The enzyme fumarase catalyzes the reversible hydration of fumarate to malate. The reaction catalyzed by fumarase is critical for cellular energetics as a part of the tricarboxylic acid cycle, which produces reducing equivalents to drive oxidative ATP synthesis. A catalytic mechanism for the fumarase reaction that can account for the kinetic behavior of the enzyme observed in both isotope exchange studies and initial velocity studies has not yet been identified. In the present study, we develop an 11-state kinetic model of the enzyme based on the current consensus on its catalytic mechanism and design a series of experiments to estimate the model parameters and identify the major flux routes through the mechanism. The 11-state mechanism accounts for competitive binding of inhibitors and activation by different anions, including phosphate and fumarate. The model is identified from experimental time courses of the hydration of fumarate to malate obtained over a wide range of buffer and substrate concentrations. Further, the 11-state model is found to effectively reduce to a five-state model by lumping certain successive steps together to yield a mathematically less complex representation that is able to match the data. Analysis suggests the primary reaction route of the catalytic mechanism, with fumarate binding to the free unprotonated enzyme and a proton addition prior to malate release in the fumarate hydration reaction. In the reverse direction (malate dehydration), malate binds to the protonated forms of the unbound enzyme are able to associate with either malate or fumarate and proposed a six-state mechanism, including a protonated enzyme-malate complex (4), shown in Fig. 1B.

Later, Rose et al. (5–7) proposed a mechanism involving a stepwise interconversion of substrate- and product-free enzyme forms associated with proton transfers and conformational changes. This “slow recycling” of the enzyme, as part of the process of recovering from dehydration to be able to initiate a forward reaction, suggests that a malate-specific isofrom first undergoes proton transfer and a conformational change to generate a nonspecific state. The different forward and reverse reaction mechanisms described by Rose et al. (5–7) are represented in the consensus mechanism of Fig. 1C, in which all reaction steps are reversible. This mechanism is tested here for its ability to explain the kinetic data.

Data from previous kinetic studies (measurements of quasi-steady initial reaction rates at various reactant concentrations) have been analyzed using mechanisms entirely different from those suggested by the isotope exchange studies. Studies by Albery et al. (8, 9) and Hil and Teipel (10) have yielded the following conclusions: at low fumarate concentrations (<1 mM), the enzyme exhibits simple Michaelis-Menten kinetics; at intermediate concentrations of fumarate (~0.001–0.033 M), allosteric activation of the enzyme apparently by binding of substrate to the regulatory site, is observed; and at concentrations of 0.1 M and higher fumarate, apparent inhibition takes place. These observations have been interpreted to reveal negative cooperativity (10, 11). Indeed, the enzyme occurs as a tetrameric complex in mammals with additional sites that can bind both reactants (12). In addition, several anions, including inorganic phosphate, have the capacity to activate the enzyme, presumably through allosteric mechanisms. However, in certain concentrations (less than ~5 mM) inorganic phosphate acts as an inhibitor. It is not clear if fumarate, malate, phos-
Catalytic Mechanism and Kinetic Parameters for Fumarase

To malate (MAL) through the biochemical reaction (i.e. involving biochemical reactants that are sums of species).

\[
\text{FUM} \rightleftharpoons \text{MAL} \quad \text{(Eq. 1)}
\]

The corresponding reference chemical reaction is as follows.

\[
\text{FUM}^2^- + \text{H}_2\text{O} \rightleftharpoons \text{MAL}^2^- \quad \text{(Eq. 2)}
\]

The standard Gibbs free energy is computed as the sum of the free energies of formation of all species.

\[
\Delta G^0 = \Delta G_{\text{MAL}} - \Delta G_{\text{FUM}} - \Delta G_{\text{H}_2\text{O}} \quad \text{(Eq. 3)}
\]

Thermodynamic data estimated independently of this study are listed in Table 1.

