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Mechanism of the Aconitate Isomerase Reaction*

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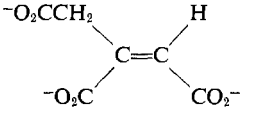
ABSTRACT: The mechanism of the interconversion of *cis*- and *trans*-aconitate catalyzed by aconitate isomerase has been studied. Nuclear magnetic resonance spectra of *trans*-aconitate prepared from *cis*-aconitate in D_2O with aconitate isomerase indicated the incorporation of 1 μatom of deuterium/μmole 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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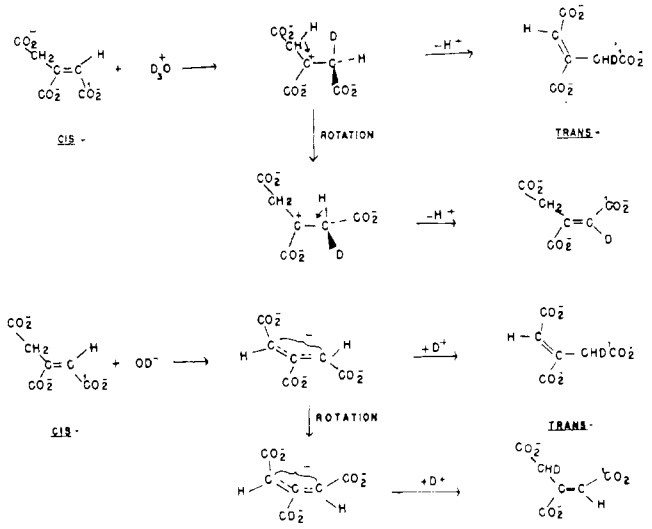

FIGURE 1: Scheme for the acid- and base-catalyzed isomerization of *cis*-aconitate in D_2O.

D_2O are illustrated. An acid-catalyzed carbonium ion mechanism is characterized by the addition of D^+ to the double bond to form a positively charged intermediate, which can break down to *trans*-aconitate either by the elimination of H^+ from C-4 (an allylic rearrangement), or by a rotation about C-2,C-3, and the subsequent elimination of H^+ from C-2. An addition–elimination mechanism would be identical with that shown for a carbonium ion mechanism, except for the formation of C–X at carbon-3 instead of a positive charge, where X is a nucleophile.

A base-catalyzed carbanion mechanism involves the loss of a proton at C-4; the resulting intermediate can decompose to *trans*-aconitate either by the incorporation of D^+ at C-2 (an allylic rearrangement) or rotation about C-2,C-3, and the incorporation of D^+ at C-4.

The present investigation was carried out with the objective of determining the following with regard to the mechanism of aconitate isomerase: (1) the position and stereochemistry of isotopic hydrogen incorporated into *cis*- and *trans*-aconitate, and whether this incorporation is a necessary part of the reaction pathway for isomerization; (2) whether double-bond migration is an integral part of isomerization; (3) the number of bases on the enzyme surface involved in C–H bond cleavage; and (4) whether isomerization occurs *via* a carbanion, carbonium ion, or addition–elimination mechanism.

Materials

The preparation and kinetic properties of aconitate isomerase are described elsewhere (Klinman and Rose, 1970).

All chemicals were obtained commercially unless otherwise noted. *cis*- and *trans*-Aconitate and *cis*-aconitate anhydride (Calbiochem) were neutralized with KOH in the preparation of stock solutions. TOH and D_2O (General Dynamics, 99.7%) were distilled prior to use. Aconitase (EC 4.2.1.3) was prepared according to the method of Morrison (1954); prior to use, it was activated (Rose and O'Connell, 1967) and then eluted from a Sephadex G-25 column to remove excess Fe^{2+} and cysteine. Aconitase was assayed either by the decrease in absorbance at 260 nm with *cis*-aconitate as substrate or by the increase in absorbance at 340 nm with citrate as substrate by coupling the reaction to isocitrate dehydrogenase and the reduction of TPN^+. Aspartase (EC 4.3.1.1) was prepared from *Pseudomonas putida*[1] and had a specific activity of 200 units/mg. Oxaloacetate:glutamate transaminase (EC 2.6.1.1) was prepared according to the method of Jenkins *et al.* (1959). Other enzymes obtained commercially from Boehringer-Mannheim were isocitrtae dehydrogenase (EC 1.1.1.42) (2 units/mg), glutamate dehydrogenase (EC 1.4.1.2) (45 units/mg), malate dehydrogenase (EC 1.1.1.3.7) (720 units/mg), citrate synthase (EC 4.1.3.7) (70 units/mg), acetate kinase (EC 2.7.2.1) (170 units/mg), and phosphotransacetylase (EC 2.3.1.8) (1000 units/mg). Fumarase (EC 4.2.1.2) (350 units/mg) was obtained from Calbiochem. A unit of enzyme activity refers to micromoles of product produced per minute.

