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New Concepts in Biochemistry

Steady State Enzyme Velocities That Are Independent of [Enzyme]: An Important Behavior in Many Membrane and Particle-Bound States[†]

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ABSTRACT: The popular paradigm for biological education in kinetics involves descriptions that are appropriate for soluble enzymes. Derivations seldom present the assumptions on which the fundamental parameter of these kinetics, the site rate constant, is based. This omission can create difficulty for understanding situations where the assumptions are invalid. Membrane- and particle-bound enzyme systems provide several examples. In fact, biological organisms show macroscopic design and enzyme expression levels which suggest utilization of alternative kinetic mechanisms. The role of substrate affinity and enzyme inhibitors is greatly altered, with correlated impact on biomedical and biotechnological designs. Enzymes may perform functions such as isolation of cell contents from the environment, an action that is usually reserved for membranes. These properties can be mimicked but never perfectly replicated in purified systems. This presentation provides a description of some of these behaviors for membrane- or particle-bound enzymes, using an approach that is closely correlated with the manner in which steady state enzyme kinetics are typically presented.

In what might be defined as classical enzyme kinetics, the type that is readily available in textbook form, reactions begin with the interaction of an enzyme [E] with one of its substrates or regulators. The velocity expression for every step of the reaction pathway contains some form of the enzyme and is characterized by a first- or second-order rate constant, k. Enzyme constants such as K_M, k_{cat}, apparent K_M, V_{max}, K_I, K_S, and cooperativity coefficient describe reaction velocity at any substrate or regulator concentration. These constants describe "site" properties of the enzyme. While membranes may influence these sites, the following description deals with cases where the enzyme site may be removed from the velocity expression altogether. Distinction of site kinetics from all other types is quite important.

With a standard educational background, our experience in study of membrane-bound enzymes was one of great difficulty in providing quantitative descriptions of reaction behavior. Since all reactions show saturation with respect to substrate, a first impulse is to use some form of K_M. However, closer inspection usually reveals steps in the reaction that complicate this constant. Experimental error can conceal nonlinearity of steady state kinetic plots, and the assay may limit the range of conditions that can be employed. As a result, K_M and other parameters of classical enzyme kinetics become default parameters used in reporting enzyme behavior; they are much overused for membrane-bound enzymes. A similar conclusion has been reached for phospholipase enzymes (*1*, *2*).

Others have suggested that, *in vivo*, Michaelis−Menten kinetics could be the exception (*3*). While theoretical alternatives to site behavior have been described for receptor−ligand binding, with somewhat lesser application to

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enzymes (*e.g.* refs 4−11), they are underdeveloped when compared with classical enzyme kinetics and are virtually unaddressed in textbooks. Greater development is dependent on identification of more examples, which depends on greater awareness of these behaviors. This concept describes how the view of an enzyme, the role of substrate affinity and inhibitors, along with almost any other property, can be dramatically altered by the context in which the enzyme is studied. Enzyme expression levels and structural features of organisms may complement novel enzyme kinetic states. They permit enzymes to serve novel functions, including the membrane-like action of isolating cell contents. There are many ways in which membranes remove the enzyme from the velocity expression. This short presentation outlines three general types.

I. Enzyme Kinetics without Site Behavior. (1) Reactions that Are Limited by Substrate Flux to the Enzyme-Containing Particle or Surface

Figure 1 contrasts the behavior of the enzyme, alkaline phosphatase, in *Escherichia coli* (Figure 1, panels A and B) with that of the same amount of free enzyme (Figure 1B, inset). The behaviors differ greatly except for V_{max} (intercept on the horizontal axis, Figure 1B). A brief theoretical outline emphasizes how this behavior arises. Literature citations should be consulted for more complete descriptions.

