Adolfsen, R., and Moudrianakis, E. N. (1971b), *Biochemistry 10*, 440.
Bennun, A., and Racker, E. (1969), *J. Biol. Chem. 234*, 1325.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Farron, F., and Racker, E. (1970), *Biochemistry 9*, 3829.
Higashi, T., Bogin, E., and Brodie, A. F. (1970), *Arch. Biochem. Biophys. 136*, 331.
Howell, S., and Moudrianakis, E. N. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1261.
Ishikawa, I. (1970), *J. Biochem. (Tokyo) 67*, 297.
Kagawa, Y., and Racker, E. (1966), *J. Biol. Chem. 241*, 2475.
Pinchot, G. B. (1953), *J. Biol. Chem. 205*, 65.
Pinchot, G. B. (1957), *J. Biol. Chem. 229*, 1.
Schneider, W. C. (1957), *Methods Enzymol. 3*, 680.
Shibko, S., and Pinchot, G. B. (1961a), *Arch. Biochem. Biophys. 93*, 140.
Shibko, S., and Pinchot, G. B. (1961b), *Arch. Biochem. Biophys. 93*, 257.
Yamashita, S., and Ishikawa, S. (1965), *J. Biochem. (Tokyo), 57*, 232.

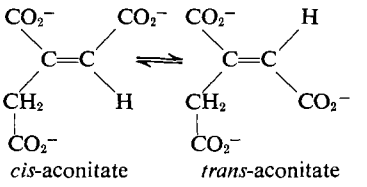
Purification and Kinetic Properties of Aconitate Isomerase from *Pseudomonas putida**

Judith P. Klinman† and Irwin A. Rose

ABSTRACT: Aconitate isomerase isolated from *Pseudomonas putida* grown on *trans*-aconitate has been partially purified 45-fold to a specific activity of 116 IU/mg. The enzyme, which catalyzes the interconversion of *cis*- and *trans*-aconitate, has a maximum rate at pH 8.5 and a molecular weight approximated to be 78,000 ± 10,000 by Sephadex chromatography. In assays initiated with aconitate isomerase the initial velocity falls off rapidly with time; when the enzyme is preincubated, and assays initiated with substrate, the initial velocity is invariant with time but depressed. The addition of 20% glycerol both diminishes the rapid fall off in velocity and gives increased activity with preincubated enzyme. The relationship between enzyme concentration and activity is linear for assays initiated with enzyme in the presence of 20% glycerol, whereas preincubation of aconitate isomerase gives rise to a nonlinear relationship. The K_m of *cis*-aconitate for preincubated enzyme is found to be inversely dependent on enzyme concentration: over a 4-fold range in enzyme concentration, the value of the K_m was 1.90–1.05 mM in the presence of 20% glycerol and 25.0–9.0 mM in the absence of glycerol. The observed relationship between enzyme activity and concentration under the different assay conditions is analyzed and shown to be consistent with a dissociation of enzyme into inactive subunits ($\eta = 2$).

An inhibitor of aconitate hydratase, *trans*-aconitic acid (Anfinsen, 1955), has been found to occur in a number of plant materials (Lippmann, 1879; Beath, 1926; Roberts and Martin, 1954; MacLennon and Beevers, 1964; Burau and Stout, 1965). Rao and Altekar (1961) reported on the presence of an induced enzyme, aconitate isomerase, in a fluorescent pseudomonad grown on a medium containing *trans*-aconitate as the sole carbon source. The presence of considerable amounts of aconitate isomerase in the leaf extracts of sugar cane has also been reported (Altekar *et al.*, 1965). In the case of the bacterial enzyme, aconitate isomerase was prepared free of aconitate hydratase by ammonium sulfate precipitation and shown to catalyze the conversion of *trans*-aconitate into *cis*-aconitate. As originally reported, the assay requirements of aconitate isomerase included an SH and Fe^{2+} requirement.

Aconitate isomerase catalyzes the interconversion of reaction 1. In this paper, the first of a series of three papers, we describe the partial purification of aconitate isomerase and some of its



cis-aconitate *trans*-aconitate (1)

kinetic properties. The second paper considers the mechanism of action of this enzyme. The third paper describes the use of aconitate isomerase to determine the steric course of the electrophilic replacement reactions catalyzed by citrate synthase, ATP citrate lyase, and citrate lyase.

Experimental Section

All chemicals were obtained commercially and were reagent grade unless otherwise noted. Glycerol was spectroquality and was obtained from Matheson Coleman & Bell. Dialysis tubing (Visking) was washed in boiling 0.1 M EDTA (pH 9) and stored in 0.01 M EDTA (pH 7). Bovine serum albumin was obtained from Pentex Biochemical Co. and ribonuclease was obtained from Worthington Biochemicals.

Determinations of pH were carried out on a radiometer (Type TTTlc) equipped with an expanded-scale attachment. Routine spectrophotometric determinations were carried out on Beckman DU spectrophotometers fitted with Gilford light

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TABLE 1: Summary of Enzyme Purification.

