Proton Transfer in Catalysis by Fumarase†

Irwin A. Rose,* Jessie V. B. Warms, and Donald J. Kuo

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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ABSTRACT: Using 3T[^{14}C]malate it was possible to show intermolecular T-transfer to unlabeled fumarate. The rate of dissociation of ET derived from the malate was not rapid, only about as fast as required for k_{cat}^{M}. Because of the slow dissociation of ET derived from T-malate, the awkward complex ET·malate is readily formed. As shown by the effect of added malate on the partition of ET, otherwise captured by fumarate, ET·malate must be functional. Its rate of dissociation to E·M determines the V/K_m value of malate. Hydrogen dissociation of the complex ET·F was linearly related to the concentration and basicity of the buffer provided, varying from <10% to >60% of the overall rate with alkyl phosphonates. Partition of EH·F to free malate or fumarate occurs in a ratio ~2:1 at both low and high buffer. This agrees well with the comparison of the equilibrium exchange rates: malate with [^{18}O]water to malate with [^{14}C]-fumarate [Hansen, J. N., Dinovo, E. C., & Boyer, P. D. (1969) *J. Biol. Chem. 244,* 6270–6279]. Therefore, the abstracted hydroxyl group is fully exchanged from the enzyme when the bound hydrogen and fumarate return to malate and must be much more accessible to the medium than the abstracted proton. The fact that buffer increases the rate of proton transfer to the medium in the central complex makes it appear that a proton relay connects the active site donor with a remote site that interfaces with the ultimate proton source, water.

That the elements of water, H^+ and OH^-, used to hydrate the double bond of fumarate do not enter and leave together as a single water molecule was shown by Hansen et al. (1969), who found that exchange between the C-3 proton of malate and solvent was much slower than ^{18}OH/malate exchange during the early stages of malate → fumarate conversion. Although pointing to a carbonium ion intermediate, isotope-exchange studies at equilibrium showed that H^+/malate exchange was slower than fumarate/malate exchange (Hansen et al., 1969; Berman et al., 1971). Therefore, a basic residue for proton abstraction from malate and the need for a separate proton dissociation step in each reaction cycle were indicated. Evidence for a carbanion mechanism (strong inhibition by relevant nitroalkane anions; Porter & Bright, 1980) requires that the proton abstraction step is the first chemical step of the dehydration reaction. In this case, the absence of back labeling of malate by solvent protons indicates that the bound proton is stabilized through all stages of the reaction following its initial abstraction. That proton dissociation follows the release of fumarate was inferred from equilibrium exchange studies in which the proton exchange from malate decreased relative to fumarate/malate exchange at higher (fumarate + malate)$_{eq}$ concentrations (Hansen et al., 1969). This decrease in relative rate was attributed to intermolecular proton transfer, thereby conserving the hydrogen isotope. However, this effect was reversed above 5 mM fumarate (with 22 mM malate), indicating the possibility of more complex mechanisms for achieving dissociation than direct solvolysis of the donor proton at the EH state. Indeed, the 2–3 fold increase in V_{max} found with anions such as phosphate, citrate, and borate, in the mid 1950s by Massey (1953) and Alberty et al. (1954), may be related to buffer catalysis of proton transfer between solvent and the proton donor site rather than formation of new complexes. Also, a role for malate in proton dissociation may be anticipated.

To learn about the reactivity of EH, EH·F, and EH·M, we have used tracer amounts of doubly labeled 3T[^{14}C]malate to generate ET + [^{14}C]fumarate and unlabeled fumarate ± malate to determine their separate effects on the fate of the T. The appearance of T in water is followed along with the formation [^{14}C]fumarate as a measure of the total free ET formed. Transfer of T to unlabeled fumarate is calculated from the decrease in T-water relative to [^{14}C]fumarate formed. It is possible to calculate the rate ET → T^+ by determining the concentration of added unlabeled fumarate needed to trap half of this T. The effect of unlabeled malate at concentrations competitive with the fumarate is followed in the same way. A malate-dependent mechanism for proton dissociation avoids dead-end inhibition by malate, were it to bind before dissociation of the proton coming from the preceding reaction cycle.

Reviews of the fumarase mechanism (Anderson, 1991; Cleland, 1977; Rose, 1970) summarize isotope effect and isotope-exchange results that show the chemical interconversion on the enzyme to be at equilibrium. While it is common to assume that product release is rate-determining for V_{max}, we have evidence, to be presented in a subsequent paper, that shows the importance of free enzyme recycling. The present paper concerns interactions of reactants with E and E–H.

EXPERIMENTAL PROCEDURES

Methods

Partition of T from T[^{14}C]2S,3R-Malate. Intermolecular transfer is studied by generation of ET from a mixture of

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* Address correspondence to this author at Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111 [telephone (215) 728-2190; fax (215) 728-2412].

[1] Abbreviations: F and M refer to fumarate or fumarate concentration and malate or malate concentration depending on usage. F* and M* refer to ^{14}C-labeled F and M. T refers to ^{3}H which appears in malate, TM, or in water, Tw. TM* refers to T[^{14}C]malate. Tw, arising from particular reaction steps of Schemes I and II, is designated by the number of that step, except in the case of T_1 which is derived by the identical step as T_3 (Scheme I).

