SUBSTRATE ACTIVATION IN THE TRYPSIN-CATALYZED HYDROLYSIS OF BENZOYL-L-ARGININE p-NITROANILIDE*

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Received September 14, 1970

SUMMARY. Substrate activation was observed in the tryptic hydrolysis of benzoyl-L-arginine p-nitroanilide in the range of substrate concentration higher than 5 X 10^{-4}M. While a similar phenomenon has been reported on the tryptic hydrolysis of p-toluenesulfonyl-L-arginine methyl ester or others (1,2,3,4), this observation is the first instance detected for the amide substrate. Succinylated trypsin did not show the phenomenon, though the chemical modification intensified the enzymatic activity toward this amide substrate.

BAPA**, one of the most suitable chromogenic substrate for trypsin of the amide type (5), was available only in the DL-form, until the recent success by Nishi et al.(6) in search for a new synthetic route to the L-antipode accompanying no racemization. By using the DL-preparation, the direct precise analysis of kinetic parameters of trypsin-catalyzed hydrolysis of this substrate has been impossible, because the D-isomer behaves as a competitive inhibitor and the racemate has only a limited solubility.

The present study was initiated in an effort to determine the accurate kinetic parameters with L-BAPA, examining whether the simple Michaelis-Menten kinetics is applicable throughout a wide range of substrate concentration or not.

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* This work was presented in part before the 23rd Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1970.
** Abbreviations used: BAPA, α-N-benzoyl-arginine p-nitroanilide; BAEE, α-N-benzoyl-L-arginine ethyl ester; BAA, α-N-benzoyl-L-arginine amide; TAME, α-N-p-toluenesulfonyl-L-arginine methyl ester; BCAT, α-N-Benzyloxycarbonyl-L-arginine p-toluidide; BA, α-N-benzoyl-L-arginine; TA, α-N-p-toluenesulfonyl-L-arginine; NPAB, p-nitrophenyl p-amidinobenzoate.

MATERIALS AND METHODS

Twice crystallized bovine pancreatic trypsin was obtained from Worthington (lot TRL-8GA) and used either directly or after partial purification by the method of Ganrot (7). In the latter case, the effluent fraction containing high specific activity from a column of Bio-Gel P-30 was immediately used for the kinetic analysis.

Succinylated trypsin was prepared by treating the commercial trypsin (1% in 0.05M Na-borate buffer-0.02M $CaCl_2$-0.1M KCl, pH 8.0) with a 500-fold molar excess of succinic anhydride at 4° for 1 hour. The medium pH was kept at 8.0 during the reaction by addition of 5N NaOH from a Radiometer pH-stat titrator Model TTT1c. After the treatment, the enzyme derivative was freed from the excess reagent and by-products by gel-filtration through Sephadex G-25 with 0.01M ammonium acetate (pH 8) as eluant and lyophilized.

L-BAPA was kindly donated by Prof. J. Noguchi of Hokkaido University. BA and TA were the products of the Foundation for Promotion of Protein Research in the Institute for Protein Research, Osaka University. NPAB was synthesized by Dr. K.Tanizawa of this department.

The initial rates of trypsin- and succinyltrypsin-catalyzed hydrolyses of L-BAPA were assessed by tracing the increase in absorbance at 410nm continuously with a Hitachi 124 or 356 recording spectrophotometer, in a manner similar to that of Method I by Erlanger et al.(5). The reaction conditions are given in the legend of Fig. 1. Tryptic activity toward BAPA was also measured under the inhibitory action of BA or TA, by Method II of Erlanger et al.(5) with slight modifications, and K_i values of the inhibitors were determined by Dixon's method (8,9).

The normality (N) of enzyme solution was measured by the method of Tanizawa et al.(10) using NPAB as an active-site titrant at pH 8.2 and the protein molarity was estimated from the absorbance at 280nm by taking $E(1\%) = 15.4$ and molecular weight = 23,800 (11). The ratio of normality/molarity was 0.60 for the commercial trypsin and 0.70 for the purified one.