Step	Sp Act. (units/mg)		Total Units	Yield	-Fold Purification	
Crude	(2.6)[a]	1.3	3400	100		
$(NH_4)_2SO_4$ (30–50%)	(11.0)	5.5	3400	100	4.2	
G-100		10.8	1000	29	8	(4)
$(NH_4)_2SO_4$ (30–50%)		26.2	900	26	20	(10)
Hydroxylapatite		116.0	406	12	89	(45)

[a] The values in parentheses are corrections for the difference in the assay conditions for steps 1–2, compared to steps 3–5. The enzyme at steps 1 and 2 was assayed after a preincubation period in the assay mixture: the velocity of preincubated enzyme has been found, from studies on the most purified enzyme fraction, to be depressed by a factor of two relative to the velocity obtained in assays initiated with enzyme.

source stabilizers and readout accessories. Kinetic studies were carried out on a Cary 16 recording spectrophotometer. For the spectrophotometric studies, constant temperature was maintained with a circulating P. M. Tamson water bath with a Neslab portable bath cooler attachment. The concentration of protein solutions was carried out with Amicon Diaflo chambers (filters, PM-10) under a stream of prepurified nitrogen.

Bacterial Growth Conditions. The growth conditions are an adaptation of those described by Altekar and Rao (1962). *Pseudomonas putida* A 3.12, a generous gift of Dr. H. P. Meloche, was grown in a medium containing per liter, 7.5 g of *trans*-aconitate (Calbiochem), 2 g of ammonium nitrate (Merck), 1 g of potassium dihydrogen phosphate (J. T. Baker), 0.5 g of magnesium sulfate heptahydrate (J. T. Baker), and 0.01 g of ferric chloride (Fisher). *trans*-Aconitic acid was neutralized with potassium hydroxide and autoclaved separately. Ammonium nitrate and potassium dihydrogen phosphate were autoclaved together, as were magnesium sulfate heptahydrate and ferric chloride.

A 125-ml flask containing 20 ml of the medium was inoculated from a stock agar slant and shaken rapidly at 25° for 24 hr. To each liter of medium in 2-l. flasks, 1 ml of this liquid culture was added. The cells were grown at 25° with rapid shaking until the optical density of the cultures was approximately 2.5 absorbance units at 660 nm in 1-cm path-length cuvets. It was observed that the specific activity of aconitate isomerase decreases with time from cultures of *P. putida* in late stationary phase. For this reason it is important to harvest cells from growing cultures at the completion of log phase.

The cells were harvested by centrifugation at 10,000 rpm for 15 min. Approximately 3.5 g of wet cells were obtained per liter of medium.

Assay of Aconitate Isomerase. The standard assay is based on the differential absorbance of *cis*- and *trans*-aconitate at 260 nm. At 25°, pH 8, and 0.1 ionic strength, $\Delta\epsilon$(*trans*–*cis*-) = 0.6 optical density unit, mM^{-1} cm^{-1}. A unit is defined as 1 μmole of *trans*-aconitate formed per min. The standard assay used throughout the purification of the enzyme is carried out in 2-mm path-length cuvets containing in 0.5 ml: 50 μmoles of Tris-chloride, pH 8 (Sigma); 20% glycerol (spectroquality; Matheson Coleman & Bell); 1 μmole of DTT[1] (Calbiochem); and 5 μmoles of *cis*-aconitate. Previous authors (Rao and Altekar, 1961) described aconitate isomerase as requiring both Fe^{2+} and SH in the assay mixture; we have found that dialysis against 1 mM EDTA for 12 hr does not result in any loss of activity of aconitate isomerase, suggesting that the native enzyme may not have a metal requirement although it may contain tightly bound metal ion(s), which are inaccessible to large chelators. The previously reported need for Fe^{2+} (Rao and Altekar, 1961) may be due to the requirements of the coupling enzyme, aconitase (Dickman and Clautier, 1950; Morrison, 1953).

Protein concentration was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin used as standard.

Results

Enzyme Purification. All procedures were carried out at 0–4°. The purification scheme is summarized in Table I. Cells (34.5 g) from 10 l. of culture were washed with 0.9% sodium chloride and suspended in 345 ml of 0.02 M potassium phosphate (pH 7) and 5 mM cysteine. The suspended cells were sonicated in two parts for 5 min each with a Bronson sonicator. The extracts were centrifuged at 18,000 rpm for 20 min. The supernatants were retained.

To the crude supernatant,[2] 59 g of ammonium sulfate was added with stirring. After centrifugation, an additional 45 g of ammonium sulfate was added to the supernatant. The resulting precipitate was dissolved in 13 ml of 0.1 M potassium phosphate (pH 7), 20% glycerol, 1 mM EDTA, and 1 mM DTT. This 30–50% cut contained aconitate isomerase and was free of aconitase.

A Sephadex G-100 column (2.5 × 45 cm, void volume 70 ml), which had been preequilibrated with 0.1 M potassium phosphate (pH 7), 1 mM EDTA, and 1 mM DTT, was charged with 3400 units of enzyme in 13 ml. The buffer with which the column was equilibrated was used to elute the column. Aconitate isomerase was obtained in a volume of 83–95 ml, representing a peak of specific activity.

To a volume of 16 ml, containing the pooled fractions from the gel filtration step, 2.8 g of ammonium sulfate was added. After centrifugation, another 2.0 g of ammonium sulfate was added to the supernatant. The precipitate so obtained (30–50% cut) was dissolved in 1 ml of 10 mM imidazole chloride

[1] Abbreviation used is: DTT, dithiothreitol.

