How Fumarase Recycles after the Malate → Fumarate Reaction. Insights into the Reaction Mechanism†

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ABSTRACT: Recycling of yeast fumarase to permit repetition of its reaction chemistry requires two proton transfers and two conformational changes, in pathways that are different in detail but thematically similar in the two directions. In the malate → fumarate direction, simple anions such as acetate accelerate the fumarate-off step producing $E_{H(f)}$, a fumarate-specific isoform that retains the C3R-proton of malate. Fumarate specificity is shown with *S*-2,3-dicarboxyaziridine, which is competitive vs fumarate and noncompetitive with malate as substrate. The steady-state level of $E_{H(f)}$, based on K_{ii} (*S*-2,3-dicarboxyaziridine), is increased by D_2O and decreased by imidazole acting as a general acid for conversion of $E_{H(f)}$ to $E^H_{H(f)}$. $E^H_{H(f)}$ is fumarate-specific as shown by the inhibition pattern with ClO_4^-. The pK_a of this step is ∼7.25 based on the pH dependence of K_{ii} (ClO_4^-). A conformational change occurs next as shown by high sensitivity of k_{cat} but not k_{cat}/K_m, to the microviscosogen, glycerol, and change to a nonspecific isoform, $E^H_{H(mf)}$, probably the same species formed in the fumarate → malate direction from malate-specific intermediates by a different conformational change. Malate enters the cycle by reaction with $E^H_{H(mf)}$ and returns to $E^H_{(m)}$·malate after a second conformational change. When fumarate-off is slow, as in low anion medium, malate itself becomes an activator of malate → fumarate. This effect occurs with changes in inhibition patterns suggestive of the bypass of the slow $E_{(f)} \rightarrow E_{(mf)}$ conversion in favor of direct formation of $E_{(mf)}$ when free fumarate is formed. 3-Nitro-2-hydroxypropionate, a strong inhibitor of fumarase [Porter, D. J. T., and Bright, H. J. (1980) *J. Biol. Chem. 255*, 4772−4780] in its carbanion form, is competitive with *both* malate and fumarate. Therefore, 3-nitro-2-hydroxypropionic acid interacts with $E^H_{H(mf)}$ and not with $E_{(m)}$ or $E_{(f)}$ isoforms. Occurrence of two different conformational changes in the recycling process suggests that the reaction chemistry employs a two-step mechanism. The specificity of inhibition for $E^H_{H(mf)}$ is consistent with the expected intermediate of a carbanion mechanism, E^H_H· carbanion$^-$. The proton transfers and conformational changes of recycling occur in the same sequence that is expected for this reaction chemistry. Several examples of ligand-activated conformational changes are reported.

For any enzyme to undergo multiple turnovers requires an ability to recover from changes experienced in the course of the reactions performed. Generally, all protons that were acquired from the substrate or used to form the product must go to or come from solvent during the recycling phase. All structural changes in the enzyme must be "reversed" without participation of the reaction that promoted the changes in the first place. We know very little of the details of the recycling processes for any enzyme, but anticipate that efficient and orderly mechanisms must have evolved as part of the story of each of these extraordinary catalysts. Our purpose in these studies of fumarase has been to determine if its recycling is well orchestrated or chaotic and whether it has anything to tell us about the reaction itself.

The choice of fumarase (FumII) for these studies is based on a series of observations that show recycling rather than the reaction to be rate determining for k_{cat}. Malate (M)[1] that was recovered after only a small conversion to fumarate (F) in [^{18}O]water was found to be significantly labeled (*1*). Therefore, the product complex, E·H_2O·F, must return readily to M after H_2O exchange in competition with dissociation of F. Second, the β proton abstracted from M was easily captured by F and its analogues, indicating slow recycling of the conjugate acid group formed in the reaction (*2*). Third, a significant D_2O effect on k_{cat} noted in 1960 (*3*)

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[1] Abbreviations: M, malate; F, fumarate; PMA, pyromellitic acid (1,2,4,5-tetracarboxybenzene); DCAZ, S-2,3-dicarboxyaziridine; NHP, 3-nitro-2-hydroxypropionic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid. endo and exo refer to the position of a recycling step in the cycle, whether prior to or after the product release step, respectively. $\overset{\sim}{\rightarrow}$ refers to a step that brings a change in specificity; i.e., a conformational change.

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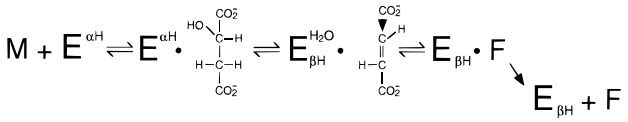
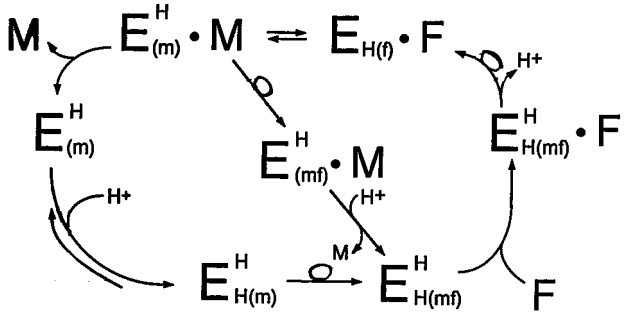
could not be originating from the fast reaction chemistry and might arise in recycling the acid proton that abstracts the α-OH group of M:



More recently, the presence of slowly equilibrating F- and M-specific free enzyme isoforms was suggested by counterflow experiments (*4*, *5*). Subsequently, the finding that k_{cat} effects of viscosogens (*6*) could occur without affecting k_{cat}/K_m (*7*) was attributed to the interconversion of M-specific and nonspecific isoforms that were identified using inhibitors of the F to M reaction. Not yet determined is the target of the nitro analogs of M whose strong inhibition in the carbanion form (*8*) has dominated discussions of the fumarase mechanism.