The equilibrium constant for the reaction is computed from the standard Gibbs free energy.

\[
K_{\text{eq}} = \exp\left(-\frac{\Delta G^0}{RT}\right) \quad \text{(Eq. 4)}
\]

In our experiments, reactions are assayed in a sodium phosphate buffer. Relationships between species and reactant concentrations, which depend on the pH and concentration of metal ions that reversibly bind to biochemical species, are expressed in terms of the binding polynomials,

\[
P_{\text{FUM}} = 1 + \frac{[\text{Na}^+]}{K_{\text{Na,FUM}}}, \quad \text{P}_{\text{MAL}} = 1 + \frac{[\text{Na}^+]}{K_{\text{Na,MAL}}}, \quad \text{P}_{\text{Pi}} = 1 + \frac{[\text{Na}^+]}{K_{\text{Pi}}} \quad \text{(Eq. 5)}
\]

where \( h \) represents the hydrogen ion activity, \( h = 10^{-pH} \). Specific details on how to derive Equation 5 are provided in the supplemental material. Given these forms of the binding polynomials, relationships between reference species concentrations and reactant concentrations are as follows,

\[
[FUM^2^-] = \frac{[\text{FUM}]}{P_{\text{FUM}}}, \quad [MAL^{2-}] = \frac{[\text{MAL}]}{P_{\text{MAL}}}, \quad [\text{HPO}_4^{2-}] = \frac{[\text{Pi}]}{P_{\text{Pi}}} \quad \text{(Eq. 6)}
\]

where the concentrations [FUM], [MAL], and [Pi] are the total reactant concentrations of fumarate, malate, and inorganic phosphate, respectively. We introduce the chemical activity variables \( f, m, \) and \( p \) to account for the ionic strength of the solution at a given pH and temperature. These variables correspond to concentrations of fumarate, malate, and phosphate, respectively, weighted by an activity coefficient \( \gamma_f \) for a divalent species.

\[
f = \gamma_f \times [\text{FUM}^2^-]
\]
Catalytic Mechanism and Kinetic Parameters for Fumarase

\[ m = \gamma_2 \times [MAL^{2+}] \]

\[ p = \gamma_2 \times [HPO_4^{2-}] \quad \text{(Eq. 7)} \]

Over the temperature range \( T = 273.15 - 313.15 \) K, the effect of ionic strength \( (I) \) on activity is approximated using the extended Debye-Hückel equation (13, 14),

\[ \ln \gamma_z = -\frac{\alpha(T) z^2 I^{1/2}}{1 + B I^{1/2}} \quad \text{(Eq. 8)} \]

where \( z \) is the charge number of the species, and the parameter \( B \) is a constant equal to 1.6 M\(^{-1/2}\). The quantity \( \alpha(T) \) is an empirical function that varies with temperature,

\[ \alpha(T) = 1.10708 - (1.54508 \times 10^{-3})T \]

\[ + (5.95584 \times 10^{-6})T^2 \quad \text{(Eq. 9)} \]

where \( T \) is given in Kelvin. The ionic strength of dissolved ions in solution depends on the number \( n \) of different types of ions present, their concentration \( C_i \), and their valence \( z_i \).

\[ I = \frac{1}{2} \sum_{i=1}^{n} z_i^2 C_i \quad \text{(Eq. 10)} \]

Appropriate values for the \( pK \) values at a given ionic strength are approximated (15, 16),

\[ pK(i) = pK(i_0) - \frac{\alpha(T) (\frac{I_i^{1/2}}{I_0} - 1) I_i^{1/2}}{\log(10) (1 + B I_i^{1/2})} \sum_{j=1}^{M} \gamma_j zn \quad \text{(Eq. 11)} \]

where the sum \( \sum_{i=1}^{M} \) is over all species in a given dissociation/association reaction, \( c_i \) is the stoichiometric coefficient of species \( i \), and the \( pK(i_0) \) values are given in Table 1 for \( I_0 = 0.1 \) M.

Multistep Reversible Catalytic Mechanism—Rose et al. (6) describe two different models for the forward and the reverse reaction, positing a slightly different order in reaction processes and strictly unidirectional pathways. Here we combine the two models to obtain the fully reversible 11-state mechanism of Fig. 1C.