[2] A number of methods for removing nucleic acid were attempted, all of which gave only slightly improved A_{280} to A_{200} ratios, and in some cases decreases in specific activity. The methods tried included manganese chloride precipitation, protamine sulfate and streptomycin treatment, and DNase and RNase treatment.

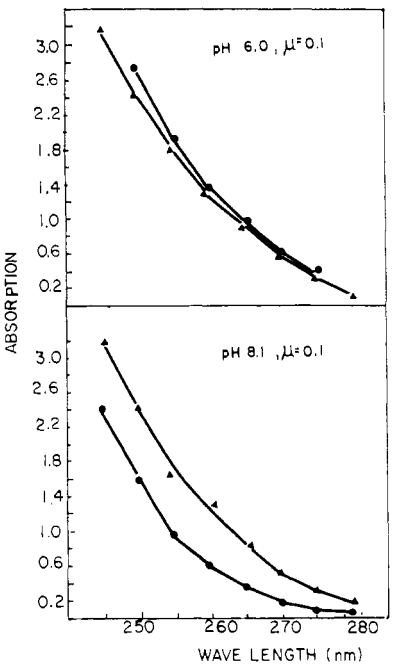


FIGURE 1: Absorption of 1×10^{-3} M *cis*-aconitate (●) and *trans*-aconitate (▲) between 245 and 280 nm at pH 6 and 8.1, $\mu = 0.1$. Buffers were imidazole chloride (pH 6) and Tris-chloride (pH 8.1). Constant ionic strength was maintained with sodium chloride.

(pH 7), 20% glycerol, 1 mM EDTA, and 1 mM DTT. The protein solution was dialyzed overnight against the same buffer.

A hydroxylapatite (Bio-Rad) column (2.5 × 4 cm), which had been preequilibrated with 1 mM potassium phosphate (pH 7), 20% glycerol, and 1 mM DTT, was charged with 900 units of enzyme. Elution was carried out with a linear gradient composed of 150 ml each of 5 mM potassium phosphate and 100 mM potassium phosphate (pH 7), both of which contained 20% glycerol and 1 mM DTT. A peak of specific activity was obtained in an effluent volume between 144 and 192 ml. The pooled fractions were concentrated by Diaflo (as described in the Experimental Section); the specific activity of this peak was 116. Disc gel electrophoresis at pH 9.5 on this sample indicated 7 protein bands. From the relative intensities of these bands, aconitate isomerase is estimated to represent between 3 and 40% of the total protein. Electrophoresis in sodium dodecyl sulfate revealed 12 bands. Further attempts at purification of this protein, including chromatography on DEAE- and CM-celluloses have been unsuccessful; however, the present level of purification is believed to be satisfactory for the experiments to be described.

To inactivate aconitase, which is present in the crude extract, aconitate isomerase was assayed at step 1 by preincubation of the enzyme for 5 min in the standard assay mixture plus 1 mM EDTA and the assay was begun by the addition of substrate. The assay of aconitate isomerase at step 2 was carried out after a 5-min preincubation of enzyme, whereas all subsequent assays were initiated by the addition of enzyme.

The purified preparation of aconitate isomerase (specific activity 116 units/mg) was stored in 0.2–0.9-ml fractions at −70°. Under these conditions the enzyme appears to be stable indefinitely. Solutions of the most purified preparation of aconitate isomerase lost activity when stored at 4°, particularly after having been dialyzed. The enzyme is susceptible to oxidation and activity can be partially restored after the addition of a mercaptan. Prior to the use of this enzyme for kinetic studies the enzyme was dialyzed overnight against a solution which was 30–50 mM Tris-chloride (pH 8), 20% glycerol, and 2 mM DTT to remove salt.

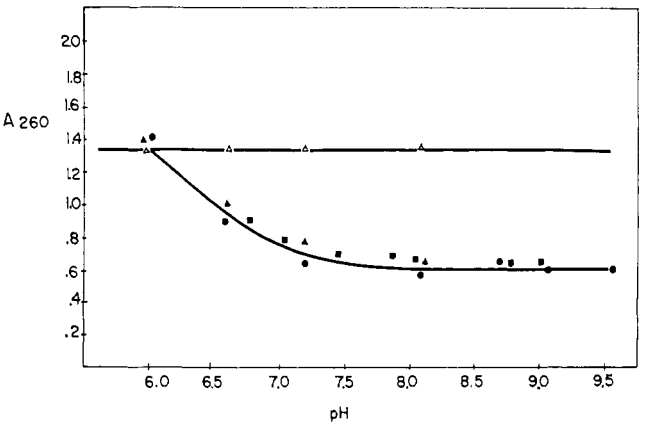
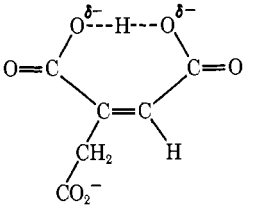


FIGURE 2: Absorption of 1×10^{-3} M *cis*- and *trans*-aconitate at 260 nm between pH 6 and 9. *trans*-Aconitate (△) was in 10% glycerol, $\mu = 0.1$. *cis*-Aconitate (■), 10% glycerol, $\mu = 0.1$; (●), $\mu = 0.1$; (▲), $\mu = 0.2$. Buffers were imidazole chloride (pH 6–7.3), Tris-chloride (pH 8.1), and sodium glycinate (pH 8.7–9.5).

