Escherichia coli Fumarase A Catalyzed Transfer of ^{18}O from C-2 and ^{2}H from C-3 of Malate to Acetylene dicarboxylate To Form ^{18}O and ^{2}H Labeled Oxalacetate

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Abstract: We have found that fumarase A can catalyze the transfer of ^{18}O from [2-^{18}O]malate and ^{2}H from (3*R*)-[3-^{2}H]malate to the carbon skeleton of acetylenedicarboxylate to form ^{18}O and ^{2}H labeled enol oxalacetate. Our data indicates that 33% of the ^{18}O mobilized from [2-^{18}O]malate and close to 100% of the ^{2}H mobilized from (3*R*)-[3-^{2}H]malate would be transferred at an infinite concentration of acetylenedicarboxylate. This is the first report of oxygen transfer by an enzyme in the hydro-lyase class, but there have been previous reports of proton transfer by enzymes of this class. The transfer of the oxygen and proton removed by fumarase A in the dehydration of malate requires that these atoms remain associated with the enzyme long enough for the four carbon substrates to interchange in the active site. With certain assumptions, the results reported in this paper allow a rough calculation of the rate constants for the dissociation of the oxygen and proton from fumarase A. The rate constant for the oxygen is between 1×10^4 and $8 \times 10^5\ s^{-1}$. Since fumarase A contains a [4Fe–4S] cluster that acts as a Lewis acid in the dehydration reaction catalyzed by fumarase A, it is likely that the oxygen is bound to the enzyme as a ligand to an iron atom in the cluster. The rate constant for the dissociation of oxygen from fumarase A compares favorably with the rate constants reported for water interchange on iron complexes. The rate constant for the dissociation of the proton from fumarase A is on the order of $7 \times 10^4\ s^{-1}$. This is similar to the rate constant for the dissociation of a proton from a carboxylic acid but higher than the rate constants for the dissociation of protons from protonated amines, protonated imidazoles, thiols, and alcohols. If the base that removes the proton from C-3 of malate in the active site of fumarase A is not a carboxylate, the dissociation of the proton from this group must be accelerated in some way by the enzyme.

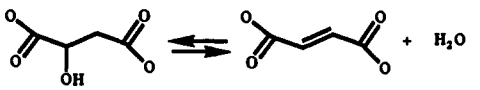
Introduction

Wild type *E. coli* contains three genes, *fumA*, *fumB*, and *fumC*, that encode proteins which catalyze the fumarase reaction shown in Scheme 1.[1] Fumarase A, the protein encoded by the *fumA* gene, has been purified and shown to contain a [4Fe–4S] cluster.[2] Fumarase A and fumarase B have the same number of amino acids and share 90% sequence homology.[3] In unpublished work, we have also obtained evidence that fumarase B contains a [4Fe–4S] cluster.

Fumarase A and B belong to the subclass of hydro-lyases that contain Fe–S clusters. Mammalian aconitase is the most thoroughly studied member of this subclass. As is the case with mammalian aconitase, the Fe–S clusters of fumarase A and B appear to participate in catalysis by acting as Lewis acids to activate the hydroxyl group of the substrate for elimination or addition depending on the direction of the reaction.[2a] Because of its sequence homology to porcine fumarase, it seems likely that fumarase C will resemble porcine fumarase in having no metal or cofactor requirements, and from recent reports this appears to be the case.[2b,4]

We have attempted to determine if fumarase A can catalyze the transfer of the oxygen from the hydroxyl group of malate to a different carbon skeleton. This possibility has been investigated previously for mammalian aconitase with negative results.[5] In spite of the failure with aconitase, we were encouraged to investigate oxygen transfer by fumarase A because its k_{cat} in the direction of hydration, $3100\ s^{-1}$,[6] is 78-fold higher than the k_{cat} of aconitase in the direction of hydration, $40\ s^{-1}$.[7] It seemed possible that the k_{cat} of fumarase A could be fast enough to allow oxygen transfer. In this paper we report that the transfer of the oxygen from the hydroxyl group of malate to the carbon skeleton of acetylenedicarboxylate occurs with good efficiency.

Scheme 1



We have also attempted to determine if fumarase A can catalyze the transfer of the 3-*pro*-*R* proton from malate to a different carbon skeleton. Proton transfer has been investigated previously for aconitase and porcine fumarase with positive results.[5,8] We report in this paper that proton transfer occurs with high efficiency in the reaction catalyzed by fumarase A.

Results and Discussion

In the experimental approach used in this paper, the products of the action of fumarase A on (2*S*)-[2-^{18}O]malate and (2*S*,3*R*)-[3-^{2}H]malate with acetylenedicarboxylate as a cosubstrate was followed by the use of a gas chromatograph coupled to a mass spectrometer (hereafter abbreviated as GC/MS). The GC/MS analysis was performed by monitoring specific ions characteristic of different isotopically labeled malate species, which were