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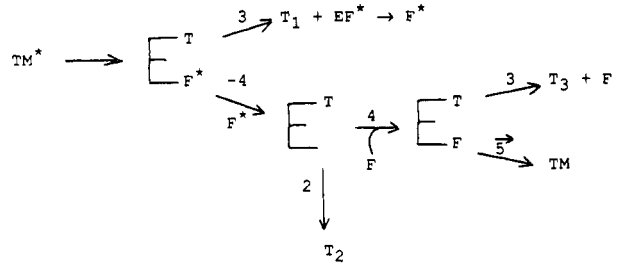
Scheme I



(3*R*)-3T- and 1,4[^{14}C]malate in the presence of varying amounts of unlabeled fumarate, TM* + F → TM + F*. Depending on the concentration of added fumarate and the occurrence of T-exchange from the various intermediates, the T that is utilized from TM* will appear in T-water, Tw, derived from the processes designated in Scheme I as T_1, T_2, and T_3, or will be captured by the added fumarate into malate as TM. Three partitions are represented: (1) T_1 vs $T_2 + T_3 + TM$ represents k_3/k_{-4}; (2) T_2 vs $T_3 + TM$ are possibilities for ET depending on k_2 vs $(k_{cat}/K_m)_F \times F$; and (3) T_3 vs TM represents the alternative routes of ET·F to fumarate and malate, respectively, $k_3/\vec{k}_5$. T_1 is an indicator of k_3 because the subsequent step, E·F* → F*, is much faster than E·F* + H^+ → HM* + E, as is shown by failure to back-label malate in T-water in the absence of fumarate. *This fact and the absence of isotope discrimination in utilization of T-malate* (Alberty et al., 1957; Fisher et al., 1955; Hansen et al., 1969) *allow the [^{14}C]fumarate and T-water produced to be used as a measure of T captured by fumarate*: *TM = F* − Tw*.

T-water, present in an acid-quenched sample, was determined by passing through a column of Dowex 1 acetate anion-exchange resin. All of the radioactivity appearing in the effluent plus water wash of the column was shown to be volatile. [^{14}C]Fumarate present in the sample was determined by cocrystallization with added fumaric acid (120 μmol) in ~0.5 mL containing 0.5 M TCA. The centrifuged crystals were washed, dissolved by heating in water and recrystallized twice before being dissolved in 0.1 N NaOH, analyzed for recovery of fumarate by absorbance at 250 nm, $\epsilon_{250} = 1450\ M^{-1}\ cm^{-1}$, and counted to determine specific activity from which was calculated total activity. Recovery of fumaric acid was usually ~50% in this process, giving fumarate without contribution from the highly labeled malate.

At equilibrium only 18% of the initial [^{14}C]malate is found as [^{14}C]fumarate, $K_{eq} = 4.4$. Therefore, the M* → F* rate requires the label in fumarate to be corrected for the extent of progress toward equilibrium, *f*, by use of

$$\%\ M^* \text{ reacted} = -18 \times \ln(1 - f) \qquad (1)$$

The distribution of Tw into T_1, T_2, and T_3 under various reaction conditions will depend on the ratio of competing rates. Reactions were terminated when more than 90% of the ^{14}C remained in the initial TM*. Therefore, correction for reutilization of captured T in TM was not considered necessary.

Determination of $\vec{k}_5/k_{-4}$ (Scheme I). [^{14}C]Fumarate was incubated with excess enzyme such that all of the label should be at the equilibrium distribution of E·F* and E·M*. Their ratio is unknown but is expected to be closer to unity than the solution ratio of 1:4.4. For simplicity, we assume 1:1. Since the interconversion of the two bound forms is rapid relative to product release and since at low buffer concentration $k_3 < k_{-4}$ (to be shown below, Figure 4), the ratio $\vec{k}_5/k_{-4}$ will determine the distribution of label after rapid mixing with unlabeled F and M:

$$\text{liberated } M^*/F^* = k_5\ E{\cdot}M/k_{-4}E{\cdot}F \cong \vec{k}_5/k_{-4}$$

That almost all of the label was bound initially was shown by equilibrium dialysis and by obtaining almost the same distribution result with 2 times as much substrate in the initial equilibrium. This pulse (7 μL at pH 7.0 and 7.8 as indicated) was diluted 40-fold through a jet mixer with a solution containing unlabeled F and M (10 mM of each) and allowed 50 ms (>10 half-lives) before a second mixing with TCA. Labeled fumarate was recovered by crystallization with carrier as above. Label in malate was calculated by difference. Control experiments were done showing that the correct solution equilibrium could be obtained by this method when malate and fumarate were omitted from the chase. Results, found in Table I, show that the liganded enzyme at equilibrium dissociates malate and fumarate in a ratio of 2:1 over a range of conditions.

Table I: Release of (M*/F*) from $(EM^*/EF^*)_{eq}$[a]

pH	buffer (mM)	M*/F*
7.0	imidazole acetate (10)	2.04 (2.5 °C), 2.61 (36 °C)
7.8	propyl phosphonate (40)	2.10, 2.27
7.8	Tris acetate (10)	1.85, 1.91

[a] A pulse of 7 μL containing fumarase (~180 μM) and [^{14}C]fumarate (37 μM) at pH 7.0 or 7.8 was mixed at 22 °C (except as noted) with 280 μL of similarly buffered solution containing 10 mM each of unlabeled malate and fumarate through a jet mixer. Quenching was with TCA after 50 ms. Controls (no M or F in the chase) gave M*/F* = 4.4, 4.2, and 4.3 in the three experiments. Extent of variation in duplicates is shown at pH 7.8. Hansen et al. (1969) report a ratio of 1.6 for [^{18}O]water to [^{14}C]fumarate exchange into malate at equilibrium (0.1 M malate and 0.073 M fumarate in 0.1 M Tris acetate at pH 7.3). Berman et al. (1971) report ratios of ~2.0 at pH 7.0 and ~1.6 at pH 7.8.

A comparison of the ratio $\vec{k}_5/k_{-4}$ with the ratio of equilibrium exchange rates found by Hansen et al. (1969) and Berman et al. (1971) for malate/^{18}O water and malate/fumarate indicates the extent to which the –OH group of the primary intermediate complex E·H·OH·F equilibrates with solvent before partitioning to M and F. The close agreement (shown in Table I) means that whenever malate binds to the enzyme, it almost fully exchanges its C_2–OH before dissociating to free malate.

