Biochemistry **1993,** *32,* 8504–8511

Role of Conformational Change in the Fumarase Reaction Cycle[†]

Irwin A. Rose,* Jessie V. B. Warms, and Richard G. Yuan

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

Received November 4, 1992; Revised Manuscript Received April 27, 1993

ABSTRACT: Activation of fumarase by high concentrations of either malate or fumarate, often referred to as negative cooperativity, can be explained without assuming additional sites of substrate action or subunit–subunit interactions. The following observations support a model based on a rate-dependent recycling of free enzyme through a sequence of conformational states that differ in substrate specificity and catalytic activity: (1) Displacement from equilibrium of a radiolabeled malate/fumarate probe is readily induced by moderate concentrations of either substrate. This phenomenon, called substrate-induced countertransport, indicates that the steady-state ratio of free enzyme forms is very dependent on substrate concentration. (2) Related to this, the back-labeling that can be observed with either ^{14}C product with either substrate in the steady state is more rapid than expected for a single free enzyme state model. (3) Fumarate, more strongly than malate, shows competitive effects as a product. This may reflect a higher affinity of fumarate for an isoform that also reacts with malate. (4) P_i, an activator of fumarase at midrange substrate concentration, overcomes strong competitive inhibition by fumarate of the M → F reaction and increases recycling as shown by its effect on counterflow. To the extent that these effects are due to buffer activation, they suggest that proton transfer between solvent and the enzyme site is important in determining the recycling rate. (5) Transaconitate, a competitive inhibitor, overcomes counterflow induced by either substrate, indicating that recycling events occur in the enzyme–transaconitate complex.

Fumarase, a homotetrameric protein, is significant among simple enzymes (a one substrate/one product reaction without cofactors) in the many *ad hoc* adjustments that have been required to explain aspects of its initial rate behavior. Additional sites have been proposed for both substrates to explain higher than expected rates at high concentrations, negative cooperativity (Alberty & Bock, 1953; Alberty et al., 1954; Hasinoff & Davey, 1986; Keruchenko et al., 1992), although binding studies have failed to detect more than one site per subunit (Teipel & Hill, 1968). Furthermore, a number of small anions such as inorganic phosphate (Massey, 1953; Alberty et al., 1954) have the unusual property of acting both as weak competitive inhibitors at low substrate concentration and as activators at high substrate concentration. Anion activation discovered for fumarase from *Escherichia coli* (Mann & Woolf, 1930), liver and muscle (Clutterbuck, 1928), and yeast enzymes (Keruchenko et al., 1992) appears to supplement the cooperative effect of high substrate, lowering the apparent K_m of substrate needed for activation. The present paper offers an explanation for these phenomena based on the observation that interconversion of substrate- and product-free enzyme forms is stepwise and rate-limiting for the reaction cycle. We conclude that the apparent negative cooperativity is not due to multiple binding sites for substrates but rather to changes in the interconversion steps that become rate-determining at different substrate concentrations. A similar explanation for apparent negative cooperativity with monomeric enzymes has been proposed by Cornish-Bowden and Cardenas (1987) and may explain supersaturation effects observed with other multimeric enzymes.

The concept that substrates induce structural changes in the course of binding to an enzyme and that different conformations result when products bind has often been verified by a variety of structural methods. Less clear in general is whether there are enzyme conformations in solution that are specific for the binding of substrate and others for the binding of product. The interconversion of these free enzyme forms would have to occur in each catalytic cycle. It seems likely that if substrate-specific conformers exist, their spontaneous interconversion might be slower than the changes that occur after the substrate is bound, lacking the inducing interactions that are provided by substrates. Rapidly reversible interactions at the active site with a variety of compounds might increase the rate of critical interconversion steps and therefore activate the catalytic cycle. Such substrate mimicking effects might be likened to substrate-induced transitions.

The fact that the fumarase reaction is close to being substrate-diffusion-limited at *low* substrate concentration (Alberty & Pierce, 1957), true for many enzymes, provides no information about the rate of the overall cycle, V_{max}, which must include the rate constants for the recycling steps. Isotope exchange studies with fumarase (Hansen et al., 1969; Berman et al., 1971) indicate that the reaction chemistry is rapid, a conclusion supported by failure to find kinetic isotope effects as the enzyme is usually studied (Anderson, 1991; Cleland, 1977; Blanchard & Cleland, 1980; Rose, 1970). Without evidence to suggest slow recycling it is usual in such cases to assume that product release steps are rate-limiting for V_{max}.

Recent studies (Rose et al., 1992) have concluded that both the donor-protonated, E^H, and unprotonated forms of fumarase are able to form competent complexes with either malate or fumarate (Scheme I).[1] The pathway using steps a–d, in the square, should be dominant at low substrate concentrations.

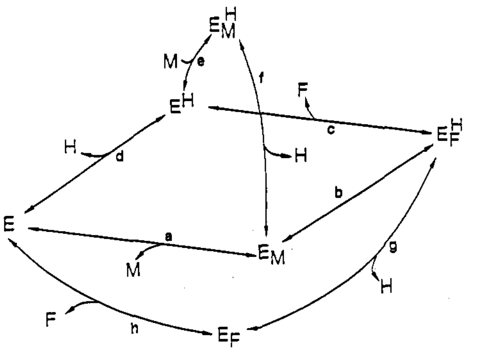
[†] This work was supported by National Institutes of Health Grants GM-20940 (I.A.R.), CA-06927, and RR-05539 (Institute for Cancer Research), and by an appropriation from the Commonwealth of Pennsylvania.

* Address correspondence to Dr. Irwin A. Rose, Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111. Telephone: 215-728-2190. Fax: 215-728-2412.

[1] Abbreviations: M, L-malate; F, fumarate; M*, [^{14}C]malate; F*, [^{14}C]fumarate; T, ^{3}H; S, substrate, P, product; S*, labeled substrate; P*, labeled product.

0006-2960/93/0432-8504$04.00/0

Scheme I



At very low substrate concentration product forms of the enzyme will have time to equilibrate before the next reaction cycle. From intermolecular tritium-transfer experiments, the dissociation of ET was found to be only $\sim 1.5k_{\text{cat}}$ or $\sim 600\ \text{s}^{-1}$ which is much too low to account for the turnover rates that are achieved at supersaturating levels of either substrate. With $(V/K)_{\text{M}} \approx 10^7\ \text{M}^{-1}\ \text{s}^{-1}$ (earlier estimates were done before fumarase was known to be a homotetramer), $\text{E}^{\text{H}}\cdot\text{M}$ would be formed from E^{H} at >0.1 mM malate. If such a complex were formed, it would have to be competent since neither substrate shows inhibition at very high concentration (Scott & Powell, 1948). The pathway efbc was indeed shown to occur when malate was found to compete with fumarate for ET, resulting in diversion to [T]water rather than to [T]malate. The partition of ET was consistent with the Haldane equation; *i.e.*, it was based on the relative V/K_{m} values of the two substrates. From this it was presumed that the malate-activated deprotonation is a component of the M → F conversion pathway at high malate. Both the efbc and hgba pathways are high substrate pathways in which proton dissociation/association occurs from liganded enzyme complexes. Scheme I does not explain activation by high substrate however. After all, Michaelis–Menten kinetics is commonly observed in random order pathways.