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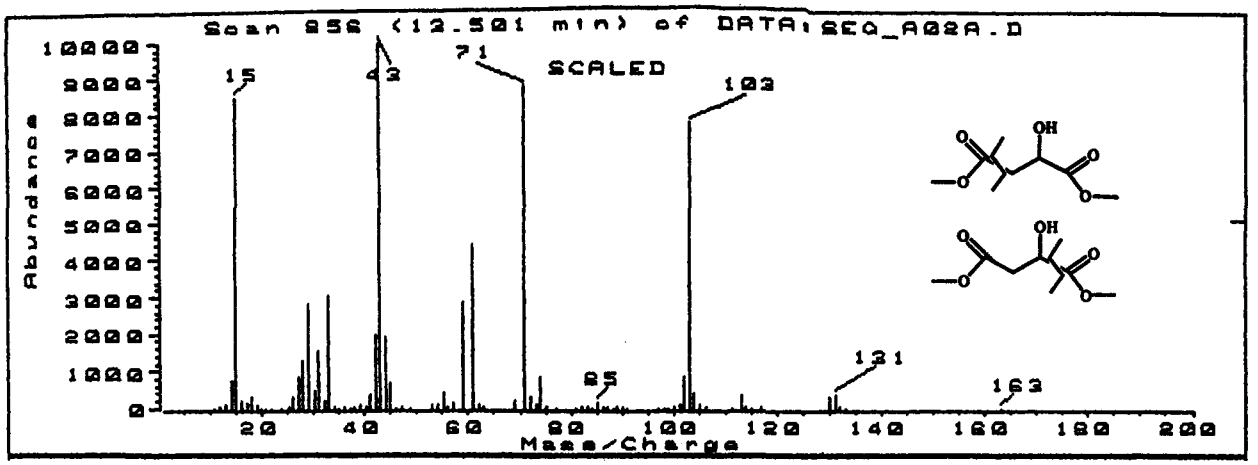


Figure 1. The mass spectrum of dimethyl malate acquired on the mass spectrometer used in these experiments. Note the prominent ion with m/z = 103 and the less prominent ions at m/z values on either side of 103. The ion with m/z = 103 can be generated by the loss of either carboxylmethyl group from dimethyl malate as shown in the inset placed in the figure.

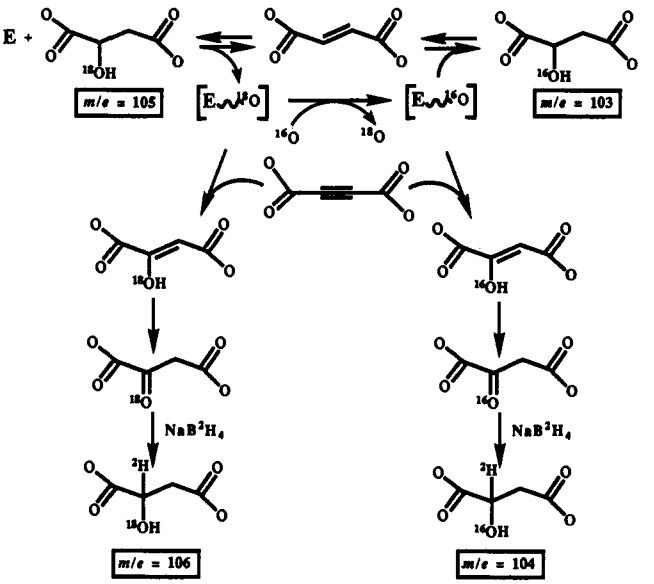
analyzed as the dimethyl esters. The mass spectrum of the dimethyl ester of normal malate is shown in Figure 1. It contains a prominent ion with a m/z of 103. This ion arises from the loss of one of the carboxymethyl groups as depicted in the inset of Figure 1. The corresponding ion in the mass spectra of [2-^{2}H]-malate and [3-^{2}H]malate had a m/z of 104, the corresponding ion in the mass spectra of [2-^{18}O]malate had a m/z of 105, and the corresponding ion in the mass spectra of [2-^{2}H,2-^{18}O]malate had a m/z of 106. The key to the experimental approach used in this paper was the independent measurement of the amounts of these four malate species in the presence of each other by monitoring the corresponding ions referred to above.

The analysis of the different species of malate in each other's presence is complicated by the fact that the mass spectra of each of these species show, besides the prominent ion with the m/z values indicated, smaller amounts of other ions with m/z values close to that of the prominent ion. This phenomenon is readily seen in Figure 1. The ions with a m/z of 104 and 105 can be explained by the natural abundance of ^{13}C, ^{2}H, and ^{18}O in the samples; however, the ions with a m/z of 102 and 106 cannot be explained in this way, so they must arise from a fragmentation pattern slightly different from that depicted in Figure 1.

Since the ions with a m/z to either side of the prominent ion of the isotopically labeled malate species mentioned above can have the same m/z values as the prominent ion of another isotopically labeled malate species in the mixture to be analyzed, corrections need to be made. To be able to make the corrections, in each set of experiments, samples of dimethyl malate containing a natural abundance of isotopes were run on the GC/MS, and the relative signals at a m/z of 1 less (102), 1 more (104), 2 more (105) and 3 more (106) than the predominant ion with a m/z of 103 were measured. In a typical experiment the relative amounts of the ions with m/z values of 102, 103, 104, 105, and 106 were respectively 8.96, 100, 5.20, 0.722, and 0.574. From these values correction factors were calculated and used to determine what portion of the amount detected at each m/z monitored in the experiment was due to the isotopically labeled species whose prominent ion had that value of m/z and what amount was due to "spill over" into that m/z value from other malate species.

Strategy for Detecting ^{18}O Transfer from [2-^{18}O]Malate. In the experiments to detect the transfer of ^{18}O from malate to a different carbon skeleton, (2*S*)-[2-^{18}O]malate was used as the substrate from which ^{18}O was mobilized, and acetylenedicarboxylate was used as the substrate to which it could be transferred. Acetylenedicarboxylate was chosen as cosubstrate instead of fumarate for the following reasons: (1) the k_{cat} of fumarase A for the hydration of acetylenedicarboxylate, 3000 s^{-1}, is almost as high as the k_{cat} for the hydration of fumarate, 3100,[6,9] (2) the equilbrium of the reaction in which acetylenedicarboxylate is hydrated lies far in the hydration direction (i.e., the hydration is essentially unidirectionally), whereas the hydration of fumarate does not, and (3) use of acetylenedicarboxylate in conjunction with NaB^{2}H$_4$ precluded the need for ^{13}C label in either the donor or acceptor substrate (which would have been necessary if fumarate had been used).

