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Perspective

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Kinetic Solvent Viscosity Effects as Probes for Studying the Mechanisms of Enzyme Action

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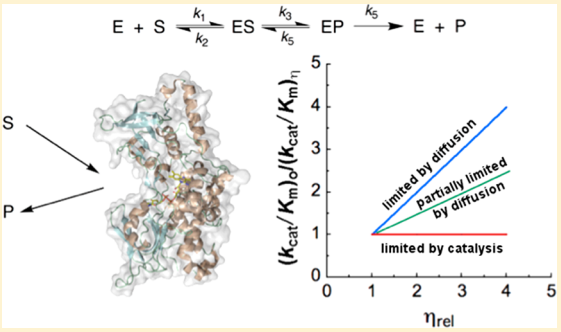
Supporting Information

ABSTRACT: The study of enzyme reaction mechanisms is fundamentally important to our understanding of biochemistry, cellular metabolism, and drug development. This Perspective focuses on the use of kinetic solvent viscosity effects (KSVEs) to study enzyme reactions. This technique is easily implemented and uses steady-state kinetic analyses to probe whether substrate binding is diffusion-controlled and whether product release is the rate-limiting step in the catalytic cycle. In addition, KSVEs can identify isomerization steps that are important for catalysis. The use of KSVEs in combination with other techniques, such as kinetic isotope effects, pH effects, and site-directed mutagenesis, can provide a detailed view of the mechanism of enzyme action. We present the basic theory, important experimental considerations, and potential outcomes and briefly discuss some examples from the literature. The derivation of the equations that are important for data analysis is also presented.



The study of enzyme function requires implementation of a number of techniques that, if properly executed and analyzed, can provide detailed descriptions of the structural changes and the chemical and kinetic steps required to complete the catalytic cycle.[1−3] X-ray crystallography has provided atomic resolution of the overall structure of enzymes, the architecture of active sites, and has revealed important interactions with substrates, inhibitors, and other protein partners. In some cases, the structures of reaction pathway intermediates have been obtained.[4,5] Enzyme structural characterization has also benefited from nuclear magnetic resonance (NMR) studies, where conformational changes can be monitored and modeled.[6−8] Steady-state and rapid reaction kinetic experiments provide a measurement of the rate constants of the various steps in the catalytic cycle and chemical steps can be probed by altering the chemical nature of the substrate, i.e., through the use of substrate analogues or altering solution conditions.[9,10] Kinetic isotope effects (KIEs) can provide information about the rate-determining step for overall turnover of enzymes and the structure of the transition state.[11] Studying the effect of pH on the kinetic parameters of enzymes can provide valuable information about the ionization state of the substrate or the protein residues involved in binding or catalysis.[10−13] Site-directed mutagenesis is now an essential tool for elucidating the roles of amino acid residues in the active site of enzymes through comparative analyses of the structure and function of mutant and wild-type enzymes.[14−17] Various computational approaches that include quantum mechanics/molecular mechanics and molecular dynamics, among others, have now been routinely added to the enzymologist's toolbox to complement experimental approaches.[18−21]

An effective, but relatively underutilized, kinetic technique for probing the mechanism of an enzyme is the kinetic solvent viscosity effect (KSVE) study. KSVEs exploit the fact that for a chemical process to occur in enzymatic reactions, except for electron transfer reactions, the substrate must first reversibly bind to the enzyme and form an enzyme−substrate complex (Scheme 1, steps k_1 and k_2). The enzyme−substrate complex is then converted to an enzyme−product complex through the chemical step of catalysis (k_3), with the subsequent release of the reaction product from the enzyme active site (k_5). To fully understand KSVEs, it is important to highlight that even the

Scheme 1. Simplest Kinetic Mechanism for an Enzyme (E) with a Single Substrate (S) and Product (P), in Which the Kinetic Steps of Substrate Association (k_1) and Dissociation (k_2), Reversible Catalysis (k_3 and k_4), and Product Release (k_5) Are Explicitly Shown

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \xrightarrow{k_5} E + P$$

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simplest enzyme kinetic mechanism should be represented by a three-step model, with substrate binding, catalysis, and product release (Scheme 1), rather than the impactful, but overly simplified, two-step model, in which the steps of catalysis and product release are considered as a single kinetic step.

Movement of both the enzyme and the substrate in solution follows the basic principles of molecular diffusion. The second-order rate constant between two molecules, such as the enzyme and the substrate (k_1 in Scheme 1, with units of M^{-1} s^{-1}), can be represented by the Smoluchowski equation (eq 1)

$$k_1 = 4\pi N_0 r_0 (D_A + D_B) \quad (1)$$

where N_0 is Avogadro's number, $D_A + D_B$ is the sum of the diffusion coefficients of the two molecules, and r_0 is the sum of the radii of the two reacting molecules.[22] The diffusion coefficient D_i, as defined by the Stokes–Einstein equation for a spherical particle with radius r_i, is inversely proportional to the frictional force experienced by the particle in solution, which, in turn, depends on solvent viscosity (eq 2)

$$D_i = \frac{k_B T}{6\pi\eta r_i} \quad (2)$$

where k_B is Boltzmann's constant, T is the absolute temperature, and η is the solution viscosity.[23] Thus, the diffusion of enzymes and substrates in solution to form an enzyme–substrate complex is inversely proportional to the solution viscosity as stated by the Stokes–Einstein equation (eq 2).

Kramers postulated that the diffusion of molecules is inversely proportional to the friction imposed by solvent viscosity, thereby making the kinetic steps defined in Scheme 1 by rate constants k_1, k_2, and k_5 sensitive to solvent viscosity.[24] Conversely, the chemical step of catalysis (k_3) is typically assumed to be independent of solvent viscosity, although examples of protein motion proportional to the rate of fluctuation of the solvent have been documented in both hydride and proton transfer enzymatic reactions.[25,26] When two small molecules are of equal size, eq 1 is approximately 10^9–10^{10} M^{-1} s^{-1} in water at temperatures commonly encountered by enzymes.[27] This value represents an upper limit for diffusion-limited reactions. Because enzymes and substrates are large molecules, their diffusion is slower and upper limit values for diffusion-limited enzymatic reactions are typically in the range of 10^8–10^9 M^{-1} s^{-1} or even lower (see case studies). Knowles introduced the term "evolutionary perfect enzymes" for enzymes that catalyze reactions with k_{cat}/K_m values of 10^9–10^{10} M^{-1} s^{-1}.[28] In evolutionary perfect enzymes, the chemical steps of catalysis (k_3 in Scheme 1, with units of s^{-1}) have been optimized and are much faster than the interaction between the enzyme and substrates (k_1[S] and k_2 in Scheme 1, with units of M^{-1} s^{-1} and s^{-1}, respectively); thus, the reaction is said to be diffusion-controlled.[10] Similarly, the release of products from the enzyme active site (k_5 in Scheme 1, with units of s^{-1}) can determine the rate for the overall turnover of the enzyme when the chemical step of catalysis is much faster than product release.

