediate, a conclusion supported by the potent inhibition of aconitase by nitronate analogues of the carbanions of citrate and isocitrate (Schloss et al., 1980). To gain further information about the mechanism of this reaction, we have examined the pH dependence of hydration and dehydration reactions with the natural substrates and with the alternate substrates  $\alpha$ -methyl-*cis*-aconitate and *threo*- $\text{D}_3$ - $\alpha$ -methylisocitrate. We have also determined the primary deuterium isotope effects with methyl substrates. Experimental results are compared for beef liver (cytoplasmic), beef heart (mitochondrial), and *Saccharomycopsis lipolytica* (yeast) aconitases.

#### Experimental Procedures

**Materials.** *threo*-DL-Isocitrate, *threo*- $\text{D}_3$ -isocitrate, tricarballic acid, *cis*-aconitate, Mes, Hepes, Tris, Caps, Ches, and Taps were from Sigma. Ethanamine, *N*-ethylmorpholine, (2-hydroxyethyl)-*N*-piperazine, ethyl cyanoacetate, and diethyl methylloxalacetate were from Aldrich. Beef liver aconitase (cytoplasmic) was isolated by the method of Henson & Cleland (1967). Beef heart aconitase (mitochondrial) was the kind gift of Drs. F. J. Ruzicka and H. Beinert.

$\alpha$ -Methyl-*cis*-aconitic anhydride was synthesized by the method of Gawron & Mahajan (1966). Tritiated  $\alpha$ -methyl-*cis*-aconitate was synthesized by including tritiated water during the last step of the synthesis of  $\alpha$ -methyl-*cis*-aconitic anhydride as described by Beach et al. (1977). In contrast to the low degree of labeling (3%) obtained by these authors, we found the product to have 31% of the specific activity of the tritiated water used in the synthesis. This degree of labeling was measured after exchange with water of volatile radioactivity (47%), the extent of which was consistent with half of the label being incorporated into the single free carboxylic acid group and the rest into the methylene position during rearrangement, as proposed by Gawron & Mahajan (1966).

*threo*- $\text{D}_3$ - $\alpha$ -Methylisocitrate was prepared enzymically from  $\alpha$ -methyl-*cis*-aconitate and purified by Dowex-1 chromatography (Schloss & Cleland, 1982).  $\alpha$ -Methylisocitrate-3-*d* was prepared by carrying out the enzymatic conversion of  $\alpha$ -methyl-*cis*-aconitate in  $>95\%$   $\text{D}_2\text{O}$ .  $\alpha$ -Methyl-*cis*-aconitic acid was found to be substantially less prone to isomerization than *cis*-aconitic acid. While solutions of the free acid of *cis*-aconitate isomerize to *trans*-aconitate with a half-time of about 1 day at room temperature in  $\text{D}_2\text{O}$ ,  $\alpha$ -methyl-*cis*-aconitate can be incubated for several days under acidic conditions without alteration of its proton NMR spectrum. Neutral solutions of either *cis*-aconitate or  $\alpha$ -methyl-*cis*-aconitate are stable indefinitely.

**Culture of *Saccharomycopsis lipolytica*.** An initial culture of this strain (ATCC 20114) was obtained from the American Type Culture Collection and maintained on YM agar (Difco) and YM broth. This organism has also been called *Candida lipolytica* by Suzuki et al. (1975a) and *Yarrowia lipolytica* by van der Walt & von Arx (1980). Induction (50–150-fold) of aconitase by the inclusion of 1 g/L sodium fluoroacetate (Fairfield Chemical Co.) during culture of *S. lipolytica*, as described by Suzuki et al. (1975a), was confirmed in pilot

growth experiments. *S. lipolytica* was grown on a large scale (200 L) by utilizing the medium described by Suzuki et al. (1975b) supplemented with  $40\ \mu\text{M}$   $\text{FeSO}_4$ . The latter was found to be necessary for maximal expression of aconitase under the conditions of fermenter growth (high cell density). After the cells (3.3 kg) were harvested by continuous-flow centrifugation, the supernatant (expected to contain highly toxic fluoroacetate and/or fluorocitrate derived from it) was concentrated to approximately 10 L in vacuo with a falling-film evaporator. The concentrate was acidified to pH 5 with HCl and reduced to 2 L over a steam bath in a hood. The concentrate was mixed with an equal volume of acetone and the precipitate removed by filtration through Celite. The acetone–water mixture was placed back on the steam bath and the remaining water removed by evaporation with repeated additions of acetone. The 471 g of light tan powder obtained showed the NMR spectrum of fluoroacetate, but not that of fluorocitrate (we thank Dr. Janos Retey for this determination).

**Purification of *S. lipolytica* Aconitase.** Purification was carried out at  $4^\circ\text{C}$ , and exposure of the enzyme to atmospheric  $\text{O}_2$  was minimized by sparging mixing vessels with  $\text{N}_2$  during column chromatography. The 50 mM Tris-HCl buffer, pH 7.4 at  $25^\circ\text{C}$ , was used throughout the purification and will be referred to as Tris buffer. Protein was determined by the biuret procedure (Gornall et al., 1949) with bovine serum albumin as a standard. To 915 g of frozen cell paste were added 2 L of Tris buffer and 30 mL of chloroform. After the thawed suspension twice was passed through a Gaulin press (10 000 psi), cell debris was removed by centrifugation at 12 000 rpm in a Sorval GSA rotor for 30 min. To the supernatant (2080 mL, 8.32 g of protein, 23 900 units) was added 1166 g of ammonium sulfate, and the precipitate was collected by centrifugation. The pellet was suspended in 380 mL of Tris buffer, 54.7 g of ammonium sulfate was added, and the solution was centrifuged. The supernatant was applied to a  $3 \times 30$  cm column of phenyl-Sepharose (Sigma). The column was eluted (2 mL/min) with a 2-L linear gradient from 56 g/L ammonium sulfate in Tris buffer to Tris buffer free of ammonium sulfate. Fractions 9–61 (646 mL, 8900 units), containing the bulk of the aconitase activity, were pooled. To the pooled fractions was added 362 g of ammonium sulfate, and the pellet was collected by centrifugation. The pellet was suspended in 58 mL of Tris buffer and passed through a  $5 \times 72$  cm column of Bio-Gel P-100 (Bio-Rad), which was eluted by reverse flow with the suspension buffer at 1.5 mL/min. Fractions 23–37 (316 mL, 1.58 g of protein, 9200 units) were pooled and applied to a  $3 \times 36$  cm column of TEAE-cellulose (U.S. Biochemical Corp.), which was eluted at 2 mL/min with a 4-L linear gradient of 0–0.5 M KCl in Tris buffer. Fractions 12–30 (455 mL, 455 mg of protein, 8700 units) were concentrated in an Amicon ultrafiltration cell by using a PM-10 membrane, dialyzed against Tris buffer, and then applied to a  $2.4 \times 23$  cm column of DEAE-Sephacel (Sigma). The enzyme was eluted with a 4-L linear gradient of 0–0.2 M KCl in Tris buffer. Fractions 40–50 (226 mL, 148 mg of protein, 7500 units, 31% yield) were concentrated to 4.5 mL in an Amicon ultrafiltration cell as above and frozen in liquid  $\text{N}_2$ .