As will be shown next, knowing that $\vec{k}_5/k_{-4} = 2$ makes it possible to calculate the other partition ratios and the absolute value of k_2.

Determination of T_1 and T_3 at High F. The experimental value Tw/TM derived from Tw/(F* − Tw) can be used to determine T_1/TM and T_3/TM when, at high F, $T_2 \sim 0$. From Scheme I

$$T_1/(T_3 + TM) \equiv k_3/k_{-4} = \vec{k}_5/k_{-4} \cdot (k_3/\vec{k}_5) \qquad (2)$$

The value $\vec{k}_5/k_{-4} \cong 2$, assuming $(E{\cdot}M^*/E{\cdot}F^*)_{eq} \sim 1$ as reported above. From eq 2

$$T_1/(T_3 + TM) = (T_1/TM)/[(T_3/TM) + 1] = 2(T_3/TM)$$

Substituting

$$T_1/TM = (Tw/TM) - (T_3/TM) \qquad (3)$$

$$(T_3/TM) = 0.25[9 + 8(Tw/TM)]^{1/2} - 0.75 \qquad (4)$$

Determination of k_2. At intermediate concentrations of F additional T will appear in the water as T_2 (Scheme I). At

F = K_c, $T_2 = T_3 + TM$ and $k_2 = (k_{cat}/K_M)_F K_c$. The corresponding value for Tw/TM is calculated as follows:

From Tw/TM as F → ∞ and eq 4, calculate $T_3/TM \equiv a$

$$\text{At low F} \quad T_1/(T_2 + T_3 + TM) = 2\,T_3/TM = 2a$$

$$T_1 = Tw - T_2 - T_3 \qquad (5)$$

Determine the ratio Tw/TM at which $T_2 = T_3 + TM$

$$Tw - T_2 - T_3 = 2a\,(T_2 + T_3 + TM)$$

$$Tw/TM = (2a + 1)T_2/TM + 2a^2 + a + 2a$$

In low buffer and high F

$$T_3/TM \cong 0.05 \text{ (Table II) and } 2a^2 \sim 0$$

Therefore $T_2/TM = (Tw/TM - 3a)/(1 + 2a)$ and

$$T_2 = T_3 + TM \text{ when } Tw/TM = 1 + 6a \quad (6)$$

The effect of a substrate analog on the partition of ET in competition with F can be quantitated and characterized by the same formalism used to evaluate T_2. If the analog does not cause T-dissociation when bound, it should have no effect on T_2/TM, although it may inhibit the rate of utilization of the labeled TM*. A ligand that binds competitively with respect to fumarate will increase the value of K_c and appear to increase k_2 if its presence on the enzyme allows the T to dissociate. Malate, by its effect on T-capture by F, will be shown to combine with ET. The new complex appears to be on the reaction path of M → F.

Materials

Pig heart fumarase was obtained from Sigma, ~450 units/mg using $A^{1\%}_{280} = 5.1$ for protein determination, or prepared by repeated crystallization according to the method of Kanarek and Hill (1964), ~600 units/mg. Standard conditions for assay were malate (50 mM) and potassium phosphate (50 mM, pH 7.9) at 25 °C. Activity was measured by following increase in absorbance at 250 nm, $\epsilon_{250} = 1450\ M^{-1}\ cm^{-1}$ (Bock & Alberty, 1953). Enzyme concentration, determined by equilibrium dialysis with excess radioactive substrate, agreed with $\epsilon^{1\%}_{280} = 5.1$ and a specific activity of 450 units/mg for the Sigma enzyme. The home-made enzyme was used only in repetition of Table II. Fluorofumarate was the generous gift of Robert L. Hill and was subsequently prepared from 2,2-difluorosuccinic acid (Raasch et al., 1959). [1,4-^{14}C]Malate was prepared by reaction from [^{14}C]fumarate and fumarase. The remaining labeled fumarate was removed by several ether extractions of the acidified solution. 3T-Malate was made in the same way by reaction in T-water. Labeled dicarboxylic acid was recovered by elution from Dowex 1 acetate with 0.1 N HCl.

All other reagents were derived commercially.

RESULTS

There are three pathways by which the enzyme used in the conversion of malate to fumarate may be recycled. Two of these are the usual alternative sequence of product release, H or F released first, shown as steps 3 and –4 in Scheme I. A third path will be shown to use malate itself to facilitate recycling of the EH form: EH + M → EH·M → EM + H$^+$.

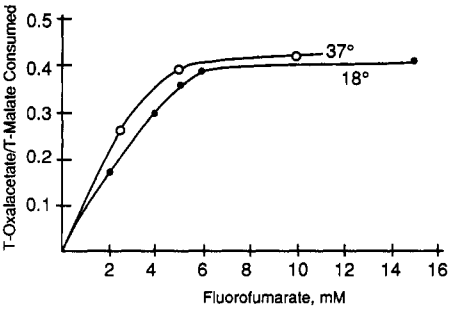
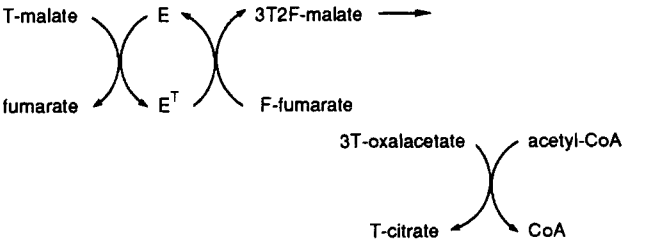


FIGURE 1: Intermolecular T-transfer to fluorofumarate. Twenty-microliter incubations at 18 or 37 °C contained triethanolamine acetate (50 mM, pH 8.0), 3T-malate (2.8 nmol, 1.3 × 10^4 cpm), F-fumarate (5 mM), citrate synthase (2.5 units), acetyl-CoA (7.5 mM), and fumarase (0.045 unit). Stop with TCA at 60 min. Determine T-water and T-citrate as counts not retained or retained on Dowex 1 acetate. Reaction was complete since no further T appeared in water following additional incubation with a large amount of fumarase. Without F-fumarate all the counts were in T-water. Without enzyme all the counts were retained by the column.