As is true for all reversible reactions, the central complex of reactants, in this case $\text{E}^{\text{H}}\cdot\text{F}$ and E·M, must partition in forming product and re-forming substrate. In the case of fumarase, malate and fumarate are formed in a ratio of ~2/1 (Rose et al., 1992) as determined from the partition of enzyme-bound ^{14}C-labeled reactants dissociating into a mixture of unlabeled substrates. This partition is similar to that reported for ^{18}O exchange from C-2 of malate relative to fumarate formation (Hansen et al., 1969; Berman et al., 1971), implying that –OH isotope exchange with water from E·malate is virtually complete before the 2 to 1 partition of the intermediate to free malate and fumarate occurs. These observations have led us to expect that instead of product release the recycling of nonliganded enzyme conformers, isoforms, may contribute substantially in determining V_{max} of reaction.

Three properties of the fumarase reaction cycle suggest the occurrence and importance of conformational isomerization in the recycling step(s) of the heart muscle enzyme. The first of these, substrate-induced countertransport (Britton, 1973), requires that substrate-specific isoforms of free enzyme in the reaction paths interconvert slowly enough for their relative concentration to be driven out of equilibrium by the passage of substrate to product. Countertransport will be demonstrated if when a probe solution of labeled substrates, malate and fumarate in this case at equilibrium, is added to a solution of enzyme that is catalyzing net product formation, the isotope ratio changes in reverse of the mass flow. Displacement of the tracer ratio from its equilibrium requires that the product and substrate react with different enzyme forms and that these forms are displaced from their equilibrium position by the net flux. Therefore, when studied under conditions that depend on the complete reaction sequence, it would seem that M* and F* are competing for different enzyme forms in the recycling process. The basis for this difference is not contained within the processes shown in Scheme I in which E and E^{H} are shown to react with both M and F.

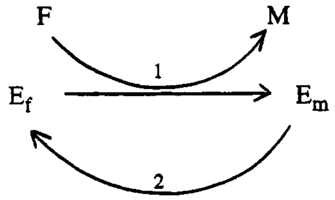
The observation of isotopic counterflow not only depends on slow recycling but also anticipates that the interconverting species are specific or relatively specific with respect to the two substrates. Real specificity would be shown if either M or F or both acted as noncompetitive inhibitors (Segel, 1975). In all the extensive kinetic literature on fumarase, one report (Taraszka & Alberty, 1964), not further pursued, suggests the importance of product accumulation terms other than V/K_{m} of product to the rate equation. The relevance of this study is difficult to evaluate on the basis of the data to be presented and in view of the low concentration range over which inhibition effects were studied by these researchers. As will be shown, the demonstration of conformational isomerization by absolute free enzyme specificity is not trivial in cases of apparent negative cooperativity. Nevertheless, evidence is offered that both fumarate and malate are noncompetitive product inhibitors by demonstrating that they cause back-labeling of substrates malate and fumarate, respectively, that cannot be prevented by substrate at high concentration. Changes in the ratio of back-labeling to net flux normalized for reactant concentration are suggestive of additional isoforms with differing substrate specificity ratios.

A consequence of the slow rate of conformational isomerization that occurs in every cycle is the observation that both V_{max} and V/K_{m} values associated with fumarate and malate depend on the concentration ranges over which these parameters are measured. This property will be interpreted as a reflection of changes in the distribution of free enzyme isoforms away from their equilibrium ratio and toward different steady-state distributions depending on the concentration of substrate and activators that are present.

METHODS

Theory

Isotope Counterflow Method of H. G. Britton (1973). It may be suggested in some cases that among the multiple forms of an enzyme in solution, varying in conformation, hydration, proton distribution, *etc.*, that different forms are generated in the forward and reverse directions of the reaction at the time of formation of product. In the case of fumarase, name the families E_{f} and E_{m} arising from the conversions M → F and F → M, respectively. This assumption is not inconsistent with conformational changes that may be *synchronous* with binding of either substrate. These same induced changes, also occurring *during* free product formation, together with the steps of catalysis make up the steps that are used for M/F isotope exchange at equilibrium. The isotope countertransport method of Britton (1973) depends on the possibility that in the direction F → M the ratio $(\text{E}_{\text{m}}/\text{E}_{\text{f}})_{\text{ss}}$ may be significantly greater than $(\text{E}_{\text{m}}/\text{E}_{\text{f}})_{\text{eq}}$ as would be the case when the first-order recycling rate $\text{E}_{\text{m}} \rightarrow \text{E}_{\text{f}}$ is not very much more rapid than the second-order counterflow rate $\text{E}_{\text{m}} + \text{M}^* \rightarrow \text{E}_{\text{f}} + \text{F}^*$. This is illustrated in simplified form in Scheme II. As long as F/M is greater than the solution equilibrium ratio, 0.22/1, and the recycling rate constants are not too rapid, eq 1 should be valid, and $k_{-1}\text{M}^*\text{E}_{\text{m}}$ will be greater than $k_1\text{F}^*\text{E}_{\text{f}}$. Therefore, Scheme II



$(M^*/F^*)_{ss}$ should be less than $(M^*/F^*)_{eq}$. An equation was derived by Britton (1973) and later by Albery and Knowles

$$(E_m/E_f)_{ss} = (k_1F + k_{-2})/(k_{-1}M + k_2) > (E_m/E_f)_{eq} \quad (1)$$

(1987) that relates the changes in the two measurable ratios product/substrate and labeled product/labeled substrate to a characteristic constant, α, that can be used to calculate the slower of the rate constants, k_2 or k_{-2}, of Scheme II. Equation 2 is a reformulation of these equations to give prominence to

$$(S^*/S_0^*) - 1 = K_{eq}(1 - f)[1 - (1 - f)^{\alpha \bullet S_0/(K_{eq}+1)}] \quad (2)$$

the parameter f that represents the fraction to which substrate has progressed toward equilibrium at the time of sampling, where $K_{eq} = (P/S)_{eq}$, S_0 is the initial concentration of substrate, S^*/S_0^* is the fold increase in label in S over that present in the equilibrium, and $\alpha^{-1} \equiv K_\alpha$ is a characteristic complex partition constant defined in eq 3. These equations apply to

$$K_\alpha^S(V/K_m)_S = k_2k_{-2}/(k_2 + k_{-2}) \quad (3)$$

an idealized model in which cross complexes $E_S{\bullet}P$ and $E_P{\bullet}S$ are either not formed or formed but not inhibitory. A variant of Scheme II in which more than two free enzyme states occur in a recycling sequence will result in a more complex counterflow equation as new states result in additional recycling pathways. This more complex model seems to apply to fumarase, and is responsible for very large extents of substrate activation such as ~8-fold with fumarase of *Saccharomyces cerevisiae* using high fumarate (Keruchenko et al., 1992).

