DETERMINATION OF THE INDIVIDUAL RATE CONSTANTS OF α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS WITH THE ADDED NUCLEOPHILIC AGENT, 1,4-BUTANEDIOL

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Knowledge of intermediary stage rate constants of enzyme reactions provides very valuable information about the mechanism both of separate reaction stages and of a process as a whole. Despite this, published data on values of rate constants of 'elementary' reactions even for such an amply described enzyme as α-chymotrypsin are very scanty. To some extent, this is due to the lack of a sufficiently simple and reliable method of determining the constants.

Hydrolysis of esters in the presence of α-chymotrypsin proceeds according to a mechanism involving at least three steps [1]:

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

where ES is the enzyme–substrate complex, EA is the acyl-enzyme, P_1 and P_2 are alcohol and acid hydrolysis products, respectively. Kinetics of such reactions is formally described by the usual Michaelis-Menten two-step scheme,

$$E + S \overset{K_{m(app)}}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \qquad (2)$$

There is a simple connection between the kinetic constants of (1) and (2) expressed as

$$K_{m\,(app)} = K_s \frac{k_3}{k_2 + k_3} \qquad (3)$$

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \qquad (4)$$

where K_s is a true Michaelis constant, k_2 and k_3 are rate constants for acylation and deacylation of enzyme, respectively. Upon viewing (3) and (4) it becomes evident that a separate determination of individual rate constants k_2 and k_3 cannot be achieved by conventional methods of steady-state kinetics.

At present in kinetic enzymology, two principally differing trends in determining the individual rate constants have taken shape. One is based on the study of pre-steady state period of enzyme-catalyzed reactions (methods for studying fast reactions in solution are used), and the other resorts to selective influence upon separate stages of an enzymatic process in its steady state. The stopped-flow method in its various modifications belongs to the first group [2–5]. The second group includes methods based on a selective reversible inhibition of α-chymotrypsin acylation reaction by means of Cu^{2+} ions [6] and on the influence of ionic strength of the solution on steady state kinetics α-chymotrypsin-catalyzed hydrolysis [7] and also a method which uses acceleration of the deacylation reaction in the presence of added nucleophiles [8, 9]. The latter is at present one of the simplest methods. It needs no special apparatus and permits results practically at any pH value. Kinetics of such α-chymotrypsin-catalyzed reactions in its simplest form agree with the scheme proposed by Bender et al. [8]:

$$E + S \overset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} EA \overset{k_3[H_2O]}{\underset{k_4[N]}{\rightleftharpoons}} \begin{matrix} E + P_2 \\ E + P_3 \end{matrix} \qquad (5)$$

$+P_1$

Table 1

Steady-state kinetic data. The values for kinetic constants of the L-substrates were determined from initial rate measurements, pH 7.8 (pH-stat), 25°, μ = 0.1 (KCl). The operational normality of the enzyme solutions was determined by titration with *N-trans*-cinnamoylimidazole [11].

N-Acyl	Side-chain	Ester	k_{cat} (sec^{-1})	$K_{m(app)}$ (mM)	k_2 (sec^{-1})	k_3 (sec^{-1})	K_s (mM)
Acetyl	Gly	OCH_3	0.109	862	0.49	0.14	3380
Acetyl	Gly	OC_2H_5	0.051	445	0.094	0.11	823
Benzoyl	Gly	OCH_3	0.31	4.24	0.42	1.17	5.78
Acetyl	But	OCH_3	1.41	66.7	8.81	1.68	417
Benzoyl	But	OCH_3	0.32	1.41	0.41	1.52	1.79
Benzoyl	Ala	OC_2H_5	0.069	5.97	0.069	0.6	5.97
Acetyl	Norval	OCH_3	5.08	14.3	35.6	5.93	100
Benzoyl	Norval	OCH_3	2.45	0.85	4.16[a]	5.93	1.45[a]
Acetyl	Val	OCH_3	0.173	87.7	0.98	0.21	500
Acetyl	Val	OC_2H_5	0.152	110	0.55[b]	0.21	398[b]
Acetyl	Val	i-OC_3H_7	0.096	177	0.178[b]	0.21	327[b]
Chloroacet	Val	OCH_3	0.127	43	0.32[b]	0.21	108.8[b]
Benzoyl	Val	OCH_3	0.064	4.17	0.09	0.22	5.84
Acetyl	Norleu	OCH_3	16.1	5.37	103	19.1	34.4
Acetyl	Phe	OCH_3	97.1	0.93	796	111	7.63
Acetyl	Phe	OC_2H_5	68.6	1.85	265	92.7	7.14
Acetylala(L)	Phe	OCH_3	57.3	0.296	176	85	0.909
Benzoyl	Phe	OCH_3[c]	30.7	0.0349	45.8	91.6	0.0524
Acetyl	Tyr	OC_2H_5	192	0.663	5000	200	17.2
Benzoyl	Tyr	OCH_3	90.9	0.018	364	121	0.072
Benzoyl	Tyr	OC_2H_5	85.9	0.022	249	131	0.0638
Acetylleu(L)	Tyr	OCH_3[d]	65.7	0.0192	158	113	0.0461
Furoyl	Tyr	OCH_3	50	0.417	66.7[a]	200	0.56[a]

