The Significance of Intermediary Plateau Regions in Enzyme Saturation Curves*

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ABSTRACT: An investigation has been made of the catalytic and binding parameters required to generate kinetic saturation curves possessing an intermediary plateau region. General rate equations were derived for the case in which the rate of equilibration between substrate and enzyme is rapid relative to the rate of catalysis. Analysis of the second derivative of these expressions revealed that kinetic or binding curves with pronounced intermediary plateau regions will only be produced when (1) the enzyme or enzymes present possess a total of *more than two* substrate binding sites and (2) the relative magnitude of the intrinsic catalytic or binding constants of these sites first decreases, then increases as the enzyme is saturated.

Multisite enzymes will not yield these undulating kinetic curves when their intrinsic catalytic, or binding constants progressively increase, progressively decrease or remain constant with saturation. Applying these and other diagnostic criteria, certain enzyme models could be unequivocably excluded as possible explanations for the complex kinetic curves experimentally observed.

Analysis of the shape of substrate saturation curves has often provided valuable information concerning the structure–function relationships of enzymes. Examination of the hyperbolic function observed in plots of initial velocity *vs.* substrate concentration, for example, led to the concept of an enzyme–substrate complex. (Michaelis and Menten, 1913). Later, the observation of sigmoidal-shaped binding curves suggested the presence of cooperative effects between binding sites (Bohr, 1903). Attempts to interpret the latter phenomenon in terms of intraprotein interactions provided several useful models (Monod *et al.*, 1965; Koshland *et al.*, 1966) relating subunit structure to enzyme function. One of these models (Koshland *et al.*, 1966) led to the prediction of negative cooperativity, a new mode of subunit interaction which has since been verified experimentally (Conway and Koshland, 1968).

Recent investigations of several enzymes, including phosphoenolpyruvate carboxylase (Corwin and Fanning, 1968), B ADP-glucose pyrophosphorylase (Gentner and Preiss, 1968), and cytidine triphosphate synthetase (Levitzki and Koshland, 1969) from *Escherichia coli*, glutamate dehydrogenase (LeJohn and Jackson, 1968) from Blastocladiella and glyceraldehyde 3-phosphate dehydrogenase (Gelb and Nordin, 1969) from insects, have revealed a new and anomalous type of kinetic behavior (see Figure 1). Plots of initial velocity *vs.* substrate concentration for these enzymes are hyperbolic at low substrate concentrations and sigmoidal at higher substrate levels. This transition from hyperbolic to sigmoidal behavior yields curves which contain a pronounced intermediary plateau region or "bump." Thus, unlike the purely hyperbolic curve, which possesses no points of inflection, or the purely sigmoidal curve, which possesses one point of inflection, the curves described above possess two points of inflection.

The observation of these bumpy curves by several independent investigators with a variety of enzymes suggests that this phenomenon might be widespread and indicative of some common structural properties. A study was therefore initiated to investigate the catalytic and binding parameters required to generate saturation curves with two points of inflection. The analysis which follows provides some general diagnostic aids by which the shapes of bumpy saturation curves might be related to structural features. These aids include a determination of the minimum number of binding sites and the nature of the cooperative interactions between sites which are consistent with this type of kinetic behavior.

Theory

One Ligand. The binding of a single substrate to a multisite enzyme may be expressed by the following equilibria

$$\begin{aligned} E + S &\rightleftharpoons ES & K_1 &= (ES)/(E)(S) \\ ES + S &\rightleftharpoons ES_2 & K_2 &= (ES_2)/(ES)(S) \\ ES_{i-1} + S &\rightleftharpoons ES_i & K_i &= (ES_i)/(ES_{i-1})(S) \\ ES_{n-1} + S &\rightleftharpoons ES_n & K_n &= (ES_n)/(ES_{n-1})(S) \end{aligned} \qquad (1)$$

where (S) and (E) refer to the concentration of free substrate and free enzyme, respectively, and n is the total number of substrate binding sites per molecule. In describing the binding of substrate to a multisite enzyme it will be valuable here to replace the association constants K_1, $K_2 \ldots K_n$, which include a statistical factor dependent upon the total number of binding sites, with the intrinsic binding constants K_1', $K_2' \ldots K_n'$, which express directly the inherent affinity of the individual binding sites for substrate. The relationship between the two sets of constants is given in eq 2.

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$$K_i = \frac{(n+1-i)}{i} K_i' \tag{2}$$

The initial velocity, v_0, of a reaction catalyzed by an enzyme with *one* binding site is given by $v_0 = k(\mathrm{ES})$, where k is a first-order rate constant. In the case of a multisite enzyme, v_0 maybe expressed as the sum of the individual turnover rates for each ES complex as shown in eq 3, where the values of k_i represent the average catalytic rate constants per site for a particular complex. If it is assumed that the rate of

$$v_0 = \sum_1^n i k_i(\mathrm{ES}_i) \tag{3}$$

equilibration between substrate and enzyme is rapid relative to the rate of catalysis, then (ES_i) in eq 3 is related to substrate concentration by the equilibrium expressions in eq 1. From eq 1, 2, and 3 one can thus readily derive a rate expression (eq 4), similar in form to the classical Adair equation (Adair, 1925) which describes initial velocity, v_0, as a function of substrate concentration(s). In eq 4 E_t is the total enzyme

$$v_0 = \frac{E_t \sum_1^n i k_i \psi_i (\mathrm{S})^i}{1 + \sum_1^n \psi_i (\mathrm{S})^i} \tag{4}$$

concentration and ψ_i represents a product of the intrinsic binding constants.

