THE ROLE OF DIFFUSION IN ENZYME KINETICS

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ABSTRACT A discussion is given of the general role of diffusion in enzyme kinetics based upon a rigorous theory for bimolecular association and dissociation steps that was presented previously, and criteria are formulated for the dependence of the over-all rate on medium viscosity. With these criteria it is possible to conclude that a number of enzymes will exhibit no appreciable dependence of over-all rate on the medium viscosity, quite irrespective of the as yet unmeasured rate constants for association and dissociation of enzyme and substrate. The effect of adsorbing the enzyme onto the surface of a much larger spherical colloidal particle is considered with the conclusion that the rate will either remain the same or decrease, and that its sensitivity to medium viscosity will remain the same or increase.

INTRODUCTION

The role of diffusion in enzyme kinetics has not hitherto been approached in a general way, although specific enzymes have been examined in this regard (1, 2). The development of a rigorous theory for the simultaneous treatment of bimolecular association and dissociation steps (3) has made such a general treatment feasible. (This development is described in a paper (3) henceforth referred to as paper I). Indeed it is found possible to formulate criteria for the diffusion dependence of over-all rates of enzyme action. Application of these criteria permit the conclusion that a number of known enzymes will exhibit no appreciable dependence of over-all rate on medium viscosity, irrespective of their as yet unmeasured rate constants for association and dissociation of enzyme and substrate.

Since it is well known that many metabolic enzymes are attached to particulate structures, there arises the question of how the individual rate constants and over-all rate might be affected by such changes in geometry. Here the effect of adsorbing the enzyme molecules on the surface of a much larger sphere is investigated. It is found that even though the over-all rate cannot be increased and will in general decrease by adsorbing the enzyme, the degree of diffusion control, or sensitivity of the rate to medium viscosity, will in general be increased. A specific example of enhancement of the degree of diffusion control is then considered.

DIFFUSION IN ENZYME KINETICS

We consider the usual Briggs-Haldane (4) modification of the treatments of Michaelis and Menten (5) and Henri (6). The reaction scheme is taken to be

$$E + S \underset{k_b}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P,$$

and the steady-state rate expression is given by

$$\frac{dP}{dt} = \Phi = k_f c_E c_S - k_b c_{ES} = k_3 c_{ES} \tag{1}$$

where the concentrations are in molecules/cm^3, and k_f and k_b are the steady-state rate constants derived in Equations 9 and 10 of paper I. These rate constants are given by

$$k_f = \frac{k_D k_1}{f k_1 + g k_D}; \qquad k_b = \frac{k_D k_2 e^{U(R)/kT}}{f k_1 + g k_D}, \tag{2}$$

where

$$k_D = 4\pi(R_E + R_S)(D_E + D_S);$$

$$f = R \int_R^\infty e^{U(r)/kT} \frac{dr}{r^2};$$

$$g = e^{U(R)/kT};$$

$R = R_E + R_S$ is the reaction radius, $U(r)$ is the intermolecular potential energy, kT is thermal energy, k_1 and k_2 are the intrinsic association and dissociation constants, respectively, as defined in paper I, and D_E and D_S are the diffusion coefficients of the enzyme and substrate. Furthermore, k_3 is a dissociation rate constant analagous to k_b and may also be diffusion dependent. A considerable simplification of the ensuing analysis is achieved if we consider only reactions for which k_3 is not diffusion dependent. This will include almost all reactions whose equilibria lie far to the side of the products, since the rate of association of enzyme and *products* to form ES will generally be sufficiently slow in these cases that it is not diffusion dependent, and consequently the conjugate dissociation step k_3 will not be diffusion dependent either. Only extremely rapid over-all reactions have the possibility of a diffusion-dependent k_3 when the equilibrium greatly favors the products, and such cases cannot be experimentally characterized by the usual steady-state methods in any case.