Preparation of Labeled Compounds. [1-^{14}C]CITRATE.[2] In a volume of 4 ml, 3.4 μmoles of L-[4-^{14}C]aspartate (New England Nuclear Corp., as D,L-[4-^{14}C]aspartate, specific activity 3×10^6 cpm/μmole), 7 μmoles of α-ketoglutarate, 400 μmoles of potassium phosphate (pH 7.3), and 0.45 unit of glutamate oxaloacetate transaminase were reacted. When there was no further absorbance change at 280 nm due to formation of oxaloacetate, 4 μmoles of acetyl-CoA[3] and 7 units of citrate synthase in 0.8 ml were added, and allowed to react until no further absorbance change was observed at 230 nm due to loss of thiol ester. The resulting [1-^{14}C]citrate was isolated from Dowex 1 (formate) by elution with 3 N formic acid. The position of label in the citrate was verified by its conversion into [^{14}C]glutamate. In 1 ml, 0.085 μmole of [^{14}C]citrate (255,000 cpm), 100 μmoles of Tris-chloride, 2 μmoles of $MnSO_4$, 0.2 μmole of TPN, 0.05 unit of aconitase (citrate assay), and 0.05 unit of isocitrate dehydrogenase were incubated for 40 min. After this time 100 μmoles of NH_4Cl and 0.03 unit of glutamate dehydrogenase were added and the mixture was allowed to react for another 40 min. [^{14}C]-Glutamate was obtained from the amino acid analyzer by elution with citrate buffer, pH 3.28 (0.2 M in Na^+). The specific activity of the glutamate in the peak tube was 4×10^6 compared to 3×10^6 for the citrate. The 30% increase in the specific activity of glutamate relative to citrate is attributed to experimental error. Treatment of this glutamate at pH 5.5 with ninhydrin, a reagent known to oxidatively decarboxylate α-amino acids (Van Slyke *et al.*, 1941), resulted in a 97% loss of counts, indicating their specific location at carbon-1 of glutamate.

cis- AND *trans*-[4-^{3}H]ACONITATE PREPARED WITH ACONITATE ISOMERASE. A typical preparation is described. To a 0.7-ml solution containing 100 μmoles of *cis*-aconitate, 50 μmoles of Tris-chloride (pH 8), 3 μmoles of cysteine, and tritiated water (specific activity 1.6×10^5 cpm/μmole), 0.5 ml of aconitate isomerase (8 units/ml) in 150 mM potassium phosphate (pH 7) and 20% glycerol was added. Reaction was allowed to proceed to 50% completion, as indicated by the absorbance change at 280 nm in 0.1-cm path-length cuvets, stopped by freezing, and the mixture was freeze-dried. The residue was dissolved in 1 ml of 0.1 N HCl and freeze-dried a second time. The reaction mixture (dissolved in 0.01 N HCl) was deproteinized by passage through a Dowex 50 (chloride) column. The breakthrough from this column was

[1] Unpublished procedure.

[2] The carbon skeleton of labeled citrate is numbered in such a way that dehydration by aconitase occurs at C-2,C-3, *i.e.*, in the (*pro-R*)-acetate portion of the molecule (Hanson, 1966; England and Hanson, 1969).

[3] Abbreviations used were: CoA, coenzyme A; DTT, dithiothreitol; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

concentrated, and added to a silicic acid column according to the methods of Bulen *et al.* (1952). *cis*- and *trans*-Aconitates were isolated by elution with 20% chloroform–butanol (Bulen *et al.*, 1952).

The preparation of *cis*- and *trans*-[4-^{3}H]aconitates was carried out three times with tritiated water of varying specific activity. For each preparation, at 50% conversion, the ratio of counts in *cis*-aconitate relative to *trans*-aconitate was 0.22, 0.25, and 0.28, giving an average value for the ratio (specific activity *cis*-aconitate)/(specific activity *trans*-aconitate) of 0.25. From one experiment the ratio (specific activity *trans*-aconitate)/(specific activity TOH) was found to be 0.20.

(3*S*)-[4-$^3H_2$]Citrate. The synthesis of citrate randomly tritiated at carbon-4 (in the (*pro-S*)-acetate portion of the molecule) was carried out. In a volume of 2 ml, 2 μmoles of [2-$^3H_3$]acetate (8×10^8 cpm), 4 μmoles of CoA, 8 μmoles of oxaloacetate, 100 μmoles of Tris-chloride (pH 8), 4 μmoles of DTT, 10 μmoles of $MgCl_2$, 10 μmoles of KCl, 4 μmoles of ATP, 3.4 units of acetate kinase, 10 units of phosphotransacetylase, and 28 units of citrate synthase were allowed to react for 3 hr. The sample was deproteinized by heating, and (3*S*)-[4-$^3H_2$]citrate was obtained from a Dowex 1 (formate) by elution with 3 N formic acid, following the removal of unreacted [2-$^3H_3$]acetate with 1 N formic acid.

Methods

Conversion of [1-^{14}C]Citrate into trans-Aconitate. The conversion of [1-^{14}C]citrate into *trans*-aconitate was carried out in an 0.65-ml volume containing 1 μmole of carrier citrate, 0.34 μmole of [1-^{14}C]citrate (1×10^6 cpm), 25 μmoles of Tris-chloride (pH 8), 0.02 unit of aconitase (assayed with citrate), and 0.1 unit of aconitate isomerase, which were reacted overnight. The acidified reaction mixture was deproteinized by passage through a Dowex 50 (chloride) column. Carrier *trans*-aconitate (20 μmoles) was added and *cis*- and *trans*-aconitate were separated by silicic acid chromatography, as described above.

Conversion of trans-Aconitate into Hydroxycitrate. A typical reaction is described. A 5-ml solution containing 8 μmoles of *trans*-[^{14}C]aconitate (150,000 cpm), 230 μmoles of collidine chloride (pH 7.4), 60 μmoles of OsO_4, and 9.3 mmoles of pyridine (0.75 ml) was stirred for 1.5 hr at 25°. After this time, 350 μmoles of $NaHSO_3$, dissolved in 1 ml of H_2O and 1.5 ml of pyridine, was added dropwise, and the solution stirred for another 0.5 hr. The reaction mixture was extracted with ether to remove the pyridine, and added directly to a Dowex 1 (formate) column (0.9×20 cm). The presumed hydroxycitrate was obtained by elution with 3 N formic acid. As will be shown, oxaloacetate is formed by $NaIO_4$ oxidation of this material as expected of hydroxycitrate.