Evidence for EH. Direct evidence for the sequence EH·F → EH + F was obtained by showing intermolecular T-transfer using monofluorofumarate as acceptor (Teipel et al., 1968) and T-malate as donor. The major product of the transfer, 2-fluoromalate, decomposes rapidly to oxalacetate that was recovered in citrate by coupling with acetyl-CoA and the citrate condensing enzyme:



As shown in Figure 1 about 40% of the T that was activated could be trapped. The remainder was found as T-water. The same result was obtained using transaminase and glutamate to trap the oxalacetate as aspartate or, if Dowex 1 acetate in the cold was used to quickly remove 3T-oxalacetate, once formed, from the incubation. In all cases enough fumarase was used to convert all of the T-malate in the presence of increasing amounts of F-fumarate at the time of sampling.

Failure to trap more than half of the T of the malate may relate to the kinetics of processing the ET·F-fumarate complex. The same partition ratio was found at 18 and 37 °C, suggesting that the result might not come from a competition of two rates, exchange and transfer. Marletta et al. (1982) showed that 3F-malate is formed from mono-F-fumarate at about 6% the overall rate, apparently derived from a less active complex. Assuming no barrier to its formation, this longer-lived complex would not only be less effective for T-capture but could be responsible for loss of the enzyme-bound T. According to Figure 1, about 2 mM fluorofumarate is required to capture half of the T of ET that could be trapped, much greater than the K_m of 27 μM (Teipel et al., 1968) and greater than required by fumarate.

Dissociation Rate of E–H. Fumarate itself was shown to capture T derived from T-malate. For these experiments a combination of independently labeled T and [^{14}C]malates at low concentration was used. The rate of appearance of T in water and ^{14}C in fumarate as a function of the concentration of unlabeled fumarate was used to measure the partition of

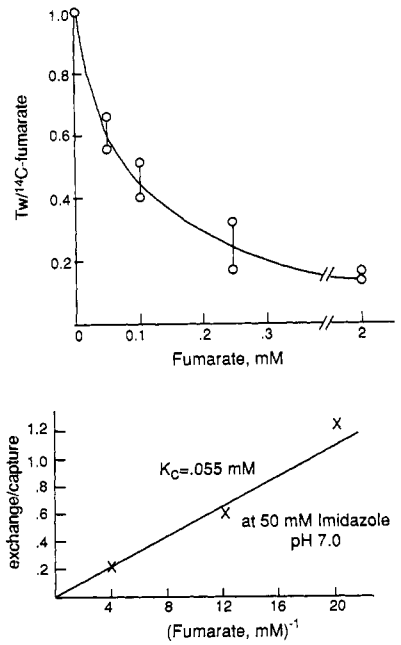


FIGURE 2: Capture of T of malate by fumarate. 3T[^{14}C]malate (6 μM, $\sim$3 $\times$ 10^4 cpm each), BSA (1 mg/mL), imidazole acetate (50 mM, pH 7.0), fumarate (as noted), and fumarase (0.6–5 milliunits) were incubated at 25 °C. Samples taken at 5 and 10 min were quenched with TCA. T in water (not absorbed by Dowex 1 acetate) and ^{14}C in fumarate (by crystallization) were determined. The data of part A (top) are used for part B (bottom) after correction for T_1 formed before ET is generated ($^2/_3$ of 15%) to determine the concentration of fumarate required to capture half the ET that could be trapped as T_3 + TM, the capture constant, K_c.

ET between Tw and TM (Scheme I). In the absence of fumarate, T-water and [^{14}C]fumarate were formed at the same rate, again indicating no discrimination between the 3H and the 3R,3T species of malate. Therefore, in all experiments with added fumarate the [^{14}C]fumarate minus T-water formed represents T trapped by the fumarate, TM. Enzyme and fumarate were increased together to compensate for the competitive effect of increasing fumarate on the utilization to the labeled malate. Many experiments of this kind were done in which pH and buffer concentration were varied. In all cases, curves similar to those in Figure 2A were obtained with variations in the limit amount that could be captured, $[TM/[^{14}C]fumarate]_{F\to\infty}$, and in the concentration required for 50% capture. Figure 2B shows the influence of F on the partition of ET at pH 7.0 as determined from Tw/TM at intermediate and high F.

Two conclusions may be drawn from these experiments. It is clear from Figure 2 that as much as 85% of the T-malate utilized can be captured at high fumarate, much more than could be trapped by F-fumarate. The observation that $\sim$15% of the T cannot be captured requires that exchange or dissociation of T occurs from the ET·F complex. There are two sites where this must occur: before F* dissociates to produce ET and after ET·F has been reconstituted with the unlabeled F (Scheme I). The first of these results in T_1 from the unavoidable partition required to form ET and will be generated in each experiment such that $k_3/k_{-4} = T_1/(F^* - T_1)$. The second source of Tw, using enough fumarate to decrease ET toward zero, is T_3, which results from the partition $k_3/\vec{k}_5 = T_3/TM$. Since $\vec{k}_5$ is $2k_{-4}$ as shown in Table I, the 15% in Tw consists of 10% as T_1 and 5% as T_3. Since 85% is present as TM, $k_3/\vec{k}_5 = 0.05/0.85 = 0.059$ and $k_3/k_{-4} = 0.10/0.90 = 0.11$.