Calculations of all the kinetic parameters were performed by a FACOM 230-60 digital computer in Hokkaido University Computing Center.

RESULTS AND DISCUSSION

The dependence of the observed initial rate of trypsin-catalyzed hydrolysis of L-BAPA upon substrate concentration was expressed in the form of Eadie plot (12) as represented in Fig. 1. The plot afforded a straight line up to the substrate concentration of 5×10^{-4}M, indicating that the enzymatic process obeys the simple Michaelis-Menten equation in this low concentration range. The constants k_{cat} and K_m(app.), which were determined by the weighted and non-linear regression method of Wilkinson (13) using the data within the range, are given in Table I.

Extension of the L-BAPA concentration beyond the above-mentioned limit produced the remarkably concaved Eadie plot as seen in Fig. 1. While this is apparently explicable as the re-

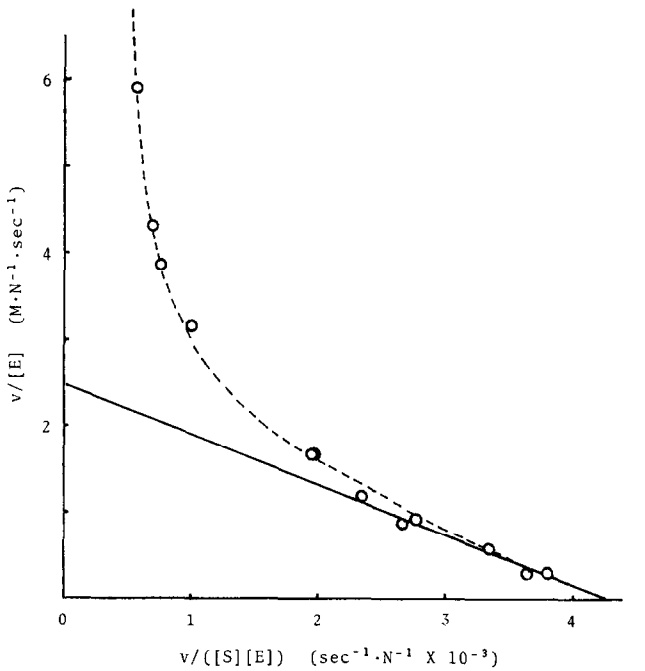

Fig. 1. An Eadie plot for the trypsin-catalyzed hydrolysis of L-BAPA (open circles) at 25° in 0.05M Tris-HCl buffer, pH 8.2, containing 0.02M $CaCl_2$ and 1% dimethylformamide. The substrate concentration was from 8.02×10^{-5}M to 1.01×10^{-2}M. The trypsin preparation purified by Ganrot's method (7) was used at the concentration (in normality) of 2.58×10^{-7}N. The solid straight line was drawn according to the simple Michaelis-Menten equation with the values indicated in Table I for K_m(app.) and k_{cat}, and the broken curve according to Eq. 2 with 364 $sec^{-1}N^{-1}$ for k_a and the above-mentioned values for the other two parameters.

sult of substrate activation, another interpretation may be also suggested based on the presence of two or more enzyme species with different activities (14). The main cause of the phenomenon, however, is considered to be the substrate activation by the following two reasons: 1, essentially the same profile of the curved Eadie plot was obtained before and after partial purification of the enzyme preparation; 2, succinylated trypsin afforded the straight Eadie plot in the entire range of substrate concentration used as shown in Fig. 2. We have previously found that the reaction of succinic anhydride on trypsin intensifies the enzymatic activity toward BAPA at a fixed concentration and that acylation of exposed tyrosyl residues appears to be fundamental to the increase in activity, which can be reversed by deacylation with hydroxylamine (pH 8.0)(15). The increase is now known to be explained only in terms of k_{cat}.