Scheme 1[a]



[a] Recycling options in the F → M direction. [F enters by reaction with nonspecific $E^H_{H(mf)}$].

Recent studies of recycling in the F → M direction are summarized in Scheme 1 (*7*). Recycling includes all the steps required to form E_H·F from the product complex E^H·M. Two pathways were found depending on whether a conformational change (⇢) preceded or followed release of M. Release of M is activated by a variety of simple anions such as acetate, Cl^-, SO_4^{2-}, and ClO_4^- in order of increasing sensitivity. Fumarate itself is an activating anion which explains the apparent cooperative kinetics with F in the absence of another activating anion. When M-off is rapid, an M-specific isoform $E^H_{(m)}$ is generated. This is followed by equilibrated protonation of the *β*-subsite, pK_a 6.75. The bis-protonated enzyme $E^H_{H(m)}$ undergoes a glycerol-sensitive conversion to a nonspecific isoform $E^H_{H(mf)}$. This conformational change is activated by P_i, N_3^-, and high Cl^- as shown by increase in k_{cat} with a decreased concentration of $E^H_{(m)}$. The specificity of intermediates was established by inhibition patterns with either M or F as substrate:mesotartrate, citrate, transaconitate, and pyromellitic acid (PMA) are competitive inhibitors with M indicating interaction with $E_{(m)}$, $E_{(mf)}$, or both. With F as substrate, these compounds produce a slope effect consistent with competition for $E_{(mf)}$ but also an intercept effect showing that they interact with a species with which F does not react, i.e., $E_{(m)}$. In a medium low in anions, glycerol was found to decrease $(k_{cat}/K_m)_F$ as well as k_{cat}. It was inferred that the glycerol-sensitive step preceded M-off when that step is slow. The expectation that $E_{(mf)}$ is then the initial free-enzyme product is confirmed in the present paper by studying the change in patterns of inhibition as a function of anion concentration.

As shown in Scheme 1, F enters the cycle by reaction with $E^H_{H(mf)}$. A proton transfer in the conversion of $E^H_{H(mf)}$·F to $E_{H(f)}$·F is responsible for $^D(k_{cat}/K_m)_F \approx 2$. There was no indication of a conformational change in this phase of the cycle from these studies.

In the present M → F study, two successive F-specific intermediates and a second conformational change are found on the way to $E^H_{H(mf)}$. We conclude that the sequence of interconversions of recycling intermediates that occurs in the absence of M and F resembles the sequence of conformational changes and H^+-transfers that occurs in the reaction chemistry through a carbanion intermediate.

EXPERIMENTAL PROCEDURES

All rates were measured at 25 °C in 1 mL containing cuvettes with 1 cm path length. Absorbance changes at λ = 240−270 nm due to F were recorded in the Spectronic 1001 of Milton Roy Co. The ϵ_λ values of Alberty et al. (*9*) were used. Rates of M → F were measured during the linear initial phase, usually within 2 min of addition of ∼1 pmol of fumarase. They are displayed in double reciprocal plots $(\Delta/\text{min})^{-1}$ vs $(\text{M, mM})^{-1}$. Effects of inhibitors and activators are characterized by their effects on intercept (as K_{ii}) and slope (as K_{is}) of linear double reciprocal plots. Care was taken to avoid long extrapolations in determining intercepts. Exposure of stock solutions to glass electrodes was avoided to restrict the introduction of Cl^-. Anions were used as their sodium salts.

The position of a step in the cycle whether prior to product release (an endo recycling step) or after product release and prior to binding of substrate (an exo recycling step) was determined by whether effectors of the step altered the slopes of double reciprocal plots. Thus, glycerol, a microviscosogenic reagent, increased the intercept and not the slope and, therefore, was concluded to act exo.

Materials. Yeast fumarase was prepared and generously provided by Dr. J. Keruchenko of the Institute of Biochemistry RAS, 33 Lenisky pr, 117071, Moscow, Russia. Many of the physical properties have been reported (*10*). *S*-2,3-Dicarboxyaziridine (DCAZ) was obtained from the laboratory of Dr. N. Yumoto, Institute of Microbial Chemistry, Microbial Chemistry Research Foundation, Tokyo, Japan. 3-Nitro,2-*R*,*S*-hydroxypropionate (NHP) was the generous gift of Dr. Dennis Flint, DuPont Research Center, Wilmington, DE. In using NHP, the carbanion was allowed to form at the final incubation pH for at least 1 h at 25 °C. $(NHP)^-$ was determined using ϵ_{243nm} = 9360 M^{-1} cm^{-1} according to Porter and Bright (*8*). The total carbanion concentration was divided by four to approximate the 2-R-isomer with the effective ionization stereochemistry.

The D_2O used throughout was the low T product of Aldrich.