Different Types of Rate Constants. All catalysis begins with arrival of substrate at the enzyme, a process described by "flux" from bulk solution [flux $= J = 4\pi Drc$ (*5*), where c is the concentration of substrate in bulk solution and D and r are defined below]. While flux is the first component of any rate description, it only contributes to rate limitation in nonhomogeneous solutions, such as those created by an enzyme that depletes substrate levels in its vicinity. If flux is at equilibrium (substrate concentration in the vicinity of the enzyme is similar to that in bulk solution), it does not contribute to the rate-limiting step. Since the latter state applies to all soluble enzymes, classical steady state derivations skip a description of flux altogether and proceed to the use of familiar first- and second-order rate constants. We found that the dominance of this practice in biochemical descriptions created a hurdle for understanding flux-dependent reactions. We therefore found it informative to describe all kinetic states on the basis of k_{obs} (eq 1), the complete description of rate constant, which can be applied to both flux-limited and flux-independent rates.

$$k_{obs} = (4\pi N_{av} Dr/1000)(Ns/(Ns + \pi r)) \qquad (1)$$

Rate constants actually arise from two terms, one for collision of the particles and another for binding to the enzyme site. For spherical particles, collision can be given by Smoluchowski theory [$k_{coll} = 4\pi N_{av} Dr/1000$, N_{av} is Avogadro's number, D is the sum of the diffusion constants of the colliding particles, and r is the sum of their radii (*12*)] and the binding event by a term described by Berg and Purcell [*5*; probability of capture $= Ns/(Ns + \pi r)$]. Site properties are contained in s, the effective capture radius of an individual site (units = centimeters/site).

Rate Constants for Individual Enzymes Are All Site Rate Constants. The simplicity of rate constants in classical enzyme kinetics is provided by the fact that the second term of eq 1 is always an extreme case ($\pi r \gg Ns$). The enzyme captures few of the substrates with which it collides and solution homogeneity is maintained. The units of k_{obs} cancel to give the rate constant in eq 2. Enzyme particle (r) disappears from the velocity expression except as it contributes to D. If the particles are of very unequal size, the

$$k_{obs} = (4N_{av} D/1000)(Ns)$$

$$k_{obs(per\ site)} = (4N_{av} D/1000)(s) \qquad (2)$$

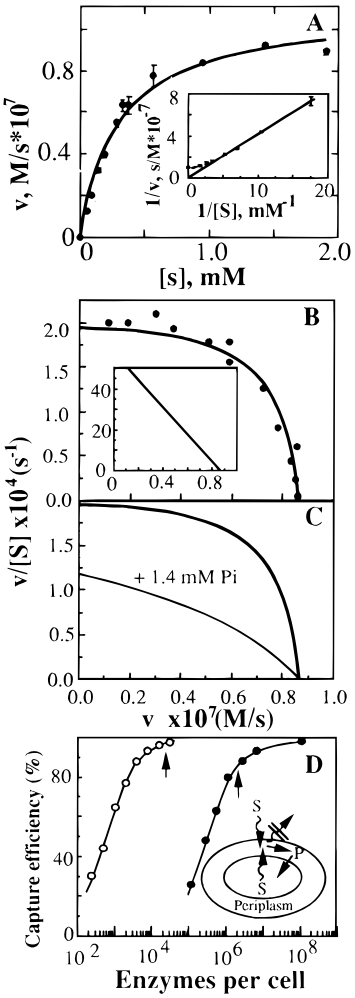

FIGURE 1: Collision- or transport-limited enzyme-catalyzed reaction. (Panel A) The kinetic result for alkaline phosphatase in wild-type strain K12 *E. coli* (data from ref 14). Substrate concentrations = 0.057−1.9 mM *o*-nitrophenylphosphate, 7.5×10^7 bacteria/mL and 2.17×10^4 enzymes per bacterium. The solid line is fit by the Michaelis−Menten relationship (apparent $K_M = 310\ \mu$M). (Inset) The results plus data for 3.8 and 20 mM substrate, plotted according to Lineweaver−Burk. (Panel B) Data plotted according to Eadie−Hofstee. The solid line is the best fit curve, calculated by eq 4 (*14*). Outer membrane permeability = $(f(r)) = 0.000\ 39$. (Inset) Result for the same amount of free enzyme ($K_M = 15\ \mu$M). (Panel C) The behavior of the uninhibited bacterial enzyme (bold line) versus activity in the presence of 1.4 mM phosphate (narrow line). (Panel D) The percentage of substrate capture as a function of enzymes per cell. Alkaline phosphatase reaction with *o*-nitrophenylphosphate (○, parameters from Figure 1B and Martinez et al. (*14*)] and β-lactamase reaction with cephaloridine (●) are shown. Parameters for β-lactamase: $k_{cat} = 98$/s, $K_M = 1$ mM, D for cephaloridine $= 7.5 \times 10^{-6}$ cm^2/second and $f(r) = 0.0045$ (values are from ref 16). The arrows show the actual number of enzymes per cell. Inset, possible functions of abundant enzyme in preventing substrate (S) escape from the periplasm.