Although the substrate activation of trypsin has been described on TAME and BAEE (1,2,4), no precedent for it has appeared on the amide (including peptide and anilide) substrate. The unique behavior of this p-nitroanilide substrate might be due to its similarity to esters in the proposed protonation mechanism

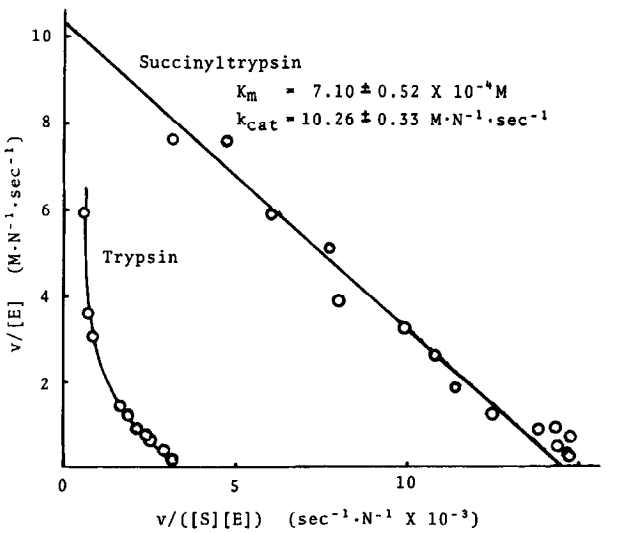

Fig. 2 Eadie plots for the succinylated trypsin- and trypsin-catalyzed hydrolyses of L-BAPA. The commercial trypsin was used in the both cases at the concentrations (in normality) of 7.74 X 10^{-8}N and 4.34 X 10^{-7}N, respectively. The substrate concentration was from 1.64 X 10^{-5}M to 2.40 X 10^{-3}M for succinylated trypsin or from 5.39 X 10^{-5}M to 1.00 X 10^{-2}M for trypsin. For the other conditions, see the legend of Fig. 1.

Table I

Steady state kinetic parameters for trypsin-catalyzed hydrolysis of L-BAPA

Trypsin preparation[a]	K_m (M X 10^4)	k_{cat}[b]	Condition
I	5.79 ± 0.60[c]	2.48 ± 0.18	pH = 8.2, T = 25°
II	6.42 ± 0.24	2.27 ± 0.06	
III[d]	9.39	0.611	pH = 8.15, T = 15°

a) I : Preparation partially purified from II by Ganrot's method(7).
II : Commercial preparation (Worthington TRL-8GA).
III: Commercial preparation (Worthington, undefined quality).

b) $M \cdot N^{-1} \cdot sec^{-1}$ for I and II, and $M \cdot M^{*-1} \cdot sec^{-1}$ for III, where N and M* denote the active-site normality and the molarity of enzyme, respectively.

c) Standard deviation.

d) Data of Erlanger et al. obtained with DL-BAPA (5).

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at the acylation step of the trypsin-catalyzed hydrolysis as discussed by Parker and Wang (16). Indeed, its k_{cat} value obtained in this study (Table I) is ranked between those of the usual ester substrate and amide substrate specific for trypsin: the value is approximately one-sixth of that of BAEE (17 and our unpublished data), and 10 and 3 times as large as BAA (18 and our unpublished data) and BCAT (19), respectively. It must be emphasized, however, that the over-all rate of tryptic hydrolysis of BAPA which is subjected to the substrate activation may be eventually controlled, for the most part, by the acylation step as in the case of the usual amide substrate.

Trowbridge et at.(1) explained the substrate activation of trypsin with TAME by the formation of a ternary complex of the enzyme with two substrate molecules at the high substrate concentration, besides of the usual binary complex, where the former decomposes to products at a much higher rate than the latter. The scheme was expressed by Eq. 1. With the data on L-BAPA

$$\frac{v}{[E]} = \frac{k_{cat}[S]/K_m + k^*_{cat}[S]^2/K_m K^*_m}{1 + [S]/K_m + [S]^2/K_m K^*_m} \qquad \text{(Eq. 1)}$$

represented in Fig. 1, operational factors K^*_m and k^*_{cat} which

are analogous to the apparent Michaelis constant and the catalytic rate constant, respectively, under the conditions of substrate activation were estimated by computer analysis from Eq. 1, employing the values indicated in Table I as those of K_m and k_{cat}. Since the estimation led to the exceedingly high values (K^*_m > 30M, k^*_{cat} > 10,000sec^{-1}), the fit to another equation (Eq. 2) of the