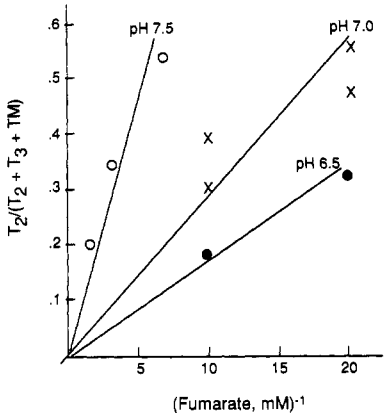


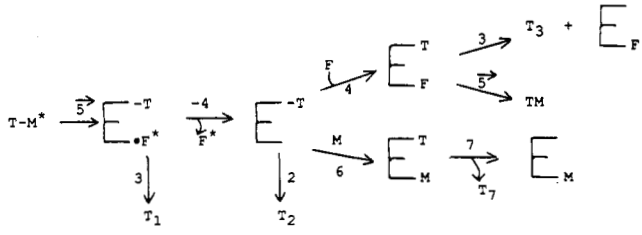
FIGURE 3: Effect of pH on T-capture. Conditions were similar to those of Figure 2, except that imidazole chloride (5 mM, at pH 6.5, 7.0, and 7.5) was the buffering agent.

Figure 2A allows an estimate of k_2 using the steady-state parameters $(k_{cat}/K_m)_F$ and a value K_c, the capture constant, the concentration of F at which $T_2 = T_3 + TM$, i.e., $T_2 = {}^1/_2(F^* - T_1)$. The value Tw/TM at which $F = K_c$ is given in eq 6. Using 50 mM imidazole adjusted to pH 7.0 with acetic acid, K_c for fumarate was $\sim$40 μM, similar to the K_m determined for fumarate under the same conditions, 80 μM. There are no primary isotope effects in the catalytic $(V/K)_M$ and $(V/K)_F$ values (Alberty et al., 1957), so that $k_2 \sim 0.5k_{cat(F\to M)}$. The ratio of maximum velocities, $V_m^{M\to F}/V_m^{F\to M}$ was determined under these conditions to be 0.35. Therefore, $k_2 \cong 1.4k_{cat(M\to F)}$ or $\sim$700 s^{-1} based on $k_{cat(M\to F)}$ = 500 s^{-1} determined. From this the loss of the abstracted proton that is required for recycling the enzyme in the M $\to$ F direction is only marginally fast enough, or possibly within experimental error of being fully rate-determining. From this it also must follow that T appears in water from ET by dissociation and not by exchange. Although this satisfying result suggests that no other pathway need by considered for recycling of the enzyme, after the dehydration of malate an alternative pathway of *equal* efficiency becomes dominant at high malate.

Figure 3 tests the influence of hydroxide concentration on ET $\to$ E + T in a very lightly buffered medium (5 mM imidazole chloride at pH 6.5, 7.0 and 7.5). The concentration of fumarate required to capture half of T_2, $T_2 = T_3 + TM$, increased about 5-fold (K_c = 34, 57, and 180 μM, respectively). When corrected for the decrease in $(V/K_m)_F$ with increasing pH (Frieden & Alberty, 1954), the increase in k_2 at pH 7.0 and 7.5 is only 1.2- and 1.8-fold relative to that at pH 6.5. Therefore, there is not much hydroxide effect on the dissociation rate at pH 7.5.

Interaction of Malate with EH Is Functional. Malate is known *not* to be inhibitory at a concentration as high as 300 mM (Anderson, 1980). In view of the calculated rate of EH $\to$ H, $\sim 10^3$ s^{-1}, the high value for $(V/K)_M \sim 10^7$ M^{-1} s^{-1} determined requires either that malate at >0.1 mM is not recognized by EH or, once formed, EH·M undergoes proton dissociation at least as rapidly as the maximum rate of its conversion to fumarate and is not a dead-end complex. In the second case one expects the fraction of the T utilized that is trapped by fumarate to be lowered when malate is available to compete for ET. To test this, malate was added in equilibrium amount, 4.4 $\times$ F, using $F > K_c$. Were malate to react with ET and simply interfere with dissociation of T, a condition that would produce inhibition of the steady-state rate of M $\to$ F, it would have *no* effect on the ratio Tw/

Scheme II



[^{14}C]fumarate formed from T[^{14}C]malate since the rate constants that determine the partition of ET would be the same as in the absence of malate. For ET·M to be functional, additional Tw would have to be produced. The magnitude of the increase can be predicted from steady-state parameters if ET·M → E·M + T, k_7, either is rate-determining for fumarate production or follows a slow step 6, Scheme II.