$$\psi_i = \prod_1^i \left(\frac{n+1-j}{j}\right) K_j' \tag{5}$$

For an enzyme possessing four binding sites eq 4, for example, would yield

$$v_0 = \frac{E_t[4k_1 K_1'(\mathrm{S}) + 12k_2 K_1'K_2'(\mathrm{S})^2 + 12k_3 K_1'K_2'K_3'(\mathrm{S})^3 + 4k_4 K_1'K_2'K_3'K_4'(\mathrm{S})^4]}{1 + 4K_1'(\mathrm{S}) + 6K_1'K_2'(\mathrm{S})^2 + 4K_1'K_2'K_3'(\mathrm{S})^3 + K_1'K_2'K_3'K_4'(\mathrm{S})^4} \tag{6}$$

It is apparent that when the intrinsic catalytic constants are all equal, *i.e.*, $k_1 = k_2 = k_i = k_n$, then $nk(E_t) = V_{max}$ and eq 4 reduces to the binding equation originally derived by Adair (1925), eq 7, where N_s is equal to the average number of

$$n\frac{v_0}{V_{max}} = N_s = \frac{\sum_1^n i\psi_i(\mathrm{S})^i}{1 + \sum_1^n \psi_i(\mathrm{S})^i} \tag{7}$$

moles of ligand bound per mole of enzyme.

More Than One Ligand. For the reaction of an enzyme with more than one substrate, or with reversible inhibitors or activators, a more generalized rate equation (eq 8) may be derived by the same treatment as described above. In this

$$v_0 = \frac{\sum_1^n \theta_i(\mathrm{S})^i}{1 + \sum_1^n \phi_i(\mathrm{S})^i} \tag{8}$$

case θ and ϕ are not as simply related to the intrinsic catalytic and binding parameters as are the constants appearing in eq 4 and 7. θ and ϕ are complex constants which will generally include terms describing the interactions of both substrate and effectors with the enzyme. If, for example, an enzyme, possessing one binding site for substrate and one binding site for the ligand, X, reacts according to the following mechanisms

$$\begin{array}{ccc} & \overset{K_1(\mathrm{S})}{\rightleftharpoons} & \overset{k_1(\mathrm{slow})}{\longrightarrow} \\ \mathrm{E} & & \mathrm{ES} \longrightarrow \text{product} \\ {\scriptstyle K_2(\mathrm{X})}\downarrow\uparrow & & \uparrow\downarrow{\scriptstyle K_3(\mathrm{X})} \\ \mathrm{EX} & \underset{K_4(\mathrm{S})}{\rightleftharpoons} & \underset{k_2(\mathrm{slow})}{\mathrm{ESX}} \longrightarrow \text{product} \end{array} \tag{9}$$

the rate equation will be given by eq 10

$$v_0 = \frac{E_t[k_1K_1 + k_2K_1K_3(\mathrm{X})](\mathrm{S})}{1 + K_2(\mathrm{X}) + [K_1 + K_1K_3(\mathrm{X})](\mathrm{S})} \tag{10}$$

If this rate equation is rewritten in the generalized form of eq 8, then θ and ϕ will have the values

$$\theta = \frac{E_t[k_1K_1 + k_2K_1K_3(\mathrm{X})]}{1 + K_2(\mathrm{X})} \tag{11}$$

$$\phi = \frac{K_1 + K_1K_3(\mathrm{X})}{1 + K_2(\mathrm{X})} \tag{12}$$

The important similarities to note between eq 4 and 8 are that (1) each is in the form of the classical Adair equation and (2) the degree of the equation, *i.e.*, the highest exponential power to which (S) is raised, is determined by the total number of substrate binding sites. It should also be noted that eq 8 may describe the kinetic behavior of a mixture of enzymes, where n, in this case, is equal to the total number of substrate binding sites contributed by all the species present.

As noted above, rate eq 4 and 8 were derived by assuming that the rates of equilibrium between substrate and enzyme were rapid relative to the rates of catalysis. If, however, the rate of the binding of substrate to enzyme is of the same order of magnitude as catalysis, then the concentrations of the ES complexes in these equations must be related to substrate concentration through a combination of kinetic constants rather than equilibrium constants. Under these conditions the rate expressions derived need not assume the form of a generalized Adair equation nor does the degree of the equation necessarily have to reflect the total number of substrate binding sites. Sanwal and Cook (1966) and Sweeney and Fisher (1968) have shown for bisubstrate reactions that when the relative rates of binding and catalysis lie within a certain range, the degree of the rate equation, with respect to one of the substrates, often exceeds that substrate's number of binding sites. The results of their analyses also pertain to the reaction of a single substrate plus effector with an enzyme possessing one substrate binding site and to the reaction of two or more molecules of the same substrate with an enzyme possessing multiple substrate binding sites. Therefore, if the rates of binding and catalyses are comparable, the kinetic

