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STEADY-STATE AND PRE-STEADY-STATE KINETICS OF THE TRYPSIN-CATALYSED HYDROLYSIS OF α-CBZ-L-LYSINE-*p*-NITROPHENYL ESTER

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Summary

The catalytic properties of bovine trypsin (EC 3.4.21.4) have been investigated using a synthetic chromogenic substrate: α-CBZ-L-lysine-*p*-nitrophenyl ester (ZLNPE). The use of ZLNPE allows the determination of trypsin down to a concentration of $2 \cdot 10^{-9}$ M.

Steady-state and pre-steady-state data have been analyzed in the framework of the minimum three-step mechanism:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E \cdot P + P_1 \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E + P_2$$

The pH-dependence of the kinetic parameters shows that at acid pH values (≃2.6) the k_{+3} step is rate limiting in catalysis, whereas for pH values higher than 4.8 k_{+2} becomes rate limiting. This change in rate-limiting step with pH illustrates the danger in the assumption that k_{cat} vs. pH profiles for protease action on substrates with good leaving groups are equivalent to k_{+3} vs. pH profiles.

The task of assaying enzymatic activity is appreciably simplified by the use of chromogenic substrates showing favourable steady-state parameters. In this framework α-benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPA) is the synthetic substrate more commonly used to study the catalytic properties of trypsin

[1]. The hydrolysis of DL-BAPA can be followed conveniently by colorimetric procedures, since one of the products, *p*-nitroaniline, is yellow. Moreover, DL-BAPA does not show spontaneous hydrolysis over a wide pH range. On the other hand, only L-BAPA is a substrate and D-BAPA is a competitive inhibitor of trypsin [2] which is not hydrolysed from the enzyme. Nevertheless, kinetic parameters of the trypsin-catalysed hydrolysis of L-BAPA show large errors because the racemization phenomenon of the substrate cannot be prevented.

It has been shown before that α-CBZ-L-lysine-*p*-nitrophenyl ester (ZLNPE) is a favourable substrate for both serinic [3—9] and thiol [10—12] proteases and it would appear to have some of the ideal properties as a chromogenic substrate for trypsin, particularly in studies on trypsin inhibitors which are in progress in our laboratories.

The potentially wide utilization of ZLNPE as a substrate stimulated interest in a detailed study of the steady-state and pre-steady-state kinetics of the tryptic hydrolysis of ZLNPE over a wide pH range. The kinetic data obtained may be fitted by the simple three-step mechanism [1] (see below) and allow the determination of the values of the individual rate constant within it.

In addition it will be shown that the use of ZLNPE as a substrate allows determination of trypsin activity to a concentration of $2 \cdot 10^{-9}$ M at pH values near 7.0.

Bovine trypsin was obtained from Sigma Chemical Co. The concentration of trypsin was determined using the extinction coefficient at 280 nm, $E^{1\%}_{1cm} = 15.4$ [13]. Synthetic molecules: α-CBZ-L-lysine-*p*-nitrophenyl ester (ZLNPE), α-benzoyl-L-arginine-*p*-nitroanilide (L-BAPA), α-benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPA), α-benzoyl-L-arginine-ethyl ester (BAEE), α-tosyl-L-arginine-methyl ester (TAME) were obtained from Sigma Chemical Co. and used without further purification.

The pH profile was investigated using the following buffers: citrate pH 2—4, acetate pH 4—6, phosphate pH 6—8, 5 all at 0.1 M. pH values were measured with a Radiometer-51 pH meter.

The assays with L-BAPA, DL-BAPA, BAEE and TAME were performed as reported previously [1,2,14,15].

The trypsin assay with ZLNPE was performed spectrophotometrically measuring the spectral changes associated with the formation of free *p*-nitrophenol. The spectral properties of ZLNPE are pH independent. The absorbance increase on hydrolysis of ZLNPE (350—400 nm) was monitored either in a double-beam spectrophotometer (Cary 118 or 219) or in a Gibson-Durrom rapid-mixing stopped-flow spectrophotometer equipped with 2 cm observation chamber. The initial velocity was corrected for the spontaneous hydrolysis of ZLNPE. Pre-steady-state and steady-state experiments were performed at 21 ± 1°C.

Pre-steady-state and steady-state parameters were evaluated as reported in literature [16—18].

In steady-state experiments ZLNPE is stable only over a limited pH range and spontaneous hydrolysis becomes considerable at pH values above 7 (Ref. 9 and Ascenzi, P., Kovacks, S., Antonini, E. and Brunori, M., unpublished data). Using catalytic concentrations of bovine trypsin, ZLNPE can be usefully

employed as a substrate only up to pH 8.2.

