Escherichia coli Fumarase A Catalyzes the Isomerization of Enol and Keto Oxalacetic Acid†

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ABSTRACT: Fumarase A, a product of the *fumA* gene of *Escherichia coli*, has been found to catalyze the isomerization of enol to keto oxalacetic acid (OAA) in addition to catalyzing the fumarase reaction. The k_{cat}/K_m for the isomerization is almost identical to that for the fumarase reaction. The isomerization reaction apparently takes place at the same active site as the fumarase reaction since both reactions show a similar sensitivity to inactivation by O_2, both reactions are strongly inhibited by 2-hydroxy-3-nitropropionate, and the isomerization reaction is inhibited by fumarate and malate. The isomerization requires the presence of a [4Fe–4S] or [3Fe–4S] cluster, perhaps for structural rather than catalytic reasons. Hydration of enol OAA to the *gem* diol has been ruled out as a possible mechanism of isomerization on the basis of the preservation of the oxygen on carbon 2 and the position of protonation on carbon 3. The isomerization is not stereospecific in the position of protonation at carbon 3 but appears to be stereoselective, with protonation preferentially occurring in the 3-*pro-S* position. Porcine fumarase, isopropyl malate isomerase, and dihydroxyacid dehydratase do not catalyze this isomerization. Fumarase A and aconitase, two enzymes with 4Fe–4S clusters that bind a linear 4-carbon dicarboxylic acid moiety in the *trans* conformation during their normal hydro-lyase reaction, do catalyze this isomerization.

It has been known for some time that an enzymatic activity exists in several organisms which catalyzes the conversion of enol to keto oxalacetic acid (herein referred to as OAA) (Annett & Kosicki, 1969; Wesenberg et al., 1976). The Nomenclature Committee of the International Union of Biochemistry has given this activity the name OAA keto–enol-isomerase (herein referred to as OAAKE isomerase) and the numerical designation EC 5.3.2.2.

None of the protein(s) responsible for the OAAKE isomerase activity had been identified until recently, when OAAKE isomerase activity from bovine heart mitochondrial matrix was shown to be associated with proteins migrating with molecular masses of 80 and 37 kDa (Belikova et al., 1988). Subsequently, the protein migrating with a mass of 80 kDa has been shown to be aconitase (Belikova et al., 1989), but the identity of the 37-kDa protein remains unknown. In addition, an OAAKE isomerase activity from porcine kidney with a molecular mass of 55 kDa has been reported (Johnson et al., 1986). The relation between the 55-kDa porcine kidney OAAKE isomerase and the 80-kDa (aconitase) and 37-kDa beef heart mitochondrial proteins is unknown. The porcine kidney OAAKE isomerase has been shown to lack stereospecificity in the protonation of carbon 3 of enol OAA (Johnson et al., 1986). The lack of stereospecificity is unusual in enzyme-catalyzed reactions.

The discovery that aconitase exhibits an OAAKE isomerase activity was unexpected and is of unknown physiological significance. Aconitase is a member of the hydro-lyase class of enzymes. Native bovine aconitase has been shown to contain a [4Fe–4S] cluster which activates the hydroxyl group of the substrate for elimination and then addition in the interconversion of citrate to isocitrate (Emptage, 1988). Aconitase is easily oxidized to a form that readily loses one Fe to give a [3Fe–4S] cluster. This form of aconitase is inactive in catalyzing the conversion of citrate to isocitrate. It was surprising then to find that not only native aconitase in the [4Fe–4S] form but also oxidized aconitase in the [3Fe–4S] form was active in the isomerization of enol to keto OAA (Belikova et al., 1989). This result brings up the question of what involvement, if any, the Fe–S cluster of aconitase has in the OAAKE isomerization reaction since both the [4Fe–4S] and [3Fe–4S] cluster forms catalyze the OAAKE isomerase reaction at similar rates.

We report here that *Escherichia coli* fumarase A, another member of the hydro-lyase class which contains a [4Fe–4S] cluster (Flint et al., 1992), also exhibits OAAKE isomerase activity. The details of our investigations on the OAAKE isomerase activity of *E. coli* fumarase A are described in this paper.

MATERIALS AND METHODS

OAA was obtained from Sigma as item number O 4126 and is identified in their 1990 catalog as *cis*-hydroxymaleic acid. ^2H_2O and $H_2{}^{18}O$ were obtained from MSD Isotopes. 2-[^{18}O]-OAA was made by the exchanging the 2-oxygen of 2-[^{16}O]-OAA in $H_2{}^{18}O$ for 30 min. 2-Hydroxy-3-nitropropionate was prepared by us. All other reagents including porcine fumarase were purchased from Sigma.

Enzyme Source. Fumarase A is the gene product of the *E. coli fumA* gene (Woods et al., 1988; Yumoto & Tokushige, 1988) and was isolated from *E. coli* strain JRG1905 as previously described (Flint et al., 1992). This strain was derived from *E. coli* strain JH400, a *fumAC fumB* triple deletion mutant, by addition of a plasmid (pGS57) carrying a copy of the *fumA* gene. The fumarase A used in these studies was purified and stored under strict anaerobic conditions. It was 80–90% pure as judged by gel electrophoresis. This purified fumarase A rapidly loses activity in air. Except where otherwise noted, all fumarase A samples used in these experiments were with active native enzyme (defined as containing an intact [4Fe–4S] cluster).

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Enzyme Assays. The assays were done in 10 mM phosphate buffer, pH 7.5, because the spontaneous rate of isomerization of OAA is conveniently low in this buffer. Enol OAA solutions (80 mM) were prepared in diethyl ether. The assays were started by the addition of aliquots of the stock enol OAA solution directly into assay buffer. The small amount of diethyl ether added to the assay solution in this way was shown in control experiments to not affect the activity of fumarase A in the fumarase or OAAKE isomerase reaction.