Scheme 2



The possible reaction paths catalyzed by fumarase A in a mixture of (2*S*)-[2-^{18}O]malate and acetylenedicarboxylate dissolved in normal water ($^{1}H_2{}^{16}O$) are illustrated in Scheme 2 where E represents fumarase A. As shown in Scheme 2, there are three possible fates for the ^{18}O once it is removed from [2-^{18}O]malate: (1) it could be added back to fumarate to reform [2-^{18}O]malate that would be available for another round of reaction, (2) it could be added to the carbon skeleton of acetylenedicarboxylate to form [2-^{18}O]enol oxalacetate, or (3) it could be released to solvent and be diluted by the ^{16}O of normal water, thereby becoming essentially unavailable for further reaction.

Once formed, [2-^{18}O]enol oxalacetate spontaneously isomerizes to its keto tautomer, and fumarase A is known to catalyze this isomerization.[9] In aqueous solutions, the carbonyl oxygen of keto oxalacetate exchanges with oxygen from water due to the formation and breakdown of the *gem*-diol. The rate of this exchange was investigated under the conditions used in this

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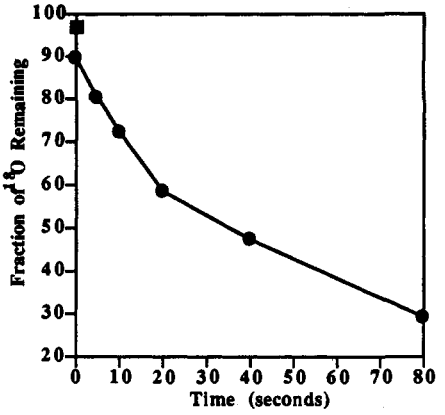


Figure 2. The exchange of ^{18}O out of [2-^{18}O]oxalacetate in $H_2{}^{16}O$. [2-^{18}O]-oxalacetate was made by dissolving oxalacetate in $H_2{}^{18}O$ and incubating the mixture for several minutes. Aliquots (10-μL) of the oxalacetate solution in $H_2{}^{18}O$ were mixed with 1-mL aliquots of 10 mM potassium phosphate buffer, pH 7.5, made up with $H_2{}^{16}O$. $NaBH_4$ (50 μL 1 M) was added at different times to individual samples (in the zero time sample, the $NaBH_4$ was added to the $H_2{}^{16}O$ before the [2-^{18}O]oxalacetate). (●) Indicates the ^{18}O enrichment of malate in the samples reduced at different times as measured by GC/MS. (■) Indicates the ^{18}O enrichment of malate formed by adding dry $NaBH_4$ to 10 μL of the [2-^{18}O]oxalacetate in $H_2{}^{18}O$. The difference between the initial enrichment of 97% ^{18}O in the [2-^{18}O]oxalacetate and the zero time value shown on the graph of 90% is due to the loss of ^{18}O that occurs in $H_2{}^{16}O$ before the reduction by $NaBH_4$. The graph shows the total loss of ^{18}O would be about 10% if the $NaBH_4$ were to be added at 2 s.

experiment. The results shown in Figure 2 demonstrate that the exchange is rapid. Left unabated, this chemical exchange would rapidly remove any ^{18}O transferred by fumarase A. The following steps were taken to minimize this exchange: (1) sufficient fumarase A was added to each sample for the reaction to be complete in 1 s, and (2) 100 mM NaB^2H_4 was added 2 s after the addition of fumarase A to reduce the oxalacetate formed to malate. The reduction prevented further chemical exchange and produced a mixture of (2*S*)- and (2*R*)-malate. Even with these precautions, the data in Figure 2 show that approximately 10% of any ^{18}O transferred would be lost from keto oxalacetate before its reduction to malate. In the calculations of the fraction of oxygen transferred a correction was made for this loss.

As illustrated in Scheme 2, the use of NaB^2H_4 as a reductant allows the individual determination of (1) [2-^{18}O]malate, the malate species initially present in the reaction mixture; (2) [2-^2H,2-^{18}O]malate, the malate species formed by the transfer of ^{18}O to acetylenedicarboxylate to from [2-^{18}O]oxalacetate that was reduced; (3) [2-^2H]malate, the malate species formed from the addition of ^{16}O (from solvent) to acetylenedicarboxylate to form normal oxalacetate that was reduced; and (4) normal malate, the malate species formed following the mobilization of ^{18}O from the [2-^{18}O]malate initially present. (It should be noted that formally fumarate would form as the ^{18}O was mobilized from malate; nevertheless, because fumarate was present in the [2-^{18}O]malate sample at a concentration in equilibrium with the malate, the equilibrium would be maintained by the fumarase A, and the net effect would be one molecule of normal malate formed for each molecule of [2-^{18}O]malate removed—see the Experimental Section for the explanation of the origin of the fumarate in the [2-^{18}O]malate sample.) The amount of ^{18}O transferred can be found from the amount of [2-^2H,2-^{18}O]malate formed, and the amount of ^{18}O mobilized and available for transfer can be found from the amount of normal malate formed. The ratio of these two amounts is the fraction of ^{18}O transferred.