Gel electrophoresis under native conditions (Davis, 1964) revealed one major and several minor protein components. Stains for enzymatic activity (Slaughter et al., 1975, 1977) indicated the major component to be aconitase. SDS gel electrophoresis (Laemmli, 1970) demonstrated the major protein component to be  $84 \pm 7$  kDa when the data were analyzed in the usual fashion or  $78 \pm 9$  kDa when the mobility

was assessed at several (7.5, 10, and 12.5%) gel concentrations (Banker & Cotman, 1972; Tracy & Chan, 1979). Both values are somewhat larger than the value previously determined by sedimentation equilibrium, 68.5 kDa (Suzuki et al., 1975b). In addition, yeast aconitase and beef heart aconitase are not resolved by SDS gel electrophoresis, implying very similar molecular weights for the two proteins. The molecular weight of beef heart aconitase has been determined to be  $83 \pm 2$  kDa by Kurtz et al. (1979) and  $81 \pm 2$  kDa by Rydén et al. (1984). SDS gels which had been stained with Coomassie brilliant blue were scanned on a Schoeffel SD 3000 spectrodensitometer at 540 nm. On this basis, aconitase comprised at least 48% of the total protein. Assuming 48% purity and a molecular weight of 81 000, iron analyses (Beinert, 1978) indicated 5.6 mol of iron/mol of enzyme. Iron and labile sulfide analyses (Beinert, 1983) on a later preparation of similar purity gave an Fe/S<sup>2-</sup> ratio of 1.2, close to the expected value of 1.0 observed for most Fe-S proteins. The enzyme also exhibited an isotropic EPR signal with a *g* value of 2.01 similar to that seen in beef heart aconitase (Ruzika & Beinert, 1978). Again assuming 48% purity, integration of the EPR signal gave 0.04 mol of spins/mol of enzyme, which upon oxidation of the enzyme with K<sub>3</sub>Fe(CN)<sub>6</sub> (3-fold excess at 0 °C) increased to 0.91 mol of spins/mol of enzyme with concomitant inactivation of the enzyme. The correlation between the increase in the *g* = 2.01 EPR signal and loss of activity agrees with the results obtained on beef heart aconitase by Emptage et al. (1983a). Consistent with the small EPR signal at *g* = 2.01 (only 4% oxidized), the enzyme was not activated by incubation with Fe<sup>2+</sup> and dithiothreitol, with or without S<sup>2-</sup>. However, samples of enzyme which had lost activity on storage or during purification (presumably due to air oxidation of the Fe-S cluster) could be substantially reactivated by incubation with Fe<sup>2+</sup> and dithiothreitol. Assay of the purified *S. lipolytica* aconitase in an isocitric dehydrogenase coupled assay (Rose & O'Connell, 1967) with 1 mM aconitate or 10 mM citrate as substrate (0.1 M Tris-HCl, pH 8) gave values of 108 and 12.9 units/mg, respectively. The value from following aconitate absorbance at 240 nm with 20 mM DL-isocitrate as substrate was 51 units/mg. These values were obtained on fully active enzyme. The specific activity with citrate as substrate is comparable to that reported for fully activated beef heart mitochondrial aconitase (Ruzika & Beinert, 1978), pig liver cytoplasmic enzyme (Eanes & Kun, 1974), or pig heart mitochondrial aconitase (Kennedy et al., 1972). The specific activity with isocitrate as substrate is half of the highest specific activity previously reported for *S. lipolytica* aconitase (Suzuki et al., 1975b), in agreement with the estimated purity of the preparation.

**Thin-Layer Chromatography.** Eastman 6064 cellulose thin-layer sheets were used with butanol/acetic acid/water (7:2:5) and butanol/pyridine/water (6:4:3) as solvents. Also used was Whatman DE 81 paper with 0.5 N formic acid as solvent. In all cases the free acids of compounds were chromatographed. Visualization of isocitric amide or lactone was achieved by spraying with 4 M hydroxylamine, pH 6.5, heating at 110 °C for 1 min, and overspraying with 5% FeCl<sub>3</sub> in 6 N HCl. The acid hydroxamates formed purple spots on a yellow background. Amides could also be visualized as brown spots on a white background by spraying with 0.3% ninhydrin in ethanol followed by heating for 5 min at 110 °C. Isocitrate, citrate, aconitate, their amides, and isocitric lactone could be visualized by spraying with a solution containing 1% ammonium molybdate, 0.1 N HCl, and 5% concentrated perchloric acid. After exposure to UV light (sunlight is suitable), com-

pounds appeared as blue spots on a white background.

**Synthesis of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -Monoamides.<sup>2</sup>** A 125-mL Erlenmeyer flask was flushed with anhydrous ammonia at room temperature and immersed in a dry ice-2-propanol bath. After 20 mL of liquid ammonia had been collected, 1 g (5.8 mmol) of isocitric  $\gamma$ -lactone was added. After this dissolved, the solution was incubated 2 h in the dry ice bath, and the flask was removed and the ammonia evaporated. The crystalline residue was dried overnight in a vacuum desiccator over H<sub>2</sub>SO<sub>4</sub>, Drierite, and NaOH pellets; 1.21 g (5.4 mmol, 93% yield) of hygroscopic isocitric  $\gamma$ -amide was obtained. Thin-layer chromatography with either acidic (*R<sub>f</sub>* 0.66) or basic (*R<sub>f</sub>* 0.33) solvents, or on DE 81 paper (*R<sub>f</sub>* 0.70), showed only a single amide spot contaminated with a small amount of isocitrate (*R<sub>f</sub>* 0.72, 0.34, and 0.59, respectively). No isocitric lactone (*R<sub>f</sub>* 0.77, 0.33, and 0.38) was present. Isocitrate was 6.4% of the amide preparation by assay with isocitric dehydrogenase. Acid hydrolysis of the amide liberated 94% of the theoretical amount of isocitrate.