It is not the purpose of the present investigation to develop a detailed model for the recycling pathways in either direction as a function of substrate concentration but rather to establish the presence of ligand-free enzyme states that are functional in the reaction cycle and differ in substrate specificity. Product inhibition is the classical approach to this kind of question using an equation such as eq 4 that defines competitive and

$$v_{M \rightarrow F} = V/[1 + (K_m^M/M)(1 + F/K_{is}^F) + F/K_{ii}^F] \quad (4)$$

noncompetitive binding constants for product fumarate, K_{is}^F and K_{ii}^F. Given the apparent cooperative activation by substrate concentration, both inhibition constants will be a function of substrate concentration except under the limiting conditions of low and infinite substrate at which unique K_{is} and K_{ii} values may be established, respectively. For the slow recycling model it will be important to eventually establish affinities for substrate, product, and allosteric effectors for each state and determine if any interconversions are subject to buffer catalysis.[2]

Reactions with Fumarase. The enzyme (EC 4.2.1.2) and the labeled reagents were the same as described in Rose et al. (1992). When assayed at 25 °C at pH 7.9 the enzyme from Sigma Chemical Co. gave a rate of 450 units/mg. The amount of enzyme was determined from $\epsilon_{280nm}^{1\%} = 0.51$ which was confirmed by dialysis equilibrium with excess [^{14}C]fumarate of known specific activity. This corresponds to a turnover rate of 363 s^{-1}. Standard conditions for assay in a 1-cm-path-length cuvette in the Gilford Response spectrophotometer maintained at 25 °C used malate (50 mM) and potassium phosphate (50 mM, pH 7.9). Results, similar to the ones presented with the Sigma enzyme were obtained with enzyme prepared by the method of Kanarek and Hill (1964), turnover rate of ~480 s^{-1}. Enzyme crystals, stored in ammonium sulfate, were dissolved in low buffer concentration medium, and passed twice through Sephadex G25 equilibrated with the same buffer. Reproducible results often depended on using the enzyme on the day of the desalting. Initial rate measurements were also made in [T]water in the F → M direction. A standard sample of [^{14}C]malate (500 cpm) was added after quenching with TCA. Labeled malates were recovered by elution from Dowex-1-acetate (1-mL column) with 0.1 N HCl from well-washed columns. Counts were determined after the eluates were dried *in vacuo*. The enzyme rate was calculated from the specific activity of the water, there being no T-isotope effect. All experiments reported were done at 25 °C.

Counterflow experiments were done at 25 °C in cuvettes of path length 0.1, 0.2, or 1 cm as required by the initial concentration of F used, F_0, or the amount to be formed in the reaction. A probe solution of M^*/F^* in tracer amounts at equilibrium was prepared when required by briefly incubating fumarase plus [^{14}C]fumarate, quenching with acid, and adjusting by buffer base to the desired pH. The forward reaction, usually F → M, was initiated by adding unlabeled substrate to a cuvette containing enzyme, buffer, and the probe. The absorbance at 250 nm was recorded with time as a measure of F using $\epsilon_{250} = 1450\ M^{-1}\ cm^{-1}$. Samples were taken at varying degrees of approach to solution equilibrium and total counts in fumarate determined.

*Analysis of F**. In all cases, the label in fumarate was determined in the TCA-quenched solutions after the addition of 120 μmol of carrier fumarate and three sequential crystallizations and dissolutions in warm water. The amount of fumarate recovered, dissolved in 10 mM NaOH, was determined at 250 nm. Total counts in fumarate were calculated from its specific activity, and the carrier was added. This method was found to give very reproducible assays of the equilibrium isotope ratio which at both zero time and infinite time was found to have 18.5 ± 0.3% of the ^{14}C counts present as fumarate. Recovery of fumarate was usually ~50%.

*Analysis of M**. To determine small amounts of label in malate in the presence of 100-fold larger amount of F*, three of four successive crystallizations of F* were done in ~0.3 mL of TCA (~1 M) with additions of fumaric acid (170 μmol) before each crystallization. Label in the final supernatant was compared with a suitable zero time control.

Determinations of Inhibition Constants. Inhibition was measured by following the decline in the rate of formation of product as the reaction progressed far from equilibrium or by measuring the initial rate with varying substrate. Both sets of data were interpreted using eq 4. A third method used in an attempt to distinguish between competitive and noncompetitive inhibition at high substrate concentration was to study back-labeling, F* → M*, in the presence of varying M and

---

[2] This standard equation can be applied only to limited ranges of substrate concentrations. It is preferred to equations second order in substrate that are inconsistent with the model for activation based on action of a single substrate molecule with alternative enzyme isoforms in the steady state.

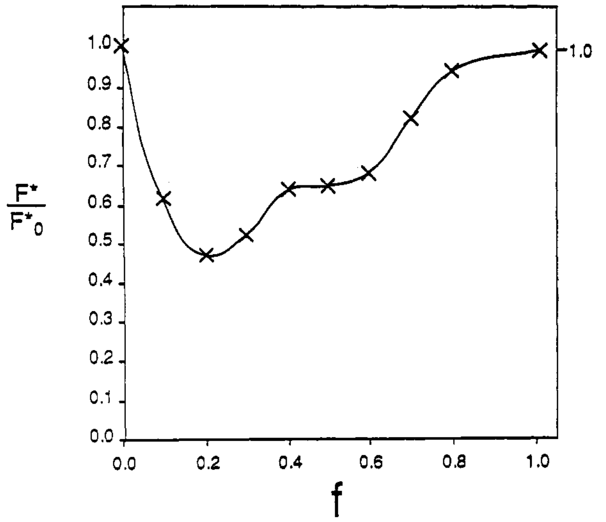


FIGURE 1: Displacement of F* from equilibrium with reaction progress due to counterflow in the M → F direction. M*/F* at equilibrium (1.5×10^5 cpm/mL) was added to 10 mM malate in 10 mM Tris–acetate, pH 7.8, at 25 °C and monitored at 250 nm. Samples were taken at various fractions, *f*, toward equilibrium and quenched with TCA, and the label in F was determined.

constant F*, or M* → F*, in the presence of varying F and constant M*. Such experiments were brief enough to preserve the initial concentration conditions. Failure to eliminate back-labeling in the limit of infinite substrate is taken as evidence for a product-specific isoform.