A simple method for studying whether enzymatic reactions are diffusion-controlled is by measuring KSVEs. In this Perspective, we present experimental considerations, data analysis and interpretation, and equations applied to enzyme mechanisms and provide examples of enzymatic reactions that are controlled by substrate diffusion, product release, or internal isomerizations of enzyme–substrate and enzyme–product complexes. As frequent users of the KSVE approach, our intent is to provide the fundamentals and applications of KSVEs to facilitate a more widespread adoption of this useful and simple kinetic technique for the study of the kinetic and catalytic mechanisms of enzymes. The work of the scientists that first introduced KSVEs for the study of enzyme mechanism, including the seminal 1982 study by Kirsch at al., is presented in the section on case studies. While the type of information that can be obtained with KSVEs is also available from other approaches, such as NMR, transient-state kinetics, or molecular dynamics simulations, the use of KSVEs on steady-state kinetics offers the advantage of being easily performed and interpreted and does not require specialized and expensive instrumentation.

SOLVENT MICROVISCOSITY AND MACROVISCOSITY

The solvent viscosity of aqueous solutions can be increased by the addition of additives, with polyhydroxylated compounds being the most effective. An important distinction exists between solvent microviscosity and macroviscosity.[29] Solvent microviscosity is defined as the resistance to motion experienced by a molecule in solution and is the relevant physical chemical parameter in the Stokes–Einstein equation (eq 2).[30] Solvent macroviscosity determines the free volume that is available to the molecule moving in solution.[29] In general, relatively small additives like glycerol, glucose, or sucrose are used to increase solvent microviscosity in KSVE experiments.[29] In contrast, polymeric additives like Ficoll-400 or polyethylene glycol (PEG) are typically used to increase solvent macroviscosity[29] and can be used as controls for KSVEs to unequivocally show that an observed solvent viscosity effect is due to diffusional processes rather than crowding effects. In agreement with this, studies have shown that additives with increased molecular weight are less able to decrease enzyme catalytic efficiency and slow the diffusion of ligands and folding processes.[31,32]

EXPERIMENT SETUP AND DATA FITTING

KSVE studies of steady-state kinetic parameters k_{cat}/K_m and k_{cat} are most commonly used because of their ease of determination and the fact that mechanistic information about the substrate binding and product release steps can be easily extracted.[33] In the example in Scheme 1, the analytical definition of k_{cat} is given by eq 3 and that of k_{cat}/K_m by eq 4. The two equations simplify to eqs 5 and 6 when the chemical step of catalysis is irreversible, i.e., when $k_4 \approx 0$, or when the enzyme–product complex has a stronger tendency to partition forward rather than reverting to the enzyme–substrate complex,[a] i.e., $k_5 > k_4$.[10,11,33] Thus, besides reporting on the chemical step of catalysis, k_{cat} informs about the kinetic step of the release of the product from the enzyme–product complex (k_5), and k_{cat}/K_m informs about the kinetic steps of substrate association to form the enzyme–substrate complex (k_1) and dissociation of the substrate from the enzyme–substrate complex (k_2). Other kinetic approaches can also be used to measure KVSEs, such as rapid kinetics or steady-state kinetics with enzymes with multiple substrates, as long as mechanistic information (for example, K_D) can be extracted from the associated kinetic parameters for analysis.

$$k_{cat} = \frac{k_3 k_5}{k_3 + k_4 + k_5} \quad (3)$$

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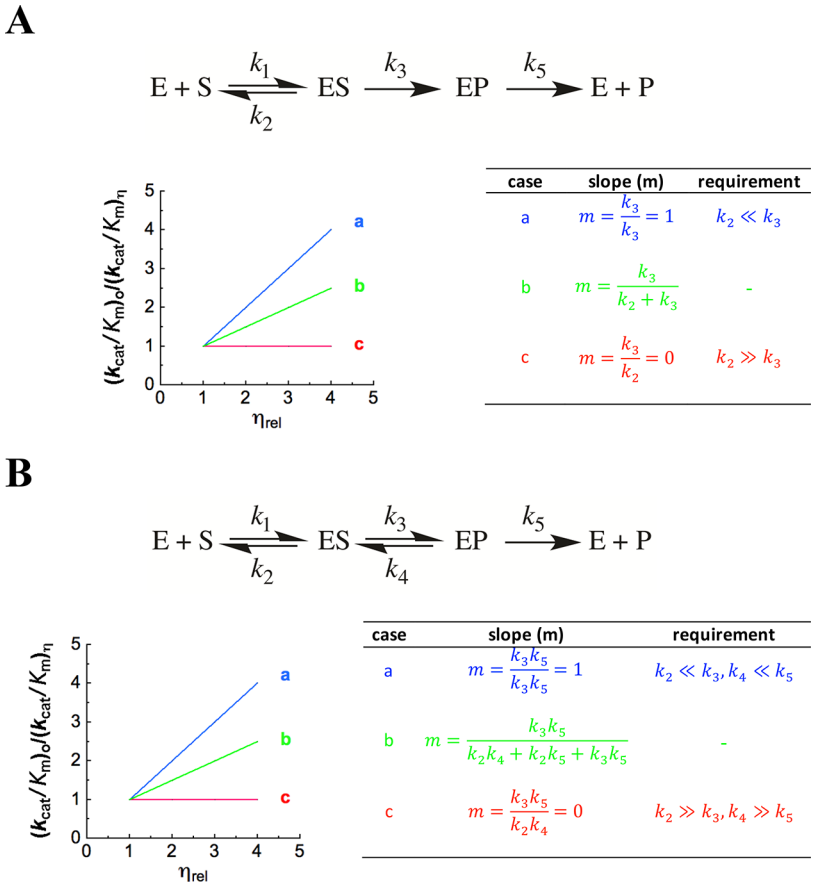

Figure 1. Plot of $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ vs η_{rel} for (A) irreversible and (B) reversible enzymatic reactions. The general analytical description for the slope is given by case b, with cases a and c representing reactions controlled by substrate diffusion and chemistry, respectively. The derivations of the analytical expressions for the slope in the various cases are presented in the Supporting Information.

$$\frac{k_{cat}}{K_m} = \frac{k_1k_3k_5}{k_2k_4 + k_2k_5 + k_3k_5} \quad (4)$$

$$k_{cat} = \frac{k_3k_5}{k_3 + k_5} \quad (5)$$

$$\frac{k_{cat}}{K_m} = \frac{k_1k_3}{k_2 + k_3} \quad (6)$$

Buffered solutions of varying solvent viscosity are prepared with the addition of glucose, sucrose, or glycerol, and their relative viscosity is calculated at the desired temperature according to the reference values of Lide at 20 °C.[34] This approach suffices for most applications without the need to use eq 7 to compute the absolute viscosity (η) after measuring the kinematic viscosity (ν) of a solvent using a Cannon–Ubbelohde Semi-Micro viscometer and its density (ρ) using a pycnometer.[26]

$$\eta = \nu\rho \quad (7)$$

The kinetic parameters of interest are then determined in buffered solvents of varying viscosity, including a reference set in the absence of the viscosigen. Caution should be exerted in cases in which the determinations are performed as a function of temperature because solvent viscosity is dependent of temperature.[26] The ratio of the solvent viscosity of the solution containing the viscosigen to the solvent viscosity of the solution without the viscosigen is defined as the relative viscosity (η_{rel}) of the solution. Typically, relative viscosities in the range of 1–4 are used because they provide a sufficient range for a meaningful interpretation of the results,[35] although smaller and larger ranges have been reported. It is also critical to use multiple microviscosigens with different chemical structures to rule out unspecific effects of the viscosigen on enzyme activity. The ratio of the value for the kinetic parameter of interest determined in the absence of viscosigen, $(k)_o$, to that in the presence of viscosigen, $(k)_\eta$, defines the normalized kinetic parameter.