A similar procedure was used to prepare a mixture of the  $\alpha$ - and  $\beta$ -amides of *cis*-aconitate. To 20 mL of anhydrous liquid ammonia in a three-necked flask, equipped with NH<sub>3</sub> and N<sub>2</sub> inlets, were added 1.02 g (6.55 mmol) of *cis*-aconitic anhydride. The bright yellow mixture was incubated for 3 h in a dry ice-2-propanol bath under a continuous flow of NH<sub>3</sub> and N<sub>2</sub>. The flask was then removed from the bath, and the sample was dried under a stream of N<sub>2</sub> for 2 h. After the sample was dried overnight in a vacuum desiccator, 1.23 g (5.91 mmol, 90% yield) of a hygroscopic white powder was obtained. Examination of aconitic amide by proton NMR in D<sub>2</sub>O gave the following:  $\delta$  3.5 (s, 2 H, allylic), 6.4 (s, 1 H, vinylic). These values are identical with those for *cis*-aconitate. When the mixed amides were incubated overnight in D<sub>2</sub>O as their free acids, the appearance of new proton NMR signals at  $\delta$  3.9 and 7.0 with concomitant drop in the signals at  $\delta$  3.5 and 6.4 was observed. Similar shifts for *cis*-aconitate correlate with its isomerization to *trans*-aconitate under acidic conditions (Pratt & Smith, 1967), confirming that the amides as prepared are *cis*. The <sup>13</sup>C NMR spectra of the mixed amides showed two major signals of similar intensity in the region expected for amide carbons (180 and 176 ppm) with four signals in the vinylic region (151, 139, 133, and 120 ppm), consistent with the presence of both  $\alpha$  and  $\beta$  amides. The mixture of amides was resolved into two UV-absorbing components by silica gel chromatography (Bulen et al., 1952). Under the assumption that these are the  $\alpha$  and  $\beta$  amides and that their extinction coefficients at 240 nm are the same, the amides are present in a 66/34 ratio. Estimation of the *cis*-aconitate contamination of the amide mixture with an aconitase-isocitric dehydrogenase coupled assay gave 4.8%, in agreement with results of silica gel chromatography (5.7%).

**Data Processing.** Computer programs of Cleland (1979) or the MLAB data modeling program (Knott, 1979) were used to fit data to the following equations.

$$v = VA/(K + A) \quad (1)$$

$$v = VA/[K(1 + F_1E_{V/K}) + A(1 + F_1E_V)] \quad (2)$$

$$y = C/(1 + H/K_1 + K_2/H) \quad (3)$$

<sup>2</sup> We define the  $\alpha$ -carboxyl of isocitrate as that primary one next to the CHOH group, the  $\beta$ -carboxyl as the secondary one, and the  $\gamma$ -carboxyl as the other primary one. The corresponding carboxyls in *cis*-aconitate are given the same designations (that is, the  $\alpha$  and  $\beta$  ones are adjacent to the double bond).

$$y = [Y_L + Y_H(K/H)] / (1 + K/H) \quad (4)$$

$$\epsilon = [A + B(H/K_3) + C[H^2/(K_2K_3)] + D[H^3/(K_1K_2K_3)]] / [1 + H/K_3 + H^2/(K_2K_3) + H^3/(K_1K_2K_3)] \quad (5)$$

In eq 1 and 2  $V$  and  $K$  are maximum velocity and Michaelis constant,  $F_i$  is fraction of deuterium label, and  $E_{V/K}$  and  $E_V$  are isotope effects minus one on  $V/K$  and  $V$ . In eq 3–5  $H$  is  $[H^+]$ , and the various  $K$  values are acid dissociation constants.  $C$  in eq 3 is the pH-independent value of  $y$ , and  $Y_L$  and  $Y_H$  in eq 4 are the constant values of  $y$  at low and high pH, respectively.  $A$ ,  $B$ ,  $C$ , and  $D$  in eq 5 are  $\epsilon$  values of the trianion, dianion, monoanion, and fully protonated forms of the compound being titrated.

## Results

**pH Dependence of Kinetic Parameters of Liver and Heart Aconitases.** Both  $V$  and  $V/K$  for citrate, isocitrate, and *cis*-aconitate were invariant over the pH range 5–9 with beef liver and beef heart aconitase (and for several of the profiles, as low as pH 4.5). The average Michaelis constants were  $210 \pm 30 \mu\text{M}$  for citrate,  $39 \pm 5 \mu\text{M}$  for isocitrate, and  $11 \pm 2 \mu\text{M}$  for *cis*-aconitate for the liver enzyme and  $8 \pm 2 \mu\text{M}$  for aconitate for the heart enzyme. Similarly, the  $K_i$  for tricarballoylate with the liver enzyme was invariant over the pH range from 5.5 to 10, with an average value of  $1.6 \pm 0.3 \text{ mM}$ . While  $V$  profiles did not appear to decrease at high pH,  $V/K$  did decrease above an apparent  $pK$  of  $9.9 \pm 0.2$  for the liver enzyme (the profiles were not extended above pH 9 with the heart enzyme), as did the  $pK_i$  for tricarballoylate. Interestingly no changes occur in  $V/K$  or  $K_i$  values at the highest  $pK$ 's of citrate or isocitrate (6.4), *cis*-aconitate (6.5), or tricarballoylate (5.8). If there is any change in the affinity of the enzyme for tricarballoylate upon its protonation to a dianion, it would seem to bind slightly tighter (data not shown).

Since the liver enzyme appears not to discriminate between dianions and trianions, the  $\gamma$ -amide of isocitrate and the mixed  $\alpha$ - and  $\beta$ -amides of *cis*-aconitate were tested for substrate activity. In neither case was a change in absorbance at 240 nm observed after reaction of the 5–6% of contaminating substrate.

**Reaction of  $\alpha$ -Methyl-*cis*-aconitate.** Beef liver aconitase (0.1 mL; 3 units based on conversion of citrate to isocitrate) was added to 10 mL of 50 mM  $\alpha$ -methyl-*cis*-aconitate, pH 7.5. After an overnight incubation, the absorbance at 240 nm was 43% of its initial value. Dowex-50- $H^+$  was added to stop the enzymatic reaction, and the pH was readjusted to 7.5 after removal of the Dowex-50 by filtration. The sample was evaporated to dryness, resuspended in  $D_2O$ , and reevaporated from  $D_2O$  several times prior to taking a 270-MHz proton NMR spectrum (Figure 1). Only peaks assignable to  $\alpha$ -methyl-*cis*-aconitate and  $\alpha$ -methylisocitrate were observed, with the latter confirmed by decoupling experiments (Figure 1). Integration of the spectrum gave a ratio of methylisocitrate/methyl-*cis*-aconitate of 1.3, indicating 43% methyl-*cis*-aconitate at equilibrium. Since the equilibrium concentrations of methyl-*cis*-aconitate determined by NMR and loss of UV absorbance agree, there appear to be no UV-absorbing contaminants in the synthetic methyl-*cis*-aconitate, and total change in UV absorbance is an accurate method for determining the equilibrium constant. The expected positions for methylcitrate (designated in Figure 1), along with the remainder of the spectrum, were examined at high sensitivity, and we estimate that we could have detected less than 0.2% methylcitrate. Similar results were obtained when the *S.*

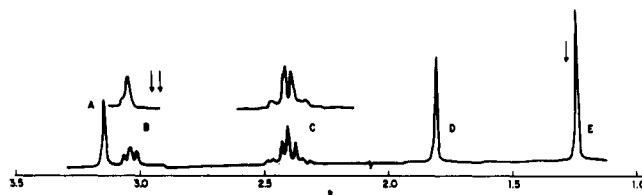


FIGURE 1: Proton NMR spectrum (270 MHz) of an equilibrium mixture of  $\alpha$ -methyl-*cis*-aconitate and  $\alpha$ -methylisocitrate in the presence of beef liver aconitase. (A) Allylic methylene protons of  $\alpha$ -methyl-*cis*-aconitate. (B) (Bottom) C-3 proton of  $\alpha$ -methylisocitrate; (top) same proton with peak C decoupled. (C) (Bottom) methylene protons of  $\alpha$ -methylisocitrate; (top) same protons with peak B decoupled. (D) Allylic methyl protons of  $\alpha$ -methyl-*cis*-aconitate. (E) Methyl protons of  $\alpha$ -methylisocitrate. The expected positions of signals for  $\alpha$ -methylcitrate are designated by arrows.