Ultraviolet Absorption Properties of cis- and trans-Aconitate. In order to study the catalytic properties of aconitate isomerase under a variety of conditions it was first necessary to determine the effect of pH, ionic strength, and solvent composition on the difference in extinction coefficient between *cis*- and *trans*-aconitate. In Figure 1, the absorption of cis and trans acids is shown in the wavelength region, 245–280 nm, at pH 6.0 and 8.1. From this figure it can be seen that the difference in absorption between the two acids is a function of both pH and wavelength and that at pH 6 the absorption of cis is actually greater than that of trans below 275 mμ, whereas at pH 8.1 the absorbance approximately doubles above 260 mμ in going from cis to trans. From Figure 2 it can be seen that the absorption of *trans*-aconitate at 260 mμ is independent of pH between pH 6 and 8.1, whereas the absorption of *cis*-aconitate decreases rapidly with pH between pH 6 and 8, the absorption leveling off after pH 8. The absorption of *cis*-aconitate is also slightly dependent on ionic strength as shown; the addition of 10% glycerol has a small effect.

The effect of pH on the absorption properties of *cis*-aconitate arises, in all likelihood, from the ionization of the third carboxyl group between pH 6 and 8, whereas *trans*-aconitate appears to be fully ionized in this pH range. Pratt and Smith (1967) studied the effect of pH (0–8) on the nuclear magnetic resonance line positions of the methylene and methine protons of *cis*- and *trans*-aconitate; they estimated pK_3 of *cis*-aconitate to be approximately 6.5 and pK_3 of *trans*-aconitate to be close to 4.

This proposed difference in the value of pK_3 between *cis*- and *trans*-aconitate is analogous to the observed differences



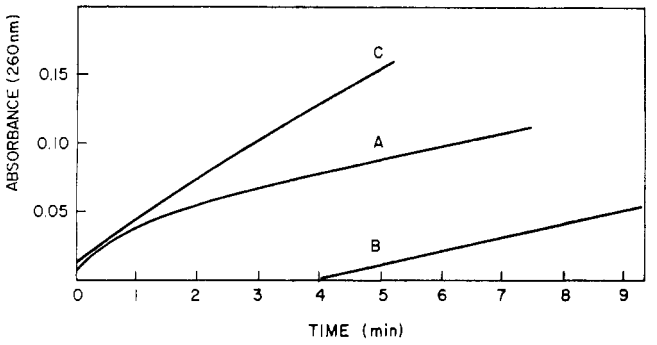


FIGURE 3: Assay of aconitate isomerase in the absence of glycerol without preincubation (curve A) and with preincubation (curve B), $T = 25°$, $\mu = 0.06$. Curve A: the assay contained in 0.5 ml, 4 μmoles of *cis*-aconitate, 15 μmoles of Tris-chloride (pH 8.1), and 1 μmole of DTT. The assay was started by the addition of 5 μl of stock enzyme. Curve B: the conditions of the assay were identical with curve A with the exception that the assay was initiated by the addition of 0.02 ml of *cis*-aconitate. Curve C: the conditions of the assay were identical with curve A except that the solvent contained 20% glycerol.

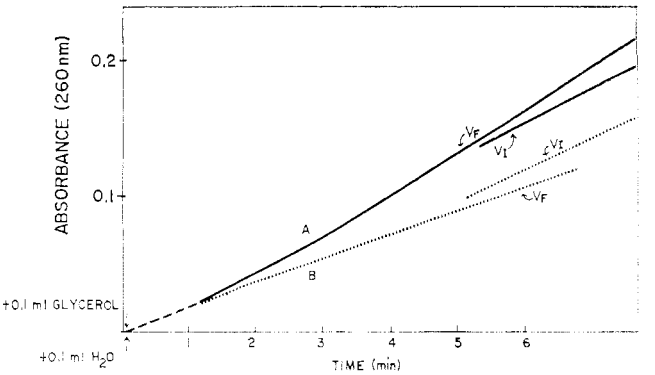


FIGURE 4: The effect of dilution with either glycerol (curve A) or water (curve B) on the activity of aconitate isomerase. The assay contained initially in 0.44 ml: 15 μmoles of Tris-chloride (pH 8.1), 1 μmole of DTT, and 10 μl of stock enzyme. The velocity obtained upon the addition of 6 μmoles of *cis*-aconitate (0.06 ml) to this reaction mixture, 4 min after the addition of enzyme, is V_I. At 2–3 min after the addition of *cis*-aconitate either 0.1 ml of glycerol (50%) (curve A) or 0.1 ml of H_2O (curve B) was added (zero time in Figure 5). The velocity 5 min after the addition of either glycerol or water (V_F) is compared to V_I.

between fumaric acid, $pK_2 = 4.5$, and maleic acid, $pK_2 = 6.6$ (Dawson *et al.*, 1959). Casciato *et al.* (1970) have concluded from a conformational analysis of crystalline potassium *cis*-aconitate that this structure is characterized by a short internal hydrogen bond in analogy to that found for potassium hydrogen maleate (Peterson and Levy, 1958).

