KINETICS OF THE CARBOXYPEPTIDASE A-CATALYZED HYDROLYSIS OF BENZYLOXYCARBONYLGLYCYL-L-PHENYLALANINE*

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Determination of K_m(app) and k_{cat} for carboxypeptidase A-catalyzed hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine is complicated because of the effect of substrate and products on the reaction. When added to a reaction mixture, benzyloxycarbonylglycine increases the rate while L-phenylalanine competitively decreases the rate of the reaction. Initial reaction rate data alone are not sufficient to resolve the problem because the substrate, benzyloxycarbonylglycyl-L-phenylalanine, also affects the rate of reaction (Whitaker, Menger and Bender, 1965). Furthermore, good initial rate data are impossible to obtain because the rate of hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine follows good first order rate kinetics even at substrate concentrations several-fold above K_m(app) (Whitaker Menger and Bender, 1965; Elkins-Kaufman and Neurath, 1948; Neurath, Elkins and Kaufman, 1947; Putnam and Neurath, 1946; Bergmann and Fruton, 1942; Hofmann and Bergmann, 1940). Data obtained with other peptide substrates (Slobin and Carpenter, 1964) indicate this may be a general feature of carboxypeptidase A-catalyzed hydrolysis of peptides.

It is the purpose of this paper to present a kinetic approach which solves the difficulties mentioned above and which gives an independent

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verification of the assumptions previously used in analyzing initial reaction rate data for the hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine by carboxypeptidase A (Whitaker, Menger and Bender, 1965).

The materials and experimental procedures were those reported previously (Whitaker, Menger and Bender, 1965). In the work under discussion here, complete reaction rate data were obtained.

On the basis of the observation that the initial concentrations of benzyloxycarbonylglycyl-L-phenylalanine, benzyloxycarbonylglycine and L-phenylalanine all modify the rate (Figures 1 and 2), the complete hydrolysis

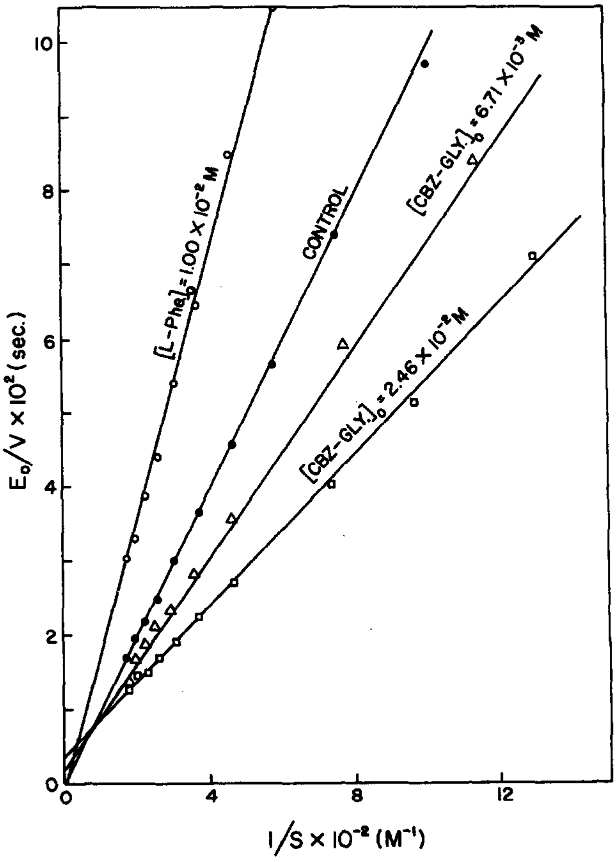

Fig. 1. Effect of added L-phenylalanine and benzyloxycarbonylglycine on rate of hydrolysis of 5.90×10^{-3}M benzyloxycarbonylglycyl-L-phenylalanine by carboxypeptidase A at pH 7.50 and 25.0°. E_0 was 3.58 to 3.89×10^{-7}M.

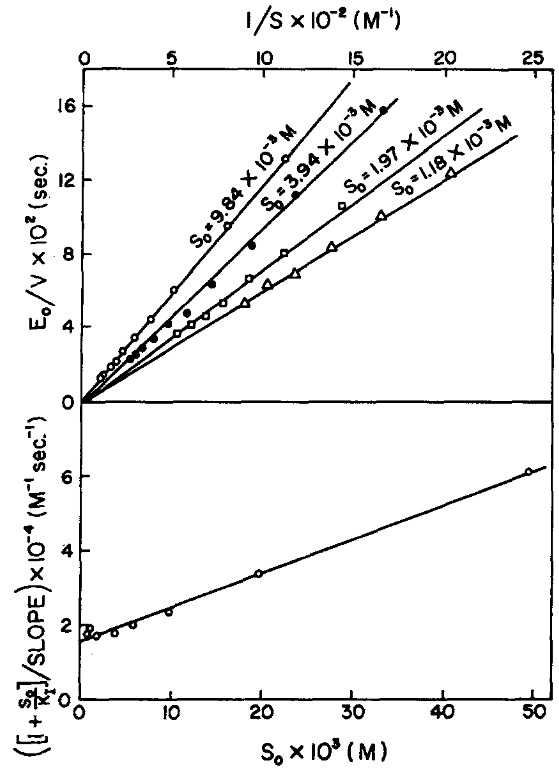

Fig. 2. Effect of initial benzyloxycarbonylglycyl-L-phenylalanine concentration on rate of hydrolysis by carboxypeptidase A at pH 7.50 and 25.0°. E_o was 1.77 to 6.13 x 10^{-7}M.

of benzyloxycarbonylglycyl-L-phenylalanine by carboxypeptidase A should be described by equations 1-4.

$$E + S \overset{K_m(\text{app})}{\rightleftharpoons} E{\cdot}S \xrightarrow{k_{cat}} E + P_1 + P_2 \tag{1}$$

$$E{\cdot}S + S \overset{K_s'}{\rightleftharpoons} E{\cdot}S_2 \xrightarrow{k'_{cat}} E{\cdot}S + P_1 + P_2 \tag{2}$$

$$E{\cdot}S + \text{CBZ gly} \overset{K_c}{\rightleftharpoons} E{\cdot}S{\cdot}\text{CBZ gly} \xrightarrow{k''_{cat}} E{\cdot}\text{CBZ gly} + P_1 + P_2 \tag{3}$$

$$E + \text{L-phe} \overset{K_i}{\rightleftharpoons} E{\cdot}\text{L-phe} \tag{4}$$

Using steady state considerations, it can be shown that equations 1-4 (and their variations with respect to binding order) lead to equation 5 which

$$V = [k_{cat}(1 + \alpha S_o + \alpha' A_o)E_oS] / \left\{ K_m(app)[1 + ((S_o - S + I_o)/K_i)] + S \right\} \quad (5)$$

covers all the special cases discussed below. α is equal to $k'_{cat}/(k_{cat}K'_s)$, α' is equal to $k''_{cat}/(k_{cat}\ K_c)$, S_o and S are the initial and time *t* benzyloxycarbonylglycyl-L-phenylalanine concentrations, respectively, A_o and I_o are the initial added concentrations of benzyloxycarbonylglycine and L-phenylalanine, respectively and V is the observed velocity of the reaction. The other symbols are defined by equations 1-4.

Rearranging equation 5 algebraically and taking the reciprocal of both sides transforms the equation into the usual Lineweaver-Burk form of the Michaelis-Menten equation.

$$E_o/V = [1 - K_m(app)/K_i]/[k_{cat}(1 + \alpha S_o + \alpha' A_o)] + \left\{ K_m(app)[1 + (S_o/K_i) + (I_o/K_i)]/[k_{cat}(1 + \alpha S_o + \alpha' A_o)] \right\} (1/S) \quad (6)$$

Equation 6 is a complex equation containing five unknowns but it can be solved. Plots of E_o/V vs 1/S for data obtained at several initial substrate concentrations and in the presence and absence of added benzyloxycarbonylglycine or L-phenylalanine are shown in Figures 1 and 2. In all cases the intercept is essentially zero. This result can be realized only if K_m(app) and K_i are essentially equal so that the intercept term of equation 6 is essentially zero. The equivalence of K_m(app) and K_i is a condition which leads to the observation of first order rate kinetics (substrate dependence) at all substrate concentrations. First order rate kinetics for benzyloxycarbonylglycyl-L-phenylalanine hydrolysis by carboxypeptidase A were always observed by the present author as well as by others (Whitaker, Menger and Bender, 1965; Elkins-Kaufman and Neurath, 1948; Neurath, Elkins and Kaufman, 1947; Putnam and Neurath, 1946; Bergmann and Fruton, 1942; Hofmann and Bergmann, 1940).

The data, therefore, can be described by a modification of equation 6 to give equation 7.

$$E_o/V = \left\{K_m(\text{app})\ [1 + (S_o/K_i) + (I_o/K_i)]/[k_{cat}(1 + \alpha S_o + \alpha' A_o)]\right\}(1/S) \quad (7)$$

Let us now consider how the five constants of equation 7 can be determined.

Determination of K_i and K_m(app). - If two complete reactions are observed under identical conditions with the exception that L-phenylalanine is added to one of the reactions (no benzyloxcarbonylglycine added), it can be shown from equation 7 that the slopes of the lines, E_o/V vs $1/S$, are related by equation 8.

$$\text{slope}_{\text{L-phe}}/\text{slope} = (K_i + S_o + I_o)/(K_i + S_o) \quad (8)$$

The average value of K_i of L-phenylalanine (and thus the K_m(app) of benzyloxycarbonylglycyl-L-phenylalanine which is assumed to be equal to the K_i of L-phenylalanine) determined by this method was 5.57×10^{-3}M. This agrèes well with K_m(app) of 5.83×10^{-3}M for benzyloxycarbonylglycyl-L-phenylalanine determined from corrected initial rate data (Whitaker, Menger, and Bender, 1965). The equivalence of the K_i of L-phenylalanine and the K_m(app) of benzyloxycarbonylglycyl-L-phenylalanine implies that the principal binding of this substrate to the enzyme involves the phenylalanine portion of the substrate. Chloroacetyl-DL-phenylalanine is bound and split readily by carboxypeptidase A (Snoke and Neurath, 1949).

Determination of α and k_{cat}. - In the absence of added L-phenylalanine and benzyloxycarbonylglycine the slope of equation 7 is given by equation 9.

$$\text{slope} = K_m(\text{app})\ [1 + (S_o/K_i)]/[k_{cat}(1 + \alpha S_o)] \quad (9)$$

By rearrangement the more useful equation 10 is obtained.

$$[1 + (S_o/K_i)]/\text{slope} = k_{cat}/K_m(\text{app}) + (k_{cat}/K_m(\text{app}))\ \alpha S_o \quad (10)$$

By determining the slopes of the plots of E_o/V vs $1/S$ for several S_o concentrations and plotting the data according to equation 10, i.e. $[1 + (S_o/$

$K_i)]$/slope vs S_o, α can be determined. A plot of such data is shown in Figure 2. α, the slope of the line divided by the intercept, is found to have a value of 58.4 ± 3.4 M^{-1}. Since K_m(app) is known ($5.57 \times 10^{-3}M$), k_{cat} can also be calculated and is found to have a value of 87.0 ± 2.7 sec^{-1} which is in reasonable agreement with a value of 106 ± 3 sec^{-1} calculated from corrected initial rate data (Whitaker, Menger and Bender, 1965). By definition $\alpha = k'_{cat}/k_{cat}\ K'_s)$. Making the assumption that the k^{exp}_{cat} of 198 sec^{-1} determined at high substrate concentrations from uncorrected initial rate data (Whitaker, Menger and Bender, 1965), is essentially k'_{cat}, K'_s is calculated to be $3.90 \times 10^{-2}M$. It is of interest that Elkins-Kaufman and Neurath (1948) found K_m(app) to be $3.7 \times 10^{-2}M$ for benzyloxycarbonylglycyl-L-phenylalanine when determined at high initial substrate concentrations (1.25×10^{-2} to $7.50 \times 10^{-2}M$).

Determination of α'. - α' is best calculated from equation 11, derived from equation 7.

$$\text{slope } A_o = K_m(\text{app})\ [1 + (S_o/K_i)]/[k_{cat}(1 + \alpha S_o + \alpha' A_o)] \qquad (11)$$

Slope A_o is determined from a plot of E_o/V vs $1/S$ for a reaction containing added benzyloxycarbonylglycine (no added L-phenylalanine). Data are presented in Table I. The average α' was found to be 57.8 ± 9.4 M^{-1}. Thus α, which reflects the activation by benzyloxycarbonylglycyl-L-phenylalanine,

Table I

Effect of Added Benzyloxycarbonylglycine on Rate of Hydrolysis of Benzyloxycarbonylglycyl-L-phenylalanine at $S_o = 5.90 \times 10^{-3}M$, pH 7.50, 25.0° and E_o of $3.58\text{-}3.89 \times 10^{-7}M$.

$[CBZ\ gly]_o$ ($\times 10^{2}M$)	slope ($\times 10^{5}M$ sec)	α'(calcd.) (M^{-1})
0.671	7.24_0	71.2
1.23	6.44_3	57.1
1.80	5.79_6	51.7
2.46	5.07_2	51.1

and α', which reflects the activation by benzyloxycarbonylglycine are the same, thus confirming an assumption made previously in order to evaluate initial reaction rate data for this system (Whitaker, Menger and Bender, 1965).

K_m(app) and k_{cat} values for the hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine by carboxypeptidase A which were obtained by two independent kinetic approaches are in reasonable agreement with each other. This not only supports the assumptions used in evaluating initial reaction rate data (Whitaker, Menger and Bender, 1965) but also indicates that a correct interpretation of the kinetic effects of substrate and products on this system, benzyloxycarbonylglycyl-L-phenylalanine - carboxypeptidase A, has been presented here.

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