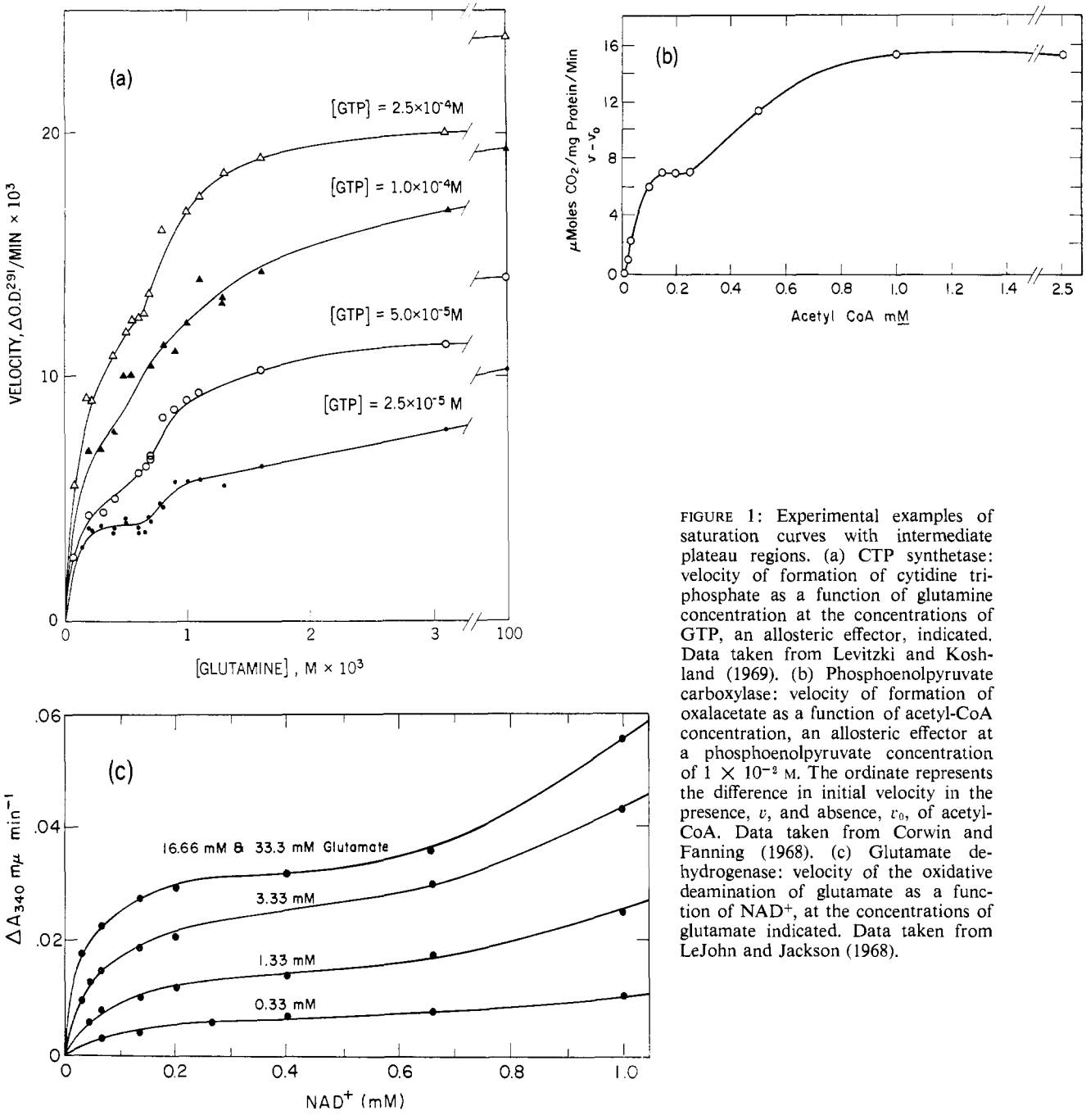

FIGURE 1: Experimental examples of saturation curves with intermediate plateau regions. (a) CTP synthetase: velocity of formation of cytidine triphosphate as a function of glutamine concentration at the concentrations of GTP, an allosteric effector, indicated. Data taken from Levitzki and Koshland (1969). (b) Phosphoenolpyruvate carboxylase: velocity of formation of oxalacetate as a function of acetyl-CoA concentration, an allosteric effector at a phosphoenolpyruvate concentration of 1×10^{-2} M. The ordinate represents the difference in initial velocity in the presence, v, and absence, v_0, of acetyl-CoA. Data taken from Corwin and Fanning (1968). (c) Glutamate dehydrogenase: velocity of the oxidative deamination of glutamate as a function of NAD^+, at the concentrations of glutamate indicated. Data taken from LeJohn and Jackson (1968).

behavior of an enzyme with a small number of substrate binding sites may resemble that expected of an enzyme possessing a larger number of such sites. The rate equations for this situation however will not be developed here since the formulation of these expressions depends upon a detailed knowledge of the catalytic mechanism of the enzyme.