The enol to keto isomerization of enol OAA was followed by measuring the change in absorbance at 260 nm when the concentration of OAA was below 0.5 mM and at 290 nm when the concentration was 0.5 mM or above. The difference in extinction coefficients between the enol and keto forms of OAA was found to be 1×10^4 M^{-1} at 260 nm and 2×10^3 M^{-1} at 290 nm. Sufficient enzyme was added in each case so the enzymatically catalyzed rate of isomerization was at least 10-fold above the spontaneous background rate. The rate of enzyme-catalyzed isomerization was determined by subtracting the spontaneous background rate from the overall rate.

GC Mass Spectral Analysis. GC/MS analysis was used to determine the content of ^2H and ^{18}O in samples of malate. Samples of malate were derivatized in acidic methanol to form the dimethyl ester. The dimethyl ester was chromatographed and a high-resolution mass spectrum was obtained using a 30-m DB-Wax column on a Varian Vista 600 gas chromatograph (temperature programmed from 60 to 220 °C at 12 °C/min) coupled to a VG Micromass 7070-HS mass spectrometer.

A major fragment in the mass spectrum of the dimethyl ester of malate is generated by the loss of a carboxyl ester. This fragment has a *m/e* of 103 in the case of the dimethyl ester of [3-$^2H_0$]malate, *m/e* of 104 in the case of the dimethyl ester of [3-$^2H_1$]malate, and *m/e* of 105 in the case of the dimethyl ester of [3-$^2H_2$] and in the case of [2-^{18}O]malate. In each case the data obtained from the GC/MS were corrected to account for the natural abundance of ^{13}C, ^{18}O, and ^2H.

Determination of Stereochemical Specificity. Porcine fumarase, which specifically removes the 3-*pro-R* proton from 2-(*S*)-malate (Hill & Teipel, 1971), was used to determine the location of ^2H in the 3-position of labeled sample of malate. One unit of porcine fumarase was added to half of each labeled sample in 10 mM phosphate buffer prepared with ^1H_2O. Incubation was continued long enough to catalyze the conversion of 100 times the amount of malate (to fumarate) present in the samples. These samples were then derivatized and analyzed by GC/MS to determine the ^2H content.

RESULTS

Determination of Isomerase Kinetic Constants and Commonality of Active Site. OAA in the crystalline form and in solution in solvents of low polarity exists predominantly in the enol form, whereas in solvents of high polarity, OAA exists predominantly in the keto form. The forms can distinguished from each other by their UV absorbance spectra (Gruber et al., 1956; Banks, 1961; Hess & Reed, 1972). Addition of OAA to water from the solid form or from a solution of OAA in a solvent of low polarity is followed by the spontaneous isomerization of the enol to the keto form. The progress of this isomerization can be monitored by the changes in the UV. The rate of isomerization of enol to keto OAA has been reported to be acid/base catalyzed (Emly & Leussing, 1981) and in our hands is considerably faster at pH 7.5 in 100 mM than in 10 mM phosphate buffer. In 10 mM phosphate buffer, the spontaneous isomerization took several minutes to reach equilibrium.

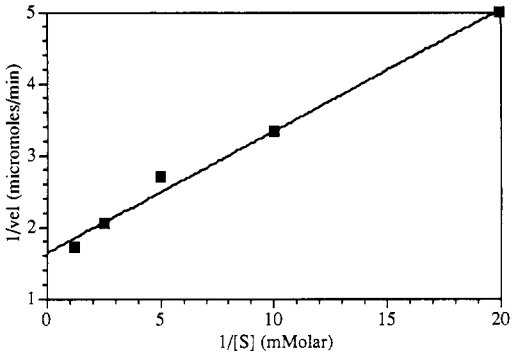


FIGURE 1: Velocity^{-1} vs [enol OAA]$^{-1}$ for the isomerase activity of a fumarase A preparation.

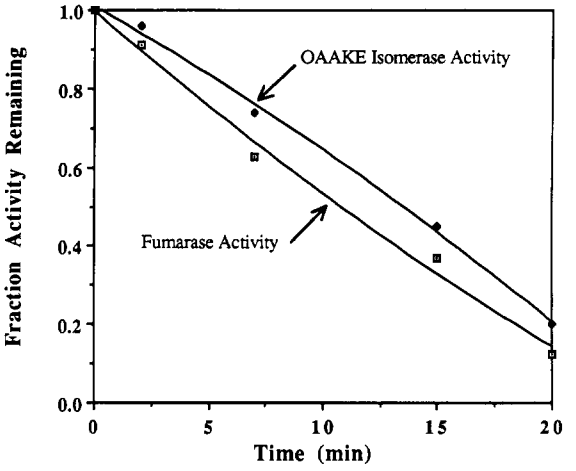


FIGURE 2: Inactivation of the isomerase and fumarase activity of fumarase A when exposed to air.

The addition of aliquots of a purified fumarase A preparation greatly accelerated the rate isomerization as monitored in the UV; however, the final UV absorbance spectrum of the OAA solution after equilibrium was reached was not affected (except for a very small effect due to addition of protein). This demonstrates a component in the fumarase A preparation can act as a catalyst for the isomerization of enol to keto OAA. The isomerization of enol to keto OAA catalyzed by the fumarase A preparation follows saturation kinetics typical of enzymatic reactions. A double-reciprocal plot of the inverse rate of isomerization vs inverse substrate concentration is shown in Figure 1.