A determination of the amount of [2-^2H,2-^{18}O]malate formed was complicated by the fact that fumarase A is not immediately inactivated upon addition of NaB^2H_4. Residual fumarase A activity catalyzed the exchange of some ^{18}O out of (2*S*)-[2-^2H,2-^{18}O]malate following the reduction of [2-^{18}O]oxalacetate to malate; however, the (2*R*)-[2-^2H,2-^{18}O]malate was not effected because the enzyme is not active on the *R* isomer.[6] Attempts were made to minimize this fumarase A catalyzed exchange, but none of the methods tried were satisfactory. In the protocol finally adopted, rather than suppressing the exchange, it was taken to completion by the further addition of porcine fumarase. The amount of (2*R*)-[2-^2H,2-^{18}O]malate that remained was measured by GC/MS. When calculating the total [2-^2H,2-^{18}O]malate originally formed in the sample, the amount of (2*R*)-[2-^2H,2-^{18}O]malate found was doubled since this enantiomer represented only one half of all the [2-^2H,2-^{18}O]malate originally formed.

Evidence for ^{18}O Transfer from [2-^{18}O]Malate to Acetylenedicarboxylate. When fumarase A was added to a mixture of [2-^{18}O]malate and acetylenedicarboxylate, the oxalacetate formed was reduced with NaB^2H_4, and the sample was analyzed by GC/MS; a substantial amount of the malate formed gave a *m/z* of 106. The amount of malate with *m/z* of 106 increased with increasing concentrations of acetylenedicarboxylate. Control samples in which fumarase A was added to a mixture of normal malate and varying concentrations of acetylenedicarboxylate, followed by reduction contained no such malate. Data from a typical experiment can be found in the supplementary material submitted with this paper. The only reasonable interpretation of these data is that [2-^2H,2-^{18}O]malate had formed when [2-^{18}O]-malate and acetylenedicarboxylate were incubated together in the presence of fumarase A. This indicates that ^{18}O transfer had taken place, and the amount transferred depended on the concentration of acetylenedicarboxylate.

As expected, the reaction went to completion in 2 s. The evidence for this was two fold: (1) the ratio of the amount of normal malate to [2-^{18}O]malate was typically >80:1 (indicating the [2-^{18}O]malate initially present had been exhausted), and (2) the amount of normal malate formed compared to the sum of the amount of [2-^2H]malate and [2-^2H,2-^{18}O]malate formed (the latter two are the products of the hydration of acetylenedicarboxylate) was close to the relative amounts of malate to acetylenedicarboxylate placed in the reaction mixture before the addition of fumarase A. This indicates that nearly all the acetylenedicarboxylate had been hydrated.

The ^{18}O in the (2*S*)-[2-^2H,2-^{18}O]malate remaining was exchanged out with porcine fumarase, and the amount of (2*R*)-[2-^2H,2-^{18}O]malate was measured. This value was doubled (to account for the (2*S*)-[2-^2H,2-^{18}O]malate that had been lost) to find the total amount of [2-^2H,2-^{18}O]malate originally formed. The fraction of ^{18}O transferred was calculated by dividing this amount by the amount of ^{18}O mobilized. The reciprocal of the concentration of acetylenedicarboxylate was plotted against the reciprocal of the fraction of ^{18}O transferred. The results from a typical experiment are shown in Figure 3. From the data in this figure, it can be calculated that at an infinite concentration of acetylenedicarboxylate 30% of the ^{18}O would be transferred. Correcting for the 10% of ^{18}O estimated to be lost from oxalacetate between transfer and reduction by NaB^2H_4, the amount actually transferred would be 33%. As far as we know, this is the first report of ^{18}O transfer by an enzyme of the hydrolyase class. It is logical to assume but hard to prove that the ^{18}O which is retained by fumarase A long enough to be transferred to acetylenedicarboxylate is bound to the active site of this enzyme as a ligand to one of the iron atoms of the Fe–S cluster.

An important question in light of these results is, why 33%? There are a number of possible explanations, some based on our experimental design, some based on the mechanism of the enzyme. With regard to experimental design, it should be noted that the acetylenedicarboxylate concentration decreased continuously during our experiments. Most of the time it was significantly lower than what was originally present, but in our plots the original concentration was used.

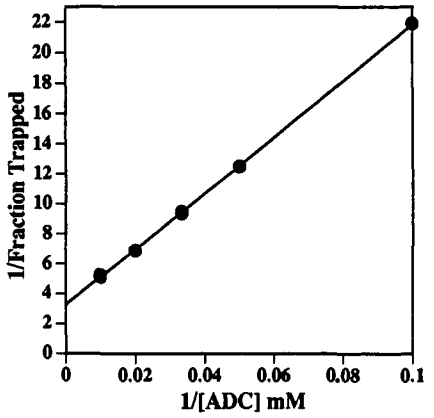
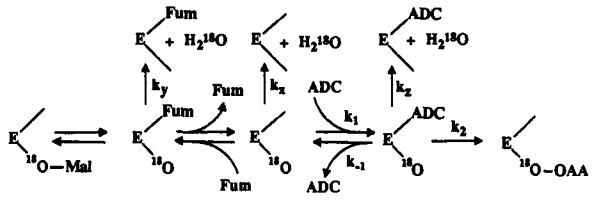


Figure 3. A double reciprocal plot of 1/[acetylenedicarboxylate] vs 1/fraction of the ^{18}O transferred. There are four data points at each concentration of acetylenedicarboxylate, each from an independently prepared sample. The scatter in the data in most cases is less than the width of the symbols used to plot the graph. (Table 1 of the supplementary material contains a typical data set.) The extrapolation to an infinite concentration of acetylenedicarboxylate predicts that at this concentration 30% of the ^{18}O mobilized from [2-^{18}O]malate would be transferred to the carbon skeleton of acetylenedicarboxylate.