$$k_{obs} = 4\pi N_{av} Dr/1000\upsilon = (4\pi N_{av} Dr/1000)[\text{particle}][\text{substrate}] \quad (3)$$

contribution of the large particle (usually the enzyme) is negligible so that k_{obs} becomes independent of the size of the enzyme particle all together. N is eliminated when reaction velocity is described per site (units = inverse molarity seconds site). The site unit is implicit in the derivation and is almost never stated.

Site Behavior Applies to Multisite and "Diffusion-Limited" Enzymes. The most efficient soluble enzymes are described as "perfect" or "diffusion-limited". However, this is diffusion of the substrate to the site, not to the particle; the enzyme does not deplete the substrate in its vicinity. The highest second-order rate constants for soluble enzymes, about 10^8 M^{-1} $second^{-1}$ (*13*), are about 1% of the rate constant for collision between a low molecular weight substrate and the entire enzyme particle (k_{coll}). Enzyme depletes substrate in its vicinity by ≤1%, a level that does not warrant concern. These enzymes can be described by classical, site rate constants.

Enzymes with multiple noncooperative substrate-binding sites are slightly more complex, but with minimal impact. For an enzyme with four highly efficient, diffusion-limited sites ($k_{obs} = 10^8$ M^{-1} $second^{-1}$ $site^{-1}$), Ns will contribute about 4% to the denominator of eq 1. Again, the enzyme captures about 4% of substrate collisions, creating a nonhomogeneous solution of this magnitude. Using eq 1 to describe this behavior, k_{obs} will no longer be a pure site term since a 4% contribution of Ns to the denominator will cancel 4% of the site units of k_{obs}. As the sites are filled, this contribution will change as N, the number of free sites, becomes 3, 2, and 1. Thus, while theory shows that any particle containing more than one site will show a different rate constant for each site, a maximum variability of 4% is usually too small to be detected and a single rate constant (eq 2) can be used for all sites, except in the case of cooperativity.

Particle-Bound Enzymes Can Offer Another Extreme. For particle-bound enzymes, the situation can change since N may be very large. If there are 10 000 sites on a particle, the reasoning given above shows that there will be 10 000 different k_{obs} values, even though the sites of all enzymes are identical. The sites differ by physical (inter-relationship in space), rather than chemical (structural) properties. Most importantly, if this includes the condition of $Ns \gg \pi r$ (eq 1), the units of k_{obs} are changed so that the enzyme site is eliminated from the rate constant (eq 3). While the units are inverse molarity seconds particle, the particle unit is implicit and is almost never stated. By analogy to flux, reactions that proceed at the particle rate constant will deplete substrate in their vicinity and are therefore limited by substrate flux.

An important feature of the resulting velocity expression (eq 3) is that it contains no saturable term. Lineweaver−Burk plots will extrapolate to zero (Figure 1A, inset) and Eadie−Hofstee will give a slope of zero (Figure 1B, at low velocity), indicating infinite V_{max}. Since velocity cannot reach infinity, the reaction must eventually become limited by site behavior. This occurs as N, the number of free sites in eq 1, decreases due to occupation by substrate. The Eadie−Hofstee plots (Figure 1B) or Lineweaver−Burk plots (Figure 1A, inset) will curve toward V_{max} as shown. Eventually, Ns (eq 1) becomes smaller than πr, and the rate constant is switched to that in eq 2 and to site behavior, where flux is no longer a rate determinant. The intervening region of substrate concentration is characterized by k_{obs} values that are partially site and particle, resulting in the continuously curved plot shown in Figure 1B.