Kinetic rate constants for this 11-state model are defined in Fig. 2A. Unbound enzyme \( E_4 \) binds \( H^+ \) and malate or fumarate in arbitrary order to ultimately form complexes \( E_5 \) and \( E_3 \). The concept of isomechanism, first introduced in 1993 by Rose et al. (4) in the context of the enzyme fumarase and further studied by Northrop and Rebholz (17, 18) in a number of publications, is the process by which the product is released from an enzyme isoform that differs from the isoform the substrate binds to. The two forms may differ both in the protonation states of acid-base groups and in their conformations. Therefore, the free enzyme must undergo an isomerization step to complete a catalytic cycle. In addition, in both forward and reverse reactions, malate- and fumarate-specific forms of the enzyme/complex, respectively annotated as “m” and “f”, require conformational changes in order for the enzyme to recover to be available for further reactions (5). The substrate-free protonated enzyme \( (E_4) \) is available to associate with either substrate (fumarate or malate). The pathway \( E_4 \rightarrow E_5 \rightarrow E_3 \) is thought to be the primary route of forward operation (net production of malate from fumarate) (5), and the pathway \( E_2 \rightarrow E_{4f} \rightarrow E_4 \) is thought to be the primary route in the reverse direction (6).

This mechanism may be simplified by assuming rapid equilibrium for \( H^+ \) binding,

\[ K_{26} = \frac{k_{62}}{k_{26}} E_{6m} = \frac{h}{K_{26}} \]

\[ K_{35} = \frac{k_{33}}{k_{35}} E_{5f} = \frac{h}{K_{35}} \]

\[ K_{14m} = \frac{k_{14m}}{k_{14f}} E_{4m} = \frac{h}{K_{14m}} \]

\[ K_{14f} = \frac{k_{14f}}{k_{14m}} E_{4f} = \frac{h}{K_{14f}} \quad \text{(Eq. 12)} \]

where the rate constant \( k_{ij} \) corresponds to the reaction \( E_i \rightarrow E_j \).

The formulation of the quasi-steady-state expression for this mechanism is detailed in the supplemental material.

Analysis of the kinetic data (see below) using this model (see supplemental material) revealed that the best fit to the data occurred when the conformation state changes were main-
The ability of the model to fit the data, as long as a constant ratio of rate constants is maintained arbitrarily close to equilibrium. (Details of these simulations are not shown here.) For example, the rate constants $k_{32}$ and $k_{23}$ could be set to arbitrarily high values without affecting the ability of the model to fit the data, as long as a constant ratio $k_{32}/k_{23}$ was maintained. Therefore, without diminishing its ability to match the measured kinetics, the model may be reduced in complexity by assuming that these steps are maintained in rapid quasi-equilibrium. Specifically, the following rapid-equilibrium assumptions are applied:

$$K_{23} = \frac{k_{32}}{k_{23}}, \quad E_1 = E_2 \frac{1}{K_{23}}$$

$$K_{55f} = \frac{k_{5f5}}{k_{55f}}, \quad E_5 = E_5K_{55f}$$

$$K_{66m} = \frac{k_{6m6}}{k_{66m}}, \quad E_6 = E_6K_{66m}$$

$$K_{44f} = \frac{k_{4f4}}{k_{44f}}, \quad E_4 = E_4 \frac{1}{K_{44f}}$$

Combining Equations 12–15, $E_2$ can be expressed as a function of $E_4$:

$$E_2 = RE_4$$  \hspace{1cm} (Eq. 16)

where

$$R = \frac{m(k_{46} + \frac{K_{44m}}{h}k_{12}) + f(k_{45} + \frac{K_{45f}/h}{K_{44f}}k_{13})}{k_{21} + K_{66m}h + \frac{h}{k_{66m}} + \frac{1}{k_{23}}(k_{31} + K_{55f}/hK_{55f})}$$  \hspace{1cm} (Eq. 17)

The total enzyme concentration is conserved.

$$E_0 = E_{1m}l_{m} + E_{1f}l_{f} + E_{2f} + E_{2j} + E_{4} + E_{4m}l_{m} + E_{4f} + E_{5} + E_{5f} + E_{6} + E_{6m}l_{m}$$  \hspace{1cm} (Eq. 18)