RESULTS

F-Specific Isoforms and Their Interconversion. S-2,3-Dicarboxyaziridine (DCAZ) was previously shown to be a competitive inhibitor with respect to F using heart fumarase (*11*). When tested in the M → F direction with the yeast enzyme at pH 7.5 and 100 mM acetate, DCAZ affects both

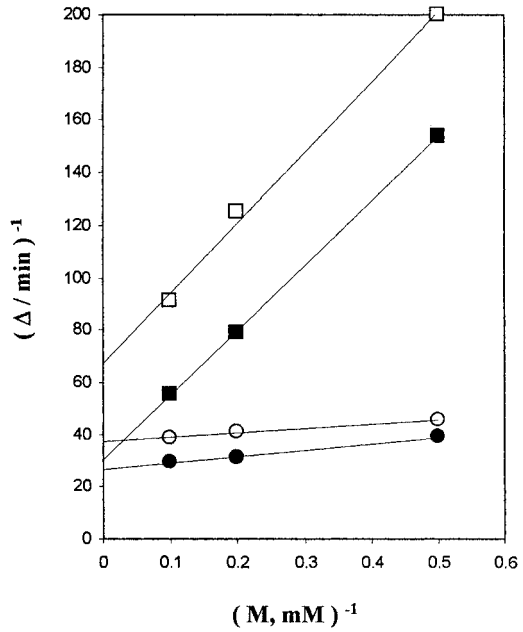


FIGURE 1: Intercept and slope effects of DCAZ and imidazole. DCAZ (20 μM) (□, ■), 100 mM imidazole (■, ●), or neither (○) in 100 mM acetate and 50 mM MOPS (pH 7.5).

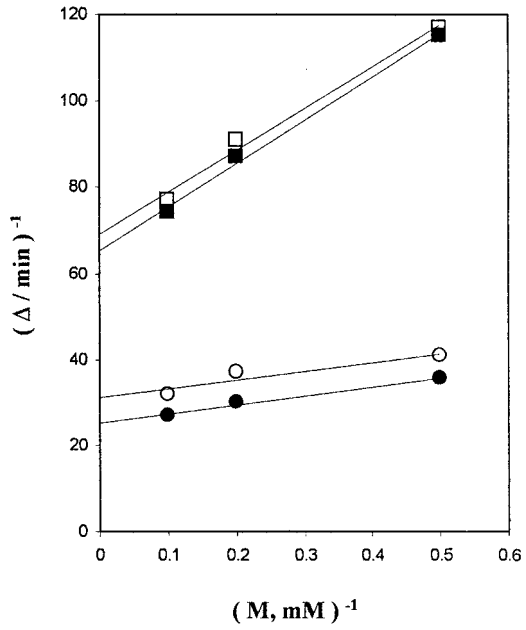


FIGURE 2: Intercept and slope effects of ClO_4^- and imidazole. ClO_4^- (30 mM) (□, ■), 100 mM imidazole (●, ■), or neither (○) in 100 mM acetate and 50 mM MOPS (pH 7.5).

slope and intercept of the v^{-1} vs M^{-1} plot (Figure 1). Thus, DCAZ interacts with both an F-specific isoform, $E_{(f)}$, and a nonspecific isoform, $E_{(mf)}$: $K_{ii} = 29$ μM and $K_{is} = 1.25$ μM, that is, it requires 29 μM DCAZ to double the intercept but only ~1 μM to double the slope. Either the affinity of DCAZ for $E_{(mf)}$ is much greater than that for $E_{(f)}$ or there is much more $E_{(mf)}$ in the steady state. Since the $C_\beta H$ of M can be captured after formation of F by an analogue of F (*2*), the first enzyme produced is designated $E_{\beta H(f)}$, indicating its specificity for F. DCAZ does not interact with any of the M-specific isoforms, $E_{(m)}$s, previously described, since when tested against F as substrate, DCAZ was strictly competitive ($K_{is} = 0.22$ μM, in 100 mM NaCl at pH 7.5).

Also shown in Figure 1 are the effects of imidazole on rate in the presence and absence of DCAZ. Imidazole alone at 100 mM is a very weak activator, ~1.3×. In the presence of 20 μM DCAZ, imidazole caused much greater activation, 2.1×, as if the step activated had become more rate limiting in the presence of inhibitor. The concentration of $E_{H(f)}$ is 10-fold decreased by the imidazole as judged by the K_{ii} ~290 μM of DCAZ. Thus, imidazole increases the recycling of $E_{H(f)}$. Absence of an effect on slope shows that imidazole acts before the M-on step of the next cycle.

To determine whether the activation by imidazole is an acid or base buffer effect, it was compared with 2-ethylimidazole, pK_a 6.9 and 8.0, respectively, at pH values of 7.5 and 8.4. At pH 7.5, both buffers produced the same 1.9-fold maximum activation, but imidazole was 2.9× more efficient, close to the 3.3× value expected for acid catalysis. At pH 8.4, the activations were about equally sensitive to the concentration of the buffers. This also suggests acid catalysis since imidazole with its ~10-fold greater acidity is only ~10% as concentrated in its acid form than is ethyl imidazole at this pH. Similar results with imidazole also indicated acid catalysis in the F → M direction (*7*). Thus, we are presented with the unusual observation of acid catalysis in both directions, suggesting different proton-transfer steps in the two directions.

A second inhibitor that reacts with $E_{(f)}$ and $E_{(mf)}$ is ClO_4^-, which in 100 mM acetate is also noncompetitive with respect to M, $K_{ii} = 31.6$ mM, $K_{is} = 7$ mM, at pH 7.5 (Figure 2). At a concentration of imidazole that decreased the inhibition of DCAZ for $E_{H(f)}$ 10-fold, the ability of ClO_4^- to inhibit was not decreased but rather increased ~25%, $K_{ii} = 23$ mM decreased from 31.6. Taken with the observation that imidazole, acting as an acid catalyst decreases $E_{H(f)}$, this suggests that protonation of the α-site without a specificity change produces the ClO_4^- target $E^H_{H(f)}$. This is confirmed by studies in D_2O and by determining the effect of pH on K_{ii} (ClO_4^-).