Radiolabeled Substrates. [^{14}C]Malate was prepared from D,L-[1-^{14}C]aspartate (5×10^6 cpm/μm from New England Nuclear) with glutamic-oxalacetic transaminase (Sigma) and α-ketoglutarate, NADH, and malate dehydrogenase (Sigma). [^{14}C]Malate was recovered on Dowex-1-chloride by elution with 50 mM HCl. It was deemed to be isotopically pure from the fraction of isotope appearing as fumarate (isolated by crystallization) after treatment with fumarase. [1,4-^{14}C]fumaric acid, 3.8×10^6 cpm/μm (Isotopes, Inc.), was purified by ether extraction from acid solution. Both ^{14}C-labeled substrates, upon incubation with fumarase, resulted in the same, equilibrium, fraction of label in fumarate of 18% at 25 °C.

RESULTS

Demonstration and Properties of Isotope Counterflow. To determine whether recycling of ligand-free enzyme, formed under conditions of net product formation, is slow relative to malate/fumarate interconversion, probe solutions, M*/F* = 4.4, were added to a reacting solution of enzyme plus substrate and samples taken to determine if changes in the amount of label in fumarate had occurred. In Figure 1 the radioactivity in fumarate is shown to decrease from its initial equilibrium even as the overall flux is in the direction of producing net fumarate from the 10 mM malate present initially. The opposite result is seen in Figure 2 in experiments initiated by F_0 at 1 and 100 mM and imidazole acetate at 10 and 50 mM, pH 7.0. In both experiments the isotope distribution returned to equilibrium when net flux reached equilibrium. Two exceptions appear in trying to relate the progress curve of Figure 2 to Britton's equation. Firstly, the displacement of F^*/F_0^* is surprisingly insensitive to progress toward equilibrium from the F → M direction. Secondly, values of the displacement with F_0 = 100 mM are significantly greater than the limit $K_{eq}(1 - f)$ above which eq 2 cannot be applied.

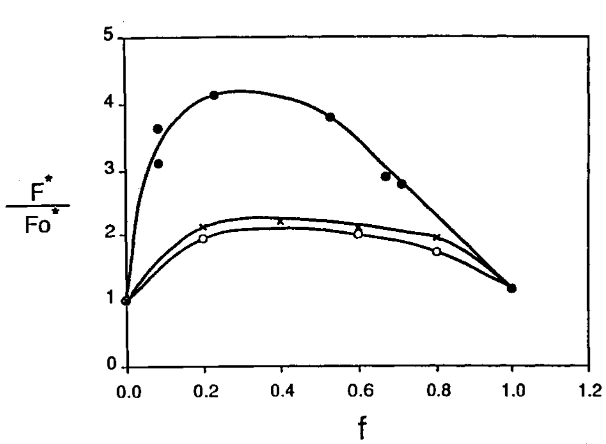


FIGURE 2: Effect of fumarate concentration and buffer on counterflow: M*/F* at equilibrium (2.5×10^5 cpm/mL), F_0 at 1 mM (×) or 100 mM (●), pH 7.0 (imidazole acetate, 10 mM), or to 1 mM F_0 in 50 mM buffer (○). Samples were analyzed for F* during approach to F/M equilibrium.

Table I: Changes in the Back-Labeling/Net Flux Ratio with Reactant Concentration and Direction of Reaction[a]

In the Direction F → M		
F/M (mM/mM)	*R*[b]	*R*·P/S[c]
10/10*	1.41	1.41
20/10*	1.69	0.84
40/10*	1.95	0.49
In the Direction M → F		
M/F (mM/mM)	*R*[b]	*R*·P/S[c]
100/10*	1.21	0.12
200/10*	1.38	0.07
400/10*	1.63	0.04

[a] Incubations at 23 °C contained the noted concentrations of substrate and 10 mM [^{14}C]malate or fumarate (10*), the products. Tris–acetate (20 mM, pH 8.5) was used. Reaction was followed at 250 nm and stopped with acid when net product formation of 0.14 mM occurred using 0.2–0.4 U of fumarase. The back-labeling rate was determined from the total counts in the substrate and the initial specific activity of the product. The ratio of fluxes normalized to initial concentrations (*R*·P/S) indicates the extent to which substrate is able to suppress back-labeling in both directions of net flux relative to the values expected for a single free enzyme form in both directions. [b] $R = [(S \rightarrow P)_{obs} + (P^* \rightarrow S^*)]/(P^* \rightarrow S^*)$ is the partition ratio of free enzyme between reaction with S and P*. The true S → P conversion is given by the loss of S corrected for the S formed from P*. [c] For a reaction in which S and P react with the same free enzyme species *R*·(P/S) should equal K_{eq}, *i.e.*, 4.4 with F → M and 4.4^{-1} or 0.22 with M → F.

Another approach, more commonly used to determine if the enzyme liberated in the forward and reverse directions of the reaction are the same, is based on a comparison of the ratios of back-labeling to the forward rate under the same conditions in both directions. In such an experiment, Table I, the partition ratios, normalized to the concentrations of reactants used, are seen to differ by at least 5–10-fold (0.22/0.04 and 4.4/0.49) in the direction of preferential back-labeling, from the predictions of a competitive model such as Scheme I.

In the counterflow experiment *any* competitive inhibitor *could* have the effect of decreasing the counterflow of the isotopic reactants *without* increasing the recycling rate. Net counterflow depends on the *ratio* of the functional enzyme families, E_f and E_m, in the steady state. Any change in reaction conditions may alter this ratio by its effect on either recycling or the chemical interconversion rate or both. A competitive inhibitor would appear to decrease counterflow without increasing the recycling rate if the change required for recycling could occur while the inhibitor was bound. The inhibitor would

Table II: Sensitivity of Counterflow to Solutes Depends on the Concentration of Substrate Used[a]

	$(F_{0.5}^* - F_0^*)/F_0^*$			
substrate	none	P_i	transaconitate	NaCl
M (1 mM)	–0.3	–0.02	–0.04	–0.23
M (200 mM)	–0.45	–0.35	–0.27	–0.45
F (0.2 mM)	1.05	.048	.055	0.44
F (40 mM)	1.26	0.56	0.58	1.02

[a] Counterflow experiments similar to those of Figures 1 and 2 were done at pH 7.8 (Tris–acetate, 20 mM) and pH 7.0 (imidazole acetate, 20 mM) with the noted initial concentrations of malate and fumarate, respectively, 0.1–0.6 unit of fumarase either with no further additions (none) or with P_i (10 mM), transaconitate (1 mM), or NaCl (50 mM). Samples were taken to determine the distribution of radioactivity at 50% toward reaction equilibrium, determined by following the absorbance at 250 nm. No counterflow corresponds to a value $(F_{0.5}^* - F_0^*)/F_0^*$ of 0.0.