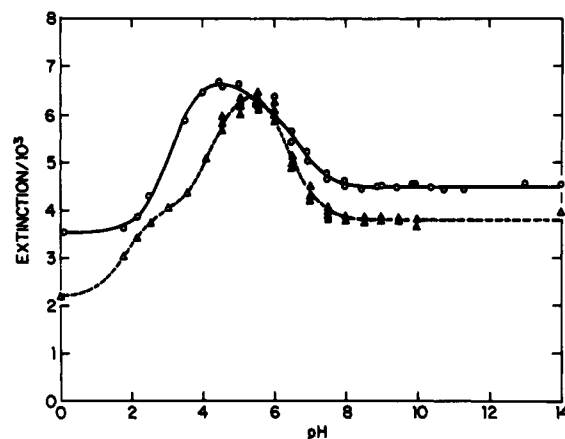


FIGURE 2: Extinction coefficients for *cis*-aconitate ( $\Delta$ ) and  $\alpha$ -methyl-*cis*-aconitate ( $\circ$ ) as a function of pH. The lines are computed from fits to eq 5 by using the parameters given in the text.

*lipolytica* aconitase was used to generate an equilibrium mixture for proton NMR analysis.

When tritiated methyl-*cis*-aconitate was incubated with beef liver aconitase for 10 times the time needed to reach equilibrium, ion-exchange chromatography revealed only two peaks identified as  $\alpha$ -methylisocitrate and  $\alpha$ -methyl-*cis*-aconitate (eluting in that order). This more sensitive analysis confirms the proton NMR results. Thus only  $\alpha$ -methylisocitrate is generated from  $\alpha$ -methyl-*cis*-aconitate by beef liver aconitase.

The extinctions of  $\alpha$ -methyl-*cis*-aconitate and *cis*-aconitate at 240 nm determined over the pH range 0–14 (Figure 2) show the presence of three ionizing groups. Fits of the data to eq 5 gave values of  $A = 4.48 \pm 0.03$ ,  $B = 6.3 \pm 0.3$ ,  $C = 6.9 \pm 0.2$ ,  $D = 3.5 \pm 0.1$ ,  $pK_1 = 3.1 \pm 0.1$ ,  $pK_2 = 4.9 \pm 0.8$ , and  $pK_3 = 6.6 \pm 0.1$  for  $\alpha$ -methyl-*cis*-aconitate and  $A = 3.78 \pm 0.02$ ,  $B = 6.8 \pm 0.1$ ,  $C = 4.0 \pm 0.1$ ,  $D = 2.2 \pm 0.1$ ,  $pK_1 = 1.9 \pm 0.1$ ,  $pK_2 = 4.3 \pm 0.1$ , and  $pK_3 = 6.39 \pm 0.04$  for *cis*-aconitate.  $A$ ,  $B$ ,  $C$ , and  $D$  are the extinction coefficients for the trianion, dianion, monoanion, and fully protonated species, respectively. The  $pK$  values for *cis*-aconitate agree well with those of 1.95, 4.3, and 6.5 determined by Pratt & Smith (1967) from proton NMR chemical shifts as a function of pH.

**Variation of Equilibrium Constant and Haldane Relationship with pH.** The pH variation of  $V$  and  $V/K$  was determined with  $\alpha$ -methyl-*cis*-aconitate and  $\alpha$ -methylisocitrate for beef liver aconitase. Although  $V$  with both substrates was largely invariant over the pH range 5–9, a small decrease was seen below pH 6. While  $V/K$  for  $\alpha$ -methylisocitrate was pH independent from pH 5 to pH 9,  $V/K$  for  $\alpha$ -methyl-*cis*-aconitate declined at low pH to a new plateau value with a  $pK$  of  $6.4 \pm 0.5$  when the data were fitted to eq 4. Calculation of  $K_{eq}$  from the  $V/K$  values for  $\alpha$ -methylisocitrate and  $\alpha$ -

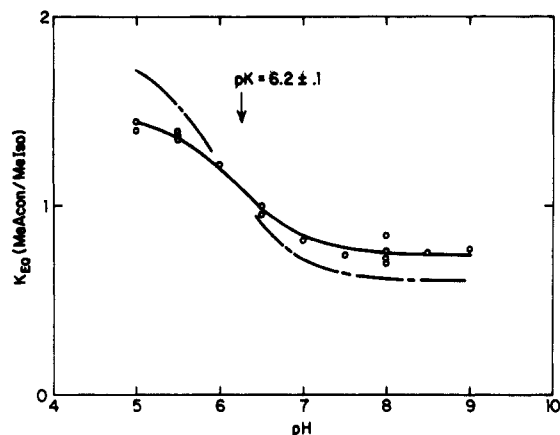


FIGURE 3: Equilibrium constant for conversion of  $\alpha$ -methylisocitrate to  $\alpha$ -methyl-*cis*-aconitate vs. pH. Values determined by change in absorbance at 240 nm in going to equilibrium (O) are compared with those determined from the Haldane relationship (---).

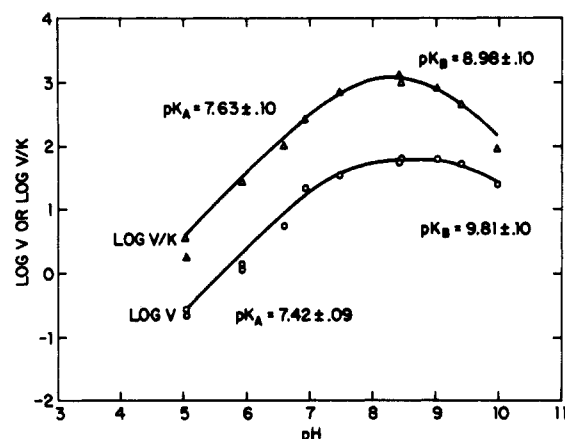


FIGURE 4:  $\log V$  (O) and  $\log (V/K)$  ( $\Delta$ ) for isocitrate vs. pH determined with *S. lipolytica* aconitase.

methyl-*cis*-aconitate using the Haldane relationship gave the pattern shown as the dashed line in Figure 3.