Conversion of Hydroxycitrate into L-Malate and Glyoxylate. The conversion of 2.5 μmoles of [^{14}C]hydroxycitrate (48,000 cpm) into oxaloacetate and L-malate was carried out in a 2-ml volume containing 200 μmoles of potassium phosphate (pH 7) and 40 μmoles of $NaIO_4$. After reaction for 4 min at 25°, excess $NaIO_4$ was reduced with 40 μmoles of ethylene glycol and 160 μmoles of cysteine. Oxaloacetate was reduced to malate with malate dehydrogenase after the addition of DPNH. The mixture was centrifuged to remove cystine, the supernatant was passed through a Dowex 50 (chloride) column, and the breakthrough, containing L-malate and glyoxylate, was stored at acid pH.

In the reaction of [^{3}H]hydroxycitrate, the oxidation is carried out on ice for 2 min. The removal of excess $NaIO_4$ and the subsequent reduction of oxaloacetate to malate must be carried out quickly to minimize enolization of the intermediate, oxaloacetate, which leads to racemization of any asymmetrically placed tritium. It would have been desirable to have malate dehydrogenase and DPNH present during the oxidation with $NaIO_4$; however, this was precluded by the rapid oxidation of DPNH under these conditions. It was found, however, that the oxaloacetate could be trapped by reaction with transaminase.

Conversion of Hydroxycitrate into L-Aspartate and Glyoxylate. A typical reaction is described. A 1.5-ml solution of 0.9 μmole of [4-^{3}H]hydroxycitrate (6400 cpm), 200 μmoles of potassium phosphate (pH 7), 90 μmoles of L-glutamate, 3 units of transaminase, and 20 μmoles of $NaIO_4$ were reacted for 2 min at 0°. Ethylene glycol (20 μmoles) and cysteine (80 μmoles) were added to oxidize unreacted periodate. The reaction mixture was added to a Dowex 50 (chloride) column (0.9×5 cm) and L-aspartate was obtained by elution with 1 N NH_4OH.

Conversion of (3S)-[4-$^3H_2$]Citrate into Tritiated trans-Aconitate (Transfer Experiment). In 1-ml volume of 97% D_2O, 0.5 μmole of (3*S*)-[4-$^3H_2$]citrate (3×10^8 cpm), 0.4 ml of 50% glycerol, 2 μmole of DTT, 80 μmoles of Tris-chloride (pD 7.7), 0.13 unit of aconitase (*cis*-aconitate assay), and 0.15 unit of aconitate isomerase were reacted until the absorbance change at 260 nm indicated 34% conversion. The reaction was terminated by the addition of 0.06 ml of 3 N $HClO_4$ and neutralized with 3 N KOH. Carrier *trans*-aconitate (20 μmoles) and carrier *cis*-aconitate (5 μmoles) were added to the deproteinized, freeze-dried reaction mixture. Tritiated *trans*-asconitate was eluted from a silicic acid column with 20% butanol–chloroform.

Conversion of L-Aspartate into L-Malate (Transfer Experiment). In a 1-ml volume, 0.05 μmole of L-[3-^{3}H]aspartate, 100 μmoles of potassium phosphate (pH 7), 0.15 μmole of DPNH, 5 μmoles of α-ketoglutarate, and 72 units of malate dehydrogenase were mixed followed by the addition of 0.1 unit of glutamate–oxaloacetate transaminase. When there was no further absorbance decrease at 340 nm, the mixture was added directly to a Dowex 1 (chloride) column. L-Malate was eluted with 5 mM HCl.

Radioactivity was measured in ethanol–toluene counting fluid with a Packard 3003 scintillation spectrophotometer. In double-labeling experiments, cross-channel counting was corrected: counts from carbon-14 overlapped in the tritium channel 73%; the overlap from tritium into the carbon-14 channel was 9%. Nuclear magnetic resonance spectra were obtained on a Varian HA-60 nuclear magnetic resonance spectrometer. Amino acid analyses were carried out on a Beckman 120C amino acid analyzer.

Results

Conversion of cis- into trans-Aconitate in D_2O. Preliminary experiments indicated the incorporation of tritium from the solvent into *trans*-aconitate formed from *cis*-aconitate with aconitate isomerase, and the question of the extent and nature of this incorporation was investigated. In 5 ml of 93% D_2O, 200 μmoles of *cis*-aconitate, 100 μmoles of Tris-chloride (pH 8), 15 μmoles of cysteine, and 6 units of aconitate isomerase were incubated at 25° for 1.5 hr. *trans*-Aconitate was purified from *cis*- on a silicic acid column. Nuclear magnetic resonance spectra of untreated *trans*-aconitate and that prepared as described above were obtained.

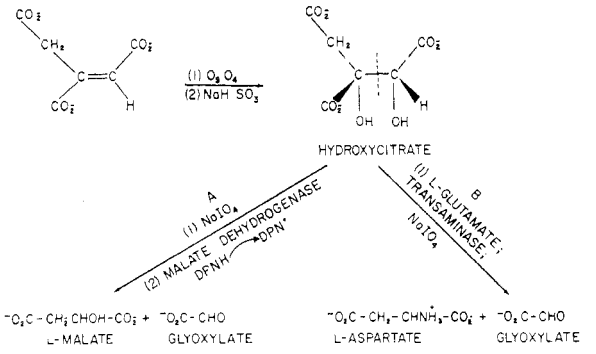

FIGURE 2: Degradation of *trans*-aconitate to either malate or aspartate *via* hydroxycitrate; only one of the pair of enantiomers of hydroxycitrate formed with OsO_4 is shown.