Scheme 3



With regard to the mechanism of the fumarase A, it should be noted that when isocitrate is bound to the aconitase, the ligands to the iron of the cluster with which the substrate interacts are three sulfides from the cluster, the hydroxyl oxygen of isocitrate, a carboxyl oxygen of isocitrate, and one water molecule.[11] Presumably immediately after a dehydration event, there are two molecules of water (or hydroxide) bound as ligands to the iron. It is known from the crystal structure that in the substrate free enzyme, only one molecule of water (or hydroxide) is bound to the iron, so one of the two water molecules present after a dehydration event must leave to generate the form of the substrate free enzyme observed in the crystal structure. It is not known if the water molecule that leaves is the one removed from the substrate, the one bound to the iron before the substrate binds, or if some fraction, F, of the time one of these water molecules leaves and another fraction, $1 - F$, of the time the other one leaves. If fumarase A similarly has two water molecules bound to its cluster after dehydration and either one can leave or they can positionally exchange, it could help explain the incomplete transfer of the ^{18}O that we observe.

The different sequences in which the water and substrate could dissociate from the enzyme are illustrated in Scheme 3. The water molecule could leave from the ternary enzyme–water–product complex, the binary enzyme–water complex, or the ternary enzyme–water–substrate complex. The dissociation of water from each of these complexes would be characterized respectively by the rate constants k_x, k_y, and k_z as shown in Scheme 3. The values of these rate constants are of interest. Although exact determinations are not possible with the data in hand, an order of magnitude estimate which provides limits to k_x is possible provided certain assumptions are made. The estimate makes use of a mathematical procedure developed by Rose *et al.*[10] in a paper on isotope trapping.

If we assume that $k_x \gg k_y$ and k_z (i.e., water bound to the cluster exchanges faster from the unliganded form of the enzyme than the forms with product or substrate present); we can use the following equation found in the last paragraph of the supplementary material of the paper by Rose *et. al.*:[10] $D/G = k_2A/k_xK_m$. Applying this equation to fumarase A, D and G become, respectively, the change in concentration of [2-^{18}O]oxalacetate and $H_2{}^{18}O$ with time, A is the concentration of ADC, k_2 and k_x are defined in Scheme 3, and K_m is the Michealis constant for acetylenedicarboxylate. $D/G = 1/5$ at the concentration of acetylenedicarboxylate, $A_{1/2}$, at which one-half of the maximal amount of ^{18}O is trapped. Therefore, we have the modified equation $k_x = 5k_2A_{1/2}/K_m$. The term k_2 is the turnover number for the fumarase A catalyzed hydration of acetylenedicarboxylate which along with K_m have been determined to be 3000 s^{-1} and 0.9 mM, respectively.[6] $A_{1/2}$ can be found from Figure 3; it is 50 mM. Substituting these values in the equation above gives a rate of 8×10^5 s^{-1} for the dissociation of ^{18}O from the substrate free form of the fumarase A–^{18}O binary complex. It must be emphasized that this is a rough estimate based on the assumption that ^{18}O does not dissociate at an appreciable rate from either ternary complex in Scheme 3. If the exchange is primarily positional between two water molecules bound to the cluster, the factor of 1/5 would not apply. In either case, an estimate of the upper limit of the rate constant for the dissociation of ^{18}O from the substrate free enzyme is given by the value calculated above. An estimate of a lower limit can be made by multiplying the k_{cat} of the enzyme for fumarate (3100 s^{-1}) by three (since only $^1/_3$ of the ^{18}O is trapped) to give the value of 9.3×10^3 s^{-1}.

The exchange rates of water from various iron complexes have been reported and are included here for comparison with the estimated rate constant given above for the dissociation of ^{18}O from fumarase A. At 25 °C the rate constant for water interchange from $[Fe(OH_2)_6]^{3+}$ is 1.6×10^2 s^{-1}, from $[Fe(OH_2)_5(OH)]^{2+}$ is 1.4×10^5 s^{-1}, and from $[Fe(OH_2)$(*o*-phenylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid)$]^{1-}$ is 1.7×10^7 s^{-1}.[12] The slower rate constant for the hexaaquairon(III) complex compared to the Fe(III) *o*-phenylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid complex and the pentaaquahydroxoiron(III) complex has been attributed to an associative interchange for the former complex and a dissociative interchange for the latter complexes. Increased electron density on the iron(III) atom that can arise from electron donation by negatively charged ligands favors the dissociative interchange. At 25 °C the rate constant for water interchange on the Fe(II) complex, $[Fe(OH_2)_6]^{2+}$, is 4.4×10^6 s^{-1},[13] so the exchange rate appears to be faster in the Fe(II) than Fe(III) oxidation state for a complex with comparable ligands. The formal oxidation state of the iron atoms of the Fe–S cluster of fumarase is 2.5. The negative charge of the sulfide ions in the cluster would be expected to be conducive to dissociative interchange. The rate of dissociation of ^{18}O from fumarase estimated above then is of the same order to somewhat slower than the known rates for water exchange on iron complexed with negative ligands. However, it should be kept in mind that similar to carbonic anhydrase II,[14] the rate of protonation of the ^{18}O-hydroxide on the cluster after elimination could be slow compared to the dissociation of water from the cluster.