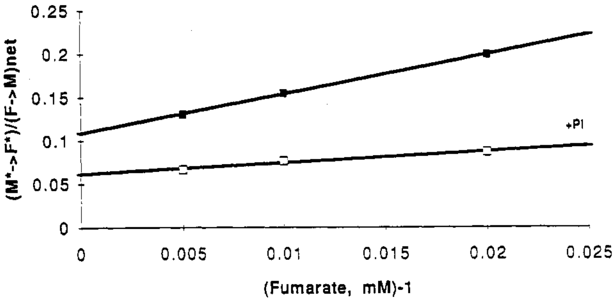


FIGURE 3: Malate is a noncompetitive product inhibitor at pH 7.0. Interconversion M* → F* as a function of F concentration: Tris–acetate (50 mM, pH 8.5), [^{14}C]malate (20 mM, 33 000 cpm/μmol), fumarate (50, 100, 200 mM), and fumarase (0.83 U/mL). P_i, when present, (□), was 20 mM. Each reaction was followed at 298 nm and quenched after formation of 1.7 mM malate. F* was determined by crystallization with carrier in acid.

appear to be a catalyst of recycling when, in fact, it was preventing reaction with substrate (being bound to the active site) while *allowing* equilibration of conformational variants to occur. This mechanism might provide the best explanation for the anticounterflow effects of the factors shown in Table II. Note that counterflow is significantly less sensitive to these factors at high substrate concentrations.

Inhibition Studies. The observation of counterflow raises the possibility that one or both of the slowly interconverting isoforms is substrate-specific. Studies were therefore conducted in both directions over the pH range 7.0–8.5, to search for malate-specific and fumarate-specific enzyme states. When assayed as an inhibitor at pH 7.0, malate appeared to be strictly competitive: Using 100 mM fumarate in [T]water and following malate formation by T incorporation, there was no evidence for a decrease in rate up to an accumulated concentration of 15 mM malate. At pH 7.8 and 25 °C comparing initial rates in the range F = 50–200 mM, with malate at 20 mM there was the suggestion of a weak intercept difference in the v^{-1}/F^{-1} plot: $K_{ii} \simeq 127$ mM, $K_{is} = 4.2$ mM, and $K_m^F = 18$ mM. This very high K_m, at least 100× greater than the classical K_m, is typical of the substrate-activated, supersaturated state. A more sensitive measure of malate-specific inhibition would be obtained by following the loss of reverse reaction, M* → F*, by increasing concentrations of fumarate. The result of such a back-labeling experiment at pH 8.5 (Figure 3) clearly indicates the presence of a product form of enzyme E_m, with which malate at 20 mM but not fumarate can react. The intercept value indicates that the partition of E_m between return to fumarate with 20 mM malate and recycling is 0.1/1. On the basis of eq 4, this would give $K_{ii}^M \simeq 200$ mM, implying a malate-specific isoform of low steady-state concentration. P_i at 20 mM decreases the lifetime of all recycling species that react with malate, either exclusively or in competition with fumarate by 2–3-fold as shown by its effect on the intercept and slope in the back-labeling experiment.

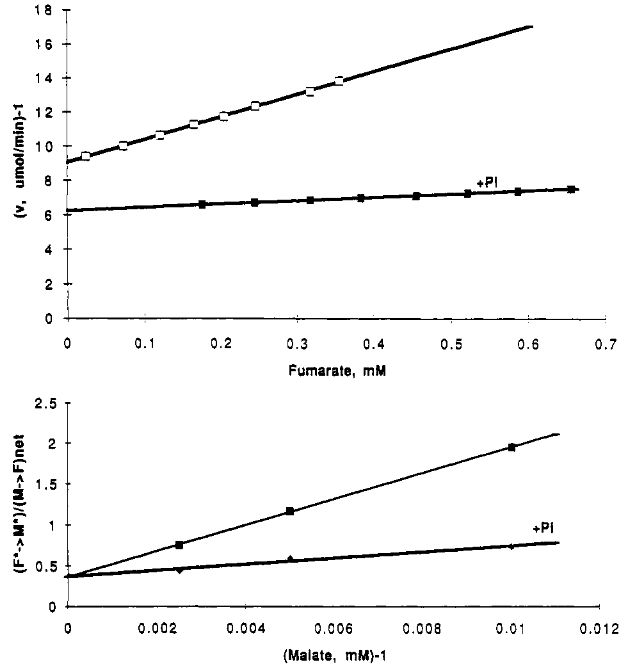


FIGURE 4: Fumarate is a non-competitive inhibitor. (A, top) Progress curve of fumarate formation: malate (50 mM), Tris–acetate (10 mM, pH 7.8), and fumarase (0.11 U), apparent K_{ii} = 0.65 mM; effect of P_i (50 mM, pH 7.8), K_{ii} = 2.9 mM. (B, bottom) Back-labeling by F* is both competitive and noncompetitive in the M → F reaction: Tris–acetate (50 mM, pH 8.5), [^{14}C]fumarate (5 mM, 10^5 cpm/μmol), malate (60, 120, 240 mM), and fumarase (0.70 U/mL). P_i, when present (□), was 50 mM. Reaction was terminated in all cases after the net formation of 0.7 μmol/mL fumarate. M* was determined after several successive removals of labeled fumarate by acid crystallization as specified in the Methods.

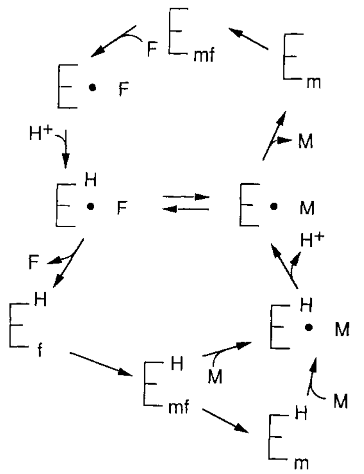
Apparent noncompetitive inhibition by fumarate was observed in the presence of 100 mM malate. The reaction rate, followed at 250 nm, was found to decrease continuously when no more than 0.1 mM fumarate had been formed. Figure 4A shows a replot of such a progress curve, v^{-1} *vs* the amount of F formed. Unexpectedly, the value of the inhibition constant, 1.2 mM in this case, was significantly a function of malate concentration even at 100 mM malate; *i.e.*, it was not safe to conclude that K_m^M/K_{is}^F was negligible with respect to 100 mM concentration of substrate in eq 4.