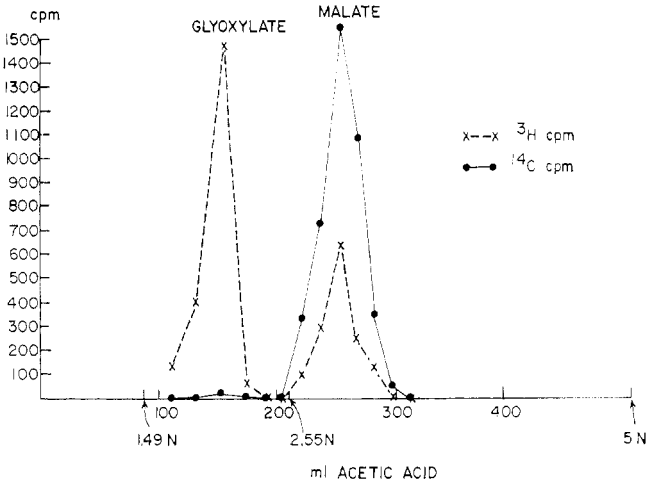

FIGURE 3: Pattern of elution from a Dowex 1 (acetate) column (0.8 × 15 cm) of the final products from the degradation of *trans*-[^{14}C]-aconitate, malate, and glyoxylate, in the presence of 13 μmoles each of carrier malate and glyoxylate, 8000 cpm of [^{3}H]glyoxylate, and 11,000 cpm of [^{3}H]malate. A convex gradient, obtained with a reservoir of 250 ml of 5 N acetic acid, and a stirred chamber containing 250 ml of 1 N acetic acid, was used to elute the acids.

These spectra of 100 μmoles of *trans*-aconitate in 0.4 ml of D_2O containing 2 mg of DSS as an internal standard were characterized by singlet resonances with τ values of 3.4 (CH) and 6.6 (CH_2). A comparison of the methylene and methine peaks indicated a decrease in the CH_2/CH ratio from two to one for *trans*-aconitate equilibrated in D_2O with aconitate isomerase compared to standard *trans*-aconitate. This indicates that the enzymatic incorporation, at equilibrium, of 1 μatom of deuterium/μmole of *trans*-, is occurring in a stereospecific manner in the position allylic to the double bond.

Conversion of [1-^{14}C]Citrate into trans-[^{14}C]Aconitate with Aconitase and Aconitate Isomerase. As illustrated in Figure 1, cis–trans isomerization of *cis*-aconitate can occur as a result of double-bond migration. In order to determine if such double-bond migration were occurring enzymatically, [1-^{14}C]citrate was synthesized and the conversion of [1-^{14}C]-citrate into *trans*-[^{14}C]aconitate with aconitase and aconitate isomerase was carried out, as described in the Materials and Methods.

The position of carbon-14 in *trans*-aconitate so obtained was determined by the degradation of *trans*-aconitate according to Figure 2A. The final products from the degradation of *trans*-[^{14}C]aconitate, L-malate, and glyoxylate, were separated on a Dowex 1 (acetate) column. In Figure 3, the pattern of elution is shown. The chemical nature of the peaks was determined enzymatically with malate dehydrogenase and glyoxylate reductase. It can be seen that carbon-14 cochromatographs almost exclusively with the tritiated malate peak. Therefore, it is concluded that in the formation of *trans*- from *cis*-aconitate with aconitate isomerase, double-bond migration occurs, *i.e.*, *trans*-5-^{14}C is formed from *cis*-1-^{14}C *via* an allylic rearrangement.

Stereochemistry of Tritium Incorporation. Although it was demonstrated that *trans*-aconitate incorporated 1 μatom of deuterium/μmole when equilibrated in D_2O in the presence of aconitate isomerase, the possibility existed that deuterium incorporation was not occurring along the reaction coordinate for isomerization; however, this possibility is precluded by the observation that isomerization occurs *via* an allylic rearrangement.

In order to determine the absolute stereospecificity of the proton activation, tritiated *cis*- and *trans*-aconitates were prepared as described in the Materials and Methods. The incorporation of counts from water into *trans*-aconitate at 50% conversion was four times greater than the incorporation into *cis*-. Initially, it was decided to determine the chirality of tritiated *cis*- and *trans*-aconitates according to the pathway illustrated in Figure 2A. Tritium at C-4 in aconitate would be converted to the C-3 position of L-malate. The stereochemistry of the dehydration of L-malate by fumarase has been shown to occur in an anti manner with the removal of the (*pro-R*)-hydrogen (Anet, 1960; Gawron *et al.*, 1961).

trans-[4-^{3}H]Aconitate (58,000 cpm) was converted into L-[3-^{3}H]malate according to the methods described in the Materials and Methods. In spite of the precautions taken to minimize enolization of intermediate oxaloacetate, 16% of the tritium initially present in hydroxycitrate was in water after reduction of oxaloacetate to malate with malate dehydrogenase. After the addition of 20 μmoles of carrier malate, L-[3-^{3}H]malate was obtained from a silicic acid column by elution with 20% butanol–chloroform. Coincidence of the acid peak, determined by titration, with the radioactive peak was observed. The malate so obtained was incubated with fumarase, giving 27% volatile counts, under conditions where a sample of (3*R*)-L-[3-^{3}H]malate was demonstrated to detritiate completely. As a test for total counts at C-3 of L-[3-^{3}H]malate, a sample was converted back into oxaloacetate with malate dehydrogenase and acetylpyridine–DPN$^+$ in glycine buffer (pH 9.5) at 37° (Söling and Holzer, 1962); under these conditions, carbon-bound tritium at carbon-3 of oxaloacetate exchanges rapidly with the solvent.[4] These results are summarized in Table I.