Tritium in water will include the irreducible minimum, T_1, and the T_3 and T_7 that result from the competition of fumarate and malate, respectively, for ET. At $F > K_c$, T_2 will become negligible. If F and M are added to the TM* in an equilibrium ratio, they should compete *equally* for ET if they compete on the basis of $(V/K)_F$·F and $(V/F)_M$·M, since at equilibrium, according to the Haldane equation for fumarase (Alberty et al., 1954), these values will be equal. Therefore, assuming k_6 or k_7 to be rate-limiting in the single turnover of malate to fumarate, T_7, the T in water due to malate should be equal to (T_3 + TM). Were a later step rate-limiting, T_7 would be greater than T_3 + TM. This expectation should hold whatever the pH of the study since the Haldane relation is known to pertain at all pHs (Alberty, 1954; Frieden & Alberty, 1955). Equations required to calculate T_1, T_3, and TM from counts in water and [^{14}C]fumarate formed are found under Methods to which is added: $T_7 = Tw - T_1 - T_3$. Results are shown in Figure 4 using 2 mM fumarate and low buffer concentration from pH 5.5 to 8.0. A low ratio of T_3/TM is evident over the whole pH range in the absence of malate and presumably in its presence as well. Adding malate at 8.8 mM increases Tw/TM significantly. The ratio $T_7/(T_3 + TM)$ was close to unity at pH 6.5–8.0, indicating that $\vec{k}_{6,7}$ is rate-determining for $(V/K_m)_M$. The possibility that the malate-dependent increase in Tw is due to malate acting as a buffer activator of $E_F^T \rightarrow E_F + T$ seems to be ruled out by negative experiments with high amounts of sodium acetate. Below pH 6.5 a step following step 7 must be rate-determining. Reproduced in Figure 4 is a plot of (V/K_m) as a function of pH due to Frieden and Alberty (1955). Of course, the (V/K_m) values in both directions are represented by the same curve when normalized for the 4.4-fold vertical displacement. It is of possible interest that both (V/K_m)s decrease below pH 6.5, the pH at which a new step subsequent to EH·M → E·M apparently becomes rate-limiting. It may be consistent with this that the ratio $T_7/(T_3 + TM)$ doubles at about pH 5.7, where V/K_m is half its maximum value. The detritiation of ET·M continues to be rate-determining for $(V/K_m)_M$ above pH 6.5 at least unitl pH 8, where V/K_m has fallen to about 10% of the value at pH 6.5 and V_{max} remains high. It would seem that the resulting large increase in the K_m of malate with pH above pH 7 (Frieden & Alberty, 1954) is due to a decrease in k_7 at high pH or to a decrease in the concentration of the reactant EH·M for step 7. Evidence for the latter will be presented in a following paper (I. Rose, J. Warms, and R. Yuan, submitted for publication).

The partition $k_3/\vec{k}_5$, measured as T_3/TM at high fumarate, is lowest at pH 6 and 6.5 at 0.02 and 10-fold higher at pH

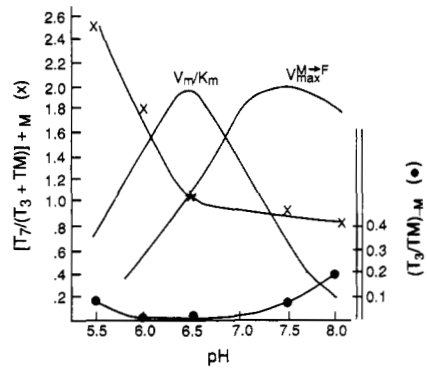


FIGURE 4: Competition by malate and fumarate for ET. Incubations at 26 °C contained 3T[^{14}C]malate and BSA as in Figure 2 with fumarate (2 mM) alone (●) or with malate (8.8 mM). Buffers, 5 mM each, were cacodylate (pH 5.5 and 6.0), imidazole (pH 6.5 and 7.5), and Tris (pH 8.0). pH adjustments were with NaOH or HCl. V_{max}^M and $(V_{max}/K_m)_M$ curves of Frieden and Alberty (1954), determined at low buffer concentrations, are reproduced here to show their sensitivities to pH in relative terms.

8, where V_{max} has just begun to decline from a peak value at 7.5 [data of Frieden and Alberty (1955) reproduced in Figure 4]. This relative increase in k_3 may indicate the beginning of hydroxide activation of the ionization of EH·F, which will next be shown to be very buffer-sensitive. However, a decrease in the concentration of E_M by a pH-sensitive equilibrium, similar to that which was just suggested for EH·M, may result in a decrease in $\vec{k}_5$.

Buffer Effects on Proton Dissociation from EH·F and EH·M. The experiments reported to this point have been done in low-buffer medium, 5–10 mM Tris acetate. Under these conditions the V_{max} values were shown to be increased by addition of buffer ions such as phosphate (Massey, 1952; Alberty et al., 1954). The most effective activation was reported to be from pH 6.5 to 8, where step 6 or 7, rate-determining for $(V/K_m)_M$, may also be rate-determining for V_{max}. The second proton dissociation step, EH·F → E·F + H, while not important at low buffer concentration, may also be activated by buffer. To this extent E, rather than EH, would be the product form of the enzyme that recycles at any concentration of malate. The same techniques used above for analyzing the malate effect were used to measure the influence of methyl phosphonate buffer ($pK_a = 7.1$) in the presence and absence of malate (Table II). Very similar results were obtained with the preparation of enzyme from Sigma and with enzyme that was used within a day of its preparation.

During the T-transfer experiment at 2 mM fumarate, i.e., $T_2 \sim 0$, the influence of buffer on the alternative dissociations from ET·F, k_3/k_{-4} (Scheme I), is calculated from $T_1/(T_3 + TM)$. A linear plot of this ratio vs buffer concentration was obtained (not shown) with ~20 mM methyl phosphonate causing a 10-fold increase. This effect is also seen in the increase in T_3/TM with increasing buffer. These buffer effects on k_3 do not correlate with their effects on V_m (Table II). The same large effects of buffer on k_3 are seen in the presence of malate as expected. One would like to be able to determine the effect of buffer on $k_{6,7}$ which, as shown in Figure 4, is rate-limiting for $(V/K_m)_M$ at low buffer. This analysis is obscured by the requirement that $(V/K_m)_M$ and $(V/K_m)_F$ must be influenced in parallel. It can be said, however, that buffer activation of k_7, if it occurred, was never so great that a subsequent step became rate-limiting for $(V/K_m)_M$. This would have caused an increase in $T_7/(T_3 + TM)$ in going to a higher buffer concentration, and this is seen only in comparing the 0 and 10 mM buffer results and none of the others. The