To estimate whether a truly fumarate-specific enzyme form exists, it was necessary to overtake the considerable competitive interaction. This was done most sensitively not by measuring the forward rate as a function of malate in the presence of fumarate but by determining the ability of [^{14}C]fumarate to label malate in the limit as malate → ∞. The experiment shown in Figure 4B with F* = 5 mM shows a positive intercept $[(F^* \rightarrow M^*)/(M \rightarrow F)]_{M\rightarrow\infty} > 0$. With P_i present the competitive aspect of back-labeling by fumarate is greatly diminished without changing the malate-insensitive ratio. From this it seems reliable to infer the presence of fumarate-specific E_f and E_f^H isoforms with a limiting value of apparent affinity for fumarate of ~17 mM, eq 4.

DISCUSSION

A model, believed to be sufficient to explain substrate-dependent activation, is given in Scheme III.[3,4] This model

Scheme III



provides an expansion of the specificity properties of E and E^H of Scheme I which does not predict counterflow and of Scheme II which does not predict activation by high concentration of substrate. In this model, degrees of substrate specificity are proposed that depend on the conformation of the active site as well as its state of protonation. Shown in Scheme III are the liganded species $E^H{\cdot}M$ and $E{\cdot}F$ that occur when malate reacts with a member of the E^H family and fumarate reacts with a member of the E family. In this model, these are functional intermediates; *i.e.*, they undergo proton loss or gain, respectively, before contributing to the central equilibrium $E{\cdot}M \rightleftarrows E^H{\cdot}F$ (Rose et al., 1992). The substrate reactivity of *each* free isoform of the two families may differ because of conformational factors such as the accessibility of interacting residues of the active site, or kinetic factors such as the rate of proton transfer required to join the central equilibrium. In cases in which affinity and rate do not increase together this model would be responsible for positive cooperativity. Depending on these properties the steady-state distribution of isoforms in each family will change with substrate concentrations. With increasing substrate the absolute concentration of all nonliganded forms will decrease, of course, and in the case of apparent negative cooperativity their distribution will shift toward members of each family that will require higher concentration for reaction with substrate and are more reactive with product.

The central complexes, $E{\cdot}M \rightleftarrows E^H{\cdot}F$, assumed to be the same when formed in either direction and believed to be at equilibrium in the steady state, are known to dissociate to M and F in a ratio of ~2 to 1 (Rose et al., 1992). The two enzyme families, E and E^H, formed by this dissociation need not be present in this same ratio if the proton transfers between the donor site and the solvent in either direction are concurrent with product release. This is clearly not the case in the direction $M \rightarrow F$ where E^H not $E + H^+$ is produced as shown by virtually complete intermolecular proton transfer, $TM^* + F \rightarrow TM + F^*$ (Rose et al., 1992). However, $E{\cdot}M$ in addition to releasing malate directly may do so by a path $E{\cdot}M + H^+ \rightarrow E^H{\cdot}M \rightarrow E^H + M$ to the extent that the donor site is maintained in the protonated state. Evidence for the reverse reaction $E^H{\cdot}M \rightarrow E{\cdot}M + H$ has already been discussed (Rose et al., 1992).

If E^H is formed as part of the process of dissociating malate from $E{\cdot}M$, we can say that it differs from E^H formed in the dissociation of F of $E^H{\cdot}F$. This latter E^H, designated E_f^H, is specific for fumarate. This was difficult to demonstrate for two reasons: E_f^H must have a short lifetime in the steady state, changing rapidly to a species with which malate can also react. This new form, E_{fm}^H or E_{mf}^H, appears to accept fumarate very well in competition with malate, causing the great sensitivity of the $M \rightarrow F$ progress curve to product inhibition (Figure 4A). It is interesting that malate has almost no effect as a competitive inhibitor.[5] This difference may reflect the above-mentioned difference in affinities for E_{mf} but also may mean that the concentration of an isoform with good affinity for malate is low in the $F \rightarrow M$ steady state. On the other hand, the ease with which malate discharges T from E^T in apparent competition with its capture by fumarate (Rose et al., 1992) provides evidence for isoforms such as E_{mf}^T derived from [T]malate and implies that they are significant components of the $M \rightarrow F$ steady state.[6] This combination of observations suggests that the slow step in recycling may be the interconversion of E_{mf} and E_m.

A very sensitive way of looking for intercept effects is based on back-labeling in the steady state. If both reactants bind functionally to the *same free enzyme form*, the ratio $R = [(S \rightarrow P)/(P^* \rightarrow S^*)]$ which represents the partition of the product form of the enzyme between reaction with S or P* should be given by $(V/K_m)_S{\cdot}S/(V/K)_P{\cdot}P$. After correcting for back-reaction to calculate $S \rightarrow P$, and normalizing the partition ratio for the concentrations of reactants used, $R(P/S)$ should be the same when determined in the two directions and should equal P/S at equilibrium. This result should be expected for Scheme I but not for Scheme II. If the substrate-specific isoforms such as E_f^H and E_m^H are not rapidly interconverted, back-labeling by product should reach a finite limit as $S \rightarrow \infty$. This can be seen by reference to Scheme II and to eq 1: The ratio of fluxes through step 1 will be $k_1SE_s/k_{-1}P^*E_p = (k_1S/k_{-1}P)(k_{-1}P + k_2)/(k_1S + k_{-2})$ which at $k_1S > k_{-2} \rightarrow 1 + k_2/k_{-1}P$. The high values of K_{ii}^F and K_{ii}^M are probably not the result of low affinity but rather the low concentrations of E_f and E_m, respectively, in the steady state which can be related to the ratio of the counterflow/recycling rates.[7]

Fumarase is probably the first enzyme found to be fully rate-limited for k_{cat} by recycling of the liberated enzyme. A number of enzymes have been studied since Britton introduced the counterflow method. Except for proline racemase (Fisher

---

[3] All reactions are reversible. M-specific and F-specific forms E_m and E_f^H, at low buffer concentration, are generated ~2/1 from the central complex with either F or M as substrate. Initially the E_f^H was defined as the form of the enzyme *produced* in the reaction $M \rightarrow F$. With the need to designate states in terms of relative specificity as well as source terms, E_{mf}^H are used interchangeably, indicating nonspecificity only, although with further information this could change. Conformational descriptors may be misleading if not applied in context. Species derived from E_f^H such as E_f and $E_f^H{\cdot}F$ are not expected to be conformationally identical. Thus, it would be grossly incorrect to assume that the rapid interconversion $E_f^H{\cdot}F \rightleftarrows E_m{\cdot}M$ is driven by the same change that occurs in the sequence $E_f^H \rightarrow E_m + H^+$. For this reason descriptors are not used for liganded enzyme forms.