Kinetic Properties of Aconitate Isomerase. GENERAL PROPERTIES. The kinetic properties of aconitate isomerase depend on the conditions of the assay. In all cases the stock enzyme is dialyzed prior to its use as described in the Experimental Section, and the assay involves a dilution of this enzyme (0.5 mg/ml) by a factor of 10–100. In Figure 3 the behavior of the enzyme is shown under two conditions. In Figure 3A the behavior of the enzyme is shown for an assay which has been initiated by the addition of enzyme; it can be seen that the initial velocity falls off rapidly with time, at a rate which is much greater than the concomitant loss of substrate. After a period of 4 min, the absorbance increase has become linear with time. In Figure 3B the assay has been initiated by the addition of substrate following a 4-min preincubation of the enzyme. The initial rate one obtains after preincubation of the enzyme is invariant with time and is equal to the rate obtained after 4 min in the assay initiated with enzyme. Although it is possible to get linear initial rates with aconitate isomerase by preincubation of the enzyme, attempts were made to determine conditions under which the initial burst rate could be studied.

A survey of inorganic salts, sulfhydryl reagents, and tricarboxyllic acid intermediates indicated that none of these reagents diminished the decay of the initial velocity of aconitate isomerase. Dialysis of the enzyme, as described in the Experimental Section, was found to have no effect on this decay, indicating that the observed kinetic properties are not due to the simple dissociation of an activator. It was found, however, that in the presence of glycerol the rate of decay of the initial rate is greatly decreased (Figure 3C). This effect of glycerol increases with increasing concentration of glycerol, leveling off above 20% glycerol. Preincubation of the enzyme for 4 min in an assay mixture containing 20% glycerol gives an initial velocity which is invariant following a small initial increase in velocity. This velocity is not equal to that which is observed in an assay initiated with enzyme after 4 min, as is the case in the absence of glycerol. It appears, therefore, that in the presence of glycerol *cis*-aconitate also slows the rate of transition to the equilibrated form of the enzyme.

The constant rate of preequilibrated enzyme is significantly greater in the presence of glycerol than the rate obtained under similar conditions in the absence of glycerol at low enzyme concentrations. Thus glycerol does more than delay the transition, it activates as well; this activation is considered in Figure 4. The procedure for the two assays illustrated was to preincubate enzyme for 4 min; the assay was initiated by the addition of substrate. Two to three minutes after the addition of substrate, either 100 μl of water (curve B) or 100 μl of 50% glycerol (curve A) were added. It can be seen from curve A that 5 min after the addition of glycerol, which results in a dilution of substrate, the velocity has increased relative to the initial velocity, in contrast to curve B.

Relationship between Enzyme Concentration and Activity. In 20% glycerol, conditions under which one can readily study the burst rate, the enzyme activity is proportional to concentration of enzyme for assays which are initiated by the addition of enzyme. This is illustrated by curve A in Figure 5. Preincubation of the enzyme for 4 min in an assay mixture containing 20% glycerol gives a lower rate at all enzyme concentrations tested (curve B) with evidence of some deviation from proportionality at the higher dilutions. When one preincubates the enzyme in an assay mixture which is free of glycerol, one obtains curve C in Figure 5; the relationship between velocity and enzyme concentration is clearly nonlinear under these conditions.

K_m of cis-Aconitate as a Function of Enzyme Concentration and Varying Assay Conditions. The effect of substrate concentration on the catalytic rate was determined under the three conditions studied in Figure 5 and with the enzyme concentration varied over the range of greatest curvature shown in curve C, 5–20 μl of enzyme stock/0.5-ml incubation volume. As summarized in Table II, the K_m of *cis*-aconitate with equilibrated enzyme was significantly lowered by either glycerol or increased enzyme concentration. Values for V_{max} are also given in Table II for both the studies in 20% glycerol; V_{max} is linearly related to enzyme concentration, but preincubation of aconitate isomerase for 4 min gives rise to a 50%

TABLE II: Values of V_{max} and K_m Obtained from Lineweaver–Burk Plots as a Function of Enzyme Concentration.

Vol of Enzyme (μl)	Dilution Factor	Conditions of Assay: Burst (20% Glycerol) V_{max} (μmole min^{-1})	Burst (20% Glycerol) K_m ($\times 10^3$), M	Preincubn (20% Glycerol) V_{max}	Preincubn (20% Glycerol) K_m	Preincubn (0% Glycerol) K_m
5	100	0.20	1.0	0.093	1.9	25
10	50	0.40	0.91	0.17	1.8	15
20	25	0.79	1.1	0.39	1.1	9

reduction in V_{max} at all enzyme concentrations. An accurate determination of V_{max} for enzyme preincubated in the absence of glycerol is difficult to obtain; due to the high K_m and the desirability to maintain a constant ionic strength (μ = 0.1), the concentration of substrate that could be used was never greater than the K_m. Approximate values for the V_{max} at the three levels of enzyme concentration given in Table II were 0.08, 0.16, and 0.32 μmole min^{-1}.

pH Dependence of Aconitate Isomerase Activity. The maximal velocity of aconitate isomerase as a function of pH is shown in Figure 6. For this study assays were carried out in 20% glycerol, and velocities were obtained from the initial burst. The assay mixtures were 0.5–10 mM in *cis*-aconitate. Maximal velocities were obtained by the extrapolation of Lineweaver–Burk plots drawn through a minimum of three points. The pH maximum of aconitate isomerase is pH 8.5. The K_m of aconitate isomerase was found to be dependent on pH and increased from 0.18 mM at pH 7.15 to 7.7 mM at pH 9.25.

