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THE HYDROLYSIS OF α-*N*-BENZOYL-L-ARGININAMIDE CATALYZED BY TRYPSIN AND ACETYLTRYPSIN

DEPENDENCE ON pH*

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SUMMARY

A newly automated method employing the Berthelot reaction is described for measurement of the rates of enzyme-catalyzed hydrolysis of amide substrates. Using this technique the pH-dependence of the hydrolysis of α-*N*-benzoyl-L-argininamide catalyzed by bovine trypsin (EC 3.4.4.4) and *N,O*-acetyltrypsin has been investigated. The apparent pK values for the free enzyme and modified enzyme have been estimated from plots of log (k_{cat}/K_m) *vs.* pH. The values for trypsin are 7.0 and 10.1 (25°, I = 0.19), while those for acetyltrypsin are 7.2 and 10.5. The apparent kinetic pK values for the enzyme-substrate complexes have been estimated from plots of log k_{cat} *vs.* pH. The values for trypsin are 6.2 and 10.4, while those for acetyltrypsin are 6.3 and 11.0. The pH-independent constants corresponding to K_m and k_{cat} for trypsin are 3.4 mM and 0.7 sec^{-1}, while those for acetyltrypsin are 2.1 mM and 1.2 sec^{-1}. The results confirm previous suggestions that acetylation of exposed tyrosyl groups increases the acylation rate constant in the trypsin-catalyzed hydrolysis of specific amide substrates and rule out altered protonic equilibria as an explanation for the enhancement of activity.

INTRODUCTION

When bovine trypsin (EC 3.4.4.4) is treated with acetylating agents under conditions which lead to modification of both accessible amino and tyrosyl hydroxyl groups, the resulting *N,O*-acetyltrypsins show increased catalytic activity with respect to the hydrolysis of specific ester and amide substrates[1-4] in the optimal pH

Abbreviations: Ac-trypsin$_{1m}$, trypsin acetylated with 0.1 M *N*-acetylimidazole as previously described[4]; BAA, α-*N*-benzoyl-L-argininamide.

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range. Acetylation of amino groups alone does not produce significant enhancement of enzymic activity.

Studies of the substrate concentration dependence of the rates of hydrolysis have shown that the effects are characterized primarily by increases in the catalytic rate constant, k_{cat}, accompanied by minor favorable changes in the apparent Michaelis constant, K_m (app). Apparent substrate activation[5-7] prevents any physical interpretation of the results with ester substrates. This complication is not observed with amide substrates[4,8,9]. Furthermore, present evidence suggests that in the case of simple amide substrates the operational constants K_m (app) and k_{cat} can be related, respectively, to the substrate-binding affinity and the specific rate constant for the rate-determining formation of an enzyme–substrate intermediate[8,10]. This is not the case when ester substrates are employed[11].

With these considerations in mind, K_m(app) and k_{cat} for the trypsin- and Ac-trypsin$_{Im}$- catalyzed hydrolysis of α-*N*-benzoyl-L-argininamide (BAA) have been compared at pH values between 6.0 and 11.0. Changes in the ionization state of the substrate are not significant in this pH range, since the pK_a of the guanidinium group is approximately 13.5[12].

MATERIALS AND METHODS

Bovine trypsin (Worthington twice crystallized, dialyzed, salt-free, and lyophilized) was purified by fractionation with 1 M NaCl–0.01 M HCl at room temperature[13]). Appropriate dilutions for concentration determinations and assays were made with water at 0°.

A single batch of Ac-trypsin$_{Im}$ containing 2.8 acetyltyrosyl groups and 6.0 free amino groups per molecule of protein was prepared and characterized as previously described[4], exhaustively dialyzed against water at 4°, and lyophilized. Samples for concentration determination were dissolved in 0.2 M NaCl at 0°. Further dilutions were prepared with the same solvent for concentration determinations and assays. Tyrosyl group deacylation is not detectable under these conditions.

α-*N*-Benzoyl-L-argininamide hydrochloride was purchased from Cyclo Chemical Corporation and Calbiochem and selected for use on the basis of low ammonia content as determined with ninhydrin-hydrindantin reagent[14]).

The buffers employed were prepared from reagent grade chemicals and selected to produce neither interference with the colorimetric ammonia determinations nor inhibition of the enzymes. Sodium phosphate was used from pH 6.0 to 7.5, triethanolamine hydrochloride from pH 7.5 to 8.7, and sodium bicarbonate–carbonate from pH 9.4 to 11.0.