The conversion of *cis*-[4-^{3}H]aconitate into L-[3-^{3}H]malate was attempted. However, in the oxidation step with $NaIO_4$, 30–40% of the tritium in hydroxycitrate was labilized, and when the malate was analyzed with fumarase it was racemic within experimental error. Presumably, the oxidation of the hydroxycitrate derived from *cis*-aconitate occurred more rapidly than that of *trans*-aconitate product, so that more time was available for enolization and racemization.

[4] The $t_{1/2}$ for hydroxide ion catalyzed enolization of oxaloacetate is 35 min at pH 9, 1.5°. In addition the tautomerization of oxaloacetate is general base catalyzed; for example, at 1.5°, $t_{1/2}$ = 2.5 min in 0.1 M imidazole, and $t_{1/2}$ = 14 min in 0.1 M PO_4^{3-} (Banks, 1961, 1962).

TABLE I: Stereochemistry of L-[3-^{3}H]Malate Derived from *trans*-[4-^{3}H]Aconitate.

Sample	% Volatile Counts (after Fumarase)[a]	% Volatile Counts (after Malate Dehydrogenase)[b]
L-[3-^{3}H]Malate	$\frac{86}{363} = 24$, $\frac{317}{1045} = 30$; Av = 27 ± 3	$\frac{210}{285} = 90$
L-[3-^{3}H]Malate + (3*R*)-L-[3-^{3}H]-Malate	$\frac{2390}{2350} = 102$	

[a] Incubations were carried out with 2.6 mM solutions of L-[3-^{3}H]malate (specific activity 1060 cpm/μmole) in 24 mM potassium phosphate (pH 7) with 4–30 units of fumarase for 1 hr at 25°. A fraction of each reaction mixture was counted and an equal fraction was distilled *in vacuo* and counted to determine volatile counts. The (3*R*)-L-[3-^{3}H]malate (7×10^6 cpm/μmole) was added as an internal control to demonstrate that fumarase was capable of full exchange of the 3-*pro*-*R* position. The counts in the control have been corrected for volatile (70 cpm) and nonvolatile (190 cpm) counts due to the L-[3-^{3}H]malate present. [b] Incubation of 0.27 μmole of L-[3-^{3}H]malate, 250 μmoles of glycine (pH 9.5), 5 mg of acetylpyridine–DPN$^+$ and 72 units of malate dehydrogenase in 0.6 ml was carried out at 37° for 1 hr. A fraction of the reaction mixture was counted, and an equal fraction distilled *in vacuo* to determine volatile counts. Counts are for 2 min.

TABLE II: Stereochemistry of L-[3-^{3}H]Aspartate Derived from *cis*- and *trans*-[4-^{3}H]Aconitates.

Sample	% Volatile Counts (after Aspartase)
L-[3-^{3}H]Aspartate (derived from *cis*-4-^{3}H)[a]	$\frac{44}{726} = 6.1$
L-[3-^{3}H]Aspartate (derived from *cis*-4-^{3}H) + 1 (3*R*)-L-[3-^{3}H]aspartate	$\frac{279}{260} = 106$
L-[3-^{3}H]Aspartate (derived from *trans*-4-^{3}H)[b]	$\frac{35}{248} = 14.1$

[a] Incubations were carried out with 0.13–0.26 mM solutions of L-[3-^{3}H]aspartate (specific activity 7100 cpm/μmole) in 140 mM Tris-chloride (pH 8), 1 mM $MgCl_2$ with 1–2 units of aspartase for 2 hr at 25°. The reaction mixtures were distilled *in vacuo* and the volatile fraction counted. The specific activity of (3*R*)-L-[3-^{3}H]aspartate present in the control was 70,000 cpm/μmole. The per cent volatile counts for the control refer to counts in (3*R*)-L-[3-^{3}H]aspartate and has been corrected for volatile (22 cpm) and nonvolatile (341 cpm) counts due to the L-[3-^{3}H]aspartate present. [b] The incubation of an 0.012 mM solution of L-[3-^{3}H]aspartate (specific activity 71,000) was identical with footnote *a*.

Another approach to the determination of the chirality of tritiated *cis*- and *trans*-aconitate resulted from the observation that oxaloacetate–glutamate transaminase remains active in the presence of $NaIO_4$. Consequently, oxaloacetate formed from the oxidation of hydroxycitrate was converted directly into L-aspartate with transaminase in the presence of periodate (Figure 2B).

The conversion of hydroxycitrate derived from both *cis*- and *trans*-[4-^{3}H]aconitate into aspartate was carried out. In the case of hydroxycitrate derived from *trans*-aconitate, approximately 19% of the counts were labilized in the oxidation step with $NaIO_4$, whereas with hydroxycitrate derived from *cis*-aconitate only 3% of the tritium was in water after the oxidation step. The reaction mixtures were added to Dowex 50 (chloride) columns, and aspartate was obtained by elution with 1 N NH_4OH. As a control, a sample of hydroxycitrate obtained from *cis*-[4-^{3}H]aconitate was oxidized in the absence of transaminase; under those conditions all of the counts appear in the breakthrough of a Dowex 50 column.

The stereochemistries of L-[3-^{3}H]aspartates obtained from *cis*- and *trans*-[4-^{3}H]aconitates were determined by treatment with aspartase under conditions where (3*R*)-L-[3-^{3}H]aspartate was demonstrated to be fully detritiated as predicted from the known stereospecificity of aspartase (England, 1958; Krasna, 1958). These results are summarized in Table II.