Results

Inflection Point Analysis. In Figure 1 are shown initial velocity *vs.* substrate concentration plots, taken from the literature, for the enzymes cytidine triphosphate synthetase (Levitzki and Koshland, 1969), phosphoenolpyruvate carboxylase (Corwin and Fanning, 1968), and glutamate dehydrogenase (LeJohn and Jackson, 1968). The kinetic behavior of these enzymes is representative of a class of proteins whose saturation curves display an intermediary plateau region. Although the absolute shapes of these curves differ, all have one feature in common. Each curve is characterized by a decreasing slope at low substrate levels, followed by an increasing slope at intermediate substrate levels, and finally a decreasing slope at high substrate levels. The changing slope of these curves generates two points of inflection, one located at the point of transition from decreasing to increasing slope and the other at the point of transition from increasing to decreasing slope.

The second derivative, with respect to (S) of the rate equations for these curves, will yield the rate of the change in

slope as a function of substrate concentration. Since the slopes of the experimental curves in Figure 1 are decreasing at both very low and very high substrate concentrations, the second derivative of these functions must be negative as $S \rightarrow 0$ and $S \rightarrow \infty$. (Although the slopes of the kinetic curves for glutamate dehydrogenase appear to be increasing at the highest substrate concentration shown in Figure 1C, kinetic measurements, reported in the same communication, show that these slopes decrease at higher substrate levels.)

In addition, the second derivative of these curves must equal zero at the two values of S which give inflection points, because for any continuous function $y = f(x)$, the values of x which give inflection points must be among the roots of $f''(x) = 0$. The type of function thus generated by the second derivative of the curves in Figure 1 is shown in a general form in Figure 2.

From the above analysis it follows that a saturation curve possessing an intermediary plateau or two points of inflection will be generated by eq 4 or 8 only if the second derivative of these expressions yields a function of the type seen in Figure 2. The second derivative of the general rate equations for a two-site and a four-site model were therefore examined.

Two-Site Model. For an enzyme or enzymes possessing a total of two substrate binding sites, eq 8 reduces to

$$v_0 = \frac{\theta_1(S) + \theta_2(S)^2}{1 + \phi_1(S) + \phi_2(S)^2} \tag{13}$$

The second derivative, with respect to S of eq 13 is given by

$$\frac{d^2v_0}{d(S)^2} = \frac{-2[\phi_2(\theta_2\phi_1 - \theta_1\phi_2)(S)^3 + 3\theta_2\phi_2(S)^2 + 3\theta_1\phi_2(S) + \theta_1\phi_1 - \theta_2]}{[1 + \phi_1(S) + \phi_2(S)^2]^3} \tag{14}$$

As discussed above, a curve of the type seen in Figure 2 will be generated by eq 14 only if (a) the value of the equation is less than zero as $S \rightarrow 0$ and $S \rightarrow \infty$, and (b) the value is equal to zero at two different values of S. Inspection of eq 14 reveals that the first criteria is satisfied when $\theta_1\phi_1 > \theta_2$ and $\theta_2\phi_1 > \theta_1\phi_2$. When condition (a) is met however, it is apparent that all of the coefficients of (S) are negative. Consequently the value of the function is less than zero at all (S) and condition (b) is not satisfied. Thus, regardless of the values selected for θ_1, θ_2, ϕ_1, and ϕ_2, eq 8 is incapable of generating a curve containing two points of inflection. Furthermore, since eq 8 is the most general rate expression for a two-site system, it may be concluded that neither a single enzyme with two substrate binding sites nor a mixture of two enzymes, each with one substrate binding site, in the presence or absence of effectors, will yield curves possessing an intermediary plateau region, provided that the rate of binding is rapid relative to the rate of catalysis.

Four-Site Model. For an enzyme possessing four substrate binding sites, rate eq 4 and 8 yield expressions containing eight independent parameters. The second derivatives of these expressions were too complex to be analyzed in the simple manner discussed above for the two-site model.

A second derivative analysis was therefore made of eq 7, which is both a binding expression and, when catalytic activity is directly proportional to saturation, a rate equation. The second derivative of eq 7 with respect to S for a four-site model is still a complicated expression and, since for the discussion to follow only the first and last few terms in the numerator of the equation are relevant, it is presented below in eq 15. The missing terms consist of the series $a_8(S)^8 + \ldots + a_3(S)^3 + \ldots + a_2(S)^2$, where the coefficients a_i consist of combinations of the equilibrium constants K_1', K_2', K_3', and K_4'.

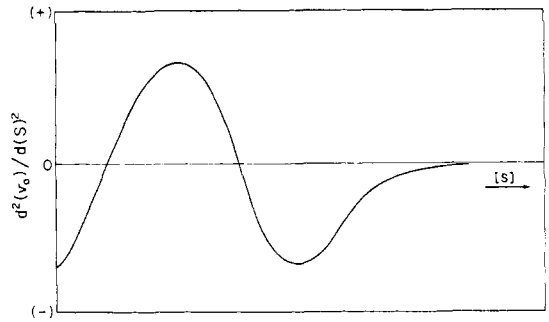

FIGURE 2: General form of the second derivative of a saturation curve with an intermediate plateau whose slope decreases at both low substrate levels and high substrate levels.

$$\frac{d^2N_s}{d(S)^2} = \frac{-8K_1[K_1'^2K_2'^3K_3'^3K_4'^2(S)^9 + \ldots + 9K_2'(2K_1' - K_3')(S) + 4K_1' - 3K_2']}{[1 + 4K_1'(S) + 6K_1'K_2'(S)^2 + 4K_1'K_2'K_3'(S)^3 + K_1'K_2'K_3'K_4'(S)^4]^3} \tag{15}$$

The behavior of eq 15 was examined for cases where (1) the affinity for substrate progressively increased with saturation, *i.e.*, $K_4' > K_3' > K_2' > K_1'$ and (2) the affinity for substrate progressively decreased with saturation, *i.e.*, $K_1' > K_2' > K_3' > K_4'$. For the case where the affinity for substrate remains constant, *i.e.*, $K_1' = K_2' = K_3' = K_4'$, eq 7 will generate a Michaelis–Menten curve which is, of course, hyperbolic and without points of inflection.