[4] No mention is made of loss of the C-2 hydroxyl group which is known to exchange rapidly probably by net dissociation, before fumarate is released. Since the ratio of $[^{18}O]$malate:H_2O/$[^{14}C]$fumarate:malate exchange ratios is ~2/1 (Hansen et al., 1969; Berman et al., 1979), similar to the M/F partition ratio of the central complexes, it must follow that the C-2-derived hydroxyl of malate exchanges from the enzyme almost completely prior to its return to malate. This conclusion may not hold at high pH where the exchange ratios become the same (Berman et al., 1971).

[5] One might think of developing this concept further by using the symbol E_{fm} to designate a state derived from E_f in an $M \rightarrow F$ steady state. The isostate model for substrate activation requires the reactivity toward product *vs* substrate to decrease the sequence $E_p > E_{ps} > E_s$.

[6] It may be important to distinguish data derived from competition experiments based on inhibition or reactant exchange rates from results of T-exchange if the tritium requires processing through a proton relay.

et al., 1986a) and triose-P isomerase (Raines & Knowles, 1987), free enzyme interconversion had not been demonstrable, allowing only lower limits to be set for a number of phosphoryl transfer enzymes (Britton & Clarke, 1968, 1972; Britton et al., 1971, 1972). Counterflow with proline racemase required proline concentration ~50× the substrate K_m, producing a ~1.4-fold displacement of the initial 1/1 ratio of label in the D,L-proline probe; $k_{recycling}/k_{cat}$ was estimated to be ~50. A variety of studies (Cardinale & Abeles, 1968; Fisher et a., 1985b) indicate that two proton transfers are required to readjust the acid/base state of the substrate-specific form in each cycle. Although two proton transfers are believed to be required to recycle proline racemase, the high ratio of recycling to k_{cat} would rule out the possibility of large effects of D_2O or buffers on k_{cat}.

Fumarase, on the other hand, with rate-limiting recycling should show clear isotope effects on k_{cat} if proton transfers were important for recycling. Significant D_2O effects have been found in cases of rate-limiting proton relays in carbonic anhydrase (Steiner et al., 1975; Pocker & Bjorkquist, 1977) and pyruvate kinase (Rose et al., 1991) as well as in proton transfer to solvent from ribonuclease (French & Hammes, 1965). That D_2O effects with fumarase are reportedly small (Thomson, 1960; Srere et al., 1961) and discrimination against T in [T]water is not seen are arguments against rate-limiting proton transfer in the recycling interconversions. In the absence of evidence to the contrary there is no reason to exclude conformational isomerization alone as the critical factor in determining k_{cat}. Perhaps the 3-fold inhibition of k_{cat} seen with viscogens (Sweet & Blanchard, 1990) and the inhibitory effects of hyperbaric pressure (Butz et al., 1988) are related to the occurrence of shape and volume changes in free enzyme recycling.

Counterflow is not particularly sensitive to ionic strength at high or low substrate as shown with 50 mM NaCl (Table II). However, transaconitate and P_i which inhibit and activate, respectively, promote recycling relative to counterflow at low but much less so at high fumarate and malate. Competitive inhibition *per se* by transaconitate cannot explain this since counterflow is dependent on the ratio of free enzyme forms. Bound transaconitate would not allow access of the active site to either M* of F*. Apparently when transaconitate dissociates, it leaves the free enzyme states closer to the equilibrium distribution. Using 0.4 mM fumarate, as in Table II, it was found that 1 mM transaconitate was sufficient to decrease the counterflow ratio by ~95%, or increase the apparent recycling rate 20-fold. Under these conditions the rate inhibitory effect of the inhibitor was about 80%. Therefore, the effect of transaconitate on recycling may be somewhat catalytic, not only passive. The lower sensitivity of counterflow to transaconitate at high concentration of malate or fumarate is not surprising since transaconitate is a competitive inhibitor. P_i is an activator of the initial rate at 1 mM malate and strongly decreases the counterflow/recycling ratio. The net effect requires P_i to activate the recycling rate. Since P_i affects the slope and not the intercept in Figure 4B, its interaction should be with E^H_{mf} and not E^H_f (see footnote 5). The nature of the activation of $E_{mf} \rightarrow E_m$ could be similar to that considered for transaconitate, by transient binding that facilitates conformational mobility. P_i is after all a weak competitive inhibitor (Alberty et al., 1954). It will be of interest to determine whether the variety of anion activators such as citrate and borate (Massey, 1954) do so by promoting recycling. Otherwise one may propose a special buffer effect of P_i.

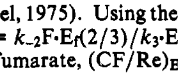
Recovery of counterflow in the presence of P_i at high malate (Table II) is predictable since $E^H_{mf} + M \rightarrow E^H{\cdot}M$ becomes the preferred path of recycling, so that whether P_i acts in the conversion $E_{mf} \rightarrow E_m$ as a buffer or as described for transaconitate, the high malate steady state would continue to show counterflow and back-labeling with F* due to the specificity of E^H_f for fumarate. Similar considerations may apply to the loss of counterflow in the F → M direction. As shown in Figure 3, P_i interacts with E_m, decreasing the back-labeling with M*. At 40 mM fumarate the reaction cycle becomes depleted of E_f and the E_m/E_f ratio should increase, becoming further displaced from equilibrium, increasing counterflow.

The possibility that nonclassical kinetic effects might result from a heterogeneity in the fumarase population such as reported by Lin et al. (1971) and Penner and Cohen (1971) on the basis of isoelectric separation experiments should be considered. Clearly this would not alter conclusions derived from the counterflow results or affect the observation of noncompetitive inhibition by fumarate by back-labeling experiments. It seems likely that microheterogeneity could lead to the appearance of substrate activation resulting from species with different kinetic constants. However, the finding of activation by P_i and high substrate effects with yeast fumarase (Keruchenko et al., 1992) makes this seem unlikely. In experiments (not shown) fumarase of yeast showed substrate-induced counterflow characteristics similar to those of the muscle enzyme. Several studies have established the single gene origin of mitochondrial and cytosolic fumarase with protein heterogeneity introduced in processing the N-terminal sequence (Wu & Tzagoloff, 1987; Boonyarat & Doonan, 1988).