Determination of  $K_{eq}$  from the drop in absorbance of  $\alpha$ -methyl-*cis*-aconitate at 240 nm gave the results in Figure 3. The equilibrium constant (expressed as  $[\alpha$ -methyl-*cis*-aconitate]/ $[\alpha$ -methylisocitrate]) changes with a  $pK$  of  $6.2 \pm 0.1$  from a value of  $1.49 \pm 0.03$  at low pH to one of  $0.74 \pm 0.02$  at high pH. These values correspond to  $\Delta pK = 0.21$ , with the  $pK$  of  $\alpha$ -methylisocitrate being lower than that of  $\alpha$ -methyl-*cis*-aconitate, which has been measured as  $6.6 \pm 0.1$  (see above). Thus, the best values for the  $pK$ 's of these two substrates are 6.3 and 6.5.

**pH Profiles with *S. lipolytica* Aconitase.** By contrast to those of the beef liver and heart aconitases,  $V$  and  $V/K$  for *S. lipolytica* aconitase exhibited bell-shaped pH profiles such as that for isocitrate in Figure 4. Data for both  $V$  and  $V/K$  for all substrates (Table I) were adequately fitted by eq 3, which assumes two ionizable groups, one of which must be protonated and the other ionized for activity. Since the highest  $pK$ 's of 6.4 for isocitrate and *cis*-aconitate and 6.5 for  $\alpha$ -methyl-*cis*-aconitate are less than the lower of the observed  $pK$  values in the pH profiles, the observed ionizations reflect essential enzymic groups. Note that no change in  $V/K$  is seen in the region of the third  $pK$  of the substrates, as was also the case with the beef enzymes. (There must be small changes in the  $V/K$  profiles of either  $\alpha$ -methyl-*cis*-aconitate or  $\alpha$ -methylisocitrate to correspond to the change in  $K_{eq}$  by a factor of 2 below pH 6.4, but the fitted  $pK$  values are dominated by

Table I: Apparent Ionization Constants for *S. lipolytica* Aconitase<sup>a</sup>

substrate or inhibitor	$V$		$V/K$ or $K_i$	
	$pK_1$	$pK_2$	$pK_1$	$pK_2$
isocitrate	$7.4 \pm 0.1$	$9.8 \pm 0.1$	$7.6 \pm 0.1$	$9.0 \pm 0.1$
isocitrate + PG <sup>b</sup>	$7.6 \pm 0.1$	$10.3 \pm 0.1$	$8.1 \pm 0.3$	$8.6 \pm 0.3$
<i>cis</i> -aconitate	$7.5 \pm 0.2$	$8.7 \pm 0.2$	$6.9 \pm 0.3$	$8.9 \pm 0.3$
<i>cis</i> -aconitate + PG <sup>b</sup>	$7.6 \pm 0.3$	$9.6 \pm 0.3$	$8.3 \pm 0.3$	$8.3 \pm 0.3$
methyl- <i>cis</i> -aconitate	$6.0 \pm 0.2$	$9.5 \pm 0.2$	$6.8 \pm 0.1$	$8.8 \pm 0.1$
methylisocitrate	$7.8 \pm 0.2$	$8.1 \pm 0.2$	$7.0 \pm 0.1$	$9.0 \pm 0.1$
tricarballoylate <sup>c</sup>			$7.7 \pm 0.2$	$9.2 \pm 0.2$

<sup>a</sup> Data were collected at 22 °C with 20 mM buffer and 0.1 M KCl. Buffers used were Mes, Hepes, Tris, Caps, Ches, Taps, ethanolamine, *N*-ethylmorpholine, (2-hydroxyethyl)-*N*-piperazine, and acetate. <sup>b</sup> Values obtained in the presence of 30% propylene glycol. The final pH of the propylene glycol solutions was determined directly; negligible perturbations were observed in the pHs of the cationic acid buffers used when propylene glycol was added. <sup>c</sup> The pH-independent minimal  $K_i$  was  $0.8 \pm 0.1$  mM.

Table II: Relative Rates Obtained for Various Substrates with Beef Liver and *S. lipolytica* Aconitases<sup>a</sup>

substrate	beef liver			<i>S. lipolytica</i>		
	rel $V$	rel $V/K$	$K_m$ ( $\mu$ M)	rel $V$	rel $V/K$	$K_m$ ( $\mu$ M)
<i>cis</i> -aconitate	1	1	4.8	1	1	18
isocitrate	0.45	0.18	12	0.39	0.16	45
citrate	0.23	0.0077	140			
methyl- <i>cis</i> -aconitate	0.40	0.22	8.9	0.062	0.0070	158
methylisocitrate	0.98	0.15	32	0.084	0.0056	268

<sup>a</sup> Values were obtained at pH 8. Similar results for *S. lipolytica* were obtained when the pH-independent values from the fitted pH profiles were used.

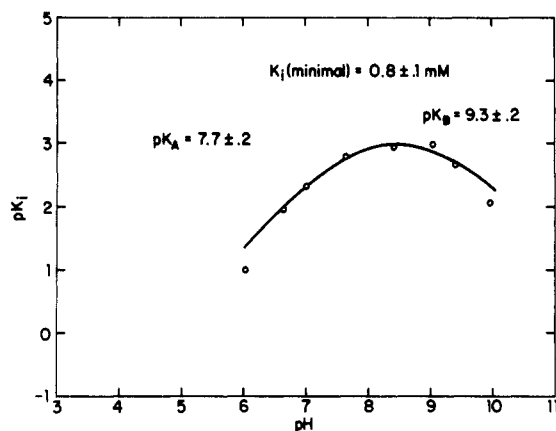


FIGURE 5:  $pK_i$  vs. pH for tricarballoylate inhibition of *S. lipolytica* aconitase.

the majority of the data which are above this pH value.) A similar pH dependence was observed for the affinity of the enzyme for the competitive inhibitor, tricarballoylate (Figure 5), suggesting that substrate binding to the incorrectly protonated forms of the enzyme does not occur. The lower  $pK$  in the tricarballoylate profile was also substantially higher than the third  $pK$  of tricarballoylate (5.8), so that groups on the enzyme are responsible for the pH variation in binding.

Solvent perturbation (30% propylene glycol) of the isocitrate and aconitate profiles (Table I) resulted in an increase in the lower  $pK$  in the  $V/K$  profiles, but in the  $V$  profile an increase occurred in the higher  $pK$ .