In the first case, where affinity for substrate progressively increased, a function of the type presented in Figure 2 may be generated by eq 15. This conclusion is reached by the following argument. Inspection of eq 15 reveals that at very low (S) the term $4K_1' - 3K_2'$ will predominate. At slightly higher (S) the term $9K_2'(2K_1' - K_3')(S)$ will predominate. At very high (S) the term $K_1'^2K_2'^3K_3'^3K_4'^2(S)^9$ will predominate. The value of the function can be negative at very low (S) when $\frac{4}{3}K_1' > K_2'$, positive at slightly higher (S) when $K_3' > 2K_2'$, and again negative at very high (S) with any values of K_1', K_2', K_3', and K_4'. Values of intrinsic binding constants may be selected such that both $K_4' > K_3' > K_2' > K_1'$ and the above conditions for two inflection points are satisfied.

Although, as shown above, a saturation curve possessing two points of inflection may, in theory, be generated when $K_4' > K_3' > K_2' > K_1'$ substitution into eq 7 of appropriate values of the intrinsic binding constants did not yield curves with an intermediary plateau regions which were visibly detectable. This result is not contradictory to the conclusion reached above, but rather indicates that the inflection point test for the detection of intermediary plateau regions is a very sensitive criterion and thus represents a necessary but not sufficient condition for demonstrating the existence of pronounced "bumps" such as seen in Figure 1.

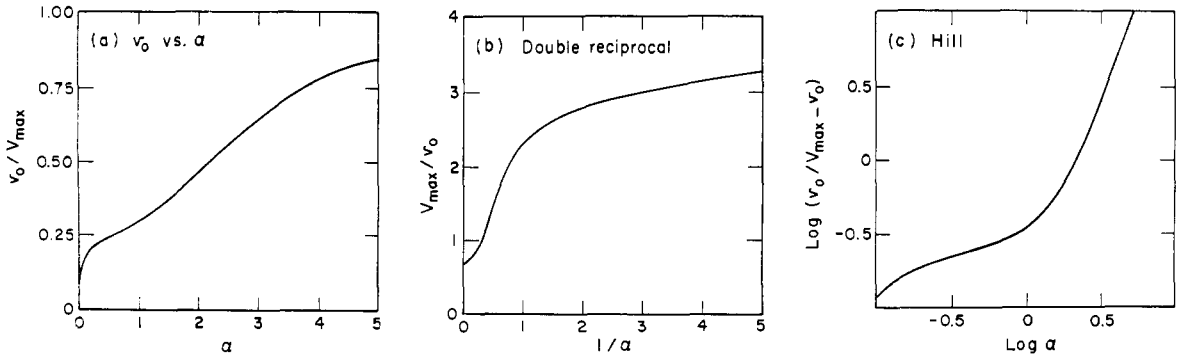

FIGURE 3: Three different plots of the same data for a curve displaying an intermediate plateau in a v_0 *vs.* (S) plot. (a) A v *vs.* (S) plot. (b) A Lineweaver–Burk plot. (c) A Hill plot. Data generated by eq 7 when $K_1' = 100$, $K_2' = 1$, $K_3' = 10$, $K_4' = 100$, and $n = 4$.

In the second case, where the affinity for substrate decreases with saturation, a situation might exist where $K_1' \gg K_2' \gg K_3' \gg K_4'$. Under these conditions the progressive binding of substrate would result in the nearly complete saturation of one binding site before an appreciable portion of another site with a lower affinity was occupied. One might intuitively reason in this case that intermediary plateau regions, associated with the saturation of each site, would be observed in a plot of N_s *vs.* (S). However, when $K_1' > K_2' > K_3' > K_4'$ all the coefficients of (S) in eq 15, including those indicated by the dots, are positive. Because the value of eq 15 is thus always less than zero, the curves generated by eq 7 in this case possess no points of inflection and are hyperbolic in shape. This result, which pertains to plots of N_s *vs.* (S) should not be confused with plots of N_s *vs.* log (S) (Koshland *et al.*, 1966). In the latter case plateau regions are produced when substrate affinity progressively decreases.

By a treatment similar to that outlined above, it may also be demonstrated that if the association constants are all equal, *i.e.*, $K_1' = K_2' = K_3' = K_4'$, saturation curves possessing an intermediary plateau region will *not* be generated when the magnitudes of the catalytic rate constants progressively increase, progressively decrease or remain constant with saturation.