(2) Biological Application of This Kinetic Behavior

Figure 1A shows the velocity versus substrate plot for alkaline phosphatase in *E. coli*. The solid line is the best fit to the Michaelis−Menten equation. While it may not appear to deviate excessively, this represents a very poor fit. The results are also plotted by Lineweaver−Burk (Figure 1A, inset) and Eadie−Hofstee (Figure 1B). These show very clearly that the Michaelis−Menten relationship was not applicable.

The solid line (Figure 1B) is the theoretical result for enzyme in the bacterium [details described by Martinez et al. (*14*)]. Briefly, eq 1 was modified to correct for the diffusion barrier provided by the outer membrane [$f(r)$, eq 4). This term varied with the porins expressed on the outer

$$k_{obs} = 4\pi N_{Av} Dr(f(r))(Nsf(s))/(Nsf(s) + \pi rf(r)) \qquad \upsilon = k_{obs}[\text{bacteria}][\text{substrate}] \quad (4)$$

membrane. The binding site radius (s) was corrected for product formation by $f(s)$, which effectively contains the Michaelis−Menten parameters, including the impact of enzyme inhibitors. The resulting relationship [eq 4, (*14*)] has all the complexity described above, including creation of two different types of rate constants, depending on the relative contribution of $Nsf(s)$ and $\pi rf(r)$ to the denominator. Support for this mechanism did not rest on single curve fits but on excellent fits for a wide variety of conditions, including a 10-fold range of enzyme level and a severalfold range in outer membrane permeability (*14*). The ability to accurately model the reaction indicated that the site rate constant (eq 2) of the enyzme was not altered by its microenvironment. The macroenvironment provided that rate was limited by particle behavior.

The behavior of the same amount of free enzyme shows that the intercept on the vertical axis was about 30-fold above (Figure 1B, inset) that for the bacterial enzyme (Figure 1B), illustrating the enormous impact that *in vivo* conditions had on reaction properties. Bacteria containing 10^5 enzymes per cell gave a difference of 350-fold, and bacteria with less permeable outer membranes gave even larger differences between free and bacterial enzyme (*15*).

This kinetic behavior has altered our view of alkaline phosphatase. For example, a typical biochemical description of this enzyme may be that it is highly efficient (K_M = 15 μM, k_{cat} = 32/second) and that competitive inhibition by phosphate (K_I = 60 μM) may serve as an example of feedback inhibition in a catabolic pathway. However, the *in vivo* system is not particularly efficient (half-maximum reaction at 300 μM substrate, Figure 1A), nonlinear Eadie−Hofstee plots abolish the use of K_M, inorganic phosphate is a very poor inhibitor (1.4 mM reduces enzyme velocity at low substrate by less than 50%, Figure 1C).

Several roles for this kinetic behavior may be suggested. Molecules that enter the periplasm from either direction will be captured by the enzyme. The bacterium may scavenge

low levels of substrate that escape from the cytoplasm (planned or otherwise) or capture substrate from the environment (Figure 1D, inset). This represents an extension of membrane function; high enzyme levels will help sequester cell contents from the surrounding medium.

Enzyme expression levels appear to target this kinetic state. Figure 1D shows that capture efficiency at low substrate concentration (1 μM) is high for wild-type levels of alkaline phosphatase [22 000 per cell, Figure 1D (*15*)]. One thousand enzymes, although providing catalytic capacity that may be sufficient for total cell phosphate utililzation, would represent under expression for purposes of efficient substrate capture (about 50%, Figure 1D). Expression of 10^5 enzymes per cell would represent over expression since the added enzymes have virtually no impact on capture efficiency. The basis for low sensitivity to product inhibition in the cell is apparent from Figure 1D. Since about 800 enzymes are sufficient to capture 50% of the substrate, 21 200 out of 22 000 enzymes must be inhibited to reduce the reaction rate by half.

