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ALTERATION OF THE KINETIC PROPERTIES OF AN ENZYME BY THE BINDING OF BUFFER, INHIBITOR, OR SUBSTRATE

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A number of the features of the catalytic properties of enzymes have been interpreted in terms of further elaborations of the simple mechanism of Michaelis and Menten.[1] Some treatments have shown that the kinetic constants for the over-all reaction cannot be interpreted in the simple way originally thought. Recently, Foster and Niemann[2] have discussed the theory for a difunctional enzyme combining with a difunctional substrate and mono- or difunctional inhibitor and have shown that the maximum initial velocity may be a measure of the equilibrium constants for various steps in the mechanism in addition to being a function of the rate constant for the breakdown of the enzyme-substrate complex. When the catalytic properties of enzymes are complicated by interaction with components of the buffer,[3–7] in addition to hydrogen ion, mechanisms may be enlarged to also take these effects into account. The mechanisms described in this paper have been developed to account for the following facts concerning the kinetics of fumarase which are not in accord with simple mechanisms used earlier. There is (1) a strong dependence of Michaelis constants and maximum initial velocities on buffer concentration and composition, figure 1 (*a*); (2) a variation of competitive inhibition constants with buffer concentration; (3) new types of inhibition, figure 1 (*b*) and (*c*); and (4) under certain conditions, markedly curved Lineweaver-Burk[8] plots, figure 1 (*d*). These effects appear to be related in that they are all accounted for by mechanisms which allow for the alteration of the properties of the enzyme by the binding of buffer, inhibitor, or even substrate in such a way that the enzymatic activity may be either enhanced or diminished. These effects are not of the nature of the ionic strength effects studied by Kistiakowsky and Shaw,[9] and such effects are neglected in the discussion of the following mechanisms.

Initial velocities for the reactions catalyzed by crystalline fumarase were obtained by recording the optical density changes at 210 to 280 mμ with a Beckman DUR spectrophotometer equipped to record optical density 0 to 0.097.[10] When fumarate was the substrate kinetic measure-