Table II: Effect of CH_3–PO_3 on Detritiation of ET·F and ET·M

additions		determined		calculated (%)					
CH_3–PO_3	F/M (mM/mM)	$v_{max}^{M\rightarrow F}$	Tw/TM	T_1	T_3	T_7	TM	$T_7/(T_3+TM)$	k_3/k_{-4}
0	2/0	1.0	0.15	8.8	4.2	0	87		
	2/8.8		0.79	8.4	2.7	33	56	0.56	0.1
10	2/0	1.4	0.9	35	13	0	52		
	2/8.8		3.5	35	6	37	22	0.9	0.54
20	2/0	1.5	1.4	43	16	0	42		
	2/8.8		4.5	43	7	32	18	1.3	0.75
40	2/0	1.6	3.3	60	17	0	23		
	2/8.8		6.6	60	11	16	14	0.67	1.5

[a] One-milliliter incubations at 25 °C contained 3T/[^{14}C]malate (~3 × 10^4 cpm each), BSA (1 mg), Tris acetate (5 mM, pH 7.8), and methyl phosphonate (pH 7.8), fumarate, and malate as noted. Fumarase (from Sigma) was added to convert about 1% ^{14}C to fumarate in 5 min. $v_{max}^{M\rightarrow F}$ was determined under the same conditions with 30 mM malate, well above its K_m of 0.67 mM in 40 mM methyl phosphonate. The values given are relative to the rate without CH_3–PO_3. [^{14}C]Fumarate and Tw were determined and values for T_1 and T_3 calculated in the absence of M using eqs 3 and 4. The percent of T in T_1 and the ratio T_3/TM were assumed not to have changed with M present. T_7 was therefore determined as 100 – (T_1 + T_3 + TM).

results of Table II were significantly the same using newly prepared enzyme.

Buffer Activation of ET·F → T⁺ + E·F Is General Base Catalyzed. To determine the character of the buffer catalysis, it was most convenient to observe effects on ET·F, i.e., using sufficient fumarate to prevent dissociation of ET. The buffers used were methyl, ethyl, and propyl phosphonate, added to 5 mM Tris acetate, final pH 7.8. Positive slopes of Tw/[^{14}C]fumarate vs buffer concentration were obtained in each case. As shown in Table III, the effect is best described as general base catalysis by the dianion form of the buffers.

DISCUSSION

The fumarase reaction cycle has seemed to be rather unapproachable following the extensive initial rate studies of Alberty and his group and kinetic isotope effect evidence that the chemical interconversion process on the enzyme is too rapid to be broken down into its component steps. Speculation that malate release might be rate-limiting was offered to explain the similar values for ^{18}O-, T-, and ^{14}C-exchange into malate at equilibrium (Hansen et al., 1969). Two suggestions from earlier works have provided insights on which the present work is based. These are the nonspecific anion activation of maximum velocities studied by Massey and Alberty's group in the mid 1950s and the extensive equilibrium isotope-exchange studies of Boyer's group during 1969–1971 that showed that exchange between water protons and the C-3 proton of malate became significantly lower than that of fumarate with malate as the concentrations of substrates at equilibrium were increased. This was attributed to the capture by free fumarate of EH competing with dissociation, a hypothesis that has been verified (Figures 1 and 2). An unexplained aspect of these earlier experiments, however, was the reversal of this effect that began above ~3 mM fumarate, when malate was present at >10 mM. Under these conditions TM → Tw exchange was decreased to about 30% of the M* → F* exchange. This is greater than the 15% found here with fumarate alone, but it is likely that the additional loss of T was due to formation of ET·M and k_7 (Scheme II). Higher concentrations of both substrates at equilibrium would have the effect of decreasing the loss of T due to trapping by fumarate and increasing the loss due to the malate. The new complex, ET·M, is functional. Its loss of T is by dissociation, not exchange, since its rate is no greater than predicted from $(V/K_m)_M$. This pathway avoids possible inhibition by malate in excess of its K_m concentration. A similar phenomenon was found for citrate and aconitase in which bound citrate allows the dissociation of EH that is formed in a previous cycle in which *cis*-aconitate is formed (Kuo & Rose, 1987).

Table III: Specific Base Activation of $E_{\cdot F}^{T} \rightarrow E_{\cdot F} + T^+$ [a]

buffer	pK_a[b]	slope[c]	origin of effect[d] base[e]	acid[f]	total[g]
CH_3–PO_3	7.10	0.073	0.85	0.16	0.28
$C_2H_5PO_3$	7.85	0.26	1.0	1.0	1.0
$C_3H_7PO_3$	8.18	0.41	1.1	2.5	1.6

[a] Incubations at 22 °C contained T[^{14}C]malate (trace), fumarate (2 mM), Tris acetate (5 mM), and the noted buffers at 0, 10, and 20 mM. The pHs were adjusted to 7.8 before fumarase was added and samples taken at early times to determine Tw/(TM + Tw). [b] Jencks and Regenstein (1968). [c] Δ(Tw/[^{14}C]fumarate)/10 mM buffer. [d] Relative to ethyl phosphonate. [e] Slope/fraction of buffer as base at pH 7.8/$10^{\Delta pK_a}$. [f] Slope/fraction of buffer as acid at pH 7.8/$10^{-\Delta pK_a}$. [g] Slope/slope for $C_2H_5PO_3$.