The dependence of the initial velocity on the concentration of ZLNPE follows simple Michaelis-Menten kinetics. Complete analysis of the time course of hydrolysis at a single initial substrate concentration yields, in accordance with simple expectation, rate values fully consistent with initial velocity estimates at different substrate concentration. Over the wide range explored ($2 \cdot 10^{-9}$ M—$1.8 \cdot 10^{-7}$ M) the initial velocity is strictly linear with enzyme concentration.

Fig. 1 shows the pH dependence of K_m and V for tryptic hydrolysis of ZLNPE. In the region explored the overall pH profiles are similar to those reported for DL-BAPA [1], L-BAPA [2], TAME [14] and BAEE [15]. The fit of the data for V with a simple pH transition yields an average pK_a value of 7.1 and k_{cat} (limiting) = 145 s^{-1}.

At very low pH ($\simeq$2.0) trypsin is inactive in the hydrolysis of ZLNPE. This effect cannot be accounted for by irreversible acid denaturation since the enzyme brought back to neutral pH shows complete recovery of the activity.

Pre-steady-state results. Several authors have presented evidence [4—7, 19—21] that trypsin, as other serinic proteases, catalyses the hydrolysis of low-molecular weight substrates 'via' a mechanism involving a minimum of two intermediates:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EP + P_1 \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E + P_2 \qquad (1)$$

In this study P_1 is *p*-nitrophenol and P_2 α-CBZ-L-lysine. The analysis of the kinetic data for the trypsin-catalysed hydrolysis of ZLNPE may be separated in two categories: (a) when the enzyme concentration $[E_0]$ is much greater than that of the substrate $[S_0]$ ($[E_0] >> [S_0]$) and (b) when $[S_0] >> [E_0]$. For these conditions the differential equations arising from the Eqn. 1 may be solved analytically as illustrated, for instance by Gutfreund [16]. It is then possible to arrive, from steady-state and pre-steady-state measurements at the determination of many of the individual constants within Eqn. 1. The results are shown in the Table I.

The determination of the kinetic parameters of Eqn. 1 is simplified by the fact the process

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot S$$

may be regarded as being at the equilibrium throughout the reaction, i.e., $k_{+2} << k_{-1}$. From a general point of view the most significant result shown in Table I is the pH dependence of the values of k_{+3} and k_{+2}, which show that at low pH (<4.8) the k_{+3} is rate limiting in catalysis, whereas at high pH k_{+2} becomes rate limiting. This illustrates the danger in interpreting k_{cat} vs. pH profiles for serinic protease action on substrates with good leaving group on the basis that deacylation is rate limiting throughout the pH range.

The catalytic parameters for the hydrolysis of ZLNPE by bovine trypsin are very favourable compared to those reported for other synthetic substrates. This point is made clear by the data reported by Table II, which gives V and K_m values for ZLNPE, BAEE, TAME, L-BAPA and DL-BAPA. It is evident

TABLE I

k_{+3}^{calc} was calculated according to following equations:

$$k_{cat} = \frac{k_{+2} \cdot k_{+3}}{(k_{+2} + k_{+3})} \quad \text{and} \quad K_m = K_s \frac{k_{+3}}{(k_{+2} + k_{+3})}$$

pH	k_{+2} (s^{-1})	K_s (μM)	k_{+2}/K_s ($\mu M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	k_{+3}^{calc} (s^{-1})	k_{+2}/k_{+3}
2.55	0.33 ± 0.03	800 ± 40	$4.1 \cdot 10^{-4}$	0.05 ± 0.05	500 ± 30	$1 \cdot 10^{-4}$	0.03 ± 0.01	11.0
2.66 *	0.395	795	$4.9 \cdot 10^{-4}$	0.033	—	—	0.015	26.3
4.80	25 ± 2	250 ± 20	0.10	10 ± 1	300 ± 20	0.04	15 ± 6	1.67
5.80	38 ± 3	200 ± 20	0.19	30 ± 3	180 ± 15	0.17	200 ± 100	0.19
6.80	83 ± 8	93 ± 10	0.89	60 ± 5	100 ± 8	0.60	600 ± 250	0.14
7.78	170 ± 15	94 ± 10	1.81	140 ± 15	89 ± 10	1.57	1600 ± 400	0.11

* From Ref. 4.

TABLE II

V and K_m values for BAEE, TAME, DL-BAPA and L-BAPA were determined in parallel with the data of ZLNPE using the same enzyme preparation. The methods used are as reported [1,2,14,15]. The values compare favourably with those reported in literature if the difference in temperature, pH and buffer composition are taken in account. Experimental conditions: phosphate buffer 0.1 M, pH 6.8, temperature = 21 ± 1°C.