As indicated in the F → M study (*7*), the release of H^+ from the α-subsite is slowed by D substitution. In the M → F direction, this isotope, derived from D_2O, should lead to an increase in $E_{H(f)}$ and decrease in $E^H_{H(f)}$ in the steady state. These changes would be seen as a decrease in K_{ii} with DCAZ and an increase in K_{ii} with ClO_4^- in D_2O. With DCAZ, K_{ii} decreased dramatically, 30 to 2 μM, a remarkable D_2O effect (Figure 3). Although D_2O had no effect on slope in the absence of DCAZ, a 3-fold slope increase is observed in its presence. It was not immediately clear how the presence of the inhibitor could transform a proton-transfer step from an exo to an endo position in the overall cycle. On the other hand, D_2O caused a small increase of 15−30% in K_{ii} of ClO_4^- without causing a significant effect on slope (not shown). Thus, the effects of D_2O on the concentrations of $E_{H(f)}$ and $E^H_{H(f)}$ were inverse, consistent with the $E_{H(f)} \rightarrow E^H_{H(f)}$.

To estimate pK_a of the α-subsite, the effect of pH on K_{ii} (ClO_4^-) was determined in the presence of imidazole (200 mM) to bring the $E_{H(f)} \rightleftarrows E^H_{H(f)}$ step close to equilibrium as judged by the effect on K_{ii} (DCAZ) (Figure 1). As shown in Figure 4, K_{ii} (ClO_4^-) increased 2-fold at pH 7.24 from a minimum value of 5.2 mM. A plot of log E_H/E^H_H vs pH has a slope of ~0.89. The K_{is} values, also shown in Figure 4,

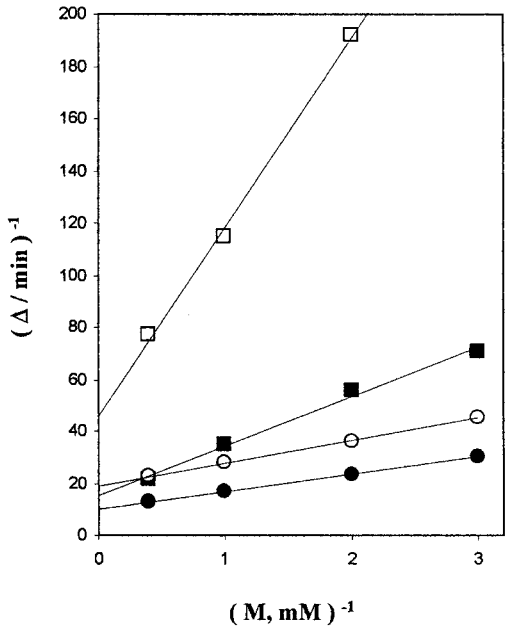


FIGURE 3: D_2O effects on intercept and slope, influence of DCAZ. DCAZ (6 μM) (□, ■) in either H_2O (■, ●) or 95% D_2O (□, ○) in 100 mM acetate and 10 mM MOPS (pH 7.5).

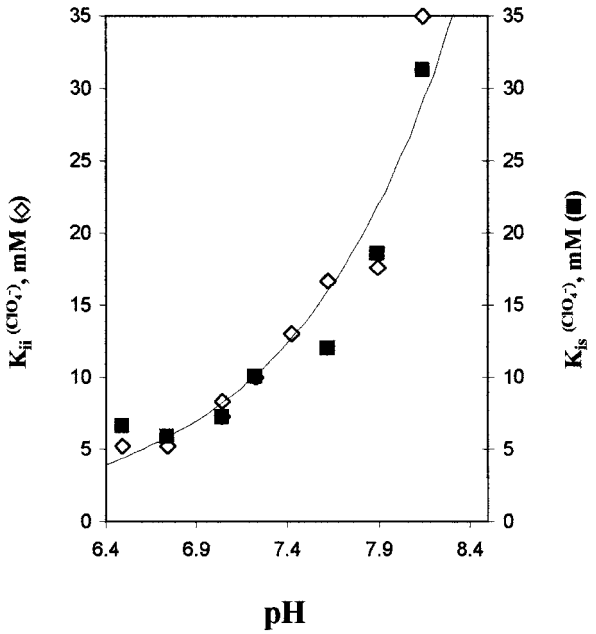


FIGURE 4: Effect of pH on K_{ii} ClO_4^- and K_{is} ClO_4^-. Inhibition constants were determined in 200 mM imidazole and 200 mM acetate adjusting pH with NaOH. The exponential best line is drawn for the K_{ii} values.

are very similar to the K_{ii} values with a similar dependence on pH and a similar pK_a.