Upon applying the conservation equations $c_E^0 = c_E + c_{ES}$ and $c_S^0 = c_S + c_{ES}$ to Equation 1 we obtain

$$\Phi = k_3 \frac{(c_E^0 + c_S^0 + K_M)}{2} \left\{ 1 - \left(1 - \frac{4 c_E^0 c_S^0}{(c_E^0 + c_S^0 + K_M)} \right)^{1/2} \right\}, \tag{3}$$

which reduces to the familiar form

$$\Phi = \frac{k_3 c_E^0 c_S^0}{K_M + c_S^0}, \tag{4}$$

when c_S^0, $K_M \gg c_E^0$. Under the assumption that k_3 is not diffusion controlled $K_M = (k_b + k_3)/k_f$ is the only part of the expression which depends upon the diffusion coefficient. Clearly, for *saturation* (i.e. $c_S^0 \gg K_M$) conditions, K_M may be neglected and diffusion plays no role in the reaction, however fast. Also, if $k_3 \ll k_b$, then $K_M \cong k_f/k_b = k_2/k_1$ so that K_M and also Φ are independent of diffusion processes. Finally, if k_f and k_b are not appreciably diffusion controlled (i.e. $fk_1 \ll gk_D$), then K_M and, hence, Φ will not be diffusion dependent. For a diffusion-dependent reaction in the absence of forces

$$K_M = \frac{k_2}{k_1} + \frac{k_3}{k_f} = K_{eq}^{-1} + \frac{k_3}{k_D}\left(\frac{k_D + k_1}{k_1}\right), \tag{5}$$

where $K_{eq} = k_1/k_2$ is the equilibrium constant for enzyme-substrate complex formation. Maximal diffusion control for a given k_3 will arise when $k_1 \gg k_D$ which is the condition that insures diffusion control of k_f and k_b; then

$$K_M = K_{eq}^{-1} + k_3/k_D. \tag{6}$$

A comparison of the experimental K_M and k_3/k_D (k_3 experimental, k_D calculated) will provide an estimate of the relative contributions of k_3/k_D and K_{eq}^{-1} to K_M. If $k_3/k_D \ll K_M$, then there is *no* diffusion dependence of the over-all reaction. However even if $k_3/k_D \cong K_M$, one *cannot* say that there *is* diffusion control of the reaction, since it is not known whether k_f is diffusion controlled. However, if it is known that $k_3 \gg k_b$, then if $k_3/k_D \simeq K_M$, it may be concluded that the reaction is diffusion dependent.

In summary it may be concluded that the usual enzyme reaction is *independent* of diffusion and, hence, the medium viscosity under the following circumstances: (*a*) $c_S^0 \gg K_M$; (*b*) $k_3 \ll k_b$; (*c*) $k_f \ll k_D$ (i.e. k_f not diffusion controlled); (*d*) $k_3/k_D \ll K_M$.

The enzyme reaction is diffusion *dependent* under the following circumstances: (*a*) $c_S^0 \lesssim K_M$ and $k_3 \gg k_b$ and $k_3/k_D \simeq K_M$ (simultaneously); (*b*) $c_S^0 \ll K_M$, $k_f \simeq k_D$ and either $k_3/k_D \simeq K_M$ or $k_3 \gg k_b$.

Laidler (7) has presented a table of some hydrolytic enzymes and their substrates for which it has been possible to show that either $k_b \gg k_3$ (i.e. $K_M = K_{eq}^{-1}$) or $k_b \ll k_3$ (i.e. $K_M = k_3/k_f$). To the former category belong the enzyme-substrate pairs listed in Table I which must now be independent of diffusion. To the second category (i.e. $k_3 \ll k_b$) belongs *carboxypeptidase*. The experimentally observed k_3 and K_M for three different substrates are given in Table II along with the appropriate k_3/k_D calculated

TABLE I

ENZYME-SUBSTRATE PAIRS FOR WHICH $k_b \gg k_3$

Trypsin	Benzoyl-L-arginine ethyl ester
α-chymotrypsin	Methyl-hydrocinnamate
α-chymotrypsin	Acetyl-L-phenylalanine ethyl ester
α-chymotrypsin	Nicotinyl-L-tryptophanamide
α-chymotrypsin	Acetyl-L-tryptophanamide
Myosin	ATP
Urease	Urea
Sucrase	Sucrose
Carbonic anhydrase	Carbon dioxide and ammonia