From the results shown in Tables I and II, the tritium incorporated with aconitate isomerase into *cis*- and *trans*-aconitates is concluded to be *pro*-*S*.

Demonstration of Transfer. From the finding that the (*pro*-*S*)-hydrogens at C-4 of both *cis*- and *trans*-aconitate are activated by aconitate isomerase, it is concluded that a 1,3-suprafacial proton activation is occurring. The possibility that a single base on the enzyme were responsible for proton activation of both substrates was investigated by carrying out experiments to determine if the transfer of tritium from (4*S*)-*cis*-[4-^{3}H]aconitate to *trans*-aconitate could be detected in the course of enzymatic isomerization.

Preliminary experiments indicated that if transfer were occurring, it was a rare event; therefore the use of *cis*-aconitate of sufficiently high specific activity to detect small amounts of transfer, was critical. For this reason it was decided to carry out the transfer experiments with (3*S*)-[4-$^3H_2$]-citrate prepared from [2-$^3H_3$]acetate (specific activity 8×10^8 cpm/μmole). It was also decided to carry out the transfer reaction in D_2O. The synthesis of (3*S*)-[4-$^3H_2$]citrate is described in the Materials and Methods section. In Figure 4 the conversion of (3*S*)-[4-$^3H_2$]citrate to *trans*-[^{3}H]aconitate, and the degradation of *trans*-aconitate to L-aspartate and glyoxylate is illustrated. The isolation of aspartate, which was tritiated, would be evidence of transfer.

The conversion of (3*S*)-[4-$^3H_2$]citrate to tritiated *trans*-aconitate was carried out to the extent of 34% conversion as described in the Materials and Methods section. Total volatile counts (2.8×10^7 cpm), represented 8.5% of the total counts. To ensure that the *trans*-[^{3}H]aconitate peak (after elution from silicic acid) was free of *cis*-[^{3}H]aconitate, the material from the former was treated with 0.1 μmole of *cis*-aconitate, 50 μmoles of Tris-chloride (pH 8), 0.2 μmole of TPN$^+$, 0.2 unit of aconitase, and 0.2 unit of isocitrate dehydrogenase to convert any contaminating tritiated *cis*-aconitate into α-ketoglutarate. The conversion of *trans*-

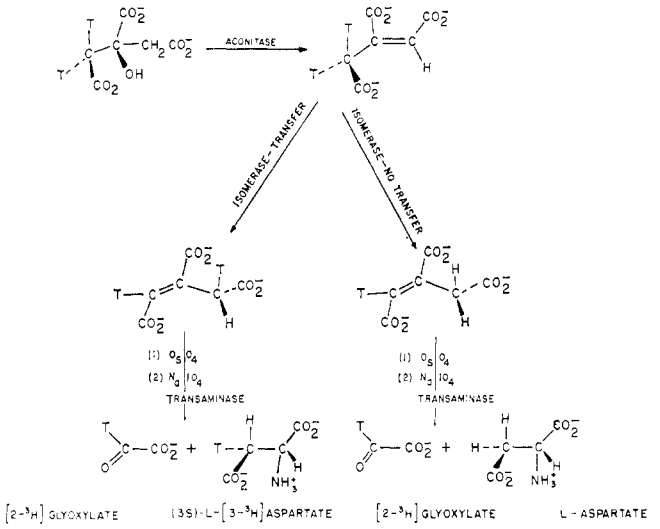

FIGURE 4: Conversion of citrate, randomly tritiated in the (*pro-S*)-acetate region, to aspartate: experiment to determine if tritium transfer occurs in the conversion of *cis*- to *trans*-aconitate with aconitate isomerase.

TABLE III: Stereochemistry of L-[3-^{3}H]Malate Derived from *trans*-[4-^{3}H]Aconitate Formed from *cis*-[4-$^3H_2$]Aconitate with Aconitate Isomerase in D_2O.[a]

Sample	% Volatile Counts (after Fumarase)
L-[3-^{3}H]Malate	$\frac{170}{614} = 27.7$
L-[3-^{3}H]Malate + (3*R*)-L-[3-^{3}H]malate	$\frac{79,000}{80,000} = 98$

[a] Incubations of 0.19–0.25 mM L-[3-^{3}H]malate, 330 mM potassium phosphate (pH 7), and 4 units of fumarase were carried out for 1 hr at 25°. The reaction mixtures were distilled *in vacuo* and both the volatile fraction and residue were counted for 2 min. The specific activity of (3*R*)-L-[3-^{3}H]malate in the control was 115,000 cpm/μmole, so that the control incubation was 1.4 mM in malate.

[^{3}H]aconitate into L-aspartate (Figure 2B) was carried out by the methods described in the Materials and Methods section, and tritiated aspartate was obtained by elution from a Dowex 50 (chloride) column.

In order to verify that the tritium detected in aspartate had arisen by way of transfer, aspartate was converted to malate, and the stereochemistry of tritium at C-3 of malate was determined. Tritium incorporated into *trans*-aconitate *via* transfer would yield a center of S chirality. Any nonenzymatic formation of *trans*-aconitate from *cis*-[4-$^3H_2$]-aconitate would give rise to malate racemic at C-3. Also, any contamination of *trans*-aconitate by unreacted *cis*-[4-$^3H_2$]aconitate would give rise to a nearly equal mixture of (3*S*)-L-[3-^{3}H]malate and (3*R*)-L-[3-^{3}H]malate. The distribution of tritium at carbon-4 of unreacted *cis*-aconitate after 10% reaction was determined by conversion of (3*S*)-[4-$^3H_2$]citrate into *trans*-[4-^{3}H]aconitate under the conditions of the transfer experiment. Unreacted citrate was purified by silicic acid chromatography, and was incubated with aconitase and aconitate isomerase to determine volatile counts: 51.2% of the total counts were volatile, indicating a 2.4% enrichment of (3*S*,4*S*)-[4-^{3}H]citrate over (3*S*,4*R*)-[4-^{3}H]citrate. One would predict very little change in this enrichment in going from 10 to 34% conversion (Melander, 1960).