Data are displayed in a plot of the normalized kinetic parameter of interest, i.e., $(k_{cat})_o/(k_{cat})_\eta$ or $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$, as a function of relative viscosity (Figures 1 and 2) and are fit with eq 8

$$\frac{(k)_o}{(k)_\eta} = m(\eta_{rel} - 1) + 1 \quad (8)$$

where m is the slope of the line and represents the degree of dependence of viscosity on the normalized kinetic parameter.[36] In some cases, the data in a plot of the normalized kinetic parameter as a function of relative viscosity significantly deviate from the expected linear correlation, demonstrating an inverse hyperbolic pattern[b] (Figure 3). This can be fit with eq 9

$$\frac{(k)_o}{(k)_\eta} = \frac{1}{1 + A\left[\frac{\eta_{rel} - 1}{(\eta_{rel} - 1) + B}\right]} \quad (9)$$

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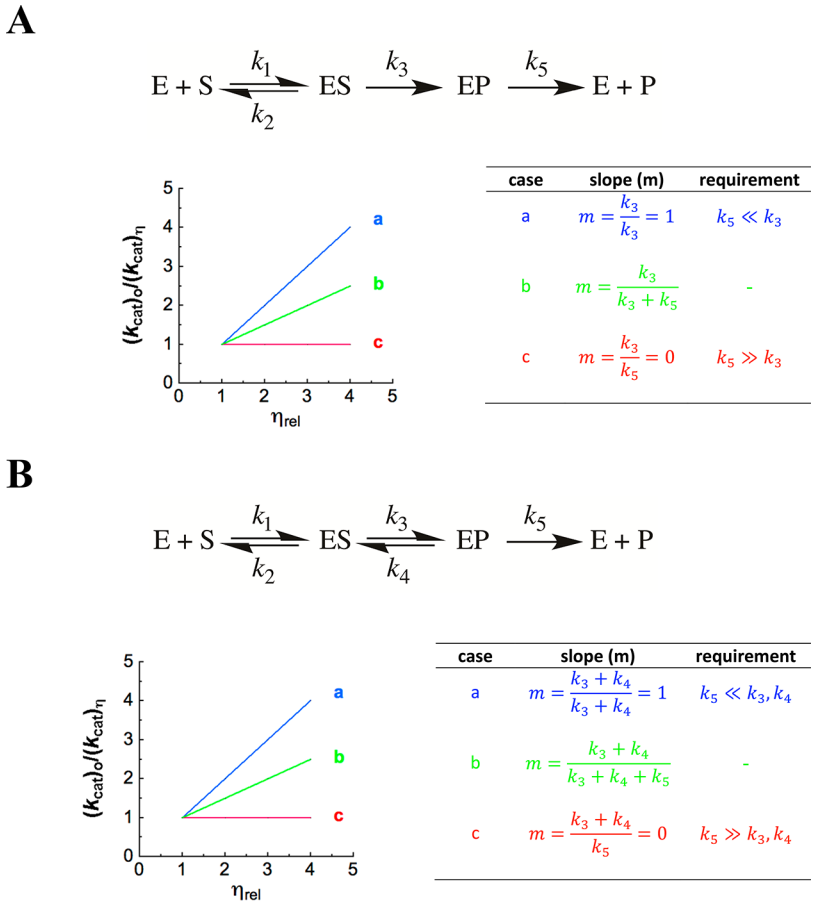

Figure 2. Plot of $(k_{cat})_o/(k_{cat})_\eta$ vs η_{rel} for (A) irreversible and (B) reversible enzymatic reactions. The general analytical description for the slope is given by case b, with cases a and c representing reactions controlled by product diffusion and chemistry, respectively. The derivations of the analytical expressions for the slope in the various cases are presented in the Supporting Information.

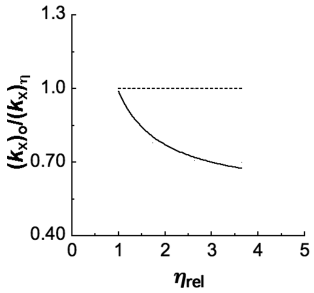

Figure 3. Plot of $(k_x)_o/(k_x)_\eta$ vs η_{rel} showing an inverse hyperbolic pattern. The solid curve was generated by fitting hypothetical data to eq 9. The dashed line represents a case lacking a solvent viscosity effect, which is equivalent to case c in Figures 1 and 2.

where A and B are parameters required to describe the hyperbolic behavior of the observed effect. It is important to note that KSVEs will not establish whether the chemical step of catalysis in an enzymatic reaction is reversible or irreversible; other approaches should be used to determine this. However, as KSVEs probe the kinetic steps of substrate binding and product release, the same conclusions about whether the enzymatic reaction is controlled by substrate diffusion, product release, or the chemical step of catalysis can be drawn irrespective of the chemistry, as illustrated in the cases below. Similarly, establishing the presence of an internal isomerization of an enzyme–substrate or enzyme–product complex does not require knowledge of whether the catalytic step in the enzymatic reaction is reversible.

DIFFUSION-CONTROLLED SUBSTRATE CAPTURE

For linear dependencies of the data in a plot of $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ versus η_{rel}, the slope of the line that fits the data (eq 8) will dictate the conclusion, with five possible outcomes.

Case a: $m = 1$. A slope of 1 in a plot of $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ versus η_{rel} is consistent with the capture of the substrate in enzyme–substrate complexes that yield products being limited fully by the rate of diffusion of the substrate (k_1) into the active site of the enzyme (Figure 1). For a diffusion-controlled reaction, indeed, all the enzyme complexes that are relevant to k_{cat}/K_m partition forward much faster than the diffusional encounter of substrate and enzyme. Consequently, an increase in solvent viscosity will result in a corresponding decrease in the rate constant for association of the substrate into the ES complex and therefore a decrease in the k_{cat}/K_m value. Mathematically, it can be shown that for an irreversible reaction the slope in a plot of $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ versus η_{rel} is given by $k_3/(k_2 + k_3)$, which simplifies to k_3/k_3 when $k_2 < k_3$ (see the Supporting Information for derivation). For a reversible reaction, the slope is given by $(k_3k_5)/(k_2k_4 + k_2k_5 + k_3k_5)$, which simplifies to k_3k_5/k_3k_5 when both $k_2 < k_3$ and $k_4 < k_5$ (see the Supporting Information for derivation). Mechanistically, beside defining the reaction as being diffusion-controlled, a slope of 1 suggests the presence of forward and,

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for reversible reactions, reverse commitments[c] to catalysis on the k_{cat}/K_m value, i.e., k_3/k_2 and $k_4/k_5 > 1$, although the commitments cannot be quantitated solely on the basis of KSVEs.