Since the fumarase A preparation used in these experiments was 80–90% pure, we attempted to determine if the OAAKE isomerase activity was associated with fumarase A or some other constituent in the sample. Three lines of evidence indicated that the fumarase and isomerase reactions are catalyzed by the same enzyme. First and most definitive is the very similar kinetics of inactivation of both the fumarase and isomerase activity of fumarase A preparation in air (see Figure 2 and associated discussion). The fumarase activity of fumarase A is known to be air-sensitive due to the oxidation of the Fe–S cluster by O_2 (Flint et al., 1992), and the isomerase activity shows a similar sensitivity. Second, we have found that the possible transition-state analog, 2-hydroxyl-3-nitropropionic acid dianion, is a strong inhibitor of the fumarase activity of fumarase A preparations (Flint, unpublished work), and we have also found it is a strong inhibitor of the isomerase reaction. For example, in the presence of 10 μM 2-hydroxy-3-nitropropionic acid dianion the initial rate of enzyme-catalyzed isomerization with 400 μM enol OAA as substrate is inhibited 96%. Third, in the presence of an equilibrium mixture of fumarate and malate in which the concentration of these two species adds up to 10 mM, the rate of enzyme-

Table I: Kinetic Constants for Hydro-Lyase and Isomerase Activities of Fumarase A, Porcine Fumarase, and Aconitase

	E. coli fumarase A (for fumarate)	porcine fumarase[a] (for fumarate)	bovine aconitase[a] (for *cis*-aconitate)
	Hydro-Lyase Activity		
K_m (M)	6×10^{-4}	5×10^{-6}	8×10^{-6}
k_{cat} (s^{-1})	3×10^{3}	3×10^{2}	4×10^{1}
k_{cat}/K_m (M^{-1} s^{-1})	5×10^{6}	6×10^{7}	5×10^{6}
	Isomerase Activity		
K_m (M)	1×10^{-4}	–	2×10^{-4}
k_{cat} (s^{-1})	3×10^{2}	<3	3×10^{1}
k_{cat}/K_m (M^{-1} s^{-1})	3×10^{6}	–	1×10^{5}

[a] Values from Belikova et al. (1988, 1989), Teipel et al. (1968), Kent et al. (1985), and Schloss et al. (1984).

catalyzed isomerization with 400 μM enol OAA as substrate is inhibited 90%. The simplest interpretation of these results is that both the fumarase and OAAKE isomerase reactions are catalyzed at the active site of fumarase A.

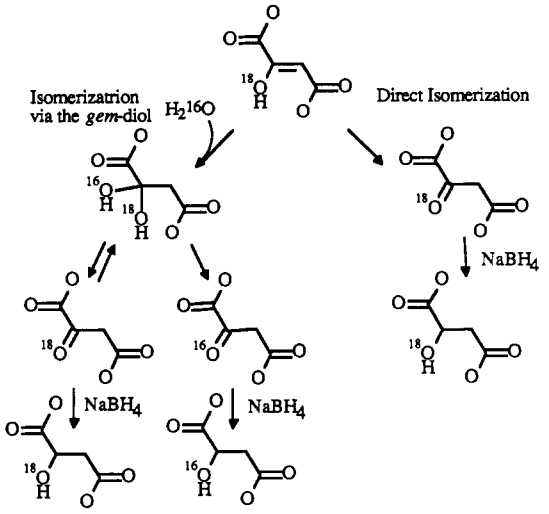
Isomerase Activity of Other Fe–S Cluster Containing and Non Fe–S Cluster Containing Hydro-Lyases. The observation of the high activity of fumarase A as an OAAKE isomerase prompted the examination of porcine fumarase for similar activity. This experiment was designed so that a level of OAAKE isomerase activity could have been detected from porcine fumarase as low as 1% of the isomerase activity of fumarase A on an equal fumarase activity basis. Under these conditions, no OAAKE isomerase activity was detected from porcine fumarase. Although porcine fumarase is a member of the hydro-lyase class like fumarase A and aconitase, it does not contain an Fe–S cluster. To further investigate the connection between Fe–S cluster containing hydro-lyases and OAAKE isomerase activity, two additional members of the hydro-lyase class that also contain a [4Fe–4S] cluster, *E. coli* dihydroxyacid dehydratase (Flint & Emptage, 1989, 1990) and yeast isopropyl malate isomerase (Emptage, 1990), were tested for OAAKE isomerase activity and neither was found to catalyze this reaction.

The kinetic constants of fumarase A determined in this laboratory for the isomerase and hydro-lyase activities and those reported in the literature for porcine fumarase and bovine aconitase are given in Table I. The K_m of fumarase A for the isomerization of enol OAA is one-sixth the K_m of fumarase A for the hydration of fumarate. The k_{cat}/K_m for the OAAKE isomerase of fumarase A is approximately equal to its k_{cat}/K_m for the hydration of fumarate.

Influence of the Fe–S Cluster on Catalysis. In the introduction it was pointed out that while aconitase catalyzes the interconversion of citrate and isocitrate only in the native [4Fe–4S] cluster containing state, it catalyzes the OAAKE isomerase reaction at a similar rate both in the native and in the oxidized [3Fe–4S] cluster containing state [which can be formed by oxidation of the native enzyme by air or $K_3Fe(CN)_6$]. In contrast to aconitase, when exposed to air, fumarase A loses both its fumarase activity and OAAKE isomerase activity at about the same rate, although the loss of isomerase activity slightly lags the loss of fumarase activity. A typical inactivation curve is shown in Figure 2.

It is not clear from studies of the type shown in Figure 2 if the [3Fe–4S] cluster containing form of fumarase A is active as an isomerase, as in the case of aconitase. The [3Fe–4S] cluster of aconitase is easily studied since it is stable in air for several hours. This is not the case with fumarase A because in air the [3Fe–4S] cluster containing form a fumarase A is readily oxidized further and loses additional Fe to form species

Scheme I



in which the cluster is even more extensively degraded (Flint et al., 1992). Unlike aconitase, the [3Fe–4S] cluster form of fumarase A does not become the predominant form when the enzyme is exposed to air.