The relative rates obtained with various substrates for *S. lipolytica* and beef liver aconitases are summarized in Table II. One striking difference between the two enzymes is that the methyl substrates are much poorer substrates for the yeast enzyme than for the beef liver aconitase. The effect involves

Table III: Isotope Effects with  $\alpha$ -Methyl Substrates<sup>a</sup>

enzyme	pH	no. <sup>b</sup>	$D(\text{Eq.P.})$ or $D(V/K)$	$DV$
liver	7.5	1	1.10	
	8.0	3	$1.15 \pm 0.02$	
	8.0	3	$1.12 \pm 0.02^c$	
	8.25	1	1.11	
	8.4	5	$1.12 \pm 0.02$	
	9.4	1	1.12	
yeast	6.0	1	$1.3 \pm 0.2$	$1.6 \pm 0.2$
	7.5	1	1.22	
	8.25	1	1.26	
	8.4	6	$1.22 \pm 0.02$	
	8.5	1	$1.3 \pm 0.1$	$1.8 \pm 0.4$
	9.4	1	1.23	

<sup>a</sup> Determinations of isotope effects were made by direct comparison of deuterated and unlabeled  $\alpha$ -methylisocitrate in water when a  $DV$  value is given or by equilibrium perturbation with deuterated  $\alpha$ -methylisocitrate in water. The values are computed for  $\alpha$ -methylisocitrate as the reactant or perturbant. <sup>b</sup> Number of determinations made. When more than one, the values are the averages  $\pm$  one standard deviation. <sup>c</sup> The perturbation was with unlabeled  $\alpha$ -methylisocitrate and  $D_2O$ .

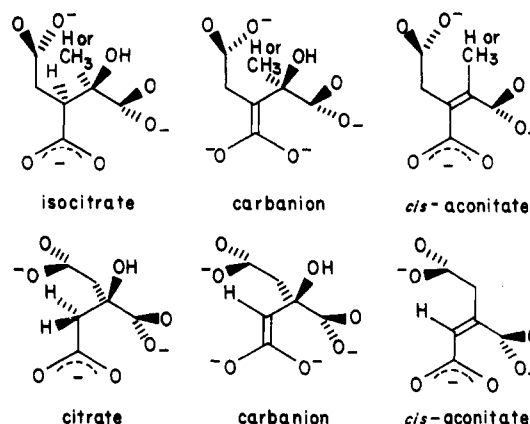
both a decrease in  $V$  and an increase in Michaelis constants.

**Isotope Effects with Beef Liver and *S. lipolytica* Aconitases.** Primary deuterium isotope effects for beef liver or yeast aconitases are summarized in Table III. These values were measured either by equilibrium perturbation using deuterated  $\alpha$ -methylisocitrate (or in one case unlabeled  $\alpha$ -methylisocitrate in  $D_2O$ ) or in several cases for the yeast enzyme by direct comparison of rates with deuterated and unlabeled  $\alpha$ -methylisocitrate. The equilibrium isotope effect for the reaction of  $\alpha$ -methylisocitrate can be calculated from the fractionation factors listed by Cleland (1980) as 1.08 in  $H_2O$  or 1.03 in  $D_2O$ . An experimental determination at pH 8 based on changes in the absorbances of  $\alpha$ -methyl-*cis*-aconitate gave  $1.15 \pm 0.02$  in  $D_2O$ ; we do not know why the value is high, but in any case the exact value assumed has no effect on the values in Table III, which are calculated as isotope effects from the  $\alpha$ -methylisocitrate side.

## Discussion

**Stereochemistry of the Reaction.** As reported by Beach et al. (1977) only  $\alpha$ -methylisocitrate is produced from  $\alpha$ -methyl-*cis*-aconitate by aconitase. Proton NMR spectra of equilibrium mixtures, anion-exchange chromatography of tritiated equilibrium mixtures, and compatibility of kinetic parameters for  $\alpha$ -methylisocitrate and  $\alpha$ -methyl-*cis*-aconitate with the observed equilibrium constant establish the absence of significant  $\alpha$ -methylcitrate. This lack of involvement of  $\alpha$ -methylcitrate requires a stereochemical explanation. Since (1) the reaction appears to involve carbanion intermediates which have largely the acicarboxylate form (Schloss et al., 1980), (2) the elimination is trans (Gawron et al., 1958, 1961), (3) the proton is conserved in the reaction from isocitrate to citrate (Rose & O'Connell, 1967), and (4) the hydroxyl group of citrate (and presumably isocitrate) became coordinated to a single Fe of the Fe-S cluster of aconitase (Emptage et al., 1983b), we believe the only reasonable stereochemical description of what happens in the active site is shown in Chart I.

On the top row we show the interconversion of isocitrate and *cis*-aconitate, and on the second row we show the interconversion of citrate and *cis*-aconitate. Note that the hydroxyl is removed from above and the proton from below the plane of the paper. The four carbons involved in the key portion of both carbanions occupy essentially isosteric positions in all structures shown and include the  $\alpha$ - and  $\beta$ -carboxyls and the

Chart I: Stereochemical Course of the Aconitase Reaction<sup>a</sup>

<sup>a</sup> An  $\alpha$ -methyl group is tolerated in the configurations shown in the top row but not in those of the lower row.

methylene carbons of *cis*-aconitate. Note that the oxygens of the carboxyl that becomes an aci-carboxylate must lie in the plane of the paper, while to avoid steric interference, those of the other carboxyl must be perpendicular to the paper.

The configuration of the other acetate arms bearing the  $\gamma$ -carboxyl are hypothetical, but those shown allow the oxygens of the  $\gamma$ -carboxyl to be isosteric in the two carbanion intermediates. The only real difference in the two carbanion structures lies in the position of the methylene group in this acetate arm and, to some extent, in the position of the carbon of the  $\gamma$ -carboxyl. Since the enzyme has an order of magnitude higher affinity for isocitrate than for citrate, and nearly 2 orders of magnitude greater affinity for the nitro analogue of the isocitrate-derived carbanion than for that of the citrate-derived carbanion (Schloss et al., 1980), it is clear that there is a strong preference for the acetate arm to assume the left hand (isocitrate-like) of the two possible configurations shown in Chart I. The binding of the  $\gamma$ -carboxyl will allow the acetate arm to assume the other configuration, but presumably when it does, a portion of the protein shifts its position in such a way that there is no room for an  $\alpha$ -methyl group. In the isocitrate-like configuration, however, the  $\alpha$ -methyl group is well removed from the carbons involved in the aci-carboxylate, and its presence can be accommodated.

**Acid-Base Chemistry of the Reaction.** An elimination reaction via a carbanion intermediate clearly requires at least two catalytic groups on the enzyme. One must be a base that abstracts a proton to form the carbanion; it must be monoprotic since the proton is conserved in the isocitrate to citrate conversion as long as sufficient *cis*-aconitate is present (Rose & O'Connell, 1967). The other group is needed to protonate or chelate the hydroxyl group when it is eliminated from the carbanion and to activate water for the reverse reaction. The observation that  $^{17}O$ -labeled citrate perturbs the EPR signal of the Fe-S cluster and has a dramatic effect on its Mössbauer spectrum (Emptage et al., 1983b) raises the possibility that the most labile iron at the corner of the cluster accepts the hydroxyl of the substrate as a ligand to facilitate its removal. The hydroxyl would still have to become protonated prior to dissociation, and since  $H_2[^{17}O]$  does not affect the EPR spectrum of the enzyme in the absence of substrate (Emptage et al., 1983b), we believe it is likely that this protonation is not spontaneous but is catalyzed by a group on the enzyme.