Case b: $0 < m < 1$. A slope between 0 and 1 in a plot of $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ versus η_{rel} is consistent with the capture of the substrate into enzyme−substrate complexes that yield products being partially limited by the rate of diffusion of the substrate (k_1) into the active site of the enzyme (Figure 1). In this case, the slope will depend on the relative magnitude of all the kinetic rate constants that contribute to k_{cat}/K_m with the exception of k_1, as illustrated in Figure 1 and demonstrated in the Supporting Information. This holds for both reversible and irreversible reactions. In addition, for irreversible reactions in which the chemical step of catalysis can be directly measured with rapid kinetics, besides defining the reaction as being partially diffusion-controlled, the slope can be used to calculate the forward commitment to catalysis on the k_{cat}/K_m value, i.e., k_3/k_2.

Case c: $m = 0$. A slope of zero in a plot of $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ versus η_{rel} is consistent with the capture of substrate into enzyme−substrate complexes that yield products being limited fully by the rate constant for the chemical step of catalysis (k_3) (Figure 1). For a reaction fully controlled by the chemical steps of catalysis, indeed, diffusion processes associated with the substrate and product associating to and dissociating from ES and EP complexes are much faster than the chemical steps of catalysis. Consequently, an increase in solvent viscosity will have no effect on k_{cat}/K_m. Mathematically, it can be shown that for an irreversible reaction the slope in a plot of $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ versus η_{rel} is given by k_3/k_2, which will tend to 0 when $k_2 > k_3$ (see the Supporting Information for derivation). For a reversible reaction, the slope is given by k_3k_5/k_2k_4, which will tend to 0 when $k_2 > k_3$ and $k_4 > k_5$ (see the Supporting Information for derivation). Mechanistically, besides defining the reaction as being fully controlled by the chemical step of catalysis, a slope of 0 is also consistent with the absence of forward and reverse commitments to catalysis on the k_{cat}/K_m value, i.e., k_3/k_2 and k_4/k_5 close to 0.

Case d: $m > 1$. Slopes of >1 in a plot of $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ versus η_{rel} are symptomatic of an effect of the viscosigen on the enzyme not being associated with diffusional processes of molecules per se, although these may still partially contribute to the general effect. Without further structural, mechanistic, or computational information, no general conclusion can be drawn because the viscosigen may have an inhibitory effect on the enzyme or may restrict movement of protein loops that are required for the capture of the substrate into enzyme−substrate complexes that yield products during the catalytic mechanism of the enzyme.

Case e: $m < 0$. Slopes of <0 in a plot of $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ versus η_{rel} are also symptomatic of an effect of the viscosigen on the enzyme not being associated with the diffusional process of the molecules, preventing a general conclusion unless further structural, mechanistic, or computational information is available. The presence of an inverse linear dependence of $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ versus η_{rel} has been associated with internal isomerizations of the enzyme−substrate complex (see below).

RATE-DETERMINING PRODUCT RELEASE

For linear dependencies in a plot of $(k_{cat})_o/(k_{cat})_\eta$ versus η_{rel}, the slope of the line that fits the data (eq 8) will dictate the conclusion, with five outcomes being possible.

Case a: $m = 1$. A slope of 1 in a plot of $(k_{cat})_o/(k_{cat})_\eta$ versus η_{rel} is consistent with the overall turnover of the enzyme being limited fully by the release of the product from the active site of the enzyme (k_5) (Figure 2). For a reaction with overall turnover being fully limited by product release, the rate constant for product release is much smaller than the rate constant for the chemical step of catalysis. Consequently, an increase in solvent viscosity will result in a corresponding decrease in the rate constant for release of the product from the EP complex and therefore a decrease in k_{cat}. Mathematically, it can be shown that for an irreversible reaction the slope in a plot of $(k_{cat})_o/(k_{cat})_\eta$ versus η_{rel} is given by $k_3/(k_3 + k_5)$, which simplifies to k_3/k_3 when $k_5 < k_3$ (see the Supporting Information for derivation). For a reversible reaction, the slope is given by $(k_3 + k_4)/(k_3 + k_4 + k_5)$, which simplifies to $(k_3 + k_4)/(k_3 + k_4)$ when $k_5 < k_3$ and $k_5 < k_4$ (see the Supporting Information for derivation). Mechanistically, besides defining the overall turnover as being fully limited by product release, a slope of 1 suggests the presence of forward and, for reversible reactions, reverse commitments to catalysis on the k_{cat} value, i.e., k_3/k_5 and $k_4/k_5 > 1$, although the commitments cannot be quantitated solely on the basis of KSVEs. For reversible reactions, either or both commitments can be present, but KSVEs will not distinguish between the two possibilities.

Case b: $0 < m < 1$. A slope between 0 and 1 in a plot of $(k_{cat})_o/(k_{cat})_\eta$ versus η_{rel} is consistent with the overall turnover of the enzyme being partially limited by release of the product from the active site of the enzyme (k_5) and the chemical step of catalysis (k_3) (Figure 2). In this case, the slope will depend on the relative magnitude of all the rate constants that contribute to k_{cat}, as illustrated in Figure 2 and demonstrated in the Supporting Information. This holds for both reversible and irreversible reactions. In addition, for irreversible reactions when the chemical step of catalysis can be directly measured with rapid kinetics, besides defining the overall turnover of the enzyme as being partially limited by product release, the slope can be used to calculate the commitment to catalysis on the k_{cat} value, i.e., k_3/k_5.

Case c: $m = 0$. A slope of zero in a plot of $(k_{cat})_o/(k_{cat})_\eta$ versus η_{rel} is consistent with the overall turnover of the enzyme being limited fully by the rate constant for the chemical step of catalysis (k_3) (Figure 2). For a reaction with overall turnover being fully limited by the chemical step of catalysis, the rate constant for product release is much faster than the rate constants for the chemical step of catalysis in the forward and reverse directions. Consequently, an increase in solvent viscosity will have no effect on k_{cat}. Mathematically, for an irreversible reaction, the slope in a plot of $(k_{cat})_o/(k_{cat})_\eta$ versus η_{rel} is given by k_3/k_5, which will tend to 0 when $k_5 > k_3$ (see the Supporting Information for derivation). For a reversible reaction, the slope is given by $(k_3 + k_4)/k_5$, which will tend to 0 when $k_5 > k_3$ and $k_5 > k_4$ (see the Supporting Information for derivation). Mechanistically, besides defining the overall turnover as being fully limited by the chemical step of catalysis, a slope of 0 also suggests the absence of forward and, for reversible reactions, reverse commitments to catalysis on the k_{cat} value, i.e., k_3/k_5 and k_4/k_5 values of close to 0.

