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DETERMINATION OF THE INDIVIDUAL RATE AND ASSOCIATION CONSTANTS OF THE HYDROLYSIS CATALYSED BY SERINE PROTEINASES BY MEANS OF ADDED NUCLEOPHILES IN EISENTHAL AND CORNISH-BOWDEN COORDINATES

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Summary

It is shown that in the three-step enzyme-catalysed hydrolysis the addition of nucleophile shifts the common intersection point in Eisenthal and Cornish-Bowden coordinates when registering the second product P_2. The different points obtained at different nucleophile concentrations are situated on a straight line with intercepts K_s on the K_m axis and $k_3[E]_o$ on the V axis. Since the Eisenthal and Cornish-Bowden method is considered as the best graphic method for determination of the kinetic parameters $\hat{K}_m$ and $\hat{V}$ of enzyme reactions, the graphic procedure proposed here for determination of k_2, k_3, k_4 and K_s by the method of added nucleophile is to be preferred. This procedure was applied for determination of the individual constants of the hydrolysis of *N*-acetylated L-amino acid methyl esters catalysed by alkaline mesentericopeptidase.

The determination of the individual rate and association constants of enzyme reactions is of great importance for establishing the mechanism of enzyme action. The method of added nucleophiles proposed by Bender et al. [1] proved to be one of the simplest methods, needing no special apparatus in the study of the individual steps of hydrolysis catalysed by serine proteinases.

An enzyme-catalysed hydrolysis involving an acyl-enzyme intermediate

can be expressed by Eqn. 1:

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{EA} \xrightarrow{k_3} E + P_2 \qquad (1)$$

where ES is the enzyme-substrate complex, EA is the acyl-enzyme, P_1 and P_2 are products formed in different reaction steps. The kinetic constants k_{cat} and K_m are related to the individual rate constants by the equations:

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \qquad (2)$$

$$K_m = K_s \frac{k_3}{k_2 + k_3} \qquad (3)$$

By introducing a nucleophile in addition to water, Eqn. 1 is expanded to Eqn. 4 in agreement with the scheme proposed by Bender et al. [1]:

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{EA} \begin{matrix} \xrightarrow{k_3} E + P_2 \\ \xrightarrow{k_4[N]} E + P_3 \end{matrix} \qquad (4)$$

where N denotes the added nucleophile (methanol, ethanol) and k_4 the rate constant of the transfer of the substrate acyl part on the nucleophile; if the nucleophile N is the same as P_1, then P_3 must be equivalent to S. The relationship between the kinetic parameters k_{cat} and K_m and the individual rate and association constants in this case is expressed by the equations:

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3 + k_4[N]} \qquad (5)$$

$$K_m = K_s \frac{k_3 + k_4[N]}{k_2 + k_3 + k_4[N]} \qquad (6)$$

$$k_3 = k_3'[H_2O]$$

Eqn. 6 holds for the case of registration of the product P_2. When plotting K_m/k_{cat} vs. [N] and $1/k_{cat}$ vs. [N], all rate and association constants, k_2, k_3, k_4, and K_s could be determined [1].

Later Berezin and coworkers [2] showed that a plot of experimental data in the Lineweaver-Burk coordinates gives straight lines, corresponding to the various nucleophile concentrations, which intersect at a common point in the upper left-hand quadrant, where the abscissa $1/[S]_o = -1/K_s$ and the ordinate $1/v = [E]_o/k_3$.

Although the most usual method of estimating enzyme kinetic parameters is the Lineweaver-Burk plot, this plot is statistically objectionable and is the worst plot of the three linear transformations of the Michaelis-Menten equation [3]. Recently Eisenthal and Cornish-Bowden proposed a method of 'direct linear plot' [4]. According to this method observations are plotted as

lines in parameter space instead of points in observation space. The kinetic parameters $\hat{V}$ and $\hat{K}_m$ can be obtained from the coordinates of the common point of intersection of lines, corresponding to each observation (s,v). This method is statistically correct and requires no calculation of any kind [5]. As Atkins and Nimmo [6] have shown, the 'direct linear plot' of Eisenthal and Cornish-Bowden is the best graphic method for fitting the Michaelis-Menten equation, and is the one to be used for data treatment.

The aim of the present paper is to show how the individual rate and association constants k_2, k_3, k_4 and K_s can be obtained in Eisenthal and Cornish-Bowden coordinates by means of added nucleophiles when registering the product P_2. The registration of P_2 is a very convenient and widely used method of following the rate of ester hydrolysis by pH-stat equipment. The method can be used not only in serine proteinase catalysis, but in a number of enzyme-catalysed reactions as expressed by Eqn. 1, where the second intermediate is hydrolysed by water to give the free enzyme and the second product P_2.

The addition of the nucleophile leads to decrease of k_{cat} and increase of K_m in accordance with Eqns. 5 and 6. In Eisenthal and Cornish-Bowden plots the common intersection point is shifted depending on the nucleophile concentration as is shown in Fig. 1. These points with coordinates $\hat{K}_m$ and $\hat{V}$ are situated on a straight line with intercepts K_s on the K_m axis and $k_3[E]_o$ on the V axis.

The equation of a straight line determined by two points (x_1y_1 and x_2y_2) of the general form:

$$\frac{y - y_1}{y_2 - y_1} = \frac{x - x_1}{x_2 - x_1} \tag{7}$$

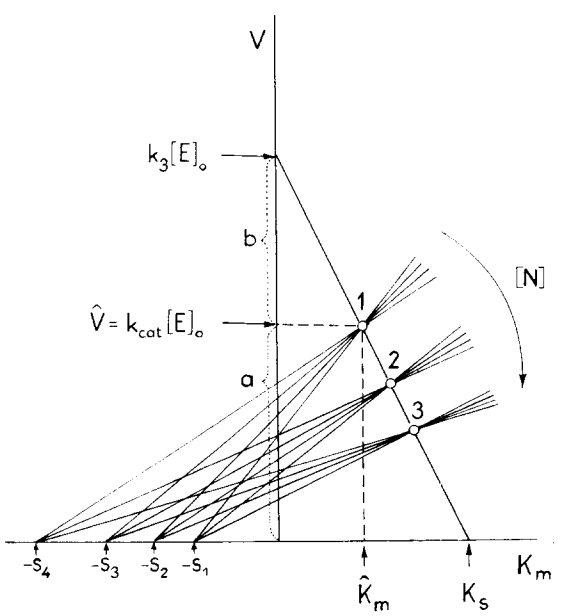


Fig. 1. Determination of the individual rate and association constants of hydrolysis in Eisenthal and Cornish-Bowden coordinates. 1, without added nucleophile; 2 and 3 with added nucleophile.

in this case can be rewritten as follows:

$$\frac{V - \hat{V}}{\hat{V}_N - \hat{V}} = \frac{K_m - \hat{K}_m}{\hat{K}_{m_N} - \hat{K}_m} \tag{8}$$

where $\hat{K}_m$ and $\hat{K}_{m_N}$ are substituted according to Eqns. 3 and 6, respectively, $\hat{V} = k_{cat}[E]_o$, respectively $\hat{V}_N = k_{cat_N}[E]_o$ (for k_{cat} and k_{cat_N} see Eqns. 2 and 5). It is easy to show that when $V = 0$, $K_m = K_s$, and when $K_m = 0$, $V = k_3[E]_o$.

The acylation rate constant, k_2, can be determined from Eqn. 2 or from the ratio of the segments a and b on the ordinate:

$$\frac{a}{b} = \frac{k_2}{k_3} \tag{9}$$

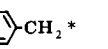
From any intersection point obtained in the presence of nucleophile ($\hat{V}_N$, $\hat{K}_{m_N}$) the rate constant k_4 can be determined according to Eqn. 5. For a sufficiently precise determination, the rate constants k_2 and k_3 should not differ by more than an order of magnitude. Otherwise, the method of added nucleophile itself is inapplicable. If $k_3 \ll k_2$, as [N] is increased the intersection point shifts in the right parallel with the K_m axis being distant at $V = k_3[E]_o$. If $k_3 \gg k_2$, the intersection point shifts down parallel with the V axis at a distance $K_m = K_s$. If at higher nucleophile concentration a non-competitive inhibiting effect occurs, the straight line, determined by the intersection points will curve left and down. If the inhibiting effect is competitive, the straight line will curve right and up.

The procedure proposed above was used for determination of the individual rate and association constants of the hydrolysis of *N*-acetylated-L-amino acid methyl esters catalysed by alkaline mesentericopeptidase by means of methanol as added nucleophile [7]. Alkaline mesentericopeptidase (EC 3.4.21.-) is a serine proteinase of bacterial origin. The enzyme behaviour is consistent with the three-step mechanism expressed by Eqn. 1 [8]. As it was shown [9,10], the rate of formation of an enzyme-substrate complex must be lower than the diffusion controlled limit and higher than the specificity constant $\frac{k_2}{K_s}$, which is equal to $\frac{k_{cat}}{K_m}$. The estimated lower limits for the formation of the alkaline mesentericopeptidase enzyme-substrate complex are much greater than k_2 and k_3, therefore the method of added nucleophile can be used.

Alkaline mesentericopeptidase was prepared in a pure state as described previously [11]. The enzyme was dissolved in 0.7 M $CaCl_2$. The normality of the stock solutions was determined by active site titration with *N-trans*-cinnamoylimidazole [12]. The synthetic method and characteristics of *N*-acetyl-L-amino acid methyl esters were reported by Jones et al. [13]. The hydrolysis of the substrates was followed by titration with 0.01 M KOH of the liberated *N*-acetyl-L-amino acids in a pH-stat 'Radiometer' at pH 8.0, 25°C, 0.1 M KCl. The steady-state rate of hydrolysis was measured at the initial time with $[S]_o$, $[N]_o \gg [E]_o$, so that the convertion of substrates does not exceed the

TABLE I

STEADY-STATE KINETIC DATA FOR THE HYDROLYSIS OF *N*-ACETYL-L-AMINO ACID METHYL ESTERS OF GENERAL FORMULA $RCH(NHCOCH_3)COOCH_3$ CATALYSED BY ALKALINE MESENTERICOPEPTIDASE. pH 8.0, 25°C, 0.1 M KCl.

R	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	K_s (mM)	k_2 (s^{-1})	k_3 (s^{-1})
CH_3	100	250	400	400	160	266
$H(CH_2)_2$	266	104	2 560	177	452	646
$H(CH_2)_3$	601	39.0	15 400	70.0	1 080	1 350
$H(CH_2)_4$	140	52.7	2 660	84.3	224	372
	210	23.5	8 940	40.9	365	494
$H(CH_2)_6$	24.1	4.07	5 920	6.75	40.0	60.5
$(CH_3)_2CH$	10.1	109	92.7	180	16.7	25.6
 *	159	14.4	11 040	23.8	262	404
$(CH_3)_2CHCH_2$	477	49.7	9 600	79.5	763	1 270

*Ethyl ester.

initial 3% of the total hydrolysis. The kinetic and association constants we determined are presented in Table I. They are in good agreement with the same constants obtained in Lineweaver-Burk coordinates.

The individual rate and association constants of the hydrolysis of *N*-acetylated-L-amino acid methyl esters have been determined also in α-chymotripsin catalysis [2]. It is worth pointing out that in α-chymotrypsin catalysis, the deacylation step is rate limiting (the ratio k_2/k_3 lies within the range 3—7), whereas in alkaline mesentericopeptidase catalysis there is no rate limiting step (the ratio k_2/k_3 lies between 0.6 and 0.8).

The procedure proposed here was used in studying the temperature dependence of k_2, k_3, k_4, and K_s of the hydrolysis of the same substrates catalysed by α-chymotrypsin [14]. The results will be published later.

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