Here the factors $I_i$ represent inhibition terms that account for dead-end binding of inhibitors to any of the 11 states in the full scheme of Fig. 2A. Each factor depends on the concentration $c_k$...
of the inhibitor $k$ and its associated dissociation constant $K_{i,k}$
according to the general form,

$$I_i = 1 + \sum_k \frac{c_k}{K_{i,k}}$$  \hspace{1cm} (Eq. 19)

where the term $I_i$ is associated with competitive binding at
enzyme state $i$. To analyze our kinetic data we assumed a general
inhibition pattern, with fumarate and phosphate potentially participating in dead-end binding to each enzyme state.

$$I_i = 1 + \frac{f}{K_{if}} + \frac{p}{K_{ip}}$$  \hspace{1cm} (Eq. 20)

This inhibition pattern assumes that fumarate and phosphate form dead-end complexes with enzyme state $E_i$. This general model includes 22 inhibition constants. In analyzing the kinetic
data below, the majority of these inhibition steps were judged
unnecessary to explain the data and have been removed from the model.

Combining Equations 12–18, we can obtain the following.

$$E_a = E_{af} \left( I_4 + \frac{1}{K_{4af}} \left( I_3 + \frac{K_{14m}}{h} I_{1m} \right) + \frac{1}{K_{af}} \left( I_{af} + \frac{K_{1af}}{h} I_{1f} \right) \right)$$

$$+ \sum_j \frac{c_j}{K_{a,j}}$$

$$F_a = \frac{\sum_j c_j}{1 + \sum_j \frac{c_j}{K_{a,j}}}$$  \hspace{1cm} (Eq. 22)

In addition to the apparent inhibitory effects, our model
assumes that binding of anions to an allosteric regulatory site is
necessary for catalysis. To model this effect, we compute the
fraction of enzyme that is catalytically active as the fraction that
is bound to any of a number of specific anions,

$$F_a = \frac{p/K_{af} + f/K_{af}}{1 + p/K_{af} + f/K_{af}}$$  \hspace{1cm} (Eq. 23)

(Allowing for activation by malate does not improve the fits to
the data.) Given the above, the net steady-state flux is computed as follows,

$$J = F_a (E_a k_{64} - E_4 m k_{46} + E_2 k_{21} - E_{1m} m k_{12})$$  \hspace{1cm} (Eq. 24)

where $E_a$ and $E_{1m}$ are computed from Equations 12, 13, 16, 17,
and 21.

**Thermodynamic Constraints on Model Parameters**—Feasible values for the rate constants are constrained by several thermodynamic relationships. First, the chemical equilibrium constant for the reaction can be shown to be equal to the following ratio.

$$K_{eq} = \frac{F_{66} k_{64} k_{46} K_{55} K_{23}}{K_{66} k_{54} k_{44} k_{55}}$$  \hspace{1cm} (Eq. 25)

Second, there exist two additional thermodynamic constraints associated with non-productive loops in the catalytic cycle.

$$\frac{k_{21} k_{44m} k_{46} K_{26}}{k_{12} k_{14m} k_{44} K_{66m}} = 1,$$

$$\frac{k_{31} k_{46} k_{45} K_{35}}{k_{13} k_{14} k_{54} K_{55}} = 1$$  \hspace{1cm} (Eq. 26)

**Alternate Formulation**—The 11-state model may be further simplified by lumping sequential step conformation states into single kinetic states. Assuming quasi-equilibrium for these conformations (Equation 13), the mechanism of Fig. 2A is equivalent to the model of Fig. 2B, in which the correspondence between rate/dissociation constants is as follows,

$$k'_{12m} = k_{12}, k'_{21m} = k_{21}$$

$$k'_{12f} = k_{13}, k'_{21f} = k_{21}$$

$$k'_{46} = k_{46} \frac{K_{4af}}{1 + K_{4af} + K_{4af} K_{46m}}$$

$$k'_{64} = k_{64} \frac{K_{66m}}{1 + K_{66m} K_{64f}}$$

$$k'_{45} = k_{45} \frac{K_{46f}}{1 + K_{46f} + K_{46f} K_{45m}}$$

$$k'_{54} = k_{54} \frac{K_{55f}}{1 + K_{55f} K_{54f}}$$

$$k'_{26} = k_{26} \frac{1 + K_{23}}{K_{23} (1 + K_{66m})}$$

$$k'_{25} = k_{25} \frac{1 + K_{23}}{(1 + K_{55f})}$$

$$k'_{54} = k_{54}$$

$$k'_{14m} = k_{14m}$$

$$k'_{14f} = k_{14f}$$

and the new expressions for inhibition constants are as follows.