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Case d: $m > 1$. Slopes of >1 in a plot of $(k_{cat})_o/(k_{cat})_\eta$ versus η_{rel} are symptomatic of an effect of the viscosigen on the enzyme not being strictly associated with the diffusional processes of molecules per se, although these may still partially contribute to the general effect. A general conclusion cannot be drawn in the absence of further structural, mechanistic, or computational information. The viscosigen may, for example, have an inhibitory effect on the enzyme that results in a decreased k_{cat}. Alternatively, the viscosigen may restrict movements of either large protein domains or protein loops that are required in the catalytic mechanism of the enzyme. The latter case was proposed with a mutant β-lactam synthase using glycerol and PEG 8000, for which a slope of 1.6 in a plot of $(k_{cat})_o/(k_{cat})_\eta$ versus η_{rel} was interpreted as being due to restricted opening of active site loops originating from the friction imposed by the high relative solvent viscosity.[37]

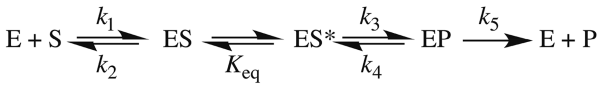
Case e: $m < 0$. Slopes of <0 in a plot of $(k_{cat})_o/(k_{cat})_\eta$ versus η_{rel} are also symptomatic of an effect of the viscosigen on the enzyme not being associated with the diffusional process of molecules. Similar to the case of slopes of >1, a general conclusion is not available in the absence of further structural, mechanistic, or computational information. This pattern has been associated with internal isomerizations of enzyme−substrate or enzyme−product complexes (see below).

■ ENZYME ISOMERIZATION

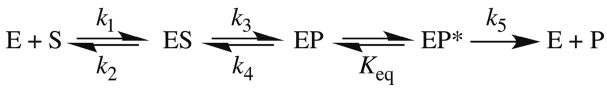
An inverse hyperbolic pattern in a plot of $(k_{cat})_o/(k_{cat})_\eta$ or $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ versus η_{rel} (Figure 3) is consistent with the presence of a solvent-sensitive internal isomerization of the enzyme−substrate or enzyme−product complex (Scheme 2).

Scheme 2. Simplest Kinetic Mechanism for an Enzyme (E) with a Single Substrate (S) and Product (P), with an Internal Isomerization of the Enzyme−Substrate Complex (A) or the Enzyme−Product Complex (B)

A



B



In some cases, an inverse linear dependence of $(k_{cat})_o/(k_{cat})_\eta$ or $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ on η_{rel} is observed if the internal equilibrium between the enzyme complexes is perturbed by very high concentrations of the viscosigen. In both cases, diffusional processes of molecules that result in the generation or decay of enzyme−substrate and enzyme−product complexes are indeed ruled out because their theoretical treatment predicts linear dependencies with slopes between 0 and +1 (see above). The hyperbolic patterns, instead, are symptomatic of an internal equilibrium of an enzyme complex that is perturbed by the presence of the viscosigen. With an inverse hyperbolic pattern, the presence of the viscosigen would favor the downstream species in the catalytic pathway involved in the internal equilibrium, as the kinetic parameter of interest is larger in the presence of the viscosigen than in its absence. The opposite would be true for a normal hyperbolic pattern, although to the best of our knowledge this pattern has not yet been reported, likely because it is difficult to distinguish it from linear patterns due to error propagation when taking the ratio of the kinetic parameter of interest. With knowledge of the steady-state kinetic mechanism of the enzyme, one could establish whether the internal isomerization occurs with the enzyme−substrate or enzyme−product complex. For example, the observation of inverse hyperbolic solvent viscosity effects on both k_{cat} and k_{cat}/K_m values with an enzyme catalyzing an irreversible reaction would be consistent with an isomerization of the enzyme−substrate complex but not the enzyme−product complex.

■ SELECT CASE STUDIES

Diffusion-Controlled Substrate Capture. By measuring the effect of solvent viscosity on k_{cat}/K_m, we can determine whether binding of a substrate to an enzyme is diffusion-controlled and, if so, the extent to which it is. Viscosity effect studies were used to establish that chymotrypsin and triosephosphate isomerase (TIM)-catalyzed diffusion-controlled reactions. The seminal work of Kirsch provided some of the key theoretical and experimental considerations that are required for KSVE studies. Kirsch's group probed binding of a peptide substrate to chymotrypsin with sucrose and Ficoll as the microviscogen and macroviscogen, respectively.[35] They tested the effect of viscosity on the kinetic parameters for chymotrypsin using peptide substrates with *p*-nitrophenyl ester and methyl esters, which displayed k_{cat}/K_M values of $\sim 10^7$ and 10^5 M^{-1} s^{-1}, respectively. In addition, they measured the effect of solution viscosity on the peptide using *p*-nitroanilide, a "poor" substrate for which the chemical step of the reaction was shown to be rate-limiting (with a k_{cat}/K_M value of ~300 mM^{-1} s^{-1}).[35] For all three peptide substrates, no major effects were observed when Ficoll was used as the macroviscogen. When sucrose was used as the microviscogen, the acylation rate constant was shown to be partially diffusion-limited with peptides containing *p*-nitrophenyl ester and, to a lesser extent, methyl esters (Figure 1, cases a and b). The k_{cat}/K_M value with the poor substrate was insensitive to solution viscosity (Figure 1, case c). The use of poor substrates helped determine whether the observed viscosity effect is due to another step in the catalytic cycle. Similarly, the lack of an effect on the k_{cat}/K_M values with the macroviscogen was consistent with the observed effect being caused by changes in substrate diffusion and not by another step.[35]

Knowles and co-workers used Ficoll, PEG, and polyacrylamide as the macroviscogens and glycerol as the microviscogen to study the reaction of TIM. In addition, a TIM mutant enzyme, where the chemical step had been shown to be the rate-limiting step, was used as the control instead of a poor substrate. They showed that binding of the substrate to TIM is diffusion-limited (Figure 1, case a) and that the enzyme represents a perfect enzyme that cannot be altered further in terms of increasing its catalytic efficiency.[29] Other examples of enzymatic reactions partially controlled by substrate binding include orotidine 5′-monophosphate decarboxylase with a number of substrates[38] and eukaryotic nitronate monooxyge-

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nase with its physiological substrate, propionate 3-nitronate, and its analogue, ethyl nitronate.[12]

The reaction of cytochrome *c* peroxidase was shown by Loo and Erman not to be diffusion controlled. They measured the effect of sucrose as the viscosigen and determined that the reaction was viscosity-independent (Figure 1, case c).[39] This is consistent with later studies that showed a conformational step that is important for electron transfer that limits the catalytic cycle.[40] Sweet and Blanchard showed that the dependence of the normalized k_{cat}/K_M of fumarase with fumarate as the substrate as a function of the relative solvent viscosity with glycerol and sucrose as the microviscogens had a slope close to 1, clearly indicating that the reaction is diffusion-limited. Again, PEG 1000 did not have any effect, and the poor substrate, tartrate, was insensitive to solution viscosity (slope of ~0).[41] Several more studies have been published using KVSE to probe binding of the substrate to enzymes.[42−50]