A Conformational Change in M → F Recycling. The ability to demonstrate $E^H_{H(f)}$ by its interaction with ClO_4^- at high concentrations of M means that a slow step intervenes between $E^H_{H(f)}$ and a species with which M can react to continue the cycle toward $E^H_{(m)}$·M. This change of $E^H_{H(f)}$ to an isoform with which both M and F can react is very sensitive to glycerol: the maximum M → F rate in the presence of 100 mM each of imidazole and acetate is slowed ~3.6- and 10-fold by 30 and 40% glycerol, respectively. The effects of glycerol on slope were <1.6-fold at M concentrations of <10 mM. This recalls the glycerol sensitivity of $E_{(m)} \rightarrow E_{(mf)}$ in the F → M direction: both occurring with changes in specificity and both with very small effects of glycerol on slope, consistent with an exo-recycling step. However, the conformational changes are not related: inorganic phosphate, N_3^-, and SCN^- are good activators of $E^H_{H(m)} \overset{o}{\rightarrow} E^H_{H(mf)}$ as shown by their effect on K_{ii} of mesotartrate. No activators have been found for $E^H_{H(f)} \overset{o}{\rightarrow} E^H_{H(mf)}$. It follows that, even at saturating M, most of the enzyme will be in the unliganded $E_{H(f)}$ and $E^H_{H(f)}$ forms, depending on pH.

Endo Conformational Changes in Recycling F → M. It was concluded previously (*7*) that a glycerol-sensitive step in the F → M direction occurs *after* the release of M from E^H·M in high-anion medium. $E^H_{(m)}$ or $E^H_{H(m)}$ are the immediate free enzyme products depending on the pH. In a low-anion medium, when M-off is slow, 15% glycerol inhibited <2-fold but caused a 3.6-fold increase in slope indicating rate limitation either before or coincident with the M-off step. Assuming the two glycerol effects to be related, it was inferred that a conformational change prior to M-off could occur when M-off is slow. The dominant slope effect indicated that the major enzyme product could react with both M and F. This inference is now confirmed by comparing the pattern of inhibition for PMA in low- and high-anion medium. PMA, although a strong anion, was chosen because its low K_{ii} allows it to be tested at low concentration. As previously shown, PMA is a sensitive noncompetitive inhibitor vs F in 100 mM NaCl, K_{ii} 27 μM. When tested with only the anions of 10 mM MOPS buffer and with <1 mM F used as substrate, K_{ii} of PMA increased to >270 μM, as though $E^H_{(m)}$ was insignificant in the steady state. At the same time K_{is} decreased from ~150 μM to ~8 μM showing that E_{mf} had emerged as a significant steady-state recycling intermediate much greater than its level in the exo pathway where it is preceded by the slow conformational change. Thus, the conformational change may occur before or after M-off and the glycerol effect on slope in low-anion medium is not related to rate-limiting diffusion in the M-off step.

Activation by Anions and by Malate. In the F → M direction, the increase in k_{cat}/K_m that results with all anions tested, including F itself, is due to activation of the M-off step (*7*). Thus, anions decreased the slope of double reciprocal plots, changed the glycerol effect from endo to exo, and as shown in the previous section, changed the nature of the first free enzyme form from E_{mf} to E_m. In the M → F direction, anions activate if they do not have distinctive inhibitory effects as with ClO_4^-. Acetate, MOPS, and N_3^- are good activators, but Cl^-, SCN^-, and dicarboxylates do not activate M → F. Azide, except for its absorbance in the ultraviolet, is preferred for its complete lack of competition with low M. Figure 5 shows 2.7× activation at 0.5 mM malate by 30 mM N_3^-. An 8-fold decrease in slope is consistent with an effect on F-off, producing $E_{H(f)}$.

Activation by M, which is most evident in low-anion medium (Figure 5), is not an anion effect in the same sense. For one thing, notwithstanding a small competitive effect of acetate at low M, 100 mM acetate still caused a 2.6× decrease in slope in the 10−40 mM range of M where the M effect on rate is maximum. This would not be expected if M itself were acting as an anion. More to the point, it is

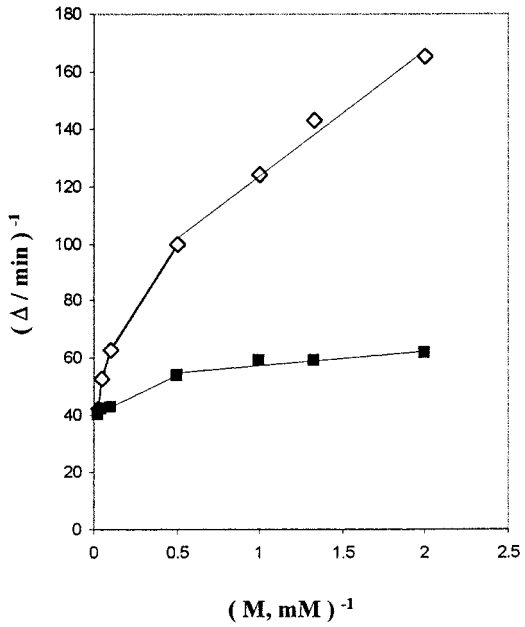


FIGURE 5: Activation by high malate and by anions (N_3^-). (◇) Cooperativity is shown with M > 2 mM in 10 mM MOPS (pH 7.4). (■) 30 mM N_3^- lowers the slope at low M showing its effect on F-off.