The proton donor site of fumarase seems to be as accessible to solvent whether the enzyme is unoccupied by substrate or has malate in a functional position. This conclusion can be drawn for ET on the basis of the low amount of fumarate required for T-capture, $k_2 \sim k_{cat(F\rightarrow M)}$ (Figures 2 and 3), and for ET·M on the basis of the prediction of its proton dissociation rate from $(V/K_m)_M$ (Figure 4). The rate of loss of T from ET·F is ~10% of the dissociation rate of F, but its rate constant cannot be given without knowing the concentration of ET·F in the steady state. Its occurrence, at high rate in the presence of 40 mM propyl phosphonate buffer, is not accompanied by significant exchange back-labeling of malate in T-water (unpublished observations), indicating a very high rate for E·F → E + F. The report by Hansen et al. (1969) that T-exchange exceeded ^{14}C-exchange by about 20% at low equilibrium concentrations of substrates is not supported by this or by the result shown in Figure 2, where at F = 0, Tw/F* = 1.

Dissociation of T from ET·F is greatly increased by buffer (Tables II and III). Therefore, the proton donor residue must be accessible to buffer ions when the active site is occupied by either substrate. The active site ternary complex E·H·OH·F must be accessible to solvent to explain the back-labeling of malate in [^{18}O]water. Hansen et al. (1969) and Berman et al. (1971) found equilibrium exchange ratios of 1.6–2 for [^{18}O]water vs [^{14}C]fumarate into malate. This is close to the partition of the central substrates complex to M*/F* (Table I), showing that in [^{18}O]water the intermediate ternary complex formed from unlabeled malate returns as [^{18}O]malate in every cycle. This can only mean that the hydroxide donor site (possibly a bound water molecule) is able to fully equilibrate with the medium in this complex. The similarity in the ^{18}O- and ^{14}C-equilibrium exchange rates is therefore based on the partition *ratio* of the central complexes and not on the rate of a single step. Absence of proton back-labeling of malate in tritiated water establishes that proton dissociation does not occur from the hydroxide-containing central complex as it does from EH·F. Any such exchange would be returned to malate with the ^{18}O. One could propose that buffer acts after the hydroxide-abstracting site has become open. The fact that malate does not block the site could be explained if

EH·M and EH·OH·F are conformationally different with respect to access to the medium.

Since OH^- and H come from opposite faces of the fumarate plane, it seems unlikely that dissociation of both occurs directly to the medium. More likely the buffers will be found to operate at a distance, presumably through a proton relay similar in function to the rate-determining proton relay established for carbonic anhydrase (Silverman & Lindskog, 1988). Pulse/chase experiments with fumarase in T-water mixed with high amounts of fumarate in normal water as the chase results in the capture of two enzyme equivalents of T (I. A. Rose and D. J. Kuo, unpublished observations). Therefore, a second proton with access to both the reaction center and the buffered medium is indicated.

Fumarase steady-state kinetics may be unusual in following different pathways depending on substrate concentration. This is not related to a second effector or cooperative effect of substrate. The kinetic order for malate is never greater than unity. The phenomenon arises when at higher concentration substrate acts before the second product, in this case H^+, has dissociated. The new complex may lose its product at an altered rate, causing stimulation or inhibition of the overall rate if a rate-limiting step is thereby altered. A further consequence of different pathways at low and high substrate concentration is that the V_{max}/K_m ratio, calculated in the usual way, contains rate constants derived from high substrate studies for V_{max}. In this case the low substrate concentration properties of the enzyme may not be correctly derived from V_m/K_m. However, it should be correct to use V_{max}/K_m to describe the competitive capacity of a substrate when the competition is done at high concentration.

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REFERENCES

Alberty, R. A., Massey, V., Frieden, C., & Fuhlbrigge, A. R. (1954) *J. Am. Chem. Soc. 76*, 2485–2493.
Alberty, R. A., Wilmer, W. G., & Fisher, H. F. (1957) *J. Am. Chem. Soc. 79*, 3973–3977.
Anderson B. (1980) *Biochem. J. 189*, 653–654.
Anderson, V. E. (1991) in *Enzyme Mechanism from Isotope Effects* (Cook, P. F., Ed.) pp 389–417, CRC Press, Boca Raton, FL.
Berman, K., DiNovo, E. C., & Boyer, P. D. (1971) *Bioorg. Chem. 1*, 234–242.
Bock, R. M., & Alberty, R. A. (1953) *J. Am. Chem. Soc. 75*, 1921–1925.
Cleland, W. W. (1977) *Adv. Enzymol. 45*, 273–387.
Fisher, H. F., Frieden, C., McKinley-McKee, J. S., & Alberty, R. A. (1955) *J. Am. Chem. Soc. 77*, 4436–4440.
Frieden, C., & Alberty, R. A. (1954) *J. Biol. Chem. 212*, 859–868.
Hansen, J. N., Dinovo, E. C., & Boyer, P. D. (1969) *J. Biol. Chem. 244*, 6270–6279.
Jencks & Regenstein (1968) *Handbook of Biochemistry*, J-150-189.
Kanarek, L., & Hill, R. L. (1964) *J. Biol. Chem. 239*, 4202–4206.
Kuo, D. J., & Rose, I. A. (1987) *Biochemistry 26*, 7589–7596.
Marletta, M. A., Cheung, Y.-F., & Walsh, C. (1982) *Biochemistry 21*, 2637–2644.
Massey, V. (1953) *Biochem. J. 53*, 67–71.
Porter, D. J. T., & Bright, H. J. (1980) *J. Biol. Chem. 255*, 4772–4780.
Raasch, M. S., Miegel, R. E., & Casstle, J. E. (1959) *J. Am. Chem. Soc. 81*, 2678–2680.
Rose, I. A. (1970) *Enzymes (3rd Ed.)* 281–320.
Silverman, D. N., & Lindskog, S. (1988) *Acc. Chem. Res. 21*, 30–36.
Teipel, J. W., Hass, M., & Hill, R. L. (1968) *J. Biol. Chem. 243*, 5681–5694.

Registry No. Fumarase, 9032-88-6; fumaric acid, 110-17-8; malic acid, 6915-15-7.