Rate-Determining Product Release. The effects of solution viscosity on k_{cat} can be analyzed as described above to establish whether product release determines the overall turnover rate of enzymes and the extent to which it does. Seminal work by Adams and Taylor focused on KSVEs on cAMP-dependent protein kinases. They used sucrose or glycerol as the microviscogen and showed that the k_{cat} values for several peptides were highly sensitive to solution viscosity with slopes close to 1, suggesting that product release is the rate-limiting step in the catalytic cycle (Figure 2, case a).[51]

Cole et al. studied the rate of human C-terminal Src kinase (Csk). They used sucrose as the microviscogen, polyethylene glycol (PEG 8000) as the macroviscogen, and ATPγS as the poor substrate.[52] Analysis of the effect of viscosity on k_{cat} resulted in a slope of 0.42, suggesting that ADP release is partially rate-limiting for overall turnover of the enzyme (Figure 2, case b).[52] Wood et al. showed that the k_{cat} values of orotidine 5′-monophosphate decarboxylase (OMPDC) from *Methanotherobacter thermoautophicus* were insensitive to solution viscosity with sucrose and Ficoll (Figure 2, case c).[38] There are several other publications reporting the use of KSVEs to probe product release,[38,53−57] with some cases in which a direct link between product release being rate-limiting for turnover and conformational changes of the enzyme has been established.

Enzyme Isomerization. By measuring the effect of solvent viscosity on k_{cat}/K_m or k_{cat} values, one can establish the presence of internal isomerizations of enzyme−substrate or enzyme−product complexes. Cook was the first to report an inverse KSVE with the NAD-malic enzyme from *Ascaris sum*.[58] With glycerol as a viscosigen, $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ decreased to ~0.6 in the presence of 9% glycerol and to ~0.2 in the presence of 18% glycerol. Similarly, $(k_{cat})_o/(k_{cat})_\eta$ had inverse values of ~0.8 with 9% glycerol and ~0.5 with 18% glycerol. These data were interpreted as reflecting changes in the relative solvent viscosity rather than in the dielectric constant or macroviscosity of the solvent based on control experiments with ethanol and Ficoll, showing no effects on the malic enzyme. The conclusion was that the KSVE with the malic enzyme most probably resulted from changes in the rate constants, i.e., equilibria, for enzyme isomerizations associated with the catalytic process. Internal isomerizations of enzyme complexes relevant to catalysis were established on the basis of the inverse linear KSVE of the steady-state kinetics with a mutant variant of choline oxidase, with E312 being replaced with aspartate,[59] and with orotidine 5′-monophosphate decarboxylase.[38]

An inverse hyperbolic dependence of $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ (Figure 3) was first reported with the flavin domain of flavocytochrome b_2, which showed a 2-fold activation by moderate concentrations of glycerol between 10 and 20%.[60] These data were interpreted with a solvent-sensitive internal equilibrium of the enzyme−lactate Michaelis complex. The use of eq 9 to fit inverse KSVE dependencies of the $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ and $(k_{cat})_o/(k_{cat})_\eta$ values was introduced in a study of D-arginine dehydrogenase with leucine as the substrate.[43] Because the k_{cat}/K_m and k_{cat} values showed inverse KSVEs (Figure 3), the possibility that an enzyme isomerization occurred on the enzyme−product complex was ruled out, and the conclusion was that the isomerization happened in the Michaelis complex, yielding an enzyme−substrate complex competent for flavin reduction. Inverse hyperbolic KSVEs were also reported with the $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ and $(k_{cat})_o/(k_{cat})_\eta$ values with eukaryotic nitronate monooxygenase and the substrate analogue ethyl nitronate.[12]

Inverse hyperbolic KSVEs (Figure 3) were observed for $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ for oxygen with the wild-type form of choline oxidase, establishing that an isomerization of the reduced enzyme is required before reaction with oxygen.[44] A follow-up study on variants of choline oxidase in which M62 and F357 were replaced with alanine showed that the inverse hyperbolic KSVE on $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ for oxygen was preserved in the M62A mutant but lost in the F357A and M62A/F357A variants, which showed a slope of zero in plots of the $(k_{cat}/K_m)_o/(k_{cat}/K_m)_\eta$ value for oxygen as a function of η_{rel}.[13] The conclusion was that F357 acts as an oxygen gate in the oxidative half-reaction of choline oxidase.

CONCLUSION

Determination of the kinetic and chemical mechanisms of enzymes is a fundamental step toward understanding the mode of action of enzymes and developing approaches for inhibitor design.[3,61,62] KSVEs provide important information regarding the relative contribution of substrate or product diffusion to and from the active site to the overall catalytic cycle. Using steady-state kinetic analyses as described here, it is possible to determine if substrate binding is diffusion-controlled, i.e., analyzing KSVE on k_{cat}/K_m, and if product release is the rate-limiting step in the catalytic cycle, i.e., analyzing the KSVE on k_{cat}. These experiments are simple to perform and straightforward to interpret and do not require specialized instrumentation or specific labeling of substrates or products. In addition, using KSVEs in combination with other techniques, such as KIEs, can be very useful for establishing what the rate-limiting step is in catalysis and identifying isomerization steps in enzyme mechanisms. We anticipate that because of its ease, KSVE will be more broadly implemented and will become a more widespread tool in the arsenal of modern enzymologists and protein chemists.

ASSOCIATED CONTENT

Supporting Information

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Derivations of the equations that describe the slope in linear plots of the normalized k_{cat}/K_m and k_{cat} values as a function of relative solvent viscosity (PDF)

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ADDITIONAL NOTES

[a]From a practical standpoint, an irreversible reaction for which $k_4 \approx 0$ and one for which $k_5 > k_4$ are practically indistinguishable; from a fundamental standpoint, they are not the same. An irreversible reaction is one for which there is no reverse flux of intermediates from EP to ES. Instead, when $k_5 > k_4$, it follows that the EP complex partitions forward to release the product rather than reverting back to the ES complex, making the flux through the reverse kinetic step k_4 negligible. Despite the reaction behaving as if it were irreversible, it is not. Indeed, one can envision altering environmental conditions to decrease the magnitude of the k_5 rate constant, thereby making k_5 comparable to k_4; this would result in the reaction behaving as if it were reversible.

[b]In principle, a normal hyperbolic pattern is possible, although it has not yet been reported. The general conclusions for such a pattern would be similar to those drawn when an inverse hyperbolic pattern is observed.

[c]A commitment to catalysis is a ratio of rate constants that provides an indication of how either the ES complex proceeds forward through k_3 rather than dissociating through k_2 (i.e., k_3/k_2 or forward commitment to catalysis) or the EP complex reverts backward through k_4 rather than proceeding forward through k_5 (i.e., k_4/k_5 or reverse commitment to catalysis).[10,11]

REFERENCES

(1) Keatinge-Clay, A. T. (2017) The Uncommon Enzymology of Cis-Acyltransferase Assembly Lines,. *Chem. Rev. 117*, 5334−5366.

(2) Walsh, C. T. (2016) Insights into the chemical logic and enzymatic machinery of NRPS assembly lines. *Nat. Prod. Rep. 33*, 127−135.