$$\frac{v}{[E]} = \frac{k_{cat}[S]/K_m + k_a[S]^2/K_m}{1 + [S]/K_m} \qquad \text{(Eq. 2)}$$

data was also examined. As mentioned by Trowbridge et al.(1), such an equation results if a path to products is provided by a bimolecular reaction between the enzyme-substrate (binary) complex and a free substrate molecule, in addition to the simple Michaelis-Menten path. A reasonable fit of the results to Eq. 2 was obtain-

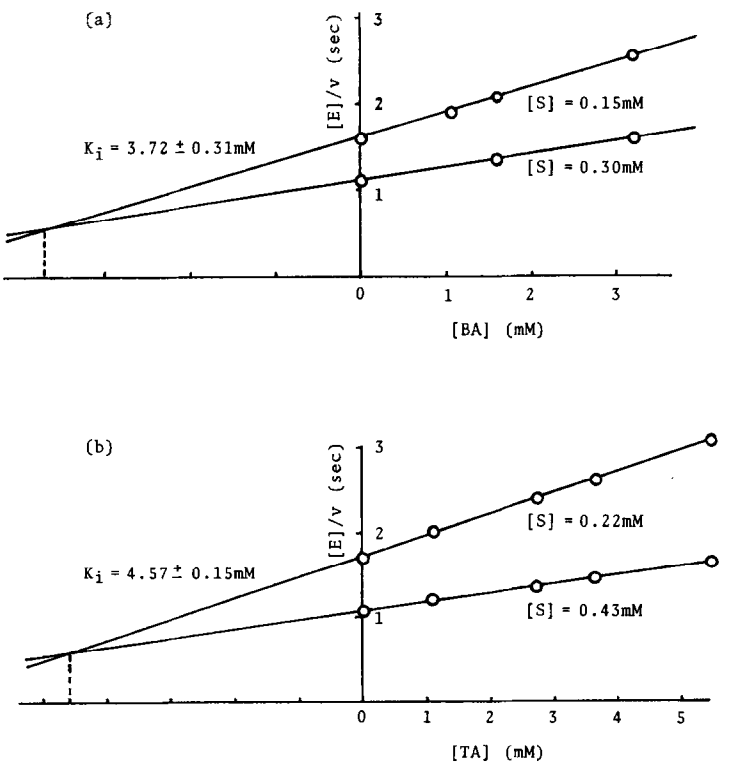

Fig. 3. Dixon plots indicating the competitive inhibition of BA (a) and TA (b) against the trypsin-catalyzed hydrolysis of L-BAPA. The commercial trypsin was used at the concentration of 1.51 X 10^{-7}N for (a) or 1.69 X 10^{-7}N for (b). The reaction medium was the same as that described in the legend of Fig. 1.

ed with the value of 364 $sec^{-1}N^{-1}$ as k_a, rate constant of the bimolecular reaction (see Fig. 1). It must be reserved, therefore, for further investigations with a single molecular species of trypsin to judge whether the substrate activation found in L-BAPA occurs by the same ternary complex mechanism as the case of TAME or not.

Although the trypsin-catalyzed hydrolysis of TAME has been reported to be susceptible also of product activation (20), that of BAPA was found inhibited competitively by BA, the product from BAPA and TA, the product from TAME as shown in Fig. 3. It is also worth noticing that acetylation of trypsin was reported to stimulate the esterolytic activity toward TAME at high concentration of substrate and so exaggerate the substrate activation of enzyme (4). The fact that the succinylated trypsin catalyzes the hydrolysis of BAPA according to the simple Michaelis-Menten kinetics (no substrate activation) with an increased k_{cat} exhibits a striking contrast to the behavior of the acetylated trypsin toward TAME.

We wish to thank Prof. J. Noguchi, Faculty of Science, Hokkaido University, for the generous gift of L-BAPA.

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