$$K'_{i(1m)} = K_{i1m}$$

$$K'_{i(1f)} = K_{i1f}$$

$$K'_{i(2)} = (1 + K_{23}) \frac{K_{i(2)} K_{i(3)}}{K_{i(2)} + K_{23} K_{i(3)}}$$

$$K'_{i(4)} = \left(1 + K_{4af} + \frac{K_{4af}}{K_{46m}}\right) K_{i(4)} K_{i(4m)} K_{i(4f)}$$
RESULTS

The model proposed in this paper is identified based on time course data obtained over a large range of pH (pH 6–8) and substrate (0.1–100 mM) and phosphate (1–100 mM) concentrations. The model is then used to investigate previously reported phenomena of effective substrate inhibition, negative cooperativity, and inhibition by phosphate.

Model Identification—Fig. 3 shows measurements of fumarate decay time courses given initial fumarate concentrations spanning 2 orders of magnitude (from ~0.1 to 100 mM initial fumarate) and obtained at five different phosphate concentrations. In each case, initial malate is zero. The 20 different progress curves provide data on reaction rate over a wide range of substrate, product, and phosphate concentration. The data in Fig. 4 represent time course measurements at zero initial malate at 100 mM total phosphate over a range of initial fumarate concentrations and pH.

In sum, the data in Figs. 3 and 4 provide the means to identify the adjustable parameters associated with either the full mechanism of Fig. 2A or the reduced mechanism of Fig. 2B. Analysis based on the full model showed that it could be reduced based on the rapid equilibrium of the conformational changes, as described under “Materials and Methods,” without affecting the fitting results.

The solid lines in Figs. 3 and 4 correspond to optimum model fits simulated using the reduced model based on Equation 32 with buffer conditions for each case reported in the legend and on the graphs. Dashed lines correspond to model fits obtained from using average parameter values from 60 independently obtained Monte Carlo samples of the optimal parameter space. Because the underlying model is nonlinear, the behavior associated with the mean values of 60 optimal parameter estimates is not necessarily optimal. Thus, the dashed line fits are slightly worse than the optimal solid line model fits. The parameter values associated with the model fits in Figs. 3 and 4 are listed in Table 2, along with the sensitivity coefficients estimated from Equation 33. Based on the 60 independent realizations of optimal parameter sets, we found that the estimated values of a given parameter were distributed symmetrically around its mean value in the logarithmic space (i.e. the parameter distributions could be explained by the log-normal distribution). Thus, the statistics in Table 2 are reported for the log_{10} of the individual parameters. The S.D. \( \sigma_{p} \) is the estimated S.D. from the 60 samples of the log_{10} of the parameter value.

Here \( K_{eq} \) is treated as an adjustable parameter, yielding an estimate of 3.5. The value computed from Equation 4 and the reported thermodynamic data in Table 1 is 3.86. The model fits and data are in good agreement with model predictions within the S.E. in the data or within 10% of the observed mean. However, some systematic discrepancy is observed on some of the data sets (e.g. Fig. 3; 1 mM fumarate, 3.2–10 mM Pi). Even in its more complex form, the model was unable to perfectly fit those data. Systematic bias in the model could be due to inadequacies in the Debye-Hückel model over the observed range of buffer conditions or incompleteness of the model. Better fits could be obtained, for example, by adding hydrogen ion binding inhibition steps, at the expense of additional complexity and adjust-
able parameters. Otherwise, the majority of parameters are estimated with reasonable sensitivities of 10% or more. Parameters $k_{12m}^0$ and $K_{14m}^0$ are assigned values of zero because optimization against the kinetic data yielded values that were effectively zero.