(3) Holdgate, G. A., Meek, T. D., and Grimley, R. L. (2017) Mechanistic enzymology in drug discovery: a fresh perspective. *Nat. Rev. Drug Discovery 17*, 115−132.

(4) Kovaleva, E. G., and Lipscomb, J. D. (2007) Crystal structures of Fe2+ dioxygenase superoxo, alkylperoxo, and bound product intermediates. *Science 316*, 453−457.

(5) Mehra-Chaudhary, R., Dai, Y., Sobrado, P., and Tanner, J. J. (2016) In Crystallo Capture of a Covalent Intermediate in the UDP-Galactopyranose Mutase Reaction. *Biochemistry 55*, 833−836.

(6) Wang, Y., Bugge, K., Kragelund, B. B., and Lindorff-Larsen, K. (2018) Role of protein dynamics in transmembrane receptor signalling. *Curr. Opin. Struct. Biol. 48*, 74−82.

(7) Rosenzweig, R., and Kay, L. E. (2014) Bringing dynamic molecular machines into focus by methyl-TROSY NMR. *Annu. Rev. Biochem. 83*, 291−315.

(8) Jiang, Y., and Kalodimos, C. G. (2017) NMR Studies of Large Proteins. *J. Mol. Biol. 429*, 2667−2676.

(9) Johnson, K. A. (1998) Advances in transient-state kinetics. *Curr. Opin. Biotechnol. 9*, 87−89.

(10) Cook, P. F., and Cleland, W. W. (2007) *Enzyme Kinetics and Mechanism*, pp 416, Garland Science, New York.

(11) Cleland, W. W. (2005) The use of isotope effects to determine enzyme mechanisms,. *Arch. Biochem. Biophys. 433*, 2−12.

(12) Smitherman, C., and Gadda, G. (2013) Evidence for a transient peroxynitro acid in the reaction catalyzed by nitronate monooxygenase with propionate 3-nitronate. *Biochemistry 52*, 2694−2704.

(13) Salvi, F., Rodriguez, I., Hamelberg, D., and Gadda, G. (2016) Role of F357 as an Oxygen Gate in the Oxidative Half-Reaction of Choline Oxidase. *Biochemistry 55*, 1473−1484.

(14) Da Fonseca, I., Qureshi, I. A., Mehra-Chaudhary, R., Kizjakina, K., Tanner, J. J., and Sobrado, P. (2014) Contributions of unique active site residues of eukaryotic UDP-galactopyranose mutases to substrate recognition and active site dynamics. *Biochemistry 53*, 7794−7804.

(15) Robinson, R., Franceschini, S., Fedkenheuer, M., Rodriguez, P. J., Ellerbrock, J., Romero, E., Echandi, M. P., Martin Del Campo, J. S., and Sobrado, P. (2014) Arg279 is the key regulator of coenzyme selectivity in the flavin-dependent ornithine monooxygenase SidA. *Biochim. Biophys. Acta, Proteins Proteomics 1844*, 778−784.

(16) Huang, Y., Zheng, X., Zhang, H., Ding, H., Guo, X., Yang, Y., Chen, X., Zhou, Q., and Du, A. (2017) Site-Directed Mutagenesis Study Revealed Three Important Residues in Hc-DAF-22, a Key Enzyme Regulating Diapause of Haemonchus contortus. *Front. Microbiol. 8*, 2176.

(17) Wagner, C. R., and Benkovic, S. J. (1990) Site directed mutagenesis: a tool for enzyme mechanism dissection. *Trends Biotechnol. 8*, 263−270.

(18) Shirey, C., Badieyan, S., and Sobrado, P. (2013) Role of Ser-257 in the sliding mechanism of NADP(H) in the reaction catalyzed by the Aspergillus fumigatus flavin-dependent ornithine N5-monooxygenase SidA. *J. Biol. Chem. 288*, 32440−32448.

(19) Boechi, L., de Oliveira, C. A., Da Fonseca, I., Kizjakina, K., Sobrado, P., Tanner, J. J., and McCammon, J. A. (2013) Substrate-dependent dynamics of UDP-galactopyranose mutase: Implications for drug design. *Protein Sci. 22*, 1490−1501.

(20) Ouedraogo, D., Souffrant, M., Vasquez, S., Hamelberg, D., and Gadda, G. (2017) Importance of Loop L1 Dynamics for Substrate Capture and Catalysis in Pseudomonas aeruginosa d-Arginine Dehydrogenase. *Biochemistry 56*, 2477−2487.

(21) van der Kamp, M. W., and Mulholland, A. J. (2013) Combined quantum mechanics/molecular mechanics (QM/MM) methods in computational enzymology. *Biochemistry 52*, 2708−2728.

(22) Von Smoluchowski, M. (1917) *Z. Phys. Chem. 92*, 129−168.

(23) Berg, O. G., and von Hippel, P. H. (1985) Diffusion-controlled macromolecular interactions. *Annu. Rev. Biophys. Biophys. Chem. 14*, 131−160.

(24) Kramers, H. (1940) Brownian motion in a field of force and the diffusion model of chemical reactions. *Physica 7*, 284−304.

(25) Heyes, D. J., Levy, C., Sakuma, M., Robertson, D. L., and Scrutton, N. S. (2011) A twin-track approach has optimized proton and hydride transfer by dynamically coupled tunneling during the evolution of protochlorophyllide oxidoreductase,. *J. Biol. Chem. 286*, 11849−11854.