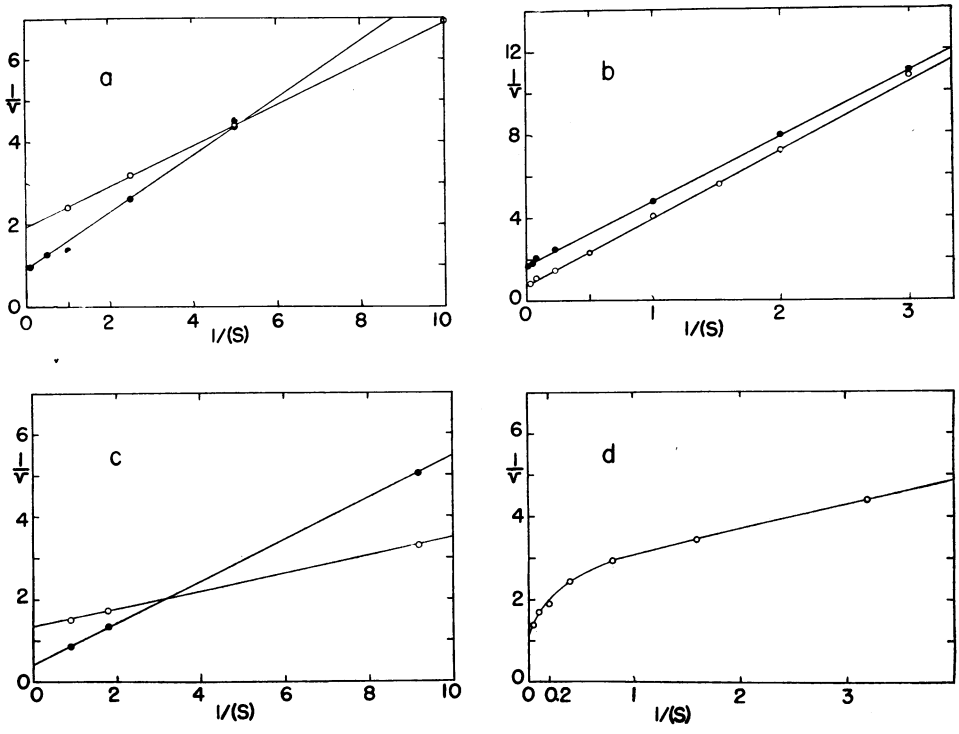

FIGURE 1

Reciprocal initial velocity versus reciprocal substrate concentration (millimolar). The velocities are in arbitrary units, and since the enzyme concentrations are not the same in (*a*), (*b*), (*c*), and (*d*), the maximum velocities may not be directly compared. (*a*) Fumarate, pH 7.3; ○, 0.015 *M* phosphate buffer; ●, 0.15 *M* phosphate buffer. (*b*) L-Malate, pH 7.5; ○, 0.05 *M* phosphate buffer; ●, 0.05 *M* phosphate buffer plus 0.1 *M* NaCl. (*c*) Fumarate, pH 8.07; ○, 0.005 *M* phosphate buffer; ●, 0.005 phosphate buffer plus 0.040 *M* succinate. (*d*) L-Malate, pH 7.4, in 0.05 ionic strength *tris*-(hydroxymethyl)-aminomethane-hydrochloric acid buffer.

ments were limited to the first 8% reaction, while only the first 2% reaction was used in the case of L-malate because of the unfavorable equilibrium.

Interaction with Buffer.—If, in addition to the basic reaction[1] between enzyme, E, and substrate, S, to yield product, P,

$$E + S \underset{}{\overset{K_{ES}}{\rightleftharpoons}} ES \xrightarrow{k_1} E + P, \qquad (I)$$

the buffer, B, can combine with the enzymatically active site

$$E + B \underset{}{\overset{K_{EB}}{\rightleftharpoons}} EB, \qquad (II)$$

or at another site at which it affects the kinetic constants,

$$E + B \underset{}{\overset{K_{BE}}{\rightleftharpoons}} BE, \qquad (III)$$

the following reactions may also be important:

$$BE + S \underset{}{\overset{K_{BES}}{\rightleftharpoons}} BES \overset{k_2}{\rightarrow} BE + P \qquad (IV)$$

$$BE + B \underset{}{\overset{K_{BEB}}{\rightleftharpoons}} BEB. \qquad (V)$$

The fact that BES is allowed to break down to yield product makes this mechanism analogous to the "non-total" inhibition discussed by Segal, Kachmar, and Boyer[11] and to the effects of "modifiers" discussed by Botts and Morales.[12] It will be assumed that in all subsequent derivations the equilibria for which the dissociation constants (K) are indicated are adjusted rapidly in comparison to the rate of appearance of product and that $(S) \gg (E)_0$, and $(B) \gg (E)_0$, where $(E)_0$ is the total molar concentration of the enzymatically active sites. It is not necessary to include steps such as ES + B $\rightleftharpoons$ BES since they are not independent of the above equilibria. For the mechanism represented by (I)–(V) the initial rate (v) of appearance of product at constant (B) will vary with substrate concentration according to the familiar expression

$$v = \frac{V'}{1 + K'_S/(S)}, \qquad (1)$$

where V', the maximum initial velocity, and K'_S, the Michaelis constant, are now given by

$$V' = k_1(E)_0 \frac{1 + k_2K_{ES}(B)/k_1K_{BE}K_{BES}}{1 + K_{ES}(B)/K_{BE}K_{BES}} \qquad (2)$$

$$K'_S = K_{ES} \frac{1 + (B)(1/K_{BE} + 1/K_{EB}) + (B)^2/K_{BE}K_{BEB}}{1 + K_{ES}(B)/K_{BE}K_{BES}}. \qquad (3)$$

Equations of this form may be used to represent the variation of V' and K'_S for fumarase such as that indicated in figure 1 (a). In sodium phosphate buffers of pH 7.3 relative values for V' for fumarate are 0.32, 0.47, 0.68, and 0.81 at 0.005, 0.015, 0.05, and 0.15 M phosphate, and the corresponding values of K'_S are 0.26, 0.38, 0.74, and 2.0 mM. It will be noted that according to this mechanism V' may be a function of buffer concentration even if $k_2 = 0$.