Molecular Weight of Aconitate Isomerase. A molecular weight determination of partially purified aconitate isomerase was made by Sephadex chromatography. The enzyme used for this determination was purified to step 2, Table I. A Sephadex G-100 column (2.5 × 45 cm) was equilibrated with 0.1 M potassium phosphate (pH 7), 1 mM DTT, and 1 mM EDTA. The standardization of the column was carried out in two separate determinations with either Blue Dextran (void volume 58 ml) or 10 mg of crystalline bovine serum albumin (mol wt 67,000, Phelps and Putnam (1960); elution volume 92 ml) and 5 mg of ribonuclease (mol wt 13,700 (Hirs *et al.*, 1956); elution volume 155 ml). The chromatography of 3 ml of aconitate isomerase (13 mg/ml) was carried out and the volume of elution was 84 ml, indicating a molecular weight of 78,000 ± 10,000.

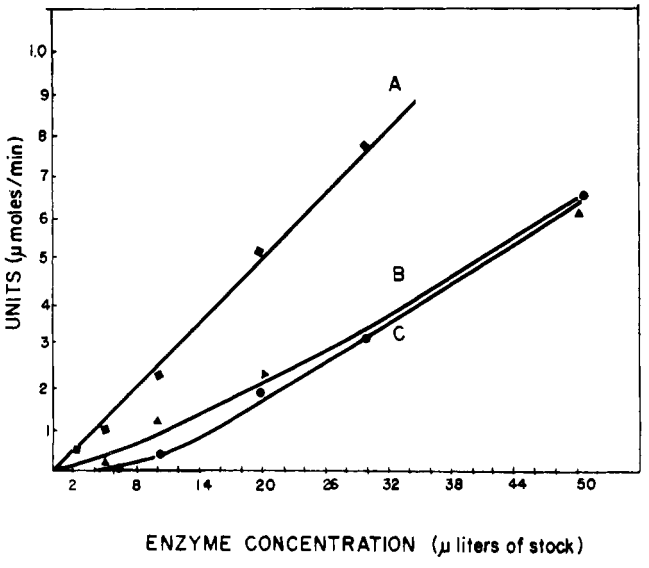


FIGURE 5: The relationship between enzyme concentration and activity. Curve A: the assay mixture contained in 0.5 ml, 4 μmoles of *cis*-aconitate, 15 μmoles of Tris-chloride (pH 8.1), 1 μmole of DTT, and 20% glycerol. T = 25°, μ = 0.06. The assay was initiated by the addition of enzyme. Curve B: the final assay mixture was the same as A. The assay was initiated by the addition of 0.02 ml of *cis*-aconitate following preincubation of the enzyme for 4 min. Curve C: the assay conditions were the same as B. The assay mixture was the same except for the exclusion of glycerol.

Discussion

The nonlinearity of the assay of aconitate isomerase with time (Figure 3, curve A) was found to be appreciably diminished by the addition of glycerol to the assay mixture (Figure 3, curve C), and it was possible to show that the initial velocity for aconitate isomerase in 20% glycerol is proportional to enzyme concentration (Figure 5, curve A). However, if aconitate isomerase is preincubated for 4 min, and the assay begun by the addition of substrate, the relationship between

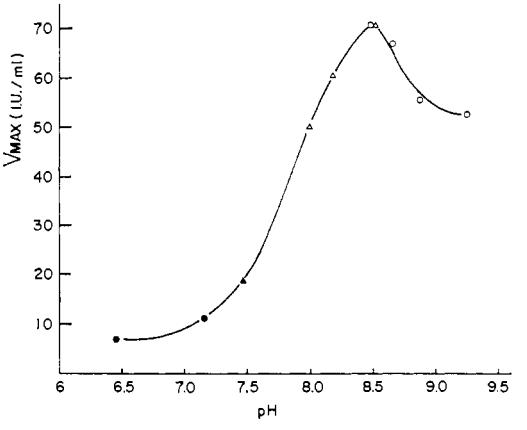


FIGURE 6: pH dependence of V_{max} of aconitate isomerase. Assays were carried out at 25°, μ = 0.1, and contained in 0.5 ml of 20% glycerol, 30 μmoles of buffer, 1 μmole of DTT, and 0.5–5 μmoles of *cis*-aconitate. Constant ionic strength was maintained with potassium chloride. Velocities were obtained from the initial burst. V_{max} was obtained by extrapolation of Lineweaver–Burk plots. Buffers were (●) imidazole chloride, (▲) *N*-Tris(hydroxymethyl)-methyl-2-aminoethanesulfonate, (△) Tris-chloride, and (○) glycine.