Strategy for Detecting ^{2}H Transfer from (3*R*)-[3-^{2}H]Malate. (3*R*)-[3-^{2}H]Malate was used as the substrate from which the ^{2}H was mobilized, and acetylenedicarboxylate was used as the substrate to which it could be transferred. The possible reaction paths catalyzed by fumarase A with (3*R*)-[3-^{2}H]malate and acetylenedicarboxylate as substrates dissolved in normal water are illustrated in Scheme 4 where E represents fumarase A. As shown in Scheme 4, there are three possible fates for the ^{2}H

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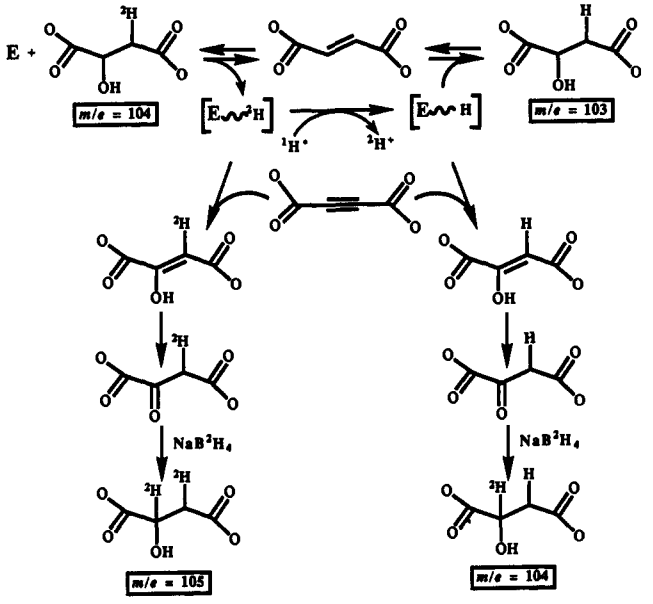
Scheme 4



bound to the active of fumarase A after abstraction from (3*R*)-[3-^{2}H]malate: (1) it could be added back to fumarate to form again (3*R*)-[3-^{2}H]malate which would be available for another round of reaction, (2) it could be added to the carbon skeleton of acetylenedicarboxylate during the hydration to form [3-^{2}H]-enol oxalacetate, or (3) it could be released to solvent and be diluted by the ^{1}H of normal water thereby becoming essentially unavailable for further reaction.

It has been previously mentioned that enol oxalacetate isomerizes to the keto form and that this isomerization is catalyzed by fumarase A. The protons on C-3 of keto oxalacetate are labile and spontaneously exchange over a period of several minutes.[9] A more severe problem as far as exchange goes is that fumarase A catalyzes the isomerase reaction in a stereoselective but not stereospecific manner.[9] Therefore, after fumarase A catalyses the transfer of ^{2}H from (3*R*)-[3-^{2}H]malate to acetylenedicarboxylate to form [3-^{2}H]oxalacetate, it can then catalyze the exchange of ^{2}H with ^{1}H from solvent. This reaction was minimized during the ^{2}H transfer experiments by keeping the concentration of acetylenedicarboxylate high. Amounts of fumarase A in combination with incubation times were chosen such that only 30–50% of the acetylenedicarboxylate was hydrated before the reaction was terminated.

As suggested by Scheme 4, the use of NaB^2H_4 as reductant allows us to independently determine the amount of [2-^{2}H,3-^{2}H]malate formed from the transfer of ^{2}H to acetylenedicarboxylate and the amount of normal malate formed. The amount of ^{2}H transferred is equal to the amount of [2-^{2}H,3-^{2}H]malate formed. The amount of ^{2}H that has been mobilized from [3-^{2}H]-malate is equal to the amount of normal malate formed (as explained in the discussion of ^{18}O transfer). The ratio of these two amounts is the fraction of ^{2}H transferred.

Evidence for ^{2}H Transfer from (3*R*)-[3-^{2}H]Malate to Acetylenedicarboxylate. When fumarase A was added to a mixture of (3*R*)-[3-^{2}H]malate and acetylenedicarboxylate, the oxalacetate formed was reduced with NaB^2H_4, and the sample was analyzed by GC/MS; a substantial amount of the malate formed gave a *m/z* of 105. The amount of malate with *m/z* of 105 increased with increasing concentrations of acetylenedicarboxylate. Control samples in which fumarase A was added to a mixture of normal malate and varying concentrations of acetylenedicarboxylate, followed by reduction contained no such malate. The only reasonable interpretation of this data is that [2-^{2}H,3-^{2}H]malate had formed by ^{2}H transfer when (3*R*)-[3-^{2}H]malate and acetylenedicarboxylate were incubated together in the presence

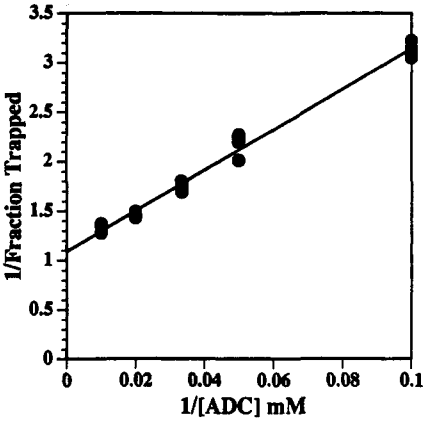


Figure 4. A double reciprocal plot of 1/[acetylenedicarboxylate] vs 1/fraction of the ^{2}H transferred. There are four data points at each concentration of acetylenedicarboxylate; each from independently prepared samples. The scatter in the data in some cases is less than the width of the symbols used to plot the graph. (Table 1 of the supplementary material contains a typical data set.) The extrapolation to an infinite concentration of acetylenedicarboxylate predicts at this concentration 92% of the ^{2}H mobilized from (3*R*)-[3-^{2}H]malate would be transferred to the carbon skeleton of acetylenedicarboxylate.

of fumarase A. This indicates that ^{2}H transfer had taken place, and the amount of transfer depended on the concentration of acetylenedicarboxylate.