β-lactamase is also a periplasmic enzyme, and its cleavage of antibiotic molecules confers antibiotic resistance. Although theoretical fits to the data were not as complete as for alkaline phosphatase (*16*), Figure 1D shows the best estimates of capture efficiency at low substrate levels for various amounts of β-lactamase. Since it is less efficient than alkaline phosphatase [K_M = 1 mM for cephaloridine (*17*)], and the substrate is more permeable to the outer membrane [$f(r)$ = 0.0045 for cephaloridine (*16*)], a higher level of β-lactamase is needed for efficient capture. Overexpression levels for alkaline phosphatase (10^5 per cell) would represent underexpression of β-lactamase, which is found at approximately the limit for protein packing in the periplasm [$\geq 10^6$ enzymes per cell; Figure 1D (*16*)].

Similar correlation of function and expression level may apply to other periplasmic enzymes. For example, 5′-nucleotidase (*18*) and trehalase (*19*) may capture and return substrates that purposely or inadvertently escape from the cytosol, before they diffuse through the outer membrane and are lost to the cell. Trehalase-deficient strains transport trehalose to the culture medium, while trehalase-positive strains do not (*19*). Methanotrophic bacteria convert methane to methanol by the cytosolic enzyme methanol monooxygenase. The next enzyme in the pathway, methanol dehydrogenase, is very abundant in the periplasm (*20*) where it may capture methanol before it escapes the cell.

(3) Related Behaviors under Flow

Descriptions of product formation have been developed for substrate flow over enzymes bound to the inner surface of a tube (*9*). For an enzyme-coated tube operating at the limit of substrate delivery to its surface, reaction product formed at the exit of the tube (Pe(D)) is described by eq 5 where D is the substrate diffusion coefficient, L is the length of the tube, r is its radius, V_f is the rate of flow, and [S] is the substrate concentration.

$$\mathrm{Pe}(D) = [DL/R^2V_f]^{2/3}[\mathrm{S}] \quad (5)$$

Like eq 4, eq 5 contains no site term and should not show saturation behavior. Velocity is described by the substrate concentration and the rate at which solvent flow and diffusion deliver it to the wall of the tube. As flow rates or substrate concentrations are increased, the delivery of substrate exceeds what the enzyme can capture and the reaction converts to eq 6 (*9*), where v_r is the steady state enzyme velocity at the bulk substrate concentration (effectively v_o of the Michaelis−Menten equation) and Pe(O) is the product concentration at the exit. Reaction time equals $2L/RV_f$.

$$\mathrm{Pe(O)} = v_r 2L/RV_f \quad (6)$$

Conversion from one limiting reaction mechanism to the other in the course of a titration includes large regions where both processes contribute to the reaction rate. This produces nonlinear Lineweaver−Burk plots (*9*). Flow-limited kinetic behaviors have been shown for a number of enzymes bound to the inner surface of flowing tubes (*9*, *21*, *22*), including recent studies of blood clotting enzymes (*23*, 24). Diffusion-limited substrate delivery to blood clotting enzymes on cell surfaces has also been detected (*25*).

Although nonlinear Lineweaver−Burk plots were characteristic of this behavior in flowing tubes, it was suggested that a limiting intercept and a limiting value for "apparent K_M" occurred (*9*). In contrast, the limiting intercept was zero for the diffusion-limited circumstance shown in Figure 1A, inset. While an apparent K_M should apply to enzymes imbedded in porous materials, the situation for which this description was first developed (*26*), we do not feel that it should apply to enzymes that are equally accessible on a surface. For example, the absence of a site in eq 5 should preclude a site parameter such as apparent K_M. We found that data may suggest a K_M for other reasons. Assay of alkaline phosphatase in *E. coli* that had been damaged by the isolation procedure gave apparent intercepts (Lineweaver−Burk plots) above zero, suggesting an apparent K_M, even though all other criteria indicated a diffusion-limited event (*15*). In this case, heterogeneity of the cell population may have created the appearance of a positive intercept. The intercept was not an important kinetic property since it was specific to the bacteria population in the isolate.