Activation by high substrate concentration is not a rare phenomenon. Some examples are aspartase (Williams & Lartigue, 1967), argininosuccinase (Carvajal et al., 1982), and phosphoglycerate kinase (Scopes, 1978; Sherman et al., 1992). These enzymes should be examined for occurrence of slow recycling of ligand-free enzyme isoforms. In addition as mentioned already, slow recycling may be the basis for positive cooperativity. This would result if the lower affinity isoforms were less reactive in product formation. A few convenient ways that slow recycling can be identified and distinguished from slow product release are the following: (1) Changes in the ratio of back-labeling to forward flux as a function of substrate concentration and direction of reaction. This method is perhaps more easily interpreted and capable of experimental manipulation than is the counterflow method. (2) True noncompetitive inhibition by the last product formed. This approach may be ambiguous in the presence of strong competitive inhibition and may be subject to medium or nonspecific binding effects and so is generally less reliable than the back-labeling method which depends on comparisons of rate ratios. (3) Substrate-induced isotope counterflow. Counterflow should be unambiguous evidence for slow

---

[7] K^F_{ii} defined in eq 4 as $M \rightarrow \infty$ is related to the counterflow/recycling ratio (CF/Re) and the dissociation constant of $E^H{\cdot}F$. For Scheme IV



where E·X represents all liganded intermediates, $K^F_{ii} = (k_2 + k_3)/k_{-2}$ and $K^M_{ii} = (k_{-1} + k_{-3})/k_1$ (Segel, 1975). Using the value for $k_{-1}/k_2 = 2$ (Rose et al., 1992) $(CF/Re)_{Ef} = k_{-2}F{\cdot}E_f(2/3)/k_3{\cdot}E_f = 2k_{-2}{\cdot}F/3k_3$. In Figure 4B as $M \rightarrow \infty$ at 5 mM fumarate, $(CF/Re)_{Ef} = 0.4$ from which $k_3/k_{-2} = 8$ mM. $(CF/Re)_{Em} = k_1{\cdot}M{\cdot}E_m/3k_{-3}E_m = k_1{\cdot}M/3k_{-3}$. In Figure 3 as $F \rightarrow \infty$ at 20 mM malate, CF/Re = 0.1 from which $k_{-3}/k_1 = 70$ mM.

recycling. It should be studied over a range of substrate concentrations in view of likely complication of cross reaction of substrate with product forms.

ACKNOWLEDGMENT

This work has been greatly facilitated by the steadfast help of a member of Secretarial Services of this Institute, Ms. Mary Williamson.

REFERENCES

Alberty, R. A., & Bock, R. M. (1953) *Proc. Natl. Acad. Sci. U.S.A. 39*, 895–900.

Alberty, R. A., & Pierce, W. H. (1957) *J. Am. Chem. Soc. 79*, 1526–1530.

Alberty, R. A., Massey, V., Frieden, C., & Fuhlbrigge, A. R. (1954) *J. Am. Chem. Soc. 76*, 2485–2493.

Albery, W. J., & Knowles, J. R. (1987) *J. Theor. Biol. 124*, 137–171.

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.

Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4506–4513.

Boonyarat, D., & Doonan, S. (1988) *Int. J. Biochem. 20*, 1125–1132.

Britton, H. G. (1973) *Biochem. 133*, 255–261.

Britton, H. G., Carreras, J., & Grisolia, S. (1971) *Biochemistry 10*, 4522–4533.

Britton, H. G., Carreras, J., & Grisolia, S. (1972) *Biochemistry 11*, 3008–3014.

Britton, H. G., & Clarke, J. B. (1968) *Biochem. J. 110*, 161–183.

Britton, H. G., & Clarke, J. B. (1972) *Biochem. J. 130*, 397–410.

Butz, P., Greulich, K. O., & Ludwig, H. (1988) *Biochemistry 27*, 1556–1563.

Cardinale, G. J., & Abeles, R. H. (1968) *Biochemistry 7*, 3970–3978.

Carvajal, N., Fernandez, M., Rodriguez, J. P., & Martinez, J. (1982) *Biochim. Biophys. Acta 701*, 408–409.

Cleland, W. W. (1977) *Adv. Enzymol. 45*, 273–387.

Clutterbuck, P. W. (1928) *Biochem. J. 22*, 1193–1205.

Cornish-Bowden, A., & Cardenas, M. L. (1987) *J. Theor. Biol. 124*, 1–23.

Fisher, L. M., Albery, W. J., & Knowles, J. R. (1986a) *Biochemistry 25*, 2538–2542.

Fisher, L. M., Belasco, J. G., Bruice, T. W., Albery, W. J., & Knowles, J. R. (1986b) *Biochemistry 25*, 2543–2551.

Hansen, J. N., DiNovo, E. C., & Boyer, P. D. (1969) *J. Biol. Chem. 244*, 6270–6279.

Hasinoff, B. B., & Davey, J. P. (1986) *Biochem. J. 235*, 891–893.

Kanarek, L., & Hill, R. L. (1964) *J. Biol. Chem. 239*, 4202–4206.

Keruchenko, J. S., Keruchenko, I. D., Gladilin, K. L., Zaitsev, V. N., & Chirgadze, N. Y. (1992) *Biochim. Biophys. Acta 1122*, 85–92.

Lin, Y.-C., Scott, C. F., & Cohen, L. H. (1971) *Arch. Biochem. Biophys. 144*, 741–748.

Mann, P. J. G., & Woolf, B. (1930) *Biochem. J. 24*, 427–434.

Massey, V. (1953) *Biochem. J. 53*, 67–71.

Penner, P. E., & Cohen, L. H. (1971) *J. Biol. Chem. 246*, 4261–4265.

Raines, R. T., & Knowles, J. R. (1987) *Biochemistry 26*,

Rose, I. A. (1970) in *The Enzymes*, (Boyer, P. D., Ed.) 3rd ed., Vol. II, pp 281–320, Academic Press, NY.

Rose, I. A., Kuo, K. J., & Warms, J. V. B. (1991) *Biochemistry 30*, 722–726.

Rose, I. A., Warms, J. V. B., & Kuo, D. J. (1992) *Biochemistry 31*, 9993–9999.

Scopes, R. K. (1978) *Eur. J. Biochem. 85*, 503–516.

Scott, E. M., & Powell, R. (1948) *J. Am. Chem. Soc. 70*, 1104–1107.

Segel, I. H. (1975) in *Enzyme kinetics*, pp 534–543, John Wiley & Sons, New York.

Sherman, M. A., Fairbrother, W. J., & Mas, M. T. (1992) *Protein Sci. 1*, 752–760.

Srere, P. A., Kosicki, G. W., & Lumry, R. (1961) *Biochim. Biophys. Acta 50*, 184–185.

Steiner, H., Jonsson, B.-H., & Lindskog, S. (1975) *Eur. J. Biochem. 59*, 253–259.

Sweet, W. L., & Blanchard, J. S. (1990) *Arch. Biochem. Biophys. 277*, 196–202.

Taraszka, M., & Alberty, R. A. (1964) *J. Phys. Chem. 68*, 3368–3378.

Teipel, J. W., & Hill, R. L. (1968) *J. Biol. Chem. 243*, 5679–5683.

Thomson, J. F. (1960) *Arch. Biochem. Biophys. 90*, 1–6.

Wu, M., & Tzagoloff, A. (1987) *J. Biol. Chem. 262*, 12275–12282.