[a] Calculated from published data for k_{cat} and $K_{m(app)}$ [12, 13] considering k_3 to be constant for all *N*-acylated derivatives of a given amino acid [14, 15].

[b] Calculated from published data for k_{cat} and $K_{m(app)}$ [16]; k_3 was determined for methyl ester. The values of the catalytic constants [16] were normalized in relation to k_{cat} of the methyl ester of *N*-acetyl-L-valine obtained in our experiments.

[c] 2% CH_3CN

[d] The individual rate constants were determined by means of added nucleophile, acetoxime [9].

where N is an added nucleophilic agent, P_3 is a product of transferring the substrate acyl part onto the nucleophile.

When registering the acid product P_2 (pH-stat), the addition of external nucleophiles changes in a general case both the catalytic constant (6) and the Michaelis constant (7):

$$k_{cat} = \frac{k_2 k_3'}{k_2 + k_3' + k_4[N]} \quad (6)$$

$$K_{m(app)} = \frac{K_s(k_3' + k_4[N])}{k_2 + k_3' + k_4[N]} \quad (7)$$

($k_3' = k_3[H_2O]$). A plot of experimental data ($1/V$ versus $1/[S]_0$), gives straight lines, corresponding to various nucleophilic agent concentrations, which intersect at a common point (figs. 1–3) in the upper left-hand quadrant at which the abscissa $1/[S]_0 = -1/K_s$ and the ordinate $1/V = [E]_0/k_3$ [9].

If the added nucleophile is an effective competitive inhibitor in an α-chymotrypsin reaction, then there will be no common intersection point (it will 'move' towards the $1/V$-axis). In our earlier paper [9] oximes were used as nucleophilic agents. In this paper we have chosen 1,4-butanediol because it contains two hydroxyl groups which weaken its competitive inhibiting effect on this reaction [10].

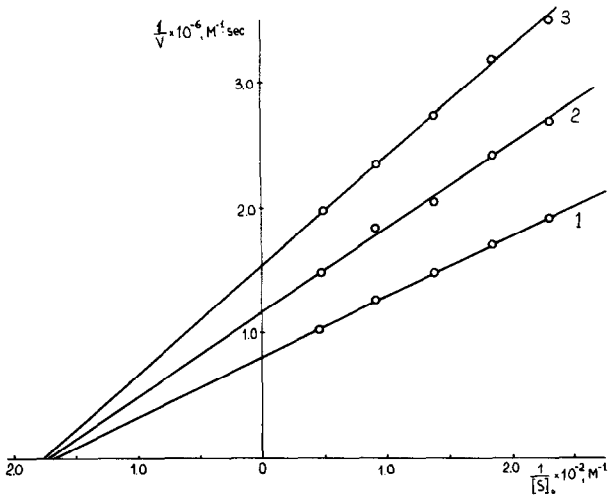

Fig. 1. The influence of 1,4-butanediol on α-chymotrypsin-catalyzed hydrolysis of *N*-benzoyl-L-alanine methyl ester. 25°, μ 0.1 (KCl), $E_0 = 1.8 \times 10^{-4}$ M. 1,4-butanediol (M): (1) 0, (2) 0.11, (3) 0.22.