The activating and inhibiting effects of several inorganic anions on both the forward and reverse reactions have been reported by Massey (19, 20) and studied later by Alberty et al. (9). As an initial guess, fumarate and phosphate were assumed to inhibit each of the enzyme states with a different inhibition constant. Although this general scheme greatly increases the number of adjustable parameters, it allows us to consider every possible inhibition pattern. The resulting optimal fits reveal that many of these inhibition steps and associated parameters can be omitted. Dead end binding inhibition reactions necessary to explain the data are found to be phosphate and fumarate binding to enzyme states 4 and 1f. Estimated binding constants for these reactions are listed in Table 2.

Model-predicted relative fluxes through the catalytic mechanism under steady-state conditions at pH 7, $I = 5$ mM, $[P_i] = 1$ mM, and fumarate and/or malate concentrations of 1 mM, are reported in Fig. 5. Given the parameter set that best fits our kinetic data (with $k_{12m}^0 = 0$), the developed model predicts that the primary reaction routes for both hydration of fumarate to malate and dehydration of malate to fumarate involve the binding/release of fumarate to a free unprotonated enzyme, $E_{1f}$. Furthermore, the model predicts that there is essentially no flux through the states 1m and 4m. This is because the best fits to the data occur when the parameter $k_{12m}^0$ approaches values arbitrarily close to zero. This observation implies that the model may be further...
reduced to a five-state model by omitting this pathway. In that case, the malate-specific unprotonated isoform would only bind to the free protonated form $E_4$. Equations 29–31 then become the following.

\[ R = \frac{k_{46}m + f\left(k_{45} + k_{12f}\right)}{h} \]  
\[ E_4 = E_0\left(\frac{K_{1af}}{h} + R_1 + I_4 + \frac{h}{K_{25}} + \frac{h}{K_{26}}I_6\right) \]  
\[ J = \frac{p/K_{2,p} + f/K_{4f}}{1 + p/K_{2,p} + f/K_{4f}} \times \left(\frac{h}{K_{4f}} - k_{46}m\right) \]  
\[ \times E_0\left(\frac{K_{1af}}{h} + \frac{f}{K_{2p(1f)}} + \frac{p}{K_{4f(1f)}} + R \right) + \left(1 + \frac{f}{K_{4f(1f)}}\right) \frac{p}{K_{4f(1f)}} \]  
\[ \frac{h}{K_{25}} + \frac{h}{K_{26}}I_6 \]  

The resulting five-state model (with additional inhibited states) is illustrated in Fig. 6 and discussed below.

**DISCUSSION**

To develop an accurate yet appropriately simple model to describe the kinetics of fumarase, the mechanism of Rose (5, 6) and Rose and Weaver (7) was used to build a kinetic model and analyze a large scale kinetic data set. Analysis of the full model of Fig. 2A revealed that a number of simplifying assumptions are justified in comparing model predictions with the kinetic data. Specifically, the reduced six-state model of Fig. 2B (which was further simplified to the five-state model of Fig. 6) is shown to capture the observed kinetics. It has been proposed that iso-mechanisms could be identified from fumarase kinetics by product inhibition (21, 22). However, those analyses were shown to be incorrect (23). Indeed, despite a comprehensive set of kinetic data and a detailed model of the mechanism, we were unable to demonstrate the influence of the isomerization steps on the kinetics. The isomerization steps are assumed to be in rapid equilibrium in our simplified five-state model.

Although results from this reduced model are emphasized, several different reduced five-state models were tested for their ability to represent the data. One alternative model was based on the assumption that the primary pathway for fumarate release is predominant and the secondary one may be neglected. Another assumed only one possible pathway for...
Catalytic Mechanism and Kinetic Parameters for Fumarase