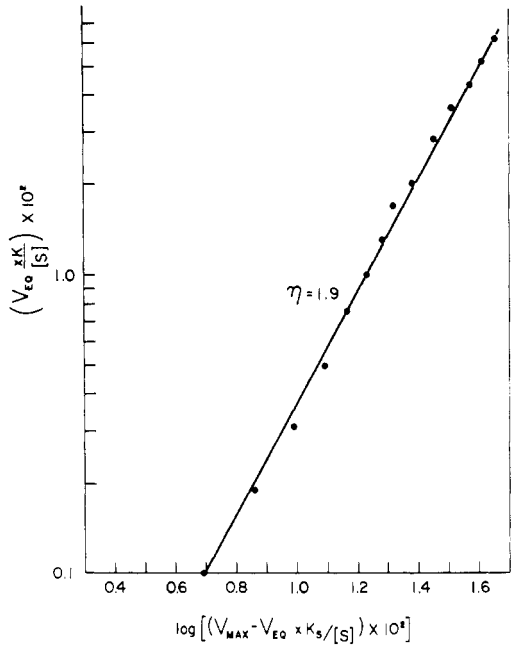


FIGURE 7: Data obtained in Figure 5 are plotted according to eq 4 to determine the order of dissociation, η, of aconitate isomerase For details, see text.

enzyme concentration and activity becomes curved (Figure 5 curves B and C), suggesting a dissociation of the enzyme into subunits.

An analysis of the difference between curves A and C in Figure 5 was made on the assumption that the nonlinearity of curve C arises from a dissociation of active enzyme, E_η, into η inactive subunits, E, upon preincubation

$$E_\eta \rightleftharpoons \eta E \tag{2}$$

$$K = [E]^\eta/[E_\eta] \tag{3}$$

The velocity at dissociation equilibrium (V_{eq}) is proportional to the concentration of $[E_\eta]$

$$V_{eq} = k[E_\eta S] = k[E_\eta][S]/K_S \tag{4}$$

where K_S is the dissociation constant of *cis*-aconitate from polymerized enzyme. The concentration of dissociated enzyme is equal to $\eta([E_T] - [E_\eta])$, where $V_{max} = k[E_T]$

$$[E_\eta] = \frac{V_{eq}}{k}\frac{K_S}{[S]}; \quad [E_T] = \frac{V_{max}}{k}; \quad \frac{[E]}{\eta} = \frac{V_{max}}{k} - \frac{V_{eq}}{k}\frac{K_S}{[S]} \tag{5}$$

Substituting velocity for enzyme concentration in eq 3, taking the log of both sides of the equation, and rearranging terms gives the following equation of a line with slope η

$$\log V_{eq}K_S/[S] = \eta \log(V_{max} - V_{eq}K_S/[S]) - \log Kk^{\eta-1}\eta^{-\eta} \tag{6}$$

In Figure 7, data obtained in Figure 5 are plotted over a 10-fold range in enzyme concentration (4–40 μl of enzyme/assay). Values for V_{eq} are obtained from curve C, values for V_{max} from curve A, K_S is 1 mM (Table II), and the substrate concentration is 8 mM at all points. A line of slope, $\eta = 1.9$, was obtained. Although some question can be raised about the use of curve A to represent V_{max}, the value of η is found to be insensitive to large changes in the V_{max} term as expected, since $[E_\eta]$ ($\propto V_{eq}K_S/[S]$) will be a small part of $[E_T]$ when $[S] > K_S$. Thus, using V_{max} values of 50 and 25% of those from curve A, gives rise to lines described by $\eta = 1.9$ and 2.0, respectively.

When a determination of both V_{max} and K_m was carried out under different assay conditions, and at several enzyme concentrations (Table II), the K_m of the preincubated enzyme was found to be higher than the K_m determined under conditions of the initial burst and to increase with decreasing enzyme concentration. This change in K_m with enzyme concentration is much greater in the absence of glycerol than in its presence. Although the V_{max} of the preincubated enzyme is almost identical in the presence or absence of glycerol, this value is approximately one-half the V_{max} under conditions of the initial burst. These differences in V_{max} suggest that the changes in aconitate isomerase which occur on preincubation of the enzyme are more complex than a simple dissociation of enzyme into subunits. It should be noted that the V_{max} of preincubated enzyme is proportional to enzyme concentration in the range studied. The changes in K_m that occur on preincubation indicate that dissociated aconitate isomerase either may not bind substrate, or does so with a low affinity. The much smaller increase in K_m in 20% glycerol medium suggests that glycerol is stabilizing the undissociated form of the enzyme. Recently, Phillip *et al.* (1970) have discussed the conversion of one form (A) of the plasmoprotein, factor V, into another oligomeric form (C) by the presence of high concentrations of glycerol.

The relationship between the reciprocals of velocity and substrate concentrations for aconitate isomerase under a variety of assay conditions has been observed to be linear. The allosteric model described by Monod *et al.* (1965) has been extended by Czerlinski (1968) to include a polymerizing model, and the saturation function for such a system involving exclusive binding of substrate to the polymerized form was derived. An examination of the reciprocal of this saturation function indicates that the extent to which V_{max}/v is concave up is dependent on the ratio of the concentration of monomer to polymerized enzyme at a given enzyme concentration. From Figure 5 and utilizing the relationships defined in eq 1–4, one concludes that for aconitate isomerase $[E]/[E_\eta]$ is large in the range of enzyme concentration (5–20 μl/assay) for which V_{max} and K_m values have been determined. The observation of linear Lineweaver–Burk plots in these studies argues against a symmetrical model for aconitate isomerase. Kirtley and Koshland (1967) have pointed out that normal Michaelis–Menten kinetics do not rule out large conformational changes under conditions where the conformational changes in a single subunit of the protein are not transmitted to neighboring subunits. The observed kinetic properties of aconitate isomerase are consistent with a dissociating model in which the subunits of aconitate isomerase are either dissimilar or form dimers in an unsymmetrical manner.