Generation of Bumpy Curves. Curves displaying pronounced plateau regions or bumps could be generated by eq 4 provided that the binding or kinetic constants chosen were such that $K_i > K_{i+1} < K_{i+2}$ or $k_i > k_{i+1} < k_{i+2}$. Under these conditions the rate equation for an enzyme possessing as little as three substrate binding sites may yield curves with intermediary plateau regions. In Figure 3 are shown a v_0 *vs.* (S) plot, together with corresponding double reciprocal and Hill plots, for an enzyme possessing four binding sites where $K_1' = 1 \times 10^2$, $K_2' = 1 \times 10^0$, $K_3' = 1 \times 10^1$, and $K_4' = 1 \times 10^2$, and $k_1 = k_2 = k_3 = k_4$. The substrate concentration range for the graphs in Figures 3, 4, 5, and 6 have been normalized such that for a given set of association constants

$$\alpha = \left(\prod_1^n K_i\right)^{\frac{1}{n}} (S) \qquad (16)$$

It may be seen that in each of the three classical methods for

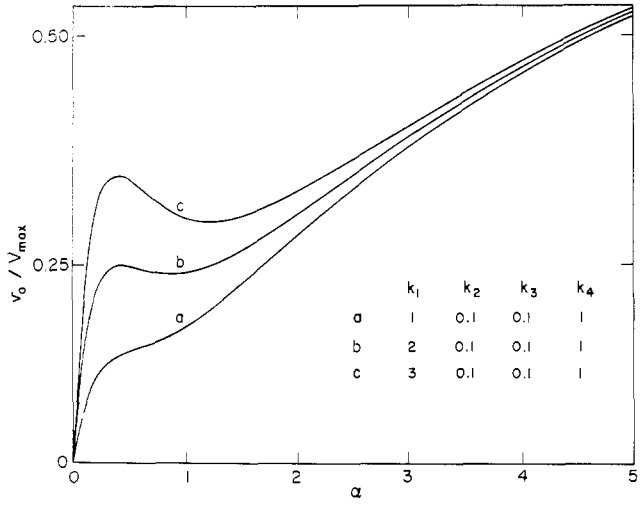

FIGURE 4: Plots of v_0 *vs.* (S) which show undulations caused by varying catalytic constants. Data generated by eq 4 when $n = 4$, $K_1' = K_2' = K_3' = K_4'$, and the values of the intrinsic catalytic constants, k, are as described in the figure.

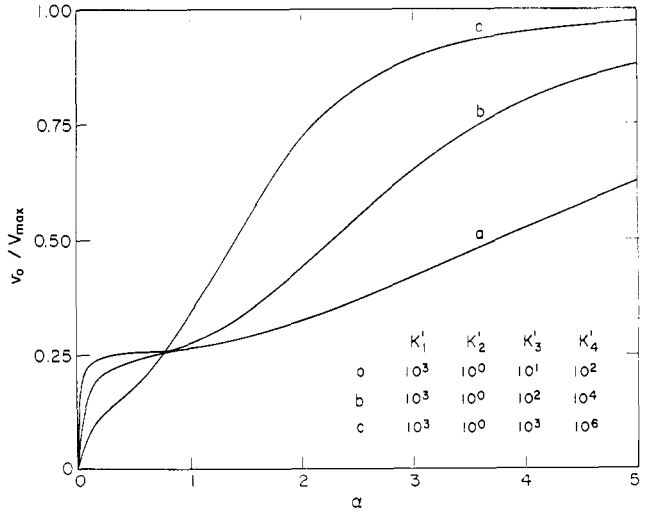

FIGURE 5: Effect on intermediate plateau regions of varying intrinsic binding constants. Data generated by eq 7 when $n = 4$, and the values of the intrinsic binding constants, K', are as described in the figure.

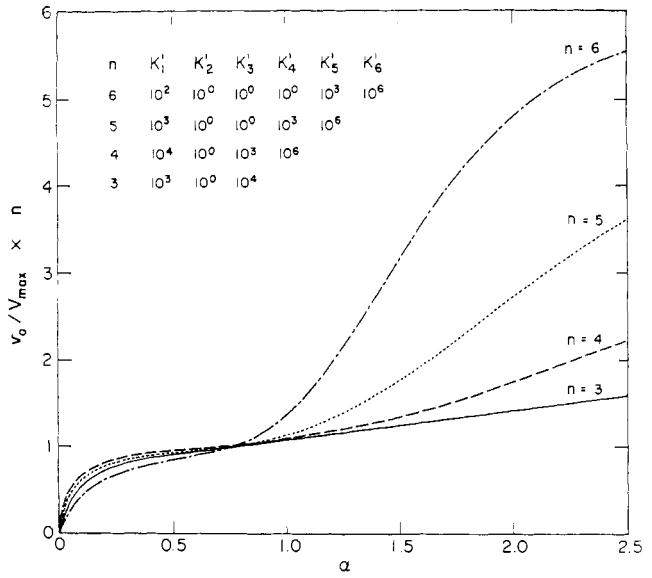

FIGURE 6: Effect on intermediate plateau regions on varying n, the number of binding sites. Data generated by eq 7 when the number of binding sites, n, and the values of the intrinsic binding constants, K', are as described in the figure.