The [3Fe–4S] cluster containing form of fumarase A can be obtained as the predominant form of fumarase A by oxidizing the enzyme with $K_3Fe(CN)_6$ under anaerobic conditions (Flint et al., 1992). When 2 equiv of $K_3Fe(CN)_6$ was added to a fumarase A preparation, all but 2% of the original fumarase activity was lost, but 23% of the original isomerase activity remained. When 3 equiv of $K_3Fe(CN)_6$ was added to a fumarase A preparation, the fumarase activity was completely lost, but 12% of the original isomerase activity remained. These results indicate that, like aconitase, the [3Fe–4S] cluster containing form of fumarase A is active as an isomerase, but the activity is lower than the [4Fe–4S] cluster containing form. The results in air suggest for fumarase A that after the cluster is oxidized past the [3Fe–4S] cluster containing form, its isomerase activity is lost. It is not known if this is the case for aconitase.

It seems unlikely that the Fe–S cluster of fumarase A and aconitase would be chemically involved in the OAAKE isomerase activity in both the [3Fe–4S] and [4Fe–4S] forms. It seems more likely the OAAKE isomerase activity is independent of the clusters in a chemical sense, but dependant on the clusters in a structural sense to maintain the other catalytic groups of the enzyme in the proper structural position. Degradation of the clusters past the [3Fe–4S] form could change the relative position of the catalytic groups at the active sites to the point they were no longer properly situated in space to catalyze the OAAKE isomerase reaction.

Mechanism of Isomerization. Fumarase A could catalyze the isomerization of enol to keto OAA either directly by acid/base-catalyzed isomerization or indirectly by hydrating enol OAA to the gem diol. The gem diol could then lose H_2O to form keto OAA while bound to the enzyme or after release into solution. These interconversions are diagrammed in Scheme I. The mechanism of isomerization has been investigated by carrying out the isomerization of [2-^{18}O]enol OAA in $H_2{}^{16}O$ and determining the fraction of ^{18}O that was retained in the keto OAA product. If direct isomerization occurs, the enrichment of the [2-^{18}O] in the keto product should be similar to that of the enol substrate. If isomerization is via the gem diol which is released by the enzyme into solution, the enrichment of the [2-^{18}O] in the keto product would be half that of the enol substrate. If isomerization is via the gem diol which loses H_2O while bound to the enzyme, the enrichment

of the [2-^{18}O] in the keto product would depend on which O was removed by the enzyme.

An important consideration in designing this experiment is the spontaneous exchange of the [2-^{18}O] in the keto product with ^{16}O from water. This exchange can be stopped by reducing OAA to malate, but it was important to determine its time course so the parameters of the experiment could be planned to minimize and correct for this exchange. The rate of exchange of ^{18}O out of [2-^{18}O]keto OAA was determined by diluting [2-^{18}O]keto OAA dissolved in $H_2{}^{18}O$ into a large excess of $H_2{}^{16}O$ buffered at pH 7.5 with 10 mM sodium phosphate, withdrawing samples at various periods of time, and immediately mixing the samples with a large excess of 50 mM $NaBH_4$ dissolved in ethanol. The malate was derivatized and the fraction containing ^{18}O was determined by GC/MS. Under these conditions the reduction of keto OAA by $NaBH_4$ appears to be complete in 2–3 s, and the half-time for exchange of the [2-^{18}O] out of keto OAA was 35 s. With this information, the following experiment was carried out to investigate the mechanism of isomerization.

A 50 mM [2-^{18}O]enol OAA solution was prepared in diethyl ether since OAA remains almost entirely in the enol form in this solvent. Aliquots of the ether solution containing 2.5 μmol of [2-^{18}O]enol OAA were withdrawn and added to each of three 1-mL samples of $H_2{}^{16}O$ containing 10 mM sodium phosphate buffer, pH 7.5, and various additional components as follows. To the first or 0 time control sample, 2 mL of 50 mM $NaBH_4$ dissolved in ethanol was added immediately before the addition of [2-^{18}O]enol OAA. To the second or 5-s control sample, no additions other than the [2-^{18}O]enol OAA were made until 5 s later, when 2 mL of 50 mM $NaBH_4$ dissolved in ethanol was added. To the third or +fumarase A sample, 2000 units of fumarase A was added before the addition of [2-^{18}O]enol OAA, which was followed 5 s later with 2 mL of 50 mM $NaBH_4$ dissolved in ethanol. The 2000 units of fumarase A in the third sample was sufficient enzyme to isomerize the enol OAA added in less than 1 s. These three samples were derivatized and analyzed by GC/MS. Scheme I shows some of the interconversions possible in this experiment.