References

Anfinsen, C. B. (1955), *Methods Enzymol. 1*, 698.

Altekar, W. W., Bhattacharyya, P. K., Rangachari, P. N., Maskati, F. S., and Rao, M. R. R. (1965), *Indian J. Biochem. 2*, 132.

Altekar, W. W., and Rao, M. R. R. (1963), *J. Bacteriol. 85*, 604.

Beath, O. A. (1926), *J. Amer. Chem. Soc. 48*, 2155.

Burau, R., and Stout, P. R. (1965), *Science 150*, 766.
Casciato, C. A., Glusker, J. P., Orehowsky, W., and Carrell, H. L. (1970), American Crystallographic Association, Winter Meeting, Tulane University, New Orleans, La., March 1–5, No. F-3.
Czerlinski, G. H. (1968), *Curr. Modern Biol. 2*, 219.
Dawson, R. M. C., Elliott, D., Elliott, W. H., and Jones, K. M. (1959), Data for Biochemical Research, Oxford, Clarendon Press, p 28.
Dickman, S. R., and Clautier, A. A. (1950), *Arch. Biochem. 25*, 229.
Hirs, C. H. W., Moore, S., and Stein, W. H. (1956), *J. Biol. Chem. 219*, 623.
Kirtley, M. E., and Koshland, D. E., Jr. (1967), *J. Biol. Chem. 242*, 4192.
Lippmann, V. (1879), *Ber. 12*, 1649.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
MacLennon, O. H., and Beevers, H. (1964), *Phytochemistry 3*, 109.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Morrison, J. F. (1953), *Biochem. J. 55*, iv.
Peterson, S. W., and Levy, H. A. (1958), *J. Chem. Phys. 29*, 948.
Phelps, R. A., and Putnam, F. W. (1960), The Plasma Proteins, Vol. 1, Putnam, F. W., Ed., New York, N. Y., Academic Press, p 143.
Phillip, G., Moran, J., and Colman, R. W. (1970), *Biochemistry 9*, 2212.
Pratt, L., and Smith, B. B. (1967), *Trans. Faraday Soc 63*, 2858.
Rao, M. R. R., and Altekar, W. W. (1961), *Biochem. Biophys. Res. Commun. 4*, 101.
Roberts, E. J., and Martin, L. F. (1954), *Anal. Chem. 26*, 815.

Mechanism of the Aconitate Isomerase Reaction*

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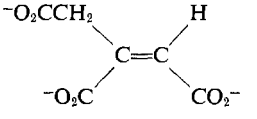
ABSTRACT: The mechanism of the interconversion of *cis*- and *trans*-aconitate catalyzed by aconitate isomerase has been studied. Nuclear magnetic resonance spectra of *trans*-aconitate prepared from *cis*-aconitate in D_2O with aconitate isomerase indicated the incorporation of 1 μatom of deuterium/μmole of aconitate at carbon-4. In order to distinguish an isomerization by facilitated rotation about the C-2,C-3 bond from an allylic rearrangement, *cis*-[1-^{14}C]aconitate was converted into *trans*-aconitate, and the trans product degraded to malate and glyoxylate. From the observation that carbon-14 was in malate, it is concluded that isomerization occurs *via* an allylic rearrangement. The stereochemistry of tritium incorporated enzymatically from water into *cis*- and *trans*-aconitate has been determined by chemical degradation and conversion of tritiated aconitates into either L-[3-^{3}H]malate or L-[3-^{3}H]aspartate, which were converted enzymatically to fumarate. For both *cis*- and *trans*-aconitate, isomerization occurs by activation of the (*pro-S*)-hydrogen at the methylene carbon, indicating a 1,3 suprafacial proton activation. The transfer of tritium from *cis*- to *trans*-aconitate in the course of enzymatic isomerization in D_2O was detected; this finding is consistent with a single catalytic base on the enzyme surface, and is discussed in terms of a carbanion mechanism.

Aconitate isomerase catalyzes the interconversion of *cis*- and *trans*-acetonitate. There are several other examples of enzymes catalyzing cis–trans isomerizations reported in the literature. These include maleyl–acetoacetate isomerase (Edwards and Knox, 1955) and maleate isomerase (Scher and Jakoby, 1969). Although the properties of these enzymes have been described (*e.g.*, both isomerases have SH requirements, and in the case of maleyl–acetoacetate isomerase glutathione is a cofactor), the details of the mechanism of action of these isomerases do not appear to have been worked out.

The structure of *cis*-aconitate is rather special in that isomerization to *trans*-aconitate can occur either by facilitated rotation about the C-2,C-3 bond, or by an allylic rearrangement.



Early in our studies on the mechanism of action of aconitate isomerase it was observed that in the enzymatic conversion of *cis*- to *trans*-aconitate in tritiated water, label was incorporated into *trans*-aconitate. There are a number of reaction pathways, all involving the incorporation of deuterium or tritium from water into *trans*-aconitate, which could describe such an isomerization; these pathways differ with respect to the charge characteristics of reaction intermediates, the position of label incorporated into *trans*-aconitate and the position of the double bond in *trans*- relative to its position in *cis*-aconitate. In Figure 1 two such reaction pathways for an acid- and base-catalyzed isomerization of *cis*-aconitate in

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