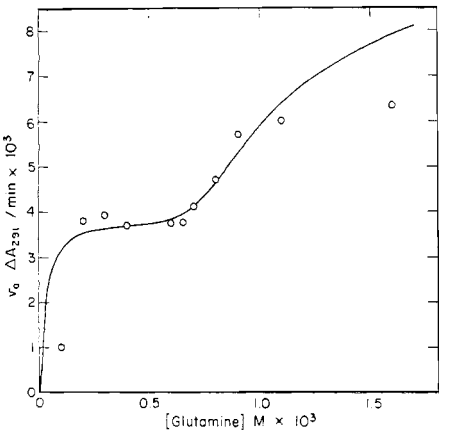

FIGURE 7: Comparison of theory and experiment for CTP synthetase. Experimental points represent rate of formation of cytidine triphosphate as a function of glutamine in the presence of 2.5×10^{-5} M GTP compared with the best theoretical curve based on eq 8 and consistent with the limitations imposed by eq 16 of Weber and Anderson (1965).

representing kinetic data the characteristic plateau region or bump is expressed. In the case of the Hill plot, however, the plateau region is not necessarily indicative of only bumpy saturation curves since quite similar curves may be generated by enzymes displaying a progressively decreasing affinity for substrate. Curves possessing intermediary plateau regions may also be generated by varying the catalytic constants k_1, k_2, k_3, and k_4 and setting $K_1' = K_2' = K_3' = K_4'$ as shown by the curves in Figure 4.

The sharpness of the plateau region, that is the rate of increase of the slope from the plateau to sigmoidal region of the curve, is dependent upon both the choice of kinetic and binding constants and the degree of the rate equation. In Figure 5 is shown the effect of increasing the magnitude of K_3' and K_4' relative to K_1' and K_2' for a four-site model. It is apparent that the slope of the sigmoidal region of the curve is increased only at the expense of reducing the plateau region. The effect of increasing the value of n, the total number of binding sites, on the shape of the saturation curve is dramatized in Figure 6. For each value of n the association constants have been selected so as to maximize the sharpness of the bump. It is evident from the shapes of the curves in Figure 6 that the rate of change in slope from plateau to sigmoidal region increases with increasing n.

The maximum rate of change in slope of a curve generated by eq 7 for a particular value of n may be determined by applying an expression derived by Weber and Anderson (1965). This expression, which sets limits on the maximum rate of change in slope as a function of the degree of saturation, is presented as

$$\frac{-n - Z}{n - N_s} < \frac{dZ}{dN_s} < \frac{n - Z}{n - N_s}\left(\frac{n}{Z} + \frac{n}{N_s} - 2\right) \qquad (17)$$

Z represents the slope of the curve in a Hill plot of the binding data. It may be seen that both the upper and lower limits of eq 17 increase with increasing n.

The limitations imposed by eq 17 were applied to one of the kinetic curves of cytidine triphosphate synthetase (Figure 1a) in order to determine whether this data could be explained by eq 7. The value of n was set equal to four, since the enzyme has been shown to be composed of four subunits (Long *et al.*, 1969). The experimental points and allowable theoretical curve are presented in Figure 7. The theoretical curve was generated by conforming as closely as possible to the experimental data yet maintaining the change of slope limitations defined by eq 17. It may be seen that while the theoretical curve approximates fairly well the plateau and early sigmoidal region of the data, the maximum rate of decrease of the curve at higher substrate levels is not consistent with the experimental points. One may, therefore, conclude that the kinetic behavior of cytidine triphosphate synthetase under certain conditions cannot be represented by eq 7 when $n = 4$ regardless of the values selected for the intrinsic binding constants K_1', K_2', K_3', and K_4'.

The above observation at first suggested that differences in the magnitude of the intrinsic catalytic constants, k_1, k_2, k_3, and k_4 might play the prominent role in determining the kinetic behavior of the enzyme. An attempt to fit the curve for cytidine triphosphate synthetase (and the kinetic curve for phosphoenolpyruvate carboxylase, Figure 1b) using eq 4, $n = 4$, in which values for the intrinsic catalytic constants were now also incorporated, was, however, largely unsuccessful. The large number of variable parameters encountered for $n > 4$ prohibited an analysis of more complex expressions. The inability to fit those saturation curves possessing the more pronounced plateau regions with eq 4, which was derived by assuming the rate of equilibration between ligand and enzyme was rapid relative to the rate of catalysis, implies that, for these enzymes, either the rate of ligand binding or the rate of conformational changes within the enzyme are comparable with or slower than the catalytic process. The rate equations for the latter situation, which would undoubtedly contain

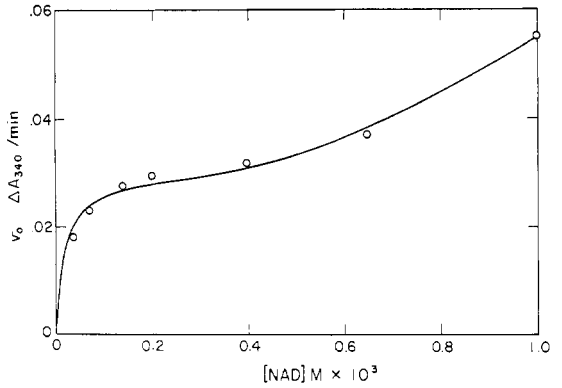

FIGURE 8: Comparison of theory and experiment for glutamate dehydrogenase. Experimental points measured for the rate of deamination of glutamate by glutamate dehydrogenase as a function of NAD^+ in the presence of 33.3 mM glutamate compared with the theoretical curve generated by eq 4, when $n = 4$, $K_1' = 100$, $K_2' = 1$, $K_3' = 10$, $K_4' = 100$ and $k_1 = k_2 = k_3 = k_4 = 2.4 \times 10^{-2}$ ΔA_{340} min^{-1} E_t^{-1}.

powers of (S) greater than four, could account for the rapidly changing slopes of these kinetic curves.