TABLE II

ENZYME-SUBSTRATE PAIRS FOR WHICH $k_b \ll k_3$; THE CORRESPONDING k_3 , k_M , AND k_3/k_D

	k_3	k_M	k_3/k_D
	sec^{-1}	M	M
Carbobenzoxyglycyl-L-tryptophan	89	5.8×10^{-3}	0.89×10^{-7}
Carbobenzoxyglycyl-L-phenylalanine	181	6.5×10^{-3}	1.81×10^{-7}
Carbobenzoxyglycyl-L-leucine	106	2.7×10^{-2}	1.06×10^{-7}

assuming a k_D of 10^9 in each case. It is seen that k_3/k_D is in general more than four orders of magnitude smaller than K_M, so that condition (*d*) above for diffusion-independent reactions is satisfied. The conclusion is, however, contingent upon a k_D of 10^9. Although at least one enzyme reaction has been observed (Strother and Ackerman, reference 2) to become viscosity dependent at the anomalously low value of $k_f \approx 5 \times 10^5$ M^{-1} sec^{-1} *at 10 times normal viscosity*, it seems highly improbable that for any system at normal viscosity k_D could be appreciably less than 10^7. Thus, it is safe to conclude that carboxypeptidase is indeed diffusion independent.

It is perhaps worthwhile to dwell upon the magnitude of the theoretical upper limit k_D for enzyme-substrate reactions. Although it is commonly assumed that a value of $k_D \gtrsim 10^9$ applies, the only experiments to date that bear upon the viscosity dependence of fast enzyme reactions (or fast reactions of any kind) are those of Ackerman and associates (2), who obtained evidence for diffusion control in the association of catalase with hydrogen peroxide, and also in the association of reduced cytochrome *c* with the hydrogen peroxide-horseradish-peroxidase complex, in solutions of about 10 times normal viscosity in spite of rate constants of only 10^7 and 10^6 M^{-1} sec^{-1}, respectively. However, in the former example it appears (8) that hydrogen peroxide has an abnormally small diffusion coefficient of about 5×10^{-7} cm^2/sec. This is about two orders of magnitude smaller than the value calculated from the Stokes-Einstein relation, which is expected to hold only for particles reasonably large compared to the solvent molecules, but which practically holds quite well in

many cases for small molecules, so that the reaction might be expected to exhibit diffusion control at a much lower rate constant. With such a diffusion coefficient one would expect a decrease in rate constant to occur after increasing the viscosity about a factor of 10, as is observed, because while $R \simeq R_{H_2O_2}$ is still small, $D \simeq D_{catalase}$ is a factor of 10 lower than what one would calculate using $R_{H_2O_2}$, so that $k_D \simeq 10^8$ M^{-1} sec^{-1} in water and $k_D \simeq 10^7$ M^{-1} sec^{-1} when the viscosity is increased by a factor of 10. The second reaction (i.e. the association of ferrocytochrome *c* and H_2O_2-peroxidase) may be an example of what can be referred to as the *small target* effect. It is easily seen that, when the value R_A (in $R = R_A + R_B$) is employed for *both* the reaction or target radius and also for the hydrodynamic radius of A in Stokes Law, with the analagous situation holding for B, then the theoretical upper limit k_D is nearly independent of R_A and R_B. However it is likely, especially for large molecules, that the target radius may be considerably smaller than the hydrodynamic radius. Thus, we may envision a small spherical target attached to the center of a fairly rigid random coil polymer. The diffusion coefficient of the target would be close to that of the whole polymer. Proper interdigitation of the polymer strands in two such molecules would permit collision and reaction of the targets. Although the target size R would still be small, the hydrodynamic radius which determines the diffusion coefficient would be very large, so that $k_D = 4\pi RD$ could well be much smaller than 10^9–10^{10}. On an intuitive basis a small site on the surface of a larger molecule would give the same kind of small target effect. It would require a hydrodynamic radius of $\sim 10^2$, 10^3 times the target radius to yield $k_D \simeq 10^7$ M^{-1} sec^{-1}. Evidently a very great deal of precision in position, and perhaps orientation as well (both types of precision constraint give small target effects), is required for the association of reduced cytochrome *c* and H_2O_2-horseradish-peroxidase, provided that the conclusion of Farwell and Ackerman (2) that the rate constant becomes viscosity dependent at $k_f \simeq 5 \times 10^5$ M^{-1} sec^{-1} and at 10 times normal viscosity is correct. The large amounts (90% w/w) of glycerol employed to increase the viscosity may be affecting either of the proteins directly or indirectly through changes in water structure. The data of Ackerman and associates are not sufficiently precise to give useful $1/k_f$ vs. η plots, where η is the medium viscosity. In the absence of long-range interactions a straight line in such a plot argues strongly that the medium viscosity changes are reflected only in the diffusion coefficients. It would be highly desirable to have this kind of data for at least a few of the fast associations that have been determined. The slope of such a plot is $(2kT/3)([1/R_A] + [1/R_B])(R_A + R_B)$ which gives information on the relative magnitudes of hydrodynamic (i.e. denominator) and target (i.e. numerator) radii, while the intercept is $1/k_1$ the reciprocal of the intrinsic bimolecular rate constant.