The  $pK$ 's of the catalytic groups are not seen in the  $V/K$  profiles for substrates or the  $pK_i$  profile for binding of an inhibitor with the beef enzyme (we will discuss a possible reason for this below), but they are seen in the  $V/K$  profiles

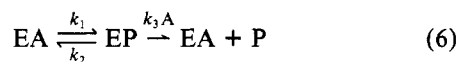


for the yeast enzyme. The  $pK$  around 7 which is elevated by propylene glycol may be a carboxyl group, as is the case with fumarase (Brant et al., 1963). Such a group in the bottom of the active site could readily sequester the proton and not exchange it with the solvent, especially if that portion of the active site is not open to the solvent, but only opens up in response to substrate binding. The proton on the carboxyl of fumarase is not exchanged with the solvent while substrates are bound (Hansen et al., 1969).

The group whose  $pK$  is 9 probably protonates the hydroxyl group of the substrates either during transfer to the iron of the Fe-S cluster or subsequent to this to allow dissociation of water. Since its  $pK$  is slightly reduced by propylene glycol, this group appears to be a cationic acid, most likely lysine. It must be kept in mind that during the hydration of *cis*-aconitate the roles of the two catalytic groups are reversed, and this lysine must deprotonate water [water coordination to the Fe-S cluster apparently occurs after *trans*-aconitate binding (Emptage et al., 1983b)], and the carboxyl group must supply the proton to the carbanion.

**Kinetic Mechanism of the Reaction.** A number of puzzling observations point to an unusual kinetic mechanism for aconitase. (1) The  $V/K$  profiles do not show any sign of the  $pK$  of the substrates, suggesting that one of the carboxyl groups can be protonated, although monoamides do not bind and are not substrates. Similarly, Dickman & Speyer (1954) reported all three monomethyl esters of *cis*-aconitate not to be substrates for aconitase. (2) For the beef enzyme, the  $V/K$  profiles are nearly pH independent and only decrease above pH 10; the value of  $V$  is totally pH independent. (3) Emptage et al. (1983a) have observed spectroscopically an apparent  $K_d$  for citrate of  $\sim 2 \mu M$ , which is 2 orders of magnitude lower than its  $K_m$  value. Later work (M. H. Emptage, unpublished data) showed that [ $^{14}C$ ]citrate is tightly bound to active aconitase and 45% remains bound during rapid passage through a Sephadex G-50 column which, in view of the turnover numbers of about  $15 s^{-1}$  for citrate and higher for the other substrates (Glusker, 1971), should have removed any ligand dissociating with this high a rate constant; 96% of the citrate was removed when the column was preincubated with  $10 \mu M$  citrate (well above the apparent dissociation constant, but only 5% of  $K_m$ ), and 99% of it was removed when the column was preincubated with 1 mM citrate ( $5K_m$ ).

These data can be explained by assuming that reactants are tightly bound to aconitase and dissociate slowly but that product release is greatly speeded up by displacement by the substrates. This mechanism in its simplest form is



where A and P are substrate and product,  $V/E_t$  is  $k_1$ ,  $V/(K_a E_t)$  is  $k_1 k_3 / (k_1 + k_2)$ , and  $K_a$  is  $(k_1 + k_2) / k_3$ . If A dissociates only slowly from EA, it is clear why EA will stay undissociated during rapid passage through a Sephadex column containing no substrate.

This mechanism can also explain the lack of pH variation in  $V/K$  profiles for the beef enzymes if  $k_3$  is pH independent and if the protonation states of groups in EA and EP are locked (that is, the  $pK$ 's are displaced by substrate binding to values that cannot be observed). To understand how  $k_3$  can be pH independent, we must consider what is involved in this step. A di- or tricarboxylic acid dissociates in steps, with one carboxyl group lifting out of the active site momentarily and then usually returning to the bound state. Only if the rest of the molecule comes loose while the first part is still unbound is the entire molecule free to dissociate away. This process

explains why low pH enhances the dissociation of malate or fumarate from fumarase (Blanchard & Cleland, 1980); protonation of the end which dissociates first prevents reassociation and facilitates total dissociation.

With aconitase we need only postulate that binding of a portion of the substrate in that part of the active site momentarily vacated by a portion of the product (the acetate arm holding the  $\gamma$ -carboxyl, for example) leads to total release of product. The rate-limiting step here will be the bimolecular reaction of substrate with the vacant portion of the active site, and the state of protonation of the rest of the substrate (especially the  $\beta$ -carboxyl, which has the highest  $pK$ ) apparently does not affect this rate. Once the product leaves, the subsequent binding of the substrate is strongly favored over its dissociation, even with the  $\beta$ -carboxyl protonated, since the interactions of the other two carboxyl groups are sufficient to hold the substrate in place until the  $\beta$ -carboxyl group loses its proton. With the monoamides or esters one carboxyl is permanently prevented from interacting properly with the enzyme, and thus no substrate activity, and only weak overall binding is seen.

With the yeast enzyme  $V$  and  $V/K$  do vary with pH, although again the third  $pK$  of the substrates does not affect  $V/K$ . The simple mechanism 6 will not explain these patterns, since if  $k_3$  is pH independent,  $V/K$  will also not vary with pH even if  $k_1$ ,  $k_2$ , and  $V/E_t$  do. Either  $k_3$  must be dependent on the state of protonation of the catalytic groups, or a more complicated mechanism is required. Alternatively, product release may not need to be facilitated by substrates with this enzyme, in which case binding would be dependent on the protonation state of the enzyme (perhaps controlled by conformation), but not that of the substrate. In any case, further work is called for to determine the rates with which reactants dissociate from the various aconitases and the degree to which mechanism 6 applies.

**Deuterium Isotope Effects.** The small deuterium isotope effects observed with the natural substrates were interpreted by Thomson et al., (1966) as evidence for a carbonium ion mechanism. However, the observed primary isotope effect is seldom equal to the intrinsic isotope effect on the bond-breaking steps because non-isotope-dependent steps are often partly or largely rate limiting (Cook & Cleland, 1981). Several lines of evidence discussed above strongly favor a carbanion mechanism for aconitase. The data in Table III show that C-H cleavage to form the carbanion intermediate is only slightly rate limiting, although 2–3 times more so with the yeast enzyme. The isotope effect with the liver enzyme in fact is only about 12% and can be determined with precision only by equilibrium perturbation. It appears pH independent, in agreement with our conclusions that only the correct protonation state of enzyme and reactants can exist in binary complexes.