Interaction with Buffer and an Inhibitor.—If an inhibitor, I, which

combines only at the enzymatically active site is added, the mechanism may be represented by (I)–(VII).

$$E + I \underset{}{\overset{K_{EI}}{\rightleftharpoons}} EI \qquad (VI)$$

$$BE + I \underset{}{\overset{K_{BEI}}{\rightleftharpoons}} BEI \qquad (VII)$$

and the initial rate reaction rate is expressed by

$$v = \frac{V'}{1 + \frac{K'_S}{(S)}\left(1 + \frac{(I)}{K'_I}\right)}, \qquad (4)$$

where the competitive inhibition constant K'_I is

$$K'_I = K_{EI}\frac{1 + (B)(1/K_{EB} + 1/K_{BE}) + (B)^2/K_{BE}K_{BEB}}{(1 + K_{EI}(B)/K_{BE}K_{BEI})}. \qquad (5)$$

Thus K'_I is not simply the dissociation constant for an EI complex, as is commonly assumed. In the case of fumarase the variation of the competitive inhibition constant for sodium *trans*-aconitate (0.46, 0.97, and 1.50 mM at 0.005, 0.015, and 0.05 M sodium phosphate buffer of pH 7.5) may readily be expressed by Eq. (5).

If the inhibitor, like the buffer, can also combine with the enzyme so that the resulting complex still acts catalytically, but with different properties, the following two steps must be added (possible complexes IEB and IEI are ignored):

$$E + I \underset{}{\overset{K_{IE}}{\rightleftharpoons}} IE. \qquad (VIII)$$

$$IE + S \underset{}{\overset{K_{IES}}{\rightleftharpoons}} IES \overset{k_3}{\rightarrow} IE + P. \qquad (IX)$$

These are steps which would probably become important in the case of a poor inhibitor which is tested at a relatively high concentration. For the mechanism represented by (I)–(IX), the variation of v with (S) is represented by Eq. (1) with

$$V' = k_1(E)_0\frac{1 + k_2K_{ES}(B)/k_1K_{BE}K_{BES} + k_3K_{ES}(I)/k_1K_{IE}K_{IES}}{1 + K_{ES}(B)/K_{BE}K_{BES} + K_{ES}(I)/K_{IE}K_{IES}} \qquad (6)$$

$$K'_S = K_{ES}\frac{1 + (B)(1/K_{EB} + 1/K_{BE}) + (B)^2/K_{BE}K_{BEB} + (I)(1/K_{EI} + 1/K_{IE} + (B)/K_{BE}K_{BEI})}{1 + K_{ES}(B)/K_{BE}K_{BES} + K_{ES}(I)/K_{IE}K_{IES}}. \qquad (7)$$

Thus the intercept of the Lineweaver-Burk plot may be either larger or smaller than in the absence of inhibitor and the inhibition may not fall into any of the recognized classes.[13] The type of inhibition represented

by Eqs. (6) and (7) is a general type which results in linear Lineweaver-Burk plots and reduces to competitive, non-competitive, uncompetitive and apparent competitive[11] cases under special conditions. In the case of fumarase the inhibition by chloride at pH 7.3, figure 1 (*b*) and, in general, by poor inhibitors such as succinate, figure 1 (*c*), does not fit into the usual classes.