Malate was obtained from aspartate in 80% yield, as described in the Materials and Methods section, and had a specific activity of 53,000 cpm/μmole. The detritiation of this malate with fumarase indicated 28% volatile counts under conditions where (3*R*)-L-[3-^{3}H]malate gave 98% detritiation (Table III). These results indicate malate obtained from the transfer experiment to be 44% enriched in the *pro-S* position at C-3.

Discussion

In this investigation it has been demonstrated that the enzymatic conversion of *cis*- to *trans*-aconitate occurs by a double-bond migration. Although the nature of the substrates of aconitate isomerase suggests a cis–trans isomerization, the enzyme catalyzes an allylic rearrangement. β-Hydroxydecanoyl thio ester dehydrase, an enzyme described by Rando and Bloch (1969), interconverts a *trans*-α,β-unsaturated thio ester and *cis*-β,γ-unsaturated thio ester; the reaction catalyzed by this enzyme could be interpreted in terms of a double-bond migration concomitant with cis–trans isomerization.

By the degradation of enzymatically tritiated *cis*- and *trans*-aconitate to either L-[3-^{3}H]malate or L-[3-^{3}H]aspartate, it has been shown that aconitate isomerase catalyzes the activation of the (*pro-S*)-hydrogen at the carbon allylic to the double-bond for both substrates. Also in the enzymatic isomerization of *cis*-[4-$^3H_2$]aconitate, tritium was shown to be transferred to *trans*-aconitate tritiated at C-4. In the case of keto steroid isomerase (Talalay and Wang, 1955; Wang *et al.*, 1963) and pyridoxamine–pyruvate transaminase (Ayling *et al.*, 1968), enzymes catalyzing allylic rearrangements, transfer has also been shown to occur. The demonstration of transfer is consistent with a single catalytic base on the enzyme surface.

The per cent of tritium transfer, shown to occur in the isomerization of *cis*-[4-$^3H_2$]aconitate in D_2O, can be calculated from the ratio of counts at carbon-4 of *trans*-aconitate relative to volatile counts, corrected for the nonproductive detritiation of *cis*-aconitate, *i.e.*, that detritiation which occurs in the absence of product formation

$$\% \text{ transfer} = \frac{(\text{cpm at C-4 of } \textit{trans}\text{-aconitate})}{(\text{cpm in } H_2O) \text{ corrected}} = \frac{(\text{specific activity of malate})(\mu\text{moles of carrier } \textit{trans}\text{-aconitate})}{0.75(\text{total volatile counts})} \quad (1)$$

From the observation that in the enzymatic conversion of *cis*- into *trans*-aconitate in tritiated water, *trans*-aconitate is labeled three times faster than *cis*-, it is estimated that 75% of the total volatile counts arise in the formation of *trans*-aconitate. Using a value for the specific activity of malate of 53,000 cpm/μmole, the per cent transfer is calculated to be 4.3%.

This value for the per cent transfer represents an upper limit, since the tritium in malate obtained from the transfer experiment was found to be a mixture of (3*S*)-L-[3-^{3}H]-malate and (3*R*)-L-[3-^{3}H]malate in a ratio of 77:28. The finding that the tritium at C-3 of malate was only 44% enriched in the *pro-S* position (as opposed to 100% enriched) may indicate either (1) considerable racemization of the intermediate oxaloacetate in the conversion of hydroxycitrate to aspartate or (2) contamination in the early stages of the transfer experiment with tritiated *trans*-aconitate formed nonenzymatically from *cis*-aconitate. If one assumes that the apparent racemization results from a contamination, one calculates a minimum value for the per cent transfer to be (4.3%)(0.44) = 1.9%, giving a range of 1.9–4.3% for the per cent transfer.

The most straightforward explanation for the observation that tritium transfer is small under the conditions described would be that the conjugate acid of a monoprotic base on the enzyme involved in C–H cleavage is exchanging tritium with the solvent at a rate which is approximately 25–50 times greater than k_2. On the assumption that this transfer is an intramolecular process, C–H-bond cleavage must precede the C–H bond-making step and the demonstration of transfer indicates a carbanion mechanism. This mechanism is illustrated in Figure 5. If transfer is occurring by an intermolecular process, a carbonium ion mechanism would be conceivable. In this study it was observed repeatedly that in the conversion of *cis*- into *trans*-aconitate in tritiated water, the incorporation of tritium into *cis*-aconitate occurs at one-third the rate of incorporation of tritium into *trans*-aconitate, under conditions where the reversal of the reaction is not significant. According to a carbonium ion mechanism, this incorporation of tritium into *cis*-aconitate must arise as a result of the back-reaction of enzyme-bound *trans*-aconitate, following a fairly rapid exchange of the conjugate acid of the base on the enzyme with solvent. However, for an intermolecular tritium transfer to be detected, one requires that the exchange out of label from tritiated enzyme in the absence of substrate to be slow. As a result of the inconsistency between a rapid exchange of the conjugate acid of the catalytic base in the presence of substrate as opposed to a slow exchange in the absence of substrate, a carbonium ion mechanism involving intermolecular transfer from a monoprotic base would appear to be a highly unlikely possibility. An alternate explanation for the observation of a small per cent transfer might be that the catalytic base on the enzyme is a polyprotic base; in such a situation, tritium in the conjugate acid would be diluted, and if there were a large tritium isotope effect, one would observe a small per cent of tritium transfer since it is likely that the base form of the enzyme would fully exchange tritium before recycling. Assuming an NH_2 group as the polyprotic base, it is possible to predict the per cent transfer one would observe for a carbanion mechanism in the absence of tritium exchange from I (Figure 5). From the observation that (specific activity *trans*-aconitate)/(specific activity TOH) = 5 for the isomerization of *cis*-aconitate in TOH, one calculates k_H/k_T = 10 for the intrinsic isotope effect. The per cent tritium transfer in H_2O is then calculated to be 5.0% assuming no exchange. Using the Swain relationship (Swain, 1958), one obtains k_H/k_D = 4.3 for the intrinsic isotope effect, and the per cent tritium transfer in D_2O is predicted to be 10.4%. This calculation assumes that the protonated amino group is free to rotate on the enzyme; any hindrance to rotation would prevent the isotopic hydrogen atoms bonded to nitrogen from