The amount of [2-^{2}H,3-^{2}H]malate was measured. The fraction of ^{2}H transferred was calculated by dividing this amount by the amount of ^{2}H mobilized from [3-^{2}H]malate. The reciprocal of the concentration of acetylenedicarboxylate was plotted against the reciprocal of the fraction of ^{2}H transferred. The results from a typical experiment are shown in Figure 4. These results show that at an infinite concentration of acetylenedicarboxylate 92% of the ^{2}H would be transferred to acetylenedicarboxylate from (3*R*)-[3-^{2}H]malate. Although these experiments were terminated while considerable acetylenedicarboxylate remained in the samples, the suppression of the isomerase activity by acetylenedicarboxylate was probably not complete. It is likely that some of the transferred ^{2}H was exchanged from C-3 of [3-^{2}H]-oxalacetate by the isomerase activity of fumarase A. The extent of this exchange was not measured, but it seems possible it could be as high as 8% of the ^{2}H transferred. If the exchange was this high, then 100% of the ^{2}H would be transferred at the limit of an infinite concentration of acetylenedicarboxylate.

A reaction path similar to that shown in Scheme 3 can be set up for the loss of the proton from fumarase A. The proton abstracted from malate could leave from the ternary enzyme–proton–product complex, the binary enzyme–proton complex, or the ternary enzyme–proton–substrate complex. Making the assumption that the proton leaves primarily from the binary enzyme–proton complex and using the procedure developed by Rose *et al.*,[10] as outlined previously for ^{18}O, we can make a rough estimate of the rate constant for the dissociation of the proton from fumarase A where the maximal transfer is 92% at infinite concentration of the trapping substrate. The modified form of the equation is $k_x = 1.2k_2K_{1/2}/K_m$. The parameters in this equation and their corresponding values are the same as given previously except for $K_{1/2}$, which was determined to be 18 mM from the data in Figure 4, and k_x, which in this case is the rate for the dissociation of the proton from fumarase A. Substituting these values in the equation above gives a rate constant for the dissociation of ^{2}H from fumarase A of 7×10^4 s^{-1}. Again, this value is subject to the same caveats described in the discussion of the rate constant for the dissociation of ^{18}O. The rate constant for the dissociation of the proton abstracted by porcine fumarase is reported to be 5×10^2 s^{-1}.[8]

It is likely that the ^{2}H that is retained by fumarase A long enough to be transferred to acetylenedicarboxylate is bound to a base in the active site of this enzyme. Rate constants for the dissociation of protons from groups which could act as bases in the active site of fumarase A are given below: carboxylic acids, 10^{5}–10^{6} s^{-1}; protonated imidazoles, 10^{3} s^{-1}; thiols, 10^{1}–10^{2} s^{-1}; protonated amines, 10^{0}–10^{1} s^{-1}; and alcohols, 10^{-3} s^{-1}.[15] The active site base of mammalian aconitase has been reported to be the deprotonated (alkoxide) form of the hydroxyl group of Ser642.[11] If the base in the active site of fumarase A that removes the proton from C-3 of malate is a carboxylate, the dissociation rate of the proton from the active site of fumarase A is near that expected for such a group. If the base is an amine, imidazole, thiolate, or alkoxide, the rate constant for dissociation of the proton from fumarase A is higher than expected. If one of the latter groups is the active site base, the enzyme must accelerate proton removal from the group (e.g., the k_{cat} of aconitase, 40^{-1}, is much faster than the rate constant for the dissociation of a proton from a serine hydroxyl). Rapid elimination of the proton from the base that abstracts it is important in order for the enzyme to catalyze the next round of dehydration.

Experimental Section

Materials. Fumarase A was purified from *E. coli* strain JRG 1905 to near homogeneity as previously described.[2a] $H_2{}^{18}O$ (95–97% enriched), $^{2}H_2O$ (99.9% enriched), and $NaB^{2}H_4$ (98% enriched) were obtained from MSD isotopes. (2*S*)-[2-^{18}O]malate and (2*S*,3*R*)-[3-^{2}H]malate were prepared by hydrating fumarate respectively in $H_2{}^{18}O$ and $^{2}H_2O$ with porcine fumarase (Sigma). After the hydration reactions had reached equilibrium, the reaction mixtures were heated at 95 °C for 5 min to inactivate porcine fumarase, then the mixtures were filtered through a membrane filter (Amicon) with a 10 000 molecular weight cut off. The filtrates were lyophilized, dissolved in $^{1}H_2{}^{16}O$, and used with the fumarate in the equilibrium mixture still present. Porcine fumarase was completely removed by the heat and filtration treatment since no exchange of the ^{18}O or ^{2}H labeled malate occurred over several weeks in $^{1}H_2{}^{16}O$. The isotopic enrichment of the [2-^{18}O]malate was found to be 92% and the [3-^{2}H]malate was 99%.

Enzyme Assays and Transfer Experiments. Fumarase was assayed spectrophotometrically in the fumarate to malate direction by following the decrease in absorbance at 300 nm (ϵ = 0.033 mM^{-1}).