DOI: 10.1021/acs.biochem.8b00232
Biochemistry 2018, 57, 3445−3453

(26) Romero, E., Ladani, S. T., Hamelberg, D., and Gadda, G. (2016) Solvent-Slaved Motions in the Hydride Tunneling Reaction Catalyzed by Human Glycolate Oxidase. *ACS Catal. 6*, 2113−2120.
(27) Alberty, R., and Hammes, G. G. (1958) Application of the Theory of Diffusion-controlled Reactions to Enzyme Kinetics. *J. Phys. Chem. 62*, 154−159.
(28) Albery, W. J., and Knowles, J. R. (1976) Evolution of enzyme function and the development of catalytic efficiency. *Biochemistry 15*, 5631−5640.
(29) Blacklow, S. C., Raines, R. T., Lim, W. A., Zamore, P. D., and Knowles, J. R. (1988) Triosephosphate isomerase catalysis is diffusion controlled. Appendix: Analysis of triose phosphate equilibria in aqueous solution by 31P NMR. *Biochemistry 27*, 1158−1167.
(30) Edward, J. T. (1970) Molecular volumes and the Stokes-Einstein equation. *J. Chem. Educ. 47*, 261.
(31) Hagen, J. S. (2010) Solvent Viscosity and Friction in Protein Folding Dynamics. *Curr. Protein Pept. Sci. 11*, 385−395.
(32) Sekhar, A., Latham, M. P., Vallurupalli, P., and Kay, L. E. (2014) Viscosity-dependent kinetics of protein conformational exchange: microviscosity effects and the need for a small viscogen. *J. Phys. Chem. B 118*, 4546−4551.
(33) Cleland, W. W. (1975) Partition analysis and concept of net rate constants as tools in enzyme kinetics. *Biochemistry 14*, 3220−3224.
(34) *Handbook of Chemistry and Physics*, CRC Press, Boca Raton, FL.
(35) Brouwer, A. C., and Kirsch, J. F. (1982) Investigation of diffusion-limited rates of chymotrypsin reactions by viscosity variation. *Biochemistry 21*, 1302−1307.
(36) Fitzpatrick, P. F., Kurtz, K. A., Denu, J. M., and Emanuele, J. F. (1997) Contrasting Values of Commitment Factors Measured from Viscosity, pH, and Kinetic Isotope Effects: Evidence for Slow Conformational Changes in the D-Amino Acid Oxidase Reaction. *Bioorg. Chem. 25*, 100−109.
(37) Raber, M. L., Freeman, M. F., and Townsend, C. A. (2009) Dissection of the stepwise mechanism to beta-lactam formation and elucidation of a rate-determining conformational change in beta-lactam synthetase. *J. Biol. Chem. 284*, 207−217.
(38) Wood, B. M., Chan, K. K., Amyes, T. L., Richard, J. P., and Gerlt, J. A. (2009) Mechanism of the orotidine 5′-monophosphate decarboxylase-catalyzed reaction: effect of solvent viscosity on kinetic constants. *Biochemistry 48*, 5510−5517.
(39) Loo, S., and Erman, J. E. (1977) The rate of reaction between cytochrome C peroxidase and hydrogen peroxide is not diffusion limited,. *Biochim. Biophys. Acta 481*, 279−282.
(40) Volkov, A. N., and van Nuland, N. A. (2012) Electron transfer interactome of cytochrome C. *PLoS Comput. Biol. 8*, No. e1002807.
(41) Sweet, W. L., and Blanchard, J. S. (1990) Fumarase: viscosity dependence of the kinetic parameters. *Arch. Biochem. Biophys. 277*, 196−202.
(42) Gadda, G., and Fitzpatrick, P. F. (2013) Solvent isotope and viscosity effects on the steady-state kinetics of the flavoprotein nitroalkane oxidase. *FEBS Lett. 587*, 2785−2789.
(43) Yuan, H., Xin, Y., Hamelberg, D., and Gadda, G. (2011) Insights on the mechanism of amine oxidation catalyzed by D-arginine dehydrogenase through pH and kinetic isotope effects. *J. Am. Chem. Soc. 133*, 18957−18965.
(44) Gannavaram, S., and Gadda, G. (2013) Relative timing of hydrogen and proton transfers in the reaction of flavin oxidation catalyzed by choline oxidase. *Biochemistry 52*, 1221−1226.
(45) Robinson, R. M., Rodriguez, P. J., and Sobrado, P. (2014) Mechanistic studies on the flavin-dependent N(6)-lysine monooxygenase MbsG reveal an unusual control for catalysis. *Arch. Biochem. Biophys. 550−551*, 58−66.
(46) Su, Q., and Klinman, J. P. (1998) Probing the mechanism of proton coupled electron transfer to dioxygen: the oxidative half-reaction of bovine serum amine oxidase. *Biochemistry 37*, 12513−12525.
(47) Glickman, M. H., and Klinman, J. P. (1995) Nature of rate-limiting steps in the soybean lipoxygenase-1 reaction. *Biochemistry 34*, 14077−14092.
(48) McKay, G. A., and Wright, G. D. (1996) Catalytic Mechanism of Enterococcal Kanamycin Kinase (APH(3′)-IIIa): Viscosity, Thio, and Solvent Isotope Effects Support a Theorell−Chance Mechanism. *Biochemistry 35*, 8680−8685.
(49) McKinnie, R. E., and Olson, J. S. (1981) Effects of solvent composition and viscosity on the rates of CO binding to heme proteins. *J. Biol. Chem. 256*, 8928−8932.
(50) Hasinoff, B. B., Dreher, R., and Davey, J. P. (1987) The association reaction of yeast alcohol dehydrogenase with coenzyme is partly diffusion-controlled in solvents of increased viscosity,. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol. 911*, 53−58.
(51) Adams, J. A., and Taylor, S. S. (1992) Energetic limits of phosphotransfer in the catalytic subunit of cAMP-dependent protein kinase as measured by viscosity experiments. *Biochemistry 31*, 8516−8522.
(52) Cole, P. A., Burn, P., Takacs, B., and Walsh, C. T. (1994) Evaluation of the catalytic mechanism of recombinant human Csk (C-terminal Src kinase) using nucleotide analogs and viscosity effects. *J. Biol. Chem. 269*, 30880−30887.
(53) Caldwell, S. R., Newcomb, J. R., Schlecht, K. A., and Raushel, F. M. (1991) Limits of diffusion in the hydrolysis of substrates by the phosphotriesterase from Pseudomonas diminuta. *Biochemistry 30*, 7438−7444.
(54) Oppenheimer, M., Valenciano, A. L., Kizjakina, K., Qi, J., and Sobrado, P. (2012) Chemical mechanism of UDP-galactopyranose mutase from Trypanosoma cruzi: a potential drug target against Chagas' disease. *PLoS One 7*, No. e32918.
(55) Arnett, S. O., Gerratana, B., and Townsend, C. A. (2007) Rate-limiting steps and role of active site Lys443 in the mechanism of carbapenam synthetase. *Biochemistry 46*, 9337−9345.
(56) Eser, B. E., and Fitzpatrick, P. F. (2010) Measurement of intrinsic rate constants in the tyrosine hydroxylase reaction. *Biochemistry 49*, 645−652.
(57) Sierks, M. R., Sico, C., and Zaw, M. (1997) Solvent and viscosity effects on the rate-limiting product release step of glucoamylase during maltose hydrolysis. *Biotechnol. Prog. 13*, 601−608.
(58) Karsten, W. E., Lai, C.-J., and Cook, P. F. (1995) Inverse Solvent Isotope Effects in the NAD-Malic Enzyme Reaction Are the Result of the Viscosity Difference between D2O and H2O: Implications for Solvent Isotope Effect Studies. *J. Am. Chem. Soc. 117*, 5914−5918.
(59) Quaye, O., Lountos, G. T., Fan, F., Orville, A. M., and Gadda, G. (2008) Role of Glu312 in binding and positioning of the substrate for the hydride transfer reaction in choline oxidase. *Biochemistry 47*, 243−256.
(60) Sobrado, P., Daubner, C., and Fitzpatrick, P. F. (2001) Probing the Relative Timing of Hydrogen Abstraction Steps in the Flavocytochrome b2 Reaction with Primary and Solvent Deuterium Isotope Effects and Mutant Enzymes. *Biochemistry 40*, 994−1001.
(61) Copeland, R. A., Pompliano, D. L., and Meek, T. D. (2006) Drug-target residence time and its implications for lead optimization. *Nat. Rev. Drug Discovery 5*, 730−739.
(62) Schramm, V. L. (2013) Transition States, analogues, and drug development. *ACS Chem. Biol. 8*, 71−81.

DOI: 10.1021/acs.biochem.8b00232
Biochemistry 2018, 57, 3445−3453