EFFECT OF ADSORBING THE ENZYME ON THE SURFACE OF A MUCH LARGER SPHERE

We consider that the total enzyme in solution is adsorbed onto the surfaces of very much larger spherical colloidal particles of radius r_0. The fraction α of the surface of

the colloid is covered by the total enzyme c_E^0, and of this fraction α, the fraction γ is free enzyme and the fraction $(1 - \gamma)$ is enzyme-substrate complex. The spherical colloid is now regarded as a single multivalent reacting species A at concentration c_A^0 (spheres/cm^3). If the concentration of substrate molecules near the surface of the sphere is $c_S(r_0)$ then the rate of the association reaction is given by the total inward current J_{in} of substrate particles across the reaction surface

$$J_{in} = \left(\frac{p\bar{v}_T}{2} c_S(r_0)\right)(\alpha\gamma 4\pi r_0^2 c_A^0). \tag{7}$$

The first factor is the current density of inward moving particles and the second factor is the reactive area. The current density is one-half the concentration of particles at r_0 times the mean thermal approach velocity times the probability p of reaction per collision with the reaction surface. By arguments similar to those presented in the derivation of Equation 21 of paper I we have for the outward current

$$J_{out} = \left(\frac{p'\bar{v}_T'}{2} \frac{3}{(4\pi R^3)}\right)(\alpha(1 - \gamma)4\pi r_0^2 c_A^0), \tag{8}$$

where p' is the probability of escape of a substrate moiety from its ES complex per collision with the reaction surface from the inside. It will be assumed that p, p', and $\bar{v}_T$ have the same values which characterize the free enzyme. If we make use of the following expressions for the intrinsic rate constants of the free enzyme (cf. Equations 17, 21–23 of paper I)

$$k_1 = \frac{p\bar{v}_T}{2} 4\pi R^2, \qquad k_2 = \frac{p\bar{v}_T}{2} \frac{3}{R}, \tag{9}$$

where $R = R_E + R_S$, the expression for the net reaction rate per unit concentration of spheres $\Phi_M = (J_{in} - J_{out})/c_A^0$ becomes

$$\phi_M = \frac{\Phi}{c_A^0} = \frac{\frac{r_0^2}{R^2} k_1 \alpha\gamma c_S(r_0) c_A^0 - \frac{r_0^2}{R^2} k_2 \alpha(1 - \gamma) c_A^0}{c_A^0}. \tag{10}$$

In the absence of long-range intermolecular forces, and in the steady state, the diffusion equation takes the form (9) (cf. also Equation 7 of paper I)

$$\frac{\phi_M}{4\pi r^2 D} = \frac{\partial c_S(r)}{\partial r}, \qquad R \leq r \leq \infty. \tag{11}$$

which is solved subject to Equation 10 and also

$$c_S(\infty) = c_S. \tag{12}$$

The solution gives

$$\Phi = \frac{4\pi D r_0 \frac{r_0^2}{R^2} k_1 \alpha\gamma c_S c_A^0 - 4\pi D r_0 \frac{r_0^2}{R^2} k_2 \alpha(1-\gamma) c_A^0}{4\pi D r_0 + \frac{r_0^2}{R^2} k_1 \alpha\gamma}$$

$$= k_f' c_S c_A^0 - k_b' c_A^0 \qquad (13)$$

where

$$k_f' = \frac{4\pi D_S r_0 \frac{r_0^2}{R^2} k_1 \alpha\gamma}{4\pi D_S r_0 + \frac{r_0^2}{R^2} k_1 \alpha\gamma}$$

and

$$k_b' = \frac{4\pi D_S r_0 \frac{r_0^2}{R^2} k_2 \alpha(1-\gamma)}{4\pi D_S r_0 + \frac{r_0^2}{R^2} k_1 \alpha\gamma}$$