II. Multistep Reactions That Include Several Sites in a Reaction Pathway

An extensive list of possibilities is illustrated by blood clotting enzymes (Figure 2). Step A shows direct substrate capture by the membrane-bound enzyme. This process dominated for membranes that had low affinity for substrate and where the enzyme was present at one or less per vesicle (*27*). The virtual absence of membrane-bound substrate required that the enzyme bind it directly from solution. Although the membrane may be a portion of the substrate binding site, the reaction should conform to Michaelis−Menten formalism since the sites have equal access to substrate and the enzyme is a component of every step in the reaction pathway.

Steps C and E (Figure 2) in the pathway do not contain the enzyme, and can be rate limiting for membranes with high affinity for substrate. For small membrane particles of 50% acidic phospholipid, the velocity of product dissociation was slow compared to enzyme catalysis at all substrate concentrations ($V_{max} < k_{cat}$, Figure 2, lower panel). Reaction velocity was determined by substrate−membrane site behavior and plot curvature arose from heterogeneity of these sites (*27*). For larger membrane particles of the same composition, substrate-membrane interaction was rate limit-

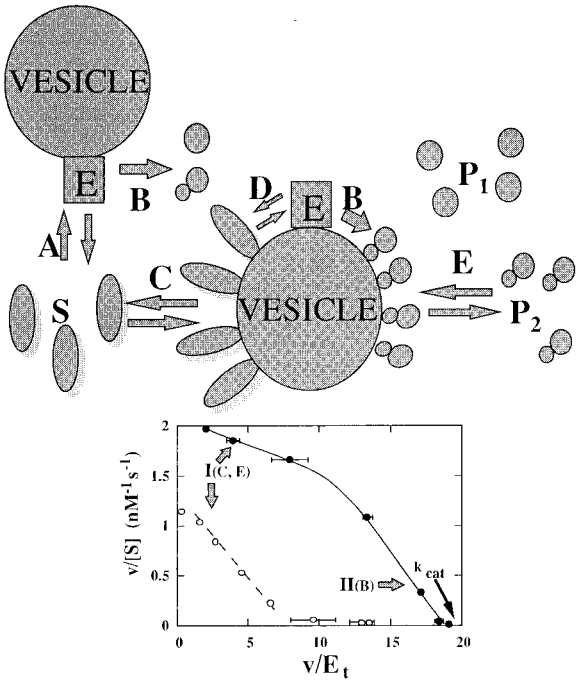

FIGURE 2: Possible kinetic models for enzyme action on membrane-bound substrates. (Upper panel) Prothrombinase (E) is shown with substrate (prothrombin, S) and products (Thrombin, P_1; and Fragment 1.2, P_2). Membranes of low affinity for substrate function by steps A and B (upper left), with direct capture of substrate by the enzyme. Membranes with high affinity for substrate can be limited by steps C, D, or E. (Lower panel) shows the kinetic results for enzyme assembled on small unilamellar vesicles of 50 percent phosphatidylserine [○ (*26*)] a reaction limited by substrate/product exchange rates (steps C and E) at all conditions. Large unilamellar vesicles containing 50% phosphatidylserine (●) were initially limited by substrate/product binding/release (steps C and E, condition I) but switched to rate limitation by catalysis (step B, condition II) at high substrate concentration.

ing at low substrate concentrations. However, the capacity of the membrane eventually exceeded that of the enzyme so that the reaction switched to be limited by k_{cat}.

Reactions that utilize steps C and D provide the condition of a reaction with reduced dimensionality. While beneficial to the circumstances in Figure 2, lower panel, it may be easy to overstate the importance of reduced dimensions; a positive impact may be limited to situations were enzymes (or receptors) are present at very low density [<100 per cell, (*28*)]. Factors that contribute to this limitation include the slow lateral diffusion of membrane-bound materials [$D = 10^{-9}$ cm^2 second^{-1}, for prothrombin (*29*)] versus that in solution [$D = 6.2 \times 10^{-7}$ cm^2 second^{-1} for prothrombin (*30*)] and the rate at which the membrane-bound protein equilibrates with the population in solution. If exchange is slow enough to allow an impact of reduced dimensionality, it can be so slow that product exchange from the membrane (Step E, Figure 2) becomes rate limiting.