Assay conditions

The rates of hydrolysis of BAA by trypsin and Ac-trypsin$_{Im}$ were studied concurrently at each pH as a function of substrate concentration. A substrate stock solution consisting of 50 mM BAA, 5 mM KCl, 85 mM NaCl, and 50 mM buffer was prepared at the desired pH (25°). The substrate concentration range was established by dilution with 5 mM KCl, 135 mM NaCl, and 50 mM buffer at the same pH and temperature. The reaction was initiated by the addition of 10–250 μl of enzyme solution to 5 ml of substrate in a jacketed vessel at 25.0°. The volumes and concentra-

tions of the enzyme solutions were chosen to provide comparable reaction rates at all pH values and substrate concentrations, and to permit monitoring of the first 15%, or less, of the reaction course. The final enzyme concentrations ranged from 0.1 to 20 μM. Four substrate concentrations in the range from 0.6 to 6.0 mM were employed. Lineweaver–Burk plots were linear over this range at all pH values, while substrate inhibition was observed at higher concentrations. Assays were carried out at least in quadruplicate at each pH and substrate concentration. Enzyme and substrate blanks were negligible under all conditions.

Colorimetric detection of ammonia

The ammonia concentrations in the assay solutions were determined by a modification of the phenol–alkaline hypochlorite method described by CHANEY AND MARBACH[15]. The procedure has been adapted for use with a Technicon Autoanalyzer. The following solutions are mixed at the stated flow rates: (1) Phenol (50 g/l), sodium nitroprusside (250 mg/l); flow rate, 2 ml/min. (2) NaOH (75 g/l), NaOCl (Fisher, N. F., 4.6%, 120 ml/l); flow rate, 0.6 ml/min. (3) BRIJ 35 solution (Fisher, 0.5 ml/l); flow rate, 1.2 ml/min. (4) Assay solution; flow rate, 0.8 ml/min. (5) Acid washed air bubbles; flow rate, 1.6 ml/min.

All components are protected from ammonia contamination by connection to a gas washing bottle containing 0.05 M H_2SO_4.

The phenol–nitroprusside solution, sample and BRIJ diluent are combined, interspersed with air bubbles, and passed through a glass mixing coil. The alkaline hypochlorite is then added followed by mixing in a second coil. The color is developed for approx. 4 min in a 42° water bath and measured at 626 nm.

NH_4Cl standard solutions ranging from 0.02–0.09 mM were interspersed between enzyme assays using identical conditions of buffer and substrate concentrations, ionic strength, pH, temperature, and flow rates. The extinction coefficient was determined at each substrate concentration employed in the kinetic assays, since it was found to be substrate concentration-dependent. The standard curves complied with the Lambert–Beer Law. No time-dependent decrease in extinction was observed when buffered standards were kept for several hours before colorimetric analysis, ruling out ammonia loss as a significant source of error in the method. The increase in absorbance from baseline to standard reading was complete in thirty seconds.

The apparent rates of ammonia release in the kinetic assays were constant for 3–5 min, the shorter times being observed at pH values of 10.6 and 11.0, where *O*-deacylation of Ac-trypsin$_{Im}$ and inactivation of trypsin become detectible.

Determination of enzyme concentrations

The normalities of active enzymes were determined by the method of CHASE AND SHAW[16].

Analysis of kinetic data

The operational constants k_{cat} and K_m(app) were determined from Eqn. 3 and the individual values of k_{obs} by the method of WILKINSON[17] using a digital computer.

THEORY

The three step kinetic mechanism illustrated in Eqn. 1 is widely accepted for the trypsin-catalyzed hydrolysis of simple amide and ester substrates[11]. In this formulation

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\qquad\qquad\qquad\qquad\quad \downarrow$$
$$\qquad\qquad\qquad\qquad\quad P_1$$

ES represents the productive form of an enzyme–substrate complex, ES' an acylenzyme intermediate, P_1 the alcohol or amine product, and P_2 the acid product. In the case of simple amide substrates, there is no evidence for the accumulation of ES'. The acylation step is rate-limiting[8], so the velocity of the reaction depends upon k_2[ES]. By definition, $k_{cat} = v_{max}/[E_0]$, and it is widely assumed that k_{cat} approximates k_2, based upon the assumption that $[ES] \to [E_0]$ when $[S] \to \infty$. This is only true when there are no pre-equilibria which significantly diminish the concentration of the productive form of ES at saturating substrate concentrations.

One possible explanation for the changes in k_{cat} and K_m(app) accompanying acetylation of the enzyme would be the alteration of protonic equilibria. Considering protonic equilibria and also that $k