Mechanism 6 predicts that the observed isotope effect in a equilibrium perturbation experiment will vary with the level of reactants present. At low A and P levels the interconversion of EA and EP comes to equilibrium, so that a value between unity and the equilibrium isotope effect will be seen. At high levels of A and P reactant release is rapid, and the isotope effects on  $k_1$  and  $k_2$  are seen, although these would probably also be reduced by internal commitments to a value less than the intrinsic isotope effect on C-H bond cleavage. In a direct comparison experiment,  $^D(V/K)$  will lie between unity and  $^D K_{eq}$ , while  $^D V$  will be the isotope effect on  $k_1$ .

With the beef enzyme, the predictions of mechanism 6 are matched fairly well, as  $^D(Eq.P.)$  values are only slightly above



the expected  $^D K_{eq}$  value. With the yeast enzyme, the values are somewhat higher for  $^D(V/K)$  or  $^D(Eq.P.)$ , suggesting that reactant release may not be as slow and thus rate limiting as with the beef enzyme. The value of  $^D V$  is now 1.6–1.8, showing that C–H bond breaking is at least partly rate limiting. However, since the isotope effect is pH independent, incorrect protonation of enzyme–reactant complexes is not possible. Such protonation would destroy the stickiness of the reactants and convert mechanism 6 into a normal one where substrate and product release were faster than the catalytic conversion. This does not occur, in agreement with our conclusions from the pH studies, but reactant release may well occur at such a rate that mechanism 6 is not totally applicable for the yeast enzyme.

**Relationships of Aconitase to Other Enzymes.** Beef mitochondrial and cytoplasmic aconitases appear to be very similar in kinetic and chemical mechanisms. Both enzymes possess an Fe-S cluster that appears to play a role in catalysis (Emptage et al., 1983b); the Fe-S cluster in cytoplasmic aconitase is very similar to that reported for the mitochondrial enzyme (Beinert & Thomson, 1983; G. Spoto & M. H. Emptage, unpublished data). Despite their similarities there are two distinct genes for human aconitase, with the mitochondrial one being on the 22nd chromosome (Sparkes et al., 1978; Meera Khan et al., 1978) and the cytoplasmic one on the 9th (Mohandas et al., 1979; Povey et al., 1976; Westerveld et al., 1978). Similarly, the *S. lipolytica* aconitase, whose physical properties are very similar to those of beef heart aconitase, appears to be another example of an "iron-sulfur hydro-lyase". Interestingly, however, a similar enzyme from *S. lipolytica*,  $\alpha$ -methylcitrate dehydratase, is not an Fe-S protein on the basis of stability, insensitivity to iron chelators, and absorption spectrum (Aoki & Tabuchi, 1981). Several other hydro-lyases exhibit properties which would suggest that, like aconitase, they may have an essential Fe-S cluster. Citraconate hydratase (Subramanian & Raghavendra Rao, 1968), mannonate dehydratase (Robert-Baudouy & Stoeber, 1973), the  $Mg^{2+}$ -independent tartrate dehydratase (Hurlbert & Jakoby, 1964, 1965), and altronate dehydratase (Smiley & Ashwell, 1960) all exhibit extreme lability which can be reversed or prevented by thiol and  $Fe^{2+}$  (with no other divalent cations able to replace  $Fe^{2+}$ ), similar to the original observation of  $Fe^{2+}$ -cysteine activation of aconitase (Dickman & Cloutier, 1950; Morrison, 1954). Isopropylmalate isomerase (Bigelis & Umbarger, 1976) and homoaconitase (Strassman & Ceci, 1966) both catalyze reactions of similar mechanism to that of aconitase and show sensitivity to some metal chelators. However, a requirement for  $Fe^{2+}$  or thiol has yet to be demonstrated for activation of these two enzymes. Finally phosphogluconate dehydratase has recently been reported to be an Fe-S protein (Scopes & Griffiths-Smith, 1984). Aconitase thus appears to be only the first well-documented example of a class of Fe-S hydro-lyases.

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## Mammalian Folyl Polyglutamate Synthetase: Partial Purification and Properties of the Mouse Liver Enzyme<sup>†</sup>

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**ABSTRACT:** Folyl polyglutamate synthetase has been partially purified from mouse liver, and the general features of this enzyme have been characterized. The purification procedure utilized fractionation with ammonium sulfate, gel filtration, and affinity chromatography on ATP-agarose and resulted in a 350-fold increase in specific activity with 8-20% recovery of enzyme activity. Enzyme could be stabilized by glycerol or by ATP, but stability was not appreciably enhanced by folate. The enzymatic reaction was completely dependent on folate, ATP, and  $Mg^{2+}$  while partial reaction rates were observed in the absence of KCl or  $\beta$ -mercaptoethanol. Highest reaction rates were observed at pH 8.2-9.5 at 37 °C. Chromatography of purified enzyme on calibrated gel filtration columns suggested a molecular weight of 65 000. Mouse liver folyl polyglutamate synthetase coupled [<sup>3</sup>H]glutamic acid to

all of the naturally occurring folates studied. Analysis of the reaction products by high-performance liquid chromatography demonstrated that several folyl oligoglutamates were formed at low substrate concentrations but that only folyl diglutamate was formed at substrate concentrations approaching saturation. Dihydrofolate, tetrahydrofolate, 5,10-methylenetetrahydrofolate, 10-formyltetrahydrofolate, and 5-formyltetrahydrofolate were the best substrates. Folic acid and 5-methyltetrahydrofolate were also substrates for this reaction, but much higher concentrations of these compounds were required to saturate the enzyme. These data suggest that all of the tetrahydrofolyl compounds (except 5-methyltetrahydrofolate) are the monoglutamyl substrates for polyglutamation in vivo and that 5-methyltetrahydrofolate is not likely to be a direct precursor for folate polyglutamates in mouse liver.

**F**olate derivatives present in mammalian cells exist predominantly as polyglutamate conjugates in which the amide linkages involve the  $\gamma$ -carboxyl rather than  $\alpha$  linkage typical of peptides (Houlihan et al., 1972; Moran et al., 1976; Noronha & Aboobaker, 1963; Shin et al., 1972b). Several lines of evidence suggest that these folyl polyglutamates are the

folate forms used as cofactors in the cell for the folate-dependent biosynthetic reactions. Thus, all of the purified folate-dependent enzymes examined to date utilize the folyl oligoglutamates at least as well as the corresponding monoglutamates [recently reviewed in McGuire & Bertino (1981)]. In addition, L1210 mouse leukemia cells have been shown to contain only polyglutamate forms of the folates even under conditions of growth-rate-limiting folate deficiency (Moran et al., 1976). This same cell line has been shown to accumulate polyglutamyl dihydrofolate but not dihydrofolate itself when exposed to cytotoxic levels of the dihydrofolate reductase inhibitor methotrexate (MTX)<sup>1</sup> (Moran et al., 1975).

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