III. Nonideal and/or Unexplained Behaviors

In addition to the rather organized events described above, there are many ill-defined events that can contribute to enzyme velocity when phospholipids are added to a reaction. Events such as membrane particle aggregation (*31*), vesicle fusion (*32*), and tight interaction of enzymes with highly curved, stressed membrane surfaces of high charge density (*33*−35) may increase or decrease enzyme activity for unknown reasons. If nonenzyme-site-related events inhibit (*36*) or stimulate (*37*) product formation, first approximations of standard kinetic plots may even suggest competitive or noncompetitive inhibition, even when the behaviors are not related to the enzyme. Whether many of these uncharacterized events signify important biological functions or just *in vitro* phenomena generally requires much further study.

Summary. Our research of membrane- or particle-bound enzymes has been confronted with the perennial problem of how to report kinetic parameters. While unique behaviors are described in the original literature and in some reviews, the developments are miniscule in comparison with the texts and literature associated with soluble enzymes. We have found the description by Berg and Purcell (*5*) to be most useful and could be modified to fit situations that involve enzymes separated by diffusion barriers. Although substrate flux is the core of these reaction states, we found that derivations based on k_{obs} (eq 1) allowed comparison with familiar steady state kinetic derivations and the assumptions inherent in classical rate constants. Equation 1 highlights the different types of rate constants, where the implicit unit determines whether classical steady state kinetic models are applicable. This concept has shown that premature use of standard steady state descriptions such as (apparent) K_M can overlook important behaviors of membrane- or particle-bound enzymes. A similar concern exists for description of binding parameters for protein−membrane association. Again, application of standard plots such as Hill or Scatchard can overlook much more interesting behaviors (*38*). Most importantly, the wrong kinetic interpretation can lead to incorrect hypotheses about the role of the enzyme, *in vivo*. For example, insight into alkaline phosphatase may alter the expected role of product inhibition. Perhaps phosphate binding to alkaline phosphatase serves another purpose such as assistance in phosphate transport, similar to the role of amino acid and carbohydrate-binding proteins in the periplasm (*39*). Enzyme expression levels and intracellular locations suggest that promotors have been selected to complement enzyme site properties and biological organisms have been designed to utilize the novel kinetic behaviors.

Virtually all surface- or particle-bound enzymes have the potential to display these mechanisms. For example, blood clotting enzymes can be limited by flow of solvent across a surface (*23*, 24), diffusion of substrate to a cell surface (*25*), or any of the steps shown in Figure 2 (*27*). Since the mechanisms are condition dependent, they can be enhanced or abolished by laboratory practices. Most often, protein isolation and reconstitution will abolish these properties since the size of the membrane and number of sites per particle will be diminished. However, low density may enhance the impact of reduced dimensionality, illustrated by substrate delivery to blood clotting enzymes from the membrane surface (Figure 2C). Even flow- and diffusion-limited reactions of these surface-bound enzymes may be exaggerated by conditions selected. Observation in a biological organism is necessary to suggest a real impact in biology.

These kinetic behaviors suggest practical limitations for bioreactors and biomedical design. For example, increased expression of enzyme in bacteria or of enzyme density in a bioreactor may have no impact on a reaction rate, unless

the substrate concentration is higher than that needed to exceed the horizontal portion of the Eadie−Hofstee plot (Figure 1B). Inhibitors of periplasmic enzymes, such as those to enzymes that create antibiotic resistance, may face the problem of alkaline phosphatase; very effective inhibitors of the free enzyme may have limited impact on the enzyme *in vivo*. Even irreversible inhibitors must inactivate a large portion of an enzyme population before significant reduction in reaction velocity is achieved. For many reasons, the potential of membrane-bound enzymes to display these unique behaviors is important to understand and test in many situations.

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