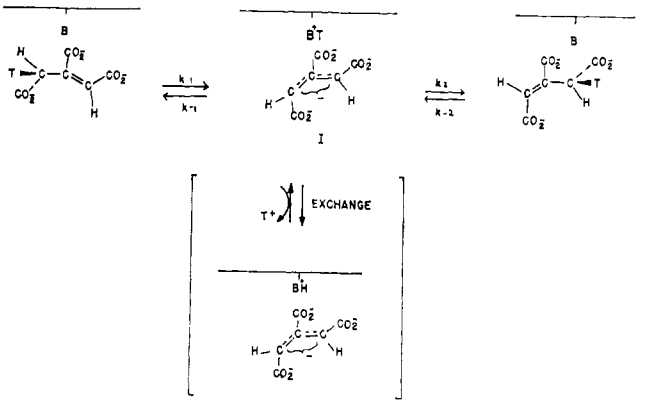

FIGURE 5: Carbanion mechanism proposed for the enzyme-catalyzed interconversion of *cis*- and *trans*-aconitate.

becoming statistically equivalent, and the predicted per cent transfer would be greater than 10.4%. The secondary isotope effect for the transfer of a tritium from a deuterated *vs.* a protonated nitrogen has been neglected in this calculation. Gold and Kessick (1965) have determined secondary kinetic isotope effects for the transfer of a proton from $H–D_2O^+$ and $H–H_2O^+$ to a carbon atom; their effects were inverse, and varied from 1.28 to 1.88 depending on the olefinic or aromatic carbon atom receiving the proton. It can be seen that secondary effects would further increase the predicted per cent transfer.

A comparison of the corrected observed values for tritium transfer in D_2O, 1.9–4.3%, and the calculated value, 10.4%, indicates that a model which assumes a nonexchanging polyprotic base is not consistent with the data. Although the effect of additional D_2O solvent effects cannot be excluded (Jencks, 1970), the finding that the tritium transfer in D_2O is appreciably less than 10.7% indicates that for a carbanion mechanism the conjugate acid of the base on the enzyme is either monoprotic and can exchange rapidly with solvent or is polyprotic and undergoes a limited exchange. The exact nature of this base will be the subject of further study.

It should be noted that an enzyme-catalyzed allylic rearrangement that has been shown to give transfer (presumed to be intramolecular), could occur *via* a carbonium ion mechanism in the event that the catalytic base on the enzyme is polyprotic. In the case of aconitate isomerase, such a mechanism is considered unlikely since (1) the observed back-labeling of *cis*-aconitate requires that the dissociation of *trans*-aconitate be partially rate-limiting for the net reaction and (2) the observed tritium transfer could arise solely by the recycling of bound *trans*-aconitate, requiring that the dissociation of *cis*-aconitate also be partially rate limiting.[5] However, as indicated from the transfer experiment, the enzymatic conversion of *cis*-[4-^{3}H]aconitate to *trans*-aconitate is characterized by a kinetic isotope effect, k_H/k_T = 2; although the observed isotope effect is small, it is unexpected for a reaction in which the rate-limiting step is a dissociation of product from the enzyme.

The mechanism of aconitate isomerase shown in Figure 5 involves deprotonation to form a carbanion. The uncatalyzed isomerization of *cis*-aconitate in base occurs very slowly. Ambler and Roberts (1948) reported a half-life for isomeriza-

[5] Only the first of these two conditions would be required for an intermolecular proton transfer.

tion of approximately 48 hr at pH 14 and 25°; from these data a pseudo-first-order rate constant of 4×10^{-6} sec^{-1} is calculated.

This slow rate of isomerization is readily understood in view of the net negative charge of −3 on *cis*-aconitate at pH's greater than 8. A turnover number of $4\text{–}50 \times 10^{2}$ sec^{-1} for aconitate isomerase is approximated from the specific activity of 120 units/mg for the best preparation, which was estimated to be between 3 and 40% pure, and from a value of 78,000 for the molecular weight (Klinman and Rose, 1971). The enzymatic acceleration is then $1\text{–}12 \times 10^{8}$. Additional factors, other than proximity effects, which may be contributing to this acceleration, are the presence of electrophyllic centers on the enzyme surface which would serve a 3-fold role in (1) neutralizing charge on the three carboxyl groups, (2) providing for delocalization of electrons from the intermediate carbanion, I in Figure 5, into the two terminal carboxyl groups, and (3) maintaining conformational rigidity so that proton transfer will occur in the conformation which gives rise to isomerization.

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