TABLE 2
Estimated model parameters

| Adjustable parameter | Optimum estimated value $p$ | $\log_{10}(p)$ | Mean of the optima ($10^p$) | Sensitivity coefficient | Unit |
|----------------------|-----------------------------|----------------|-----------------------------|------------------------|------|
| $K_{\text{f}}$ | 3.5006 | 0.5473 ± 0.0069 | 3.5266 | 9.0674 | $M^{-1}$ |
| $K_{\text{f}}$ | 0 | 0 | 0 | Not identified | $M^{-1}$ |
| $K_{\text{f}}$ | $1.91 \times 10^6$ | $6.3714 \pm 0.1535$ | $2.53 \times 10^6$ | 2.1041 | $M^{-1}$ |
| $K_{\text{f}}$ | $4.46 \times 10^5$ | $5.7131 \pm 0.1382$ | $5.45 \times 10^5$ | 1.2590 | $M^{-1}$ |
| $K_{\text{f}}$ | 54.12 | $1.7547 \pm 0.0076$ | 57.80 | 2.7144 | $M^{-1}$ |
| $K_{\text{f}}$ | $5.82 \times 10^4$ | $4.6638 \pm 0.5725$ | $9.99 \times 10^4$ | 0.2963 | $M^{-1}$ |
| $K_{\text{f}}$ | $8.28 \times 10^{-8}$ | $-7.0108 \pm 1.1345$ | $1.07 \times 10^{-6}$ | 0.0163 | $M^{-1}$ |
| $K_{\text{f}}$ | $1.81 \times 10^{-10}$ | $-9.6854 \pm 0.5363$ | $3.91 \times 10^{-11}$ | 0.2705 | $M^{-1}$ |
| $K_{\text{f}}$ | 0 | 0 | 0 | Not identified | $M^{-1}$ |
| $K_{\text{f}}$ | $7.69 \times 10^{-9}$ | $-8.4373 \pm 0.7186$ | $1.19 \times 10^{-8}$ | 0.1426 | $M^{-1}$ |
| $K_{\text{f}}$ | $31.5 \times 10^{-3}$ | $-1.4961 \pm 0.0497$ | $32.1 \times 10^{-3}$ | 0.1446 | $M^{-1}$ |
| $K_{\text{f}}$ | $0.58 \times 10^{-3}$ | $-3.2342 \pm 0.0552$ | $0.59 \times 10^{-3}$ | 0.2773 | $M^{-1}$ |
| $K_{\text{f}}$ | $14.3 \times 10^{-3}$ | $-2.2401 \pm 0.6673$ | $15.1 \times 10^{-3}$ | 0.0837 | $M^{-1}$ |
| $K_{\text{f}}$ | $41.7 \times 10^{-3}$ | $-1.2348 \pm 0.6109$ | $165.0 \times 10^{-3}$ | 0.0320 | $M^{-1}$ |
| $K_{\text{f}}$ | $4.0 \times 10^{-3}$ | $-2.3149 \pm 0.7273$ | $20.3 \times 10^{-3}$ | 0.0452 | $M^{-1}$ |
| $K_{\text{f}}$ | $90.7 \times 10^{-3}$ | $-1.1176 \pm 0.8287$ | $272.1 \times 10^{-3}$ | 0.0146 | $M^{-1}$ |

FIGURE 6. Reduced five-state mechanism adapted from the model of Fig. 2B. The pathway implicating the free unbound enzyme state $E_{\text{un}}$ is neglected. The identified inhibition pattern is also illustrated.

This value is significantly different from our estimated activation constant $K_{\text{a},p} = 0.58 \text{ mM}$, which is measured relative to the activity of the species $\text{HPO}_4^{2-}$. At pH 7 and in the limit of low fumarate concentrations, our data show an activation by phosphate with an apparent half-activation of $[\text{P}_i] \sim 0.7 \text{ mM}$. However, at phosphate concentrations higher than $\sim 7 \text{ mM}$, phosphate clearly inhibits the reaction. Note that it is difficult to directly compare our data and model with that of Alberty et al. (9) because the detailed conditions of the buffer used in the experiments of Alberty et al. are not known.