It is desired to compare the forward and backward rates contained in Equation 13 with those for the same total enzyme but no colloidal spheres. That is we wish to compare

$$k_f c_S c_A^0 \text{ with } k_f c_S c_E^0,$$

and

$$k_b' c_A \text{ with } k_b c_{ES},$$

and for the comparison we allow that the same fraction γ of total enzyme is free in both cases. It is also assumed that $R_S \ll R_E$, so that $R \cong R_E$ and $k_D = 4\pi R_E D_S$. The relation between c_E^0 and c_A^0 is obtained by considering how many enzyme molecules could possibly adsorb onto a single A particle. The area occupied by an enzyme molecule is πR_E^2 and the number of enzyme molecules per colloid is approximately

$$\frac{\alpha 4\pi r_0^2}{\pi R_E^2} = 4\alpha \frac{r_0^2}{R_E^2}.$$

Thus,

$$c_E^0 \cong 4\alpha \frac{r_0^2}{R_E^2} c_A^0. \qquad (14)$$

The forward rate in the absence of the colloidal particles may now be written as

$$\phi_f \cong \frac{4\pi R_E D_S k_1 4\alpha \frac{r_0^2}{R_E^2} \gamma c_A^0 c_S}{4\pi R_E D_S + k_1}. \qquad (15)$$

When the enzyme is adsorbed on the colloid this rate is

$$\phi_f' \cong \frac{4\pi r_0 D_S k_1 \frac{r_0^2}{R_E^2} \alpha\gamma c_A^0 c_S}{4\pi r_0 D_S + \gamma\alpha \frac{r_0^2}{R_E^2} k_1}. \tag{16}$$

The ratio is

$$\phi_f/\phi_f' \cong \frac{4}{\left(\frac{4\pi r_0 D_S + k_1 \frac{r_0}{R_E}}{4\pi R_0 D_S + k_1 \alpha\gamma \frac{r_0^2}{R_E^2}}\right)} \tag{17}$$

For reasonable coverage (i.e. $\alpha \sim 1$) and a significant fraction of the enzyme free, that is $\gamma \gtrsim \frac{1}{2}$, it will be true that $\alpha\gamma r_0/R_E \gg 1$, so that

$$\phi_f/\phi_f' \gtrsim 4. \tag{18}$$

Only if the enzyme is almost completely saturated so that $\gamma \to 0$ will ϕ_f/ϕ_f' drop below 1. Thus, the effect of adsorption is generally to reduce the forward rate, unless the enzyme is saturated, in which case the over-all or net rate has already been shown to be independent of diffusion.

By similar arguments we may arrive at

$$\phi_b/\phi_b' \cong \frac{4}{\left(\frac{4\pi r_0 D_S + k_1 \frac{r_0}{R_E}}{4\pi r_0 D_S + k_1 \alpha\gamma \frac{r_0^2}{R_E^2}}\right)} \gtrsim 4, \tag{19}$$

which is formally equivalent to Equations 17 and 18, and from which it may be inferred that the dissociation rate, too, can only be reduced by the adsorption unless the enzyme is almost entirely saturated. The over-all rate in the steady state will clearly be reduced by the factor of either Equation 17 or 19 and, thus, will also either decline or remain unchanged unless the enzyme is saturated in which event there will still be no change in over-all rate, provided that the dissociation step k_3 is not diffusion controlled. For the usual unsaturated situation $\alpha\gamma \sim 1$, and it is easily seen that

$$\left(k_1 \alpha\gamma \frac{r_0^2}{R_E^2} \Big/ 4\pi r_0 D_S\right) \gg (k_1/4\pi R_E D_S)$$

so that the degree of diffusion control or sensitivity of the rate to medium viscosity may be appreciably enhanced by the adsorption of the enzyme, despite the decline in rate. The decline in rate will be maximal for adsorption of an enzyme with strongly

diffusion-controlled association and dissociation rates. These conclusions all rest upon the assumption that the enzyme molecules are either (*a*) equally reactive over their entire surface or (*b*) adsorbed onto the colloid with random orientation, and that the free and bound enzyme molecules are distributed uniformly over the surface. For either very large or very small fraction γ of free enzyme the assumption of a homogeneous distribution breaks down because the small fraction species will be so widely separated from one another that they will develop their concentration gradients individually rather than collectively over the surface of the sphere, and their rates will be more or less the same as for the unadsorbed enzyme.