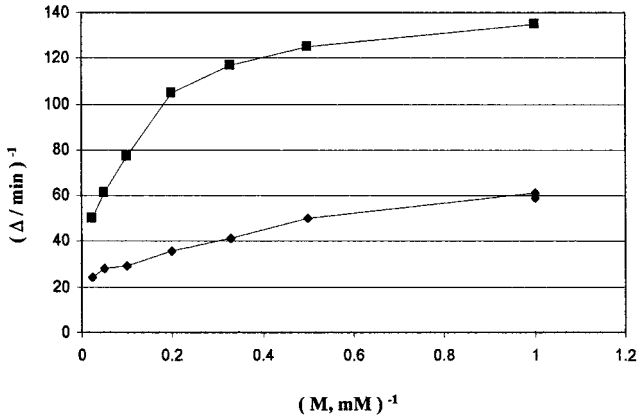


FIGURE 6: Rate-limiting proton-transfer becomes endo at high M/low salt (10 mM MOPS, pH 7.0). A higher slope effect is seen in D_2O (■) than in H_2O (◆) at M > 2 mM.

observed that high M favors the formation of $E_{(mf)}$ rather than $E_{(f)}$ as the first free-enzyme product: as shown in Figure 3 in the low M range with anions present and again in Figure 6 with only 10 mM MOPS, there is little or no slope change in D_2O, showing that proton transfer to the α-subsite occurs prior to the binding of substrate M with E_{mf}. However, at M > 2 mM where activation begins, there appears to be a 6× D_2O effect on slope (Figure 6). Apparently, H^+ transfer to the α-subsite now *follows* the binding of substrate. This would be the case if M as activator promotes the following sequence:

$$E_{H(f)}\cdot F \overset{\curvearrowright}{\rightarrow} E_{H(mf)}\cdot F \rightarrow E_{H(mf)} + F$$

This conclusion is confirmed by comparing the inhibition pattern, K_{ii}/K_{is}, ratio of DCAZ in the high M range in the presence and absence of acetate. With no other anion than the 20 mM serine buffer, pH 8.5, the ratio 190/1 (μM/μM) translates to a very low level of $E_{H(f)}$ and high $E_{H(mf)}$. With the addition of acetate (250 mM), the ratio shifts to 27/5 not far from the result at low M plus acetate. Therefore, it appears that acetate overcomes the high M effect, stimulating the F-off rate, presumably not allowing time enough for the conformational change.

Happily, the conformational change of the $E_{H(f)}$·F complex suggests an explanation for the observation made in Figure 3 of a large slope increase caused by D_2O if DCAZ is also present. Given that DCAZ is an analogue of F, $E_{H(f)}$·DCAZ will not be a dead-end complex but will be capable of a conformational change that bypasses $E^H_{H(f)}$ as follows:

$$E_{H(f)}\cdot DCAZ \overset{\curvearrowright}{\rightarrow} E_{H(mf)}\cdot DCAZ \rightarrow E_{H(mf)} + DCAZ$$

The larger slope effect with D_2O would result since the higher level of $E_{H(f)}$ in D_2O would lead to a greater rate of the above shunt and a level of $E_{H(mf)}$ that would more than replace the fall in $E^H_{H(mf)}$, formed in a slow conformational transition. A second source of the increased slope effect in D_2O could be that protonation of the α-subsite in $E_{H(mf)}$·M is now endo.

In a related experiment, the small slope effect of glycerol was found to be significantly increased in the presence of DCAZ. Glycerol would increase $E_{H(f)}$ by slowing the flux to $E^H_{H(mf)}$. $E_{H(f)}$·DCAZ would be greater in glycerol, about 70% greater based on the change in intercept with 20 μM DCAZ between 0 and 25% glycerol. This would increase the bypass of the slow $E^H_{H(f)} \overset{\curvearrowright}{\rightarrow} E^H_{H(mf)}$ step in favor of the ligand-activated conversion $E_{H(f)} \overset{\curvearrowright}{\rightarrow} E_{H(mf)}$. The increase in $E_{H(mf)}$ would result in an increase in slope, also about 70%.

Inhibition by (NHP)$^-$. Porter and Bright (*8*) found that the carbanion form of NHP was a very strong inhibitor, competitive with respect to M using the pig heart enzyme. This was interpreted in favor of a carbanion intermediate or transition state. It was of interest to determine which of the recycling intermediates has such a high affinity for $(NHP)^-$. Competitive inhibition vs M (also observed with the yeast enzyme, $K_i \approx 1.6 \times 10^{-6}$ M) would be consistent with interaction with both $E_{(m)}$ and $E_{(mf)}$ or either one alone and would only exclude the $E_{(f)}$s. As shown in Figure 7, inhibition is competitive with F also as substrate, $K_i \approx 2.1 \times 10^{-6}$ M. Therefore, $(NHP)^-$ does not interact with $E_{(m)}$, only with $E^H_{H(mf)}$. This result, competition with both substrates, confirms the conformational uniqueness of this species based on the K_{is} values observed with all the inhibitors.

DISCUSSION

The effects of anions on fumarase kinetics have a long and inconclusive history (*12*, *13*) which can now be clarified in terms of different steps in the recycling pathway that are specifically affected. The role of many simple anions on the off rate of M, first identified in 1997 (*7*) and now in the M → F direction as well, may provide another example of the importance of electrostatic attraction between substrates and active sites (*14*). The simple anions that produce k_{cat}/K_m enhancements may be acting to neutralize attractive positive charges in the region of the active site, promoting the dissociation of the anionic products. In both directions, the anion effects are maximum at low pH ~6.5 and disappear above pH 8 (not shown) consistent with this view. Although previously attributed to diffusion limitation by an associated glycerol effect on slope (*6*), this is evidently not the