Inhibition—It has been proposed that inorganic phosphate acts as an inhibitor of fumarase at low concentrations of fumarate (in the range of a few mM and less), perhaps through competitive binding to the active site (9). This observation was repeated in studies by Hasinoff and Davey (11), who investigated the fumarase kinetics at a constant ionic strength. Our model captures this effect of inhibition by inorganic phosphate at low fumarate concentrations. Furthermore, model fits require that the inhibition constants take into account the effect of the ionic strength, by assigning effective charges on each enzyme state (see Fig. 2). This phenomenon implies that variation in ionic strength is partly responsible for some of the apparent inhibition observed in previous studies.

In addition, inhibition by substrate has been reported by Alberty et al. (9) at very high fumarate (>100 mM). Although the developed model includes inhibition by fumarate via dead end binding steps, these inhibition steps do not result in an obvious or significant reduction in flux as fumarate concentration is increased over the range of 0–100 mM and under the conditions employed here.

Cooperativity—Our results are not consistent with previous reports of negative cooperativity (10, 11). In particular, Hasinoff and Davey (11) report that an apparent negative cooperativity was obtained at a constant ionic strength. However, it is not possible to directly compare our results with those of Hasinoff and Davey (11). In their experiments, Hasinoff and Davey added NaCl and NaNO$_3$ to the buffer solution to maintain a constant ionic strength, in concentrations that are not possible to directly compare our data and model with that of Alberty et al. (9) because the detailed conditions of the buffer used in the experiments of Alberty et al. are not known.
an activating effect of Cl\(^{-}\) as well as potential effects of NO\(_3\)\(^{-}\) as an inhibitor and/or activator. It is therefore not possible to unambiguously analyze those data with the model developed here. Nonetheless, it is worth noting that the steady-state flux equation in our model has the general form of Equation 1 in Ref. 11.

In the present study, in contrast to previous studies, the complete make-up of the buffer is reported for all experiments, along with estimated ionic strength based on the dissociation constants reported in Table 1. The model matches the kinetic data over ranges of fumarate and malate concentration spanning 4 orders of magnitude (from 0.1 to 100 mM) and phosphate spanning 3 orders of magnitude (from 1 to 100 mM).

**pH Effect**—Alberty et al. (9) reported experimental measurements of the pH dependence of the initial velocities at high phosphate concentration (133 mM) and for increasing fumarate concentrations. Fig. 7 plots model predictions of the net quasi-steady-state flux versus pH, under the same conditions. (Compare with Alberty et al. (9) (Fig. 1).) Here, model predictions are based on the assumption that ionic strength varies according to pH and according to the composition of the buffer. (The buffer conditions used in Ref. 9 are not reported.) Regardless, the model predictions reproduce the previously reported asymmetric bell-shaped curves, yet the predicted peaks are slightly shifted toward lower pH than in Ref. 9.

**Forward Versus Reverse Operation of the Catalytic Mechanism**—Rose et al. (5) report that the pathway \(E_1 \rightarrow E_5 \rightarrow E_2\) is the primary route of the forward operation and that the pathway \(E_2 \rightarrow E_{1f} \rightarrow E_4\) is the primary route in the reverse direction (6). As illustrated in Fig. 5, under physiological conditions, our model predicts that both pathways are active in the forward and the reverse directions, with about one-third of the flux going through the first pathway and two-thirds through the second. The relative flux values in Fig. 5 do not depend substantially on the values of parameters not sensitively estimated, with less than 10% variability predicted for the ensemble of 60 independently obtained optimal parameter sets.

**CONCLUSIONS**

In summary, an 11-state model for the reversible catalytic mechanism of fumarase was formulated in terms of chemical species and accounting for ionic strength and pH as well as dead-end binding with inhibitors and binding of anionic activators. The model was identified by fitting experimental time courses of the fumarate disappearance during fumarate hydration over a wide range of substrate and phosphate concentrations and pH. Although the model developed here is consistent with both the catalytic mechanism proposed by Rose et al. (6) and data reported here, both the present model and data are inconsistent with some of the phenomena reported in the literature, such as substrate inhibition at high fumarate concentrations and negative cooperativity.

Based on identified rapid equilibrium steps, this 11-state model is reduced to a six-state model. It is further suggested that the pathway involving the malate-specific free unprotonated enzyme state is not used in either the forward or the reverse reaction under the conditions assayed here, resulting in an effective 5-state model.

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