In the ^{18}O transfer experiments, 50 μL mixtures containing 10 mM [2-^{18}O]malate, 10 mM potassium phosphate buffer, pH 7.5, and 0–100 mM acetylenedicarboxylate were prepared in 1 mL conical polypropylene vials. Sufficient fumarase A was drawn up into a pipette to hydrate the acetylene dicarboxylic acid present in a given vial in 1 s (0.05–0.65 units of fumarase A were added depending on the amount of acetylenedicarboxylate present). The enzyme solution was carefully pipetted as a bolus onto the inside wall of the vial above the level of the substrate mixture. The vial was then vortexed, which commingled the fumarase A with the substrate mixture. Two seconds after the vortexing started, 100 μL of 50 mM $NaB^{2}H_4$ in water was added, and the vortexing continued for another 2 s. The contents of the vials were left overnight at room temperature open to the atmosphere. The H^{+} generated by H_2CO_3 formed as CO_2 diffused into the solution from the atmosphere exhausted the excess $NaB^{2}H_4$. Small pieces of solid CO_2 were added to each vial after sitting overnight, which served to adjust the pH to 7.5–8.0. Next, sufficient porcine fumarase was added to each vial to be able to interconvert all the malate and fumarate present in 1 min. Then this mixture was incubated for 100 min to insure the exchange of ^{18}O out of (2*S*)-[2-^{2}H,2-^{18}O]-malate was complete. (2*R*)-[2-^{2}H,2-^{18}O]Malate was not effected because neither fumarase A nor porcine fumarase dehydrate the 2*R* isomer of malate.[6]

In the ^{2}H transfer experiments, 50 μL mixtures containing 10 mM (2*S*,3*R*)-[3-^{2}H]malate, 10 mM potassium phosphate buffer, pH 7.5, and 0–100 mM acetylenedicarboxylate were prepared in 1-mL conical polypropylene vials. Sufficient fumarase A to hydrate the acetylenedicarboxylate in approximately 20 s was added directly to the mixtures with vortexing (0.0025–0.03 units of fumarase A were added depending on the amount of acetylenedicarboxylate present). Five seconds later, 200 μL of 50 mM $NaB^{2}H_4$ in ethanol was added with vortexing to half of the samples. The same solution was added after 10 s to the other half of the samples. The addition of ethanolic $NaB^{2}H_4$ inactivated the fumarase A present in the samples within 1 s. The samples were also left open to the atmosphere overnight to exhaust the excess $NaB^{2}H_4$. No porcine fumarase was added to these samples.

The samples from the ^{18}O transfer and ^{2}H transfer experiments were dried by lyophilizing. Methanol/(80%)H_2SO_4(20%) (250 μL) containing 10 mM tartronic acid (added as a GC/MS standard) was added to each of the dried samples. The dimethyl esters of malate and tartronate were formed by incubating the stoppered samples for 3 h at 50 °C.

Analysis by GC/MS. The esterified samples were extracted into $CHCl_3$ by adding 2 volumes of $CHCl_3$ and 1 volume of H_2O to the methanol/H_2SO_4 mixtures. The $CHCl_3$ phase was removed, and 2 μL were injected into a HP 5890 gas chromatograph using a 30 m DB-Wax column (temperature programmed 50–200 °C at 10 °C/min) that was coupled to a HP 5970A mass selective detector set to monitor ions with m/z = 89, 102, 103, 104, 105, and 106. The mass spectra of the dimethyl ester of tartronic acid had a prominent ion with a m/z of 89. The amount of tartronic acid was measured by monitoring this ion.

Indirect Determination of Acetylenedicarboxylate. To identify and use the conditions which maximized ^{18}O and ^{2}H transfer in these experiments, it was helpful to know the amount of acetylenedicarboxylate remaining after the reactions were terminated. It would have been convenient to directly determine the amount of acetylenedicarboxylate by GC/MS; however, a useful ion to monitor this quantity was sought for, but not found. Either acetylenedicarboxylate does not survive the conditions for making the dimethyl ester, or its dimethyl ester is not stable under the GC conditions used.

Although it was not possible to directly measure the amount of acetylenedicarboxylate remaining in the samples by GC/MS, an indirect measurement can be made by subtracting the amounts of the malate species formed by the hydration of acetylenedicarboxylate from the amount originally present. The use of tartronic acid as an internal standard was helpful in this analysis. In ^{18}O transfer experiments, the malate species formed from acetylenedicarboxylate were [2-^{2}H]malate and the [2-^{2}H,2-^{18}O]malate, which both gave unique ions with a m/z of 104 and 106, respectively. A sum of these two species was subtracted from the amount of acetylenedicarboxylate originally present.

In the ^{2}H transfer experiments, the malate species formed from acetylenedicarboxylate were [2-^{2}H,3-^{2}H]malate and [2-^{2}H]malate. While the ion given by [2-^{2}H,3-^{2}H]malate (m/z of 105) could only arise from acetylenedicarboxylate, the ion given by [2-^{2}H]malate (m/z of 104) could also arise from [3-^{2}H]malate remaining in the sample. The amount of [3-^{2}H]malate present was readily found by subtracting the amount of normal malate formed (m/z of 103) from the [3-^{2}H]malate originally added. Then the amount of [2-^{2}H]malate could be found by subtracting the [3-^{2}H]malate present from the m/z 104 signal. The amount of [2-^{2}H]-malate formed was added to the amount of [2-^{2}H,3-^{2}H]malate formed, and this total was subtracted from the amount of acetylenedicarboxylate originally added to find the amount of acetylenedicarboxylate remaining.

It is important to note that the amount of acetylenedicarboxylate remaining did not enter into the ^{18}O or ^{2}H transfer calculations but was merely used to estimate the progress of the reactions. This was useful in optimizing the reaction conditions.

Acknowledgment. The authors would like to thank Dr. Irwin Rose for several enlightening discussions in the course of this work and to recognize him for the inspiration he has provided to a generation of enzymologists.

Supplementary Material Available: Data from ^{18}O transfer from malate with a control experiment using ^{16}O malate (Table 1) and data from 3H transfer from malate at 5 and 10 s (Table 2) (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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