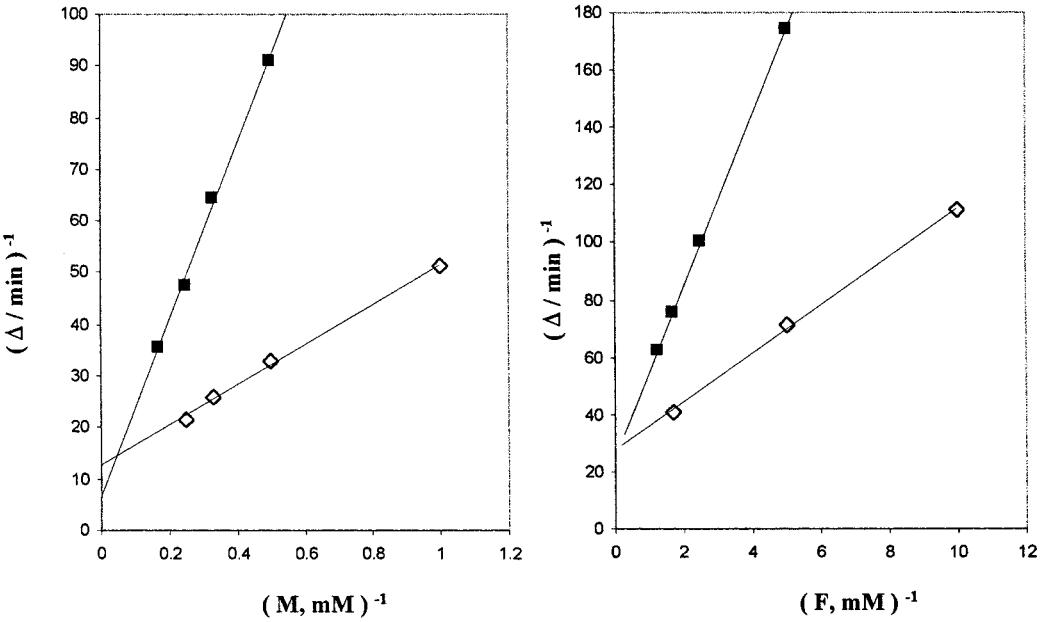


FIGURE 7: Inhibition by nitro-hydroxypropionate: 50 mM serine (pH 8.6) and 100 mM NaCl (with F) or 100 mM acetate (with M) with (■) 22 μM $(NHP)^-$ or without inhibitor (◇).

explanation since the slope effect disappears with activating anions and the glycerol effect can be explained by a conformational change that can precede product release (*7*).

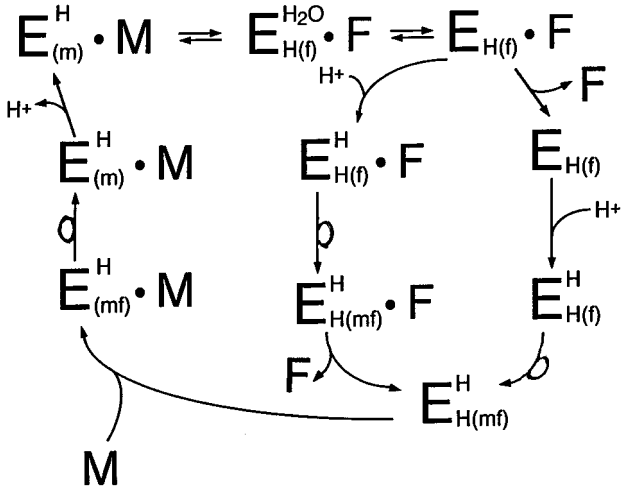
An alternate pathway for the release of F that is activated by M may have a similar electrostatic explanation: an allosteric binding site for M, ~12 Å from the active site, was identified by crystallography of *Escherichia coli* FumC (*15*, *16*), a homologue of the yeast enzyme. The addition of two negative charges close to the active site would lower the barrier to separation of F in the M → F direction. Of course, if the allosteric site were present in $E_{(mf)}$·F but not $E_{(f)}$·F or if the dissociation of F from $E_{(mf)}$·F were less dependent on the presence of anions, conditions would favor the formation of $E_{(mf)}$.

As shown previously (*7*), Cl^- is an activator of fumarase in the F → M direction by action at two sites: at <100 mM, Cl^- facilitates the M-off step as do all anions. At higher concentrations, Cl^- is one of a small group (P_i > N_3^- > SCN^- > Cl^-) that activates $E^H_{H(m)} \rightarrow E^H_{H(mf)}$ from which one would suppose that it forms complexes with both $E^H_{H(m)}$ and $E^H_{H(mf)}$. In the M → F direction, Cl^- is an inhibitor. Using acetate to activate F-off, the addition of Cl^- inhibits by interaction with $E^H_{H(f)}$ with an affinity ~15× weaker than that of ClO_4^-. As shown by the loss of inhibition at high pH, Cl^- does not interact with $E_{H(f)}$. The only other anion found to inhibit by specific interaction with $E^H_{H(f)}$ is BF_4^- (K_{ii} = 40 mM compared with 10 mM for ClO_4^- at pH 7.25) which is isomorphous with ClO_4^-.

P_i and SO_4^{2-} are competitive with M. The origin of these effects remains to be determined. Although $E_{(m)}$ is believed to be bypassed by reaction of M with $E_{(mf)}$, $E^H_{(m)}$ will be generated in the M → F reaction during the partition of the central reaction complexes. This partition, determined by dilution of labeled substrates bound to excess enzyme (*2*), was ~2/1 in favor of M. This partition provides sites for competitive inhibition other than $E_{(mf)}$ in both directions.

What supports the interpretation that rate effects with glycerol are due to viscosity effects on conformational transitions? Foremost is the observation that rate effects with glycerol are coincident with transitions between isoforms with different ligand specificities. For example, in M → F, glycerol lowers V_{max} and increases $E_{H(f)}$ and $E^H_{H(f)}$, as shown by the decrease in K_{ii} for inhibitors specific for each. Therefore, the effect of glycerol is after $E^H_{H(f)}$ at a step that will allow M to react. Absence of a glycerol effect on slope rules out nonspecific effects on substrate and product binding, as well as the possibility of diffusion-limited rates in these steps.