The factor of 4 in Equations 17–19 arises in the estimate of the number of enzyme molecules which are adsorbed on the colloid and the area which they present to the solution. It is true that roughly one-half of the area of the enzymes is blocked upon adsorption, but the individual enzymes might present a "bumpy" surface of area $2\pi R_E^2$ rather than the flat projection πR_E^2 assumed, so that the factor of 4 may be as low as 2. Also, a preferential orientation of the enzymes with sites facing outward from the colloid surface might enhance the rate by a factor or two, but probably not more. Thus, the factor of 4 may actually be as low as 1; however, the conclusion that the rates will either remain the same or decline upon adsorption is unaltered by these considerations.

Of course the presence of long-range intermolecular potentials associated with the colloid may have a pronounced effect upon the rate, but we have not attempted to evaluate this effect here.

Finally an equation is presented for γ, the fraction of enzyme molecules which are not combined with substrate in the steady state (corresponding to Equation 1):

$$\gamma = \frac{-B + (B^2 - 4AC)^{1/2}}{2A}, \tag{20}$$

where

$$A = 4k_3 \frac{r_0^2}{R_E^2} k_1 \alpha,$$

$$B = 4\pi D_S r_0 (k_1 c_S + k_2 + 4k_3) - 4k_3 \frac{r_0^2}{R_E^2} k_1 \alpha,$$

$$C = -4\pi D_S r_0 (k_2 + 4k_3).$$

It has been assumed that k_3 is unaffected by the adsorption. Equations 13 may be employed in conjunction with Equation 20 to calculate the rate in any particular case of interest. Limiting forms of interest are the following.

(*a*) $B/A \gg 1$ which will arise when

$$4\pi D_S r_0 \gg \alpha \frac{r_0^2}{R_E^2} k_1$$

(the extreme nondiffusion-controlled limit):

$$\gamma = \frac{K_m'}{K_m' + c_S}, \tag{21}$$

where $K'_m = (k_2 + 4k_3)/k_1$ is an effective Michaelis constant, and the 4 comes again from counting the number of adsorbed enzyme molecules. This is essentially the usual result for nondiffusion-controlled reactions.

(*b*) $B \approx 0 \ll AC$ which occurs for

$$4\pi D_S r_0(k_1 c_S + k_2 + 4k_3) \approx \alpha \frac{r_0^2}{R_E^2} k_1 4k_3:$$

$$\gamma = 2\left(\frac{4\pi D_S r_0(k_2 + 4k_3)}{\alpha \frac{r_0^2}{R_E^2} k_1 + 4k_3}\right)^{1/2},$$

which is seen to be diffusion dependent. The substrate concentration for which this diffusion dependence arises is found from Equation 20 to be

$$c_s = \frac{4k_3}{4\pi D_s r_0 \left(\frac{R_E^2}{\alpha r_0^2}\right)} - K'_m. \tag{22}$$

An enzyme of radius 10 A adsorbed onto a sphere of radius 1 μ (a typical bacterium) would find it possible to satisfy Equation 22 for some substrate concentration provided that $\alpha \approx 1$ and $k_1 \gtrsim 10^6(1 + k_2/k_3)$ M^{-1} sec^{-1}. Thus, an enzyme, which when free operates at a rate well below the diffusion-controlled range of $k_1 \gtrsim 10^9$ M^{-1} sec^{-1}, may exhibit a strong (i.e. one-half power) dependence of its over-all rate $\phi = k_3 \gamma c_E^0$ on the medium viscosity for some range of substrate concentrations, when it is adsorbed to moderate fractional coverage on a sphere of 1000 times its own radius.

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