A fairly good fit of the experimental data was obtained in the case of glutamate dehydrogenase (Figure 1c) with eq 7, $n = 4$. A comparison between the theoretical curve and the experimental points, measured at one concentration of glutamate, is shown in Figure 8. This result suggests that, for glutamate dehydrogenase, an initial decreasing affinity for ligand followed by an increasing affinity for ligand may account for the intermediary plateau regions experimentally observed.

Discussion

An analysis has been presented of saturation curves which possess an intermediary plateau region. This analysis has consisted of an examination of the types of functions generated by general rate equations for multisite enzymes. These equations were derived by assuming that the rate of equilibration between substrate and enzyme was rapid relative to the rate of catalysis. This assumption, in the absence of detailed information on the catalytic mechanisms of the enzymes involved, appeared to be a reasonable starting proposition since (1) the rate of binding of substrate to enzyme has, in fact, been shown to be rapid relative to the rate of catalysis for many enzymes and (2) the rate equations derived for this case are quite general and thus capable of describing a wide range of kinetic behavior.

By differentiating the general rate equations, it was found that certain enzyme models could not account for the bumpy kinetic curves experimentally observed. Firstly, it was shown that enzymes displaying saturation curves with an intermediary plateau region, must possess *more than two* substrate binding sites. Interestingly, among the enzymes reported here, whose gross physical properties are known (glutamate dehydrogenase, cytidine triphosphate synthetase, and glyceraldehyde 3-phosphate dehydrogenase), all are composed of four or more subunits and are thus likely to possess at least this number of binding sites. Secondly, it was demonstrated that saturation curves with intermediary plateau regions will *not* be produced by an enzyme possessing more than two binding sites, when the magnitude of the catalytic or binding constants of these sites progressively increases, progressively decreases, or remains constant with saturation. Bumpy curves of the type experimentally observed could, however, be generated by multisite enzymes when the relative magnitude of the catalytic or binding constants first decreased, then increased as the enzyme was saturated.

If the intermediary plateau region of these curves results from an initial decreasing affinity for ligand followed by an increasing affinity for ligand, then protein models may be postulated which can account for this anomolous behavior. An increasing affinity for ligand is usually attributed to positively cooperative interactions of the allosteric type. A decreasing affinity for ligand, however, may result from one of two causes. For a single protein species the effect may arise from ligand-induced conformational changes. These forces, which may be termed negatively cooperative interactions, have been shown to be operative in glyceraldehyde 3-phosphate from rabbit muscle (Conway and Koshland, 1968). Alternatively, a decreasing affinity for ligand may result from a mixture of two or more proteins, or subunits (as in isozymes), each with a different intrinsic binding constant. It should be noted that from an analysis of binding curves alone, the two alternative explanations are indistinguishable. Determination of protein homogeneity, however, could resolve this ambiguity.

In cases in which there is first a decrease in affinity for ligand followed by an increase in affinity for ligand, it is apparent that this behavior may also be explained in one of two ways. Firstly, if only one protein is responsible for the observed phenomena, the curve may result from ligand induced cooperative interactions, in which negatively cooperative interactions predominate at low substrate concentrations and positively cooperative interactions predominant at higher substrate levels. Secondly, the apparently fluctuating affinity for ligand may result from a mixture of two proteins, one displaying Michaelis–Menten or negatively cooperative behavior and the other positively cooperative behavior. In the latter case it is necessary that the magnitude of intrinsic binding constant(s) of the Michaelis–Menten or negatively cooperative enzyme be greater than that of the first intrinsic binding constant of the positively cooperative enzyme.

Finally it is noted that the rate of change in slope in some cases, *i.e.*, cytidine triphosphate synthetase and phosphoenalpyruvate carboxylase, cannot be explained by even the most general rate equation based upon a rapid rate of equilibration between substrate and enzyme relative to the rate of catalysis. In such cases the data provide presumptive evidence that rate-determining conformational changes or rate-determining binding steps are involved in the over-all enzymic process. In addition, it should be noted that saturation curves with intermediary plateau regions may also be generated under certain conditions for single-site enzymes when there is interaction between the substrate and a modifier (as exists, for example, between ATP and magnesium ion) (London and Steck, 1969; Atkinson, private communication).

In summary, the mathematical analysis presented above reveals that this new phenomena involving intermediary plateau regions can only be accommodated by certain models of enzyme action. The existence of such plateaus restricts the potential models and thus can be used as a diagnostic

test which provides information concerning structural and kinetic characteristics of the protein under study. Furthermore the analysis suggests that curves with even more complex relationships, *i.e.*, a pronounced maximum in the v_0 *vs.* (S) plots at intermediate ligand levels (Figure 4), are possible. These and other types of bumpy curves would most likely have been dismissed in the past due to the large number of closely spaced points required to verify such curves and due to the tendency to average out such bumps assuming they reflect experimental error. It will be of interest to see if further examples of these unusual saturation curves are discovered in the future.

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