Scheme 2[a]



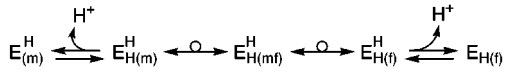
[a] Recycling options in the M → F direction [M enters by reaction with nonspecific $E^H_{H(mf)}$].

Scheme 2 shows the alternative routes from E_H·F to $E^H_{H(mf)}$: the primary route in which F-off is facilitated by anions includes the bis-protonated intermediate $E^H_{H(f)}$ which is formed in a D_2O-sensitive proton transfer to $E_{H(f)}$ activated by imidazole acting as an acid donor and followed by a slow conformational change to $E^H_{H(mf)}$ inhibited by glycerol. The secondary path that appears when F-off is not activated includes steps utilized in completion of the F → M recycling

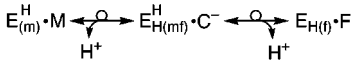
pathway (Scheme 1), in which F reacts with $E^H_{H(mf)}$ followed by a conformational change and the loss of the α-site proton to produce $E_{H(f)}$·F. This latter observation was demonstrated by a D_2O effect on the F → M rate that produced a significant increase in slope relative to the H_2O control. This result suggests that α-site protonation may precede the conformational change in the (f) → (mf) transition when it occurs prior to release of F as shown in Scheme 2. Likewise, completion of the M → F pathway occurs by reaction of M with $E^H_{H(mf)}$, the reverse of the endo sequence giving $E^H_{H(mf)}$ in Scheme 1. A functional complex must be formed with M as substrate to explain the slope effects seen with DCAZ, ClO_4^-, and NHP^- as inhibitors. There is no evidence for return by the exo route, $E^H_{H(mf)} \xrightarrow{o} E^H_{H(m)}$, etc., and no increase in the M → F rate by activators of that slow step, which is apparently bypassed even at low M.

Enzymes are said to perform two major functions in catalysis: binding the substrate which implies specificity for the substrate and lowering the energy of the transition state for conversion to an intermediate or product which implies a new specificity. The specificity change suggests that a conformational change may be coincident with all significant chemical changes. Finding that the recycling pathways of fumarase require two conformational changes in each cycle suggests that the reaction chemistry includes at least two steps. This is consistent with two steps joined by a common chemical intermediate. The state of protonation of the enzyme in the intermediate complex will depend on the reaction chemistry: E for a carbonium ion and E^H_H for a carbanion, depending on whether the hydroxyl of malate is abstracted before or after the proton. Identification of the isoform with which $(NHP)^-$ interacts strongly as E^H_H, based on the analysis of recycling in both directions, is therefore consistent with an enzyme·carbanion intermediate.

In the absence of substrates, the free enzyme oscillates through all the exo transitions of the recycling pathways:



The proton mobility in this sequence reflects the mobility in the following reaction sequence



The *β*-proton exchanges slowly in $E_{H(f)}$ as shown by its ease of capture by F and exchanges easily in $E^H_{H(m)}$, pK_a ~6.7. Thus, the *β*-subsite becomes a stronger base in the direction for abstracting the *β*-proton of malate. The α-proton is inert in $E^H_{H(m)}$ and dissociates in $E^H_{H(f)}$. Thus, the α-subsite becomes a stronger acid in the direction that would drive abstraction of the α-hydroxyl group of the carbanion intermediate. A shift in pK_a is proposed to be a major factor in promoting catalysis by a number of enzymes (*17*).

That two independent conformational changes are needed for recycling suggests that the two steps of the reaction impose structural changes on the enzyme that cannot be linked in a single step such as $E^H_{H(f)} \xrightarrow{o} E^H_{H(m)}$. The repertoires of conformational changes of which $E_{(f)}$ and $E_{(m)}$ are capable are not overlapping, except through a stable conformational state $E_{(mf)}$ which shows the transition in ligand specificity.

Several examples of ligand-activated conformational change have been observed in the present studies. The $E^H_{H(m)} \xrightarrow{o} E^H_{H(mf)}$ transition is well activated by 5 mM P_i and 10 mM N_3^- in F → M and, in unpublished results, inhibitory M analogues such as mesotartrate were shown to increase the recycling/counterflow ratio, presumably by increasing this same transition. Further examples are the activation of $E_{H(f)} \xrightarrow{o} E_{H(mf)}$ by DCAZ, and the absence of a slope effect in the F → M recycle which includes $E^H_{H(mf)}$·F $\xrightarrow{o}$ $E^H_{H(f)}$·F. The $E_{H(f)}$·F complex traverses its conformational and vibrational repertoire over two transition states to $E^H_{(m)}$·M more rapidly than the $E_{H(f)} \rightarrow E^H_{H(mf)} \rightarrow E^H_{(m)}$ conformational transitions. The effects of ligands in facilitating conformational transitions, so-called ligand catalysis, is probably due to the effect of the ligand in decreasing the time spent in unproductive conformational alternatives which, judging from the magnitude of the effects, may be considerable. Ligands that bind preferentially to conformational states not in the line of a productive sequence would be negative catalysts.

AFTERTHOUGHTS

One is struck by the similarity found in the recycling pathways in the two directions. Does this imply an evolutionary convergence? What are the relative advantages of the endo and exo systems? As an escape valve for the slow release of a product, endo-recycling steps provide a transition to less specific isoforms from which the product can be more readily released.

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