The malate in the 0 time control sample would be expected to have almost as high a degree of enrichment of ^{18}O as the original sample of [2-^{18}O]enol OAA, since the $NaBH_4$ would reduce the keto OAA almost immediately on formation so little spontaneous exchange with $H_2{}^{16}O$ would occur. The malate in the 5-s control sample would be expected to have slightly lower enrichment of ^{18}O than the 0 time control sample, since it was incubated in $H_2{}^{16}O$ for 5 s before the addition of $NaBH_4$, during which time some of the enol OAA would isomerize and the [2-^{18}O] would begin to exchange with $H_2{}^{16}O$ (but the bulk of the enol OAA would remain in the enol form since the spontaneous isomerization rate of enol OAA is such that minutes pass in 10 mM phosphate buffer, pH 7.5, before equilibrium is reached). In the +fumarase A sample there are two possibilities. If the isomerization was direct, the enrichment of the ^{18}O malate would be somewhat less that the 5-s control sample because the isomerization would have reached equilibrium in less than 1 s after addition of the enol OAA. During the next 4 s, the OAA would be primarily in the keto form and some of the [2-^{18}O] would exchange with $H_2{}^{16}O$. Nevertheless, the enrichment of ^{18}O in the keto product should be similar to the enrichment of the [2-^{18}O]enol OAA substrate because the $t_{1/2}$ for exchange is 35 s. If the gem diol is an intermediate in isomerization catalyzed by fumarase A and it is released into solution, the upper limit on the enrichment of the product keto OAA would be 50% that of the enol substrate. Since the isomerization reaction is reversible and sufficient enzyme was added to isomerize the OAA present 5 times over during the period before addition of $NaBH_4$, almost complete washout of the label would be expected in $H_2{}^{16}O$ if the isomerization was via the gem diol. This is possibility is designated in Scheme I by the reversible arrows between [2-^{18}O,2-^{16}O] gem diol and [2-^{18}O]keto OAA and a one-way arrow between the [2-^{18}O,2-^{16}O] gem diol and [2-^{16}O]keto OAA. Of course, both reactions are reversible in a kinetic sense, but in a thermodynamic sense once the ^{18}O is lost from keto OAA it is irreversible since the ^{18}O will be diluted by the excess of ^{16}O in the solvent water. If the gem diol is an intermediate in the isomerization catalyzed by fumarase A and the loss of H_2O to form keto OAA occurs on the enzyme, the [2-^{18}O]enrichment of the product keto OAA would depend on the stereochemistry of H_2O loss on the enzyme.

The ^{18}O enrichment of the malate in the 0 time control sample, the 5-s control sample, and the +fumarase A sample was found to be 94%, 84%, and 74%, respectively. These results are consistent with direct isomerization but are not consistent with the isomerization to the gem diol followed by its release into solution. The results could be consistent with the formation of the gem diol followed by the specific removal of the oxygen that was added to the form the gem diol (^{16}O in this case) during ketonization on the enzyme. Since the k_{cat}/K_m for the isomerization of enol to keto OAA is almost the same as the hydration of fumarate to malate, it seemed unlikely that the isomerization would involve the formation of the gem diol followed by ketonization on the enzyme, a reaction clearly involving more steps than the hydration of fumarate to malate. Nevertheless, it is possible to investigate this possibility by determining the position of protonation on the 3-carbon during isomerization. If the isomerization occurs by formation of the gem diol, the proton added to the 3-carbon during the formation of the gem diol should be located in the 3-*pro-R* position, since this is the location of the proton in malate formed by the hydration of fumarate by fumarase A (Flint, unpublished observations).

Stereochemistry of Protonation of Enol OAA. Since other OAAKE isomerases have been reported to lack stereochemical specificity (Johnson et al., 1986), a simple experiment was done to see if this was also the case with fumarase A. A sample of OAA was dissolved in 10 mM phosphate buffer (pH 7.5) prepared in ^1H_2O and sufficient fumarase A to accelerate the OAAKE isomerization rate 100-fold above the nonenzymatic rate was added. An identical sample without the addition of fumarase A was prepared. Both samples were then diluted 10-fold into ^2H_2O. Samples of the mixture were withdrawn periodically and mixed with an excess of ethanolic $NaBH_4$ to reduce the OAA to malate and inactivate the fumarase A. The ^{2}H content of the malate in the samples was then determined by GC/MS of the dimethyl ester. This protocol allows the enzyme-catalyzed isomerization to be followed over a period of time during which the contribution of the nonenzymatic background isomerization is insignificant (see Figure 3).

In this experiment, the number of ^{2}H in OAA should increase from zero to one if fumarase A catalyzes the addition/elimination of a proton to/from the 3-carbon with stereospecificity. The number of ^{2}H in the OAA should increase from zero to two if fumarase A lacks stereospecificity. Since this is a multiturnover experiment, if fumarase A is stereoselective but not stereospecific the number of ^{2}H should also eventually increase to two, but at a slower rate than if it lacked stereospecificity. The results from these experiments are shown in Figure 3. Fumarase A clearly catalyzes the

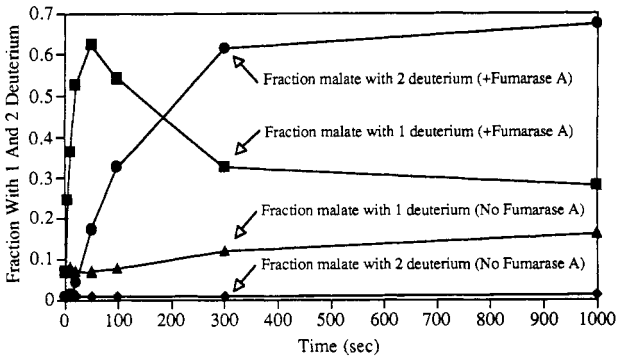


FIGURE 3: Fraction of [3-$^2H_1$]malate and [3-$^2H_2$]malate at various times after dilution into ^2H_2O. Note the comparatively slow rate of nonenzymatic incorporation. The significant level of [3-$^2H_1$]malate at zero time arises from the presence of enol OAA in equilibrium with keto OAA at the time of dilution into ^2H_2O. The enol OAA must tautomerize to the keto form before it is reduced and in the process it picks up a $^2H^+$ from solvent. The enzyme-catalyzed samples come to equilibrium with [3-$^2H_2$]malate below 100% in part because the ^2H content of the isomerization mixture is only 86% enriched so only 75% of the OAA should have two ^2H at equilibrium. In addition, the $NaBH_4$ is added in [^1H]ethanol, which further dilutes the ^2H to ^1H ratio, so the fraction of enol OAA present at the time of $NaBH_4$ addition that has one ^2H will have a small chance of picking up a second ^2H when it isomerizes.