	V (μM/min · μM)	K_m (μM)
L-BAPA	40 ± 8	300 ± 20
DL-BAPA *	35 ± 8	600 ± 30
TAME	600 ± 150	300 ± 25
BAEE	650 ± 150	300 ± 30
ZLNPE	3600 ± 300	100 ± 8

* The D-BAPA is a competitive inhibitor of trypsin [2] showing a K_i value of 400 μM at pH 6.8; phosphate buffer 0.1 M at 21 ± 1°C. The reported value K_m has been measured in the presence of a constant concentration of D-BAPA (300 μM).

under the conditions used, V is approx. 8-fold greater for ZLNPE as compared to TAME or BAEE and approx. 100-fold greater as compared to L-BAPA and DL-BAPA. From the data given above it may be calculated that the method of assay with ZLNPE at pH 6.8 allows the determination of trypsin as low as $2 \cdot 10^{-9}$ M corresponding to about 6 I.U./ml. On the other hand TAME and BAEE show a V value approx. 12-fold greater as compared to L-BAPA and DL-BAPA, but it is only possible to directly follow the hydrolysis process at 247—253 nm. The drawback of using ZLNPE as a substrate for the assay of trypsin is that reliable measurements at the lowest enzyme concentration can only be carried out at pH 7.5, i.e., over a pH region where spontaneous hydrolysis of the substrate is low.

The pre-steady-state results indicate that the kinetic data for the trypsin-catalysed hydrolysis of ZLNPE may be consistently fitted to the simple three-step mechanism (Eqn. 1) over a wide pH range between 2.6 and 7.8. However, it should be emphasized that Eqn. 1 is only minimal and other more complex models may apply as long as certain conditions are satisfied.

According to Peller and Alberty [22] the pH dependence of the ratio k_{+2}/K_s ($=k_{cat}/K_m$) should reflect ionizations of group(s) in the free enzyme. The pH dependence of the ratio k_{+2}/K_s depends on a single ionizable group with a pK_a value of 7.1 ± 0.1. It is noteworthy that the value of pK_a determined from the pH dependence of the ratio k_{cat}/K_m is in fair agreement with the corresponding value obtained from the pH dependence of the ratio k_{+2}/K_s (from the experiments with $[E_0] >> [S_0]$).

The k_{+2} vs. pH profile implicates an ionizing group with pK_a = 7.1 ± 0.1 in the E · S complex. The observed pK_a value is in fair agreement with those reported for k_{+2} in tryptic hydrolysis of *p*-nitrophenyl acetate [19] and α-(CBZ-L-alanine-*p*-nitrophenyl ester [21]. This observation implies that there is no pK_a-shift in the formation of enzyme-substrate complex. Accordingly the value of K_s is pH independent, within experimental errors, in the pH range between 5.8 and 7.8.

Additionally the pH dependence of k_{+3} reflects the ionization process of

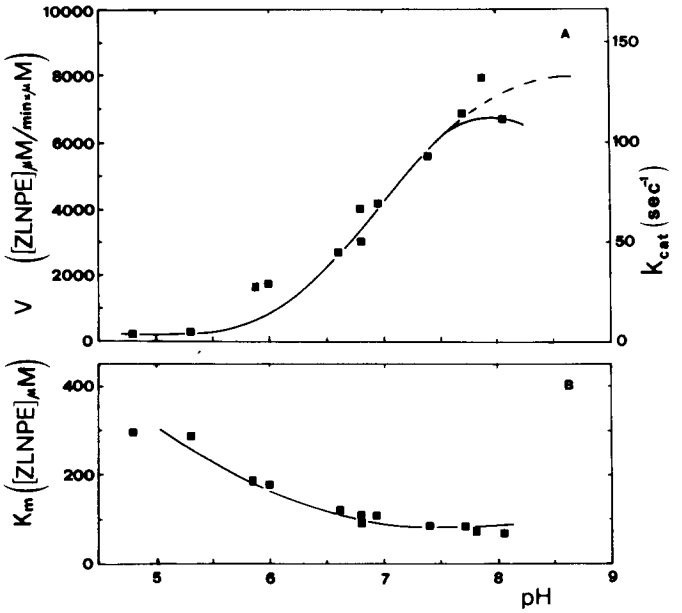

Fig. 1. pH dependence of V (A) and K_m (B) for the hydrolysis of ZLNPE catalysed by trypsin. The dotted line through the value of V is a theoretical titration curve for one ionizable group with a pK_a of 7.1 ± 0.1.

a single group with a pK_a value of 7.1 ± 0.1. The observed pK_a value is in fair agreement with those reported for k_{+3} in the tryptic hydrolysis of *p*-nitrophenyl acetate [19] and *N*-transcinnamoyl-imidazole [20].

In trypsin the ionizable group involved in acylation (k_{+2}) and deacylation (k_{+3}) steps has been found to be the His 46 present in the active site of this enzyme [23,24].

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