If steps (I)–(IX) apply and $k_3 = 0$, V may decrease with increasing (I). This is an effect which may also occur in the case of a difunctional enzyme, difunctional substrate, and monofunctional inhibitor as pointed out by Foster and Niemann.[2]

Interaction with Buffer and Substrate.—The explanation of the effects represented in figure 1 (*a*)–(*c*) in terms of alteration of catalytic properties of the enzyme by the binding of buffer or inhibitor suggests that substrate itself may have a similar effect. If this is the case, the following two steps are added to the mechanism represented by Eqs. (I)–(V):

$$\mathrm{E} + \mathrm{S} \underset{}{\overset{K_{\mathrm{SE}}}{\rightleftharpoons}} \mathrm{SE}. \qquad \text{(X)}$$

$$\mathrm{SE} + \mathrm{S} \overset{K_{\mathrm{SES}}}{\rightleftharpoons} \mathrm{SES} \overset{k_4}{\rightarrow} \mathrm{SE} + \mathrm{P}. \qquad \text{(XI)}$$

The variation of initial rate with substrate concentration is given by an equation of the type

$$v = \frac{a + b/(S)}{1 + c/(\mathrm{S}) + d/(\mathrm{S})^2} \qquad (8)$$

where a, b, c, and d are constants for a particular buffer concentration. Figure 1 (*d*) indicates that this type of equation satisfactorily represents the data for fumarase in a *tris*-(hydroxymethyl)-aminomethane chloride buffer of pH 7.5. Such activation by substrate is in contrast with the inhibition which is frequently observed at high substrate concentrations in the case of some other enzymes. As pointed out by Kistiakowsky and Rosenberg[3] this type of rate equation also results if there are two types of sites which are either different and independent or which are identical but interact in pairs. It may be very difficult to distinguish between these various possibilities. It is characteristic of Eq. (8) that straight line plots will be obtained by the Lineweaver and Burk method at very high or very low substrate concentrations, so that if experiments fall accidentally in one of these regions the data appears to conform to the simple Michaelis-Menten mechanism. This may be seen when (8) is written as

$$v = \frac{V_2 + V_1K_2/(1 - V_1/V_2)(\mathrm{S})}{1 + K_2/(1 - V_1/V_2)(\mathrm{S}) + K_1K_2/(1 - V_1/V_2)(\mathrm{S})^2} \qquad (9)$$

where
$$V_1 = k_1(\mathrm{E})_0 \frac{1 + k_2K_{ES}(\mathrm{B})/k_1K_{BE}K_{BES}}{1 + K_{ES}/K_{SE} + K_{ES}(\mathrm{B})/K_{BE}K_{BES}}$$

$$K_1 = K_{ES} \frac{1 + (\mathrm{B})(1/K_{BE} + 1/K_{EB}) + (\mathrm{B})^2/K_{BE}K_{BEB}}{1 + K_{ES}/K_{SE} + K_{ES}(\mathrm{B})/K_{EB}K_{BES}}$$

$$V_2 = k_4(\mathrm{E})_0$$

$$K_2 = K_{SE}K_{SES}[(1 - k_1/k_4)/K_{ES} + 1/K_{SE} + (\mathrm{B})(1 - k_2/k_4)/K_{BE}K_{BES}]$$

As (S) $\rightarrow$ 0, Eq. 9 becomes $v = V_1/(1 + K_1/(\mathrm{S}))$. It should be noted that V_1 and K_1 are the same as V' and K'_S given by Eqs. (2) and (3) except for the additional term K_{ES}/K_{SE} which appears in the denominator as a result of equilibrium (X).

As (S) $\rightarrow \infty$, Eq. (9) becomes $v = V_2/(1 + K_2/(\mathrm{S}))$, and it is of interest to note that according to this mechanism the limiting velocity at high substrate concentration (V_2) is independent of the buffer concentration. The importance of this type of mechanism for the control of the rate of an enzymatic reaction in vivo is that the rate may be greatly increased in the presence of a considerable excess of the substrate for the enzyme.

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