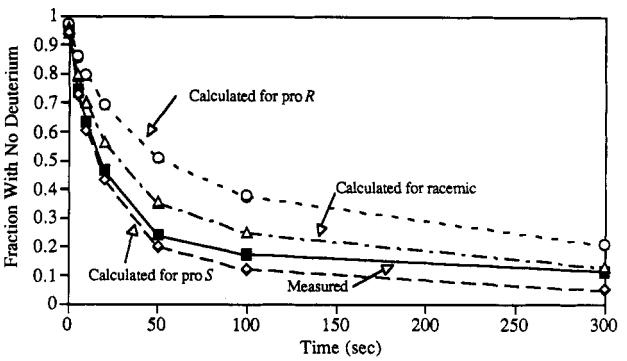


FIGURE 4: Fraction of [3-$^2H_0$]malate (no deuteriums) in the 2-(*S*)-3-(*pro-R*) exchanged-out fractions at various times after addition of fumarase A compared to the content predicted from the calculations based on the amounts of [3-$^2H_0$]malate and [3-$^2H_1$]malate in the control fractions, assuming different stereochemistries of protonation at carbon 3. The results given in this figure are averages from duplicate GC/MS determinations on each sample. The results of each pair of duplicate determinations differed from one another by <1%.

incorporation of 2 deuteriums into OAA, which means the OAAKE isomerase is not stereospecific in the addition of protons to the 3-carbon.

In experiments similar to those reported above for fumarase A, we investigated the stereospecificity of aconitase in the OAAKE isomerase reaction. In these experiments both the [3Fe–4S] and [4Fe–4S] cluster forms of aconitase catalyzed the incorporation of two ^2H into OAA, indicating aconitase also lacks stereospecificity in the OAAKE isomerase reaction (Flint, unpublished work).

Inspection of Figure 3 suggested that in the presence of fumarase A the rate of incorporation of the first ^2H was considerably faster than the second, implying that the OAAKE isomerase activity of fumarase A could be stereoselective. If the protonation were stereoselective, then the amount of one isomer of [3-$^2H_1$]OAA should predominate over the other in the early stages of the incubation. This possibility was investigated by determining the fraction of ^2H in the 3-*R* and in the 3-*S* position of OAA (as malate) at various times after addition of enzyme and OAA to ^2H_2O.

Experiments similar to those shown in Figure 3 were repeated and the samples from each time point were divided into two subsamples after the $NaBH_4$ reduction. One of the subsamples from each time point was used as the control. The other was incubated in ^1H_2O with porcine fumarase to exchange out the 3-*pro-R* proton from 2-(*S*)-malate. The latter subsamples were designated the exchanged subsamples. All the samples were derivatized and the amount of [3-$^2H_0$]-malate, [3-$^2H_1$]malate, and [3-$^2H_2$]malate in the exchanged and control subsamples were determined by GC/MS.

An internal verification that the exchange went to completion in each exchanged subsample was made by comparing the amount of [3-$^2H_2$]malate in the exchanged subsample with that in the companion control subsamples. In each case, the content of [3-$^2H_2$]malate in the exchanged subsample was half that of the corresponding control. This demonstrated that the exchange was complete since only half of the [3-$^2H_2$]-malate is in the porcine fumarase-accessible 2-*S* form.

The position of the 3-^2H in the [3-$^2H_1$]OAA (as malate) was ascertained by comparing the amount of [3-$^2H_0$]malate in the exchanged subsamples with the amount of [3-$^2H_0$]malate and [3-$^2H_1$]malate in the control subsamples. The comparison was done using three equations which separately assumed that the ^2H had been added to OAA in the 3-*pro-R* position, the 3-*pro-S* position, or equally at both positions. The equations used for the comparison are given below, where [3-$^2H_0$]malate$_{(cont)}$ and [3-$^2H_1$]malate$_{(cont)}$ are the amounts of [3-$^2H_0$]malate and [3-$^2H_1$]malate, respectively, in the control subsamples and [3-$^2H_0$]malate$_{2\text{-}(S)\text{-}3\text{-}(pro\text{-}R)\text{exchanged}}$ is the amount of [3-$^2H_0$]malate expected in the exchanged subsamples for different protonation stereochemistry.

(1) Isomerization reaction occurs with protonation in the 3-*pro-R* position:

$$[3\text{-}^2H_0]\text{malate}_{2\text{-}(S)\text{-}3\text{-}(pro\text{-}R)\text{exchanged}} = [3\text{-}^2H_0]\text{malate}_{(cont)} + (^1/_2)(0.81)[3\text{-}^2H_1]\text{malate}_{(cont)}$$

(2) Isomerization reaction occurs with protonation in the 3-*pro-S* position:

$$[3\text{-}^2H_0]\text{malate}_{2\text{-}(pro\text{-}R)\text{exchanged}} = [3\text{-}^2H^0]\text{malate}_{(cont)}$$

(3) Isomerization reaction occurs without stereospecificity:

$$[3\text{-}^2H_0]\text{malate}_{2\text{-}(S)\text{-}3\text{-}(pro\text{-}R)\text{exchanged}} = [3\text{-}^2H_0]\text{malate}_{(cont)} + (^1/_4)(0.81)[3\text{-}^2H_1]\text{malate}_{(cont)}$$

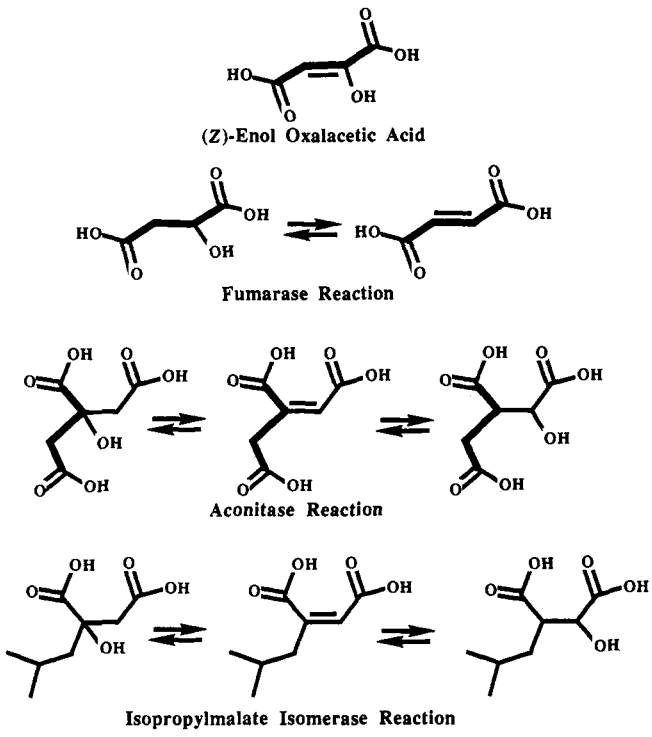
These equations take into account the fact that only half of the malate is in the porcine fumarase-accessible 2-*S* form. The factor of 0.81 enters into these equations because some of the malate is converted to fumarate during the exchange experiment; K_{equil} = [malate]/[fumarate] = 4.4.

A comparison of the measured and calculated amount of [3-$^2H_0$]malate in the exchanged subsamples at different time points is shown in Figure 4. It is obvious from Figure 4 that in the early stages of the isomerization the measured amounts of [3-$^2H_0$]malate in the exchanged subsamples most closely resembles that calculated if protonation occurs predominantly in the 3-*pro-S* position. At longer times, the measured amounts of [3-$^2H_0$]malate in the exchanged subsamples most closely resemble that calculated if the protonation is racemic. The simplest interpretation of this is that the protonation is stereoselective with protonation occurring preferentially (but not entirely) in the 3-*pro-S* position. Calculations show if the protonation occurred approximately five times in the 3-*pro-S* position for one time in the 3-*pro-R* position, a curve very similar to the measured one above would be generated.

DISCUSSION AND SUMMARY

With the results reported in this paper, fumarase A has been shown to catalyze the isomerization of enol and keto

Scheme II



OAA with a k_{cat}/K_m close to that of the hydro-lyase reaction of fumarase A. During the isomerization, the oxygen on carbon 2 is retained. This could arise by direct isomerization or by hydration of enol OAA to the gem diol followed by ketonization before the gem diol intermediate leaves the enzyme. However, if the latter mechanism is the case, stereospecific protonation at the 3-*pro-R* position would be expected since this is the position of protonation in the hydration of fumarate to malate. The results presented in this paper show the protonation is stereoselective for the 3-*pro-S* position. Since it is highly unlikely that the stereochemistry of protonation would be the opposite in the hydration of fumarate and enol OAA, the most reasonable conclusion is that the isomerization occurs directly and that the gem diol is not an intermediate.

Further evidence against the gem diol intermediate is existence of the isomerase activity of the [3Fe–4S] cluster containing form of fumarase A. This form is completely inactive in the hydration of fumarate (because it is missing the Fe that plays an essential role in the hydration reaction), so it would be highly unlikely that it could hydrate enol OAA. While the mechanism of the isomerization catalyzed by aconitase has not been investigated, it is unlikely that it would occur via the gem diol either, since aconitase is equally active as an isomerase in the [3Fe–4S] as it is in the [4Fe–4S] cluster containing form.

As shown in Scheme II, fumarase A, porcine fumarase, aconitase, and yeast isopropylmalate isomerase, four of the members of the hydro-lyase class that have been tested as isomerases, each catalyze a similar dehydration reaction on a linear four-carbon dicarboxylic acid fragment. In each of these four enzymes the elimination of the elements of water is known to be anti (Gawron & Glaid, 1955; Gawron et al., 1958; Cole et al., 1973; Gawron & Fondy, 1954; Flint, unpublished work). However, the carboxyl groups between which the dehydration occurs are trans in the case of fumarase A and porcine fumarase but cis in the case of aconitase and yeast isopropylmalate isomerase (Gross et al., 1963). Two related questions arise in connection with these four enzymes: (1) why are fumarase A and aconitase active as OAAKE isomerases and (2) why are yeast isopropyl malate isomerase and porcine fumarase inactive? The way these enzymes bind or are unable to bind enol OAA could be a key in answering these questions. We have shown (Flint et al., 1993) that enol OAA exists in the *Z* conformation. Therefore, a protein would have to productively bind (*Z*)-enol OAA to function as an OAAKE isomerase.

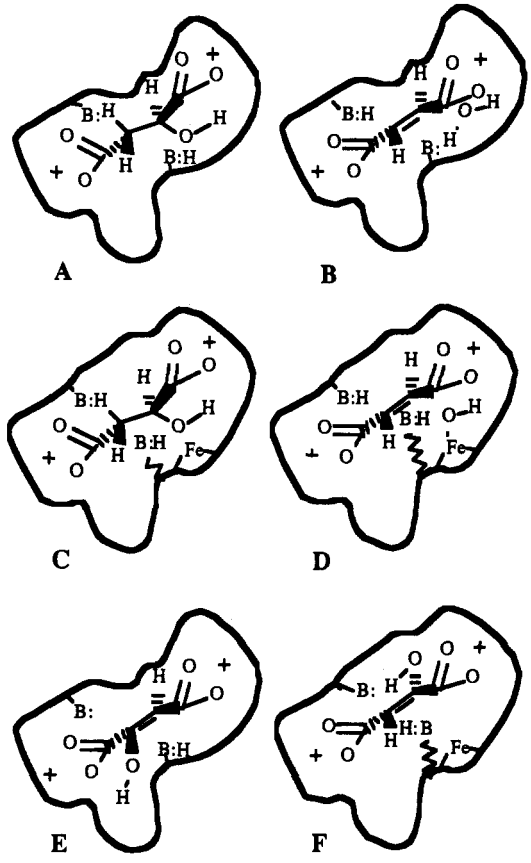
There is little question that the active site of fumarase A can bind (*Z*)-enol OAA since it is produced in its active site as a product of the hydration of acetylene dicarboxylic acid, an excellent substrate for fumarase A (Flint, unpublished work). It would be simplest to assume that (*Z*)-enol OAA is bound to the active site of fumarase A after the hydration of acetylene dicarboxylic acid so that C-2 and C-3 of (*Z*)-enol OAA are close to the same positions as those occupied the C-2 and C-3 of malate after the hydration of fumarate. If this is the case, the acidic and basic groups that normally catalyze hydration/dehydration could be well be positioned to directly catalyze tautomerization by abstracting a proton from the hydroxyl group on C-2 of enol OAA and protonating C-3.

The situation is not quite so clear with aconitase. *Z*-Enol OAA would not be expected to bind to the part of the active site of aconitase near the Fe–S cluster because the relationship of the carboxyl groups attached to the carbons which undergo hydration/dehydration is cis. However, the carboxyl group of the acetyl arm of the substrates of aconitase has been thought for some time to bind in a configuration that is trans to the carboxyl group on C-3, and more recently this has been confirmed by X-ray crystallography (Schloss et al., 1984; Lauble et al., 1992). These stereochemical relationships are shown in Scheme II with the parts of the fragments in a trans configuration shown in boldface type. It can be seen from Scheme II that it would be possible for (*Z*)-enol OAA to bind in the active site of aconitase in such a way its C-2, C-3, and hydroxyl group occupied positions similar to those occupied respectively by the C-3, C-4, and hydroxyl group of citrate when it binds. If (*Z*)-enol OAA does bind this way there is a group near the hydroxyl that normally participates in the hydration/dehydration catalyzed by aconitase that could catalyze the tautomerization of enol OAA by abstracting the proton from the hydroxyl group. Because of its location, however, protonation of C-3 does not seem possible by a group that is normally involved in the hydration/dehydration reaction, so there are no obvious candidates for protonation of C-3.

The lack of activity of yeast isopropylmalate isomerase is easy to rationalize since its active site is arranged to bind an (*E*)-enol OAA moiety, so it would not be expected to bind (*Z*)-enol OAA.

The lack of activity of porcine fumarase as an OAAKE isomerase, given the high activity of fumarase A, is difficult to rationalize. One possibility is that porcine fumarase is not able to bind (*Z*)-enol OAA in a productive orientation. A few lines of evidence suggest this might be the case. For example, porcine fumarase catalyzes the addition of a hydroxyl group to C-2 in the hydration of fluorofumarate but to C-3 in the hydration of chloro-, bromo-, and iodofumarate (Hill & Teipel, 1971). For this to occur, fluorofumarate must bind in the same orientation as malate; that is, its C-2 and C-3 occupy respectively the same positions of C-2 and C-3 of malate when it binds. In contrast chloro-, bromo-, and iodofumarate must bind in the opposite orientation of malate such that their C-2 and C-3 occupy respectively the positions C-3 and C-2 of malate when it binds. This switch in orientation is probably driven by the greater bulk of the larger halo atoms precluding

Chart I



binding in the malate orientation. It is known that porcine fumarase tolerates some bulk in substituents at C-3 of malate, but the results with the halofumarates suggest that it tolerates little bulk in substituents at C-2. For this reason, the active site of porcine fumarase may not readily bind enol OAA in a productive orientation similar to that in which malate binds because of the bulk and position of the OH group in enol OAA. These possibilities are illustrated diagrammatically in Chart I, where A and B represent respectively malate and fumarate in the active site of porcine fumarase, C and D represent respectively malate and fumarate in the active site of fumarase A, and E and F represent enol OAA in the active site of porcine fumarase and fumarase A, respectively.

Enol OAA is shown in the active site of porcine fumarase (E) in an inverted configuration compared to enol OAA in fumarase A (F) and malate bound to porcine fumarase (A) because if enol OAA binds at all to porcine fumarase, steric constraints in the active site may prevent it from binding in a similar orientation to that of malate when it binds (A). If the orientation of enol OAA in the active site of fumarase A (F) is correct, to accommodate the stereochemical findings reported in this paper, the addition of a proton to enol OAA would need to be from the opposite side of the molecule as takes place with fumarate. Perhaps this can occur via the base that normally functions to remove a proton from the H_2O whose O in association with the cluster will ultimately be added to fumarate to form malate. This possibility is suggested by having this base protonated in (F). If this is the case, the base involved in the addition of a proton to the carbon of fumarate that will eventually become C-3 of malate may remove the proton from the hydroxyl group of enol OAA, as is also suggested by (F).

This reasoning is consistent with acetylene dicarboxylate being a very poor substrate for porcine fumarase (Hill & Teipel, 1971), but an excellent substrate for fumarase A (Flint, unpublished work). The product of the hydration of acetylene carboxylic acid in each case would be enol OAA in the same orientation as malate in the active site. It seems likely that fumarase A is active as an OAAKE isomerase and an acetylene dicarboxylate hydratase because its active site can accommodate enol OAA in the same orientation as malate. In contrast, porcine fumarase is inactive as an OAAKE isomerase and poorly active as an acetylene dicarboxylate hydratase because its active site has difficulty binding enol OAA in the same orientation as malate.

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