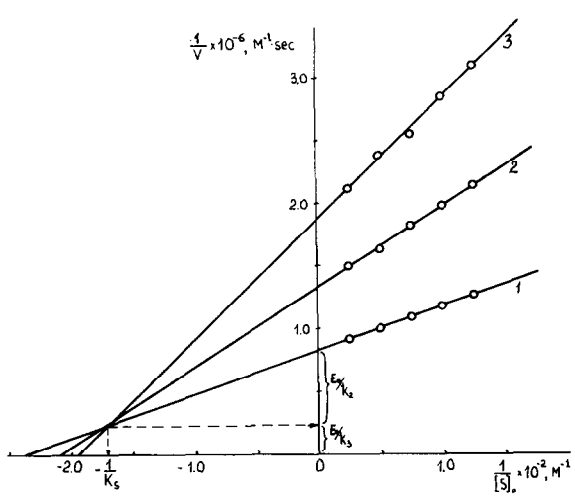

Fig. 3. The α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-phenylalanine ethyl ester in the presence of 1,4-butanediol. 25°, μ 0.1 (KCl), $E_0 = 1.03 \times 10^{-8}$ M, 1,4-butanediol (M): (1) 0, (2) 0.11, (3) 0.22.

Kinetics of α-chymotrypsin-catalyzed hydrolysis and solvolysis were determined using a Radiometer pH-stat (TTT-1c, Denmark) by titrating the acid product P_2 (scheme 5) with KOH (0.01 N). The reaction was carried out in an inert atmosphere at 25° and ionic strength 0.1 (KCl). The steady-state rate of hydrolysis was measured at the initial time. The value of the true Michaelis constant, K_s, was determined from the common intersection point of the straight lines in Lineweaver-Burk coordinates (figs. 1–3). The ratio of the rate constants of acylation and deacylation and the individual constants k_2 and k_3 were calculated from the equations (8), (9) and (10) respectively.

$$\frac{k_2}{k_3} = \frac{K_s}{K_{m(\text{app})}} - 1 \qquad (8)$$

$$k_2 = k_{\text{cat}}(1 + k_2/k_3) \qquad (9)$$

$$k_3 = k_{\text{cat}}\left(1 + \frac{1}{k_2/k_3}\right) \qquad (10)$$

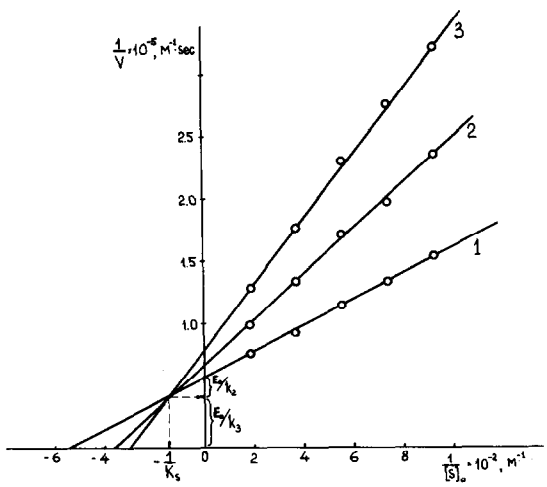

Fig. 2. The α-chymotrypsin-catalyzed hydrolysis of methyl-hippurate in the presence of 1,4-butanediol. 25°, μ 0.1 (KCl), $E_0 = 3.87 \times 10^{-6}$ M 1,4-butanediol (M): (1) 0, (2) 0.11, (3)0.22.

In table 1 some experimental results of determining rate constants of individual stages of α-chymotrypsin-catalyzed hydrolysis of the series of *N*-acylated L-amino acid esters are presented.

As can be seen figs. 1–3, in order to determine the values of the true Michaelis constant (and those of the individual constants) substrate concentrations should be around the K_s value (i.e., for $k_2 > k_3$, substrate concentrations should exceed the value of the apparent Michaelis constant, $K_{m(\text{app})}$). In this case, the common intersection point in a Lineweaver-Burk

plot will be in the upper left-hand quadrant at a sufficient distance from the $1/V$-axis. It may be seen from fig. 3 that when the values of k_2 and k_3 differ by more than one order of magnitude, their separate determination becomes rather difficult because of insufficient precision of the experiments. However, as the data in table 1 indicate, for most model α-chymotrypsin substrates the ratio of the constants k_2/k_3 lies within the range 0.5–7.0, which is in agreement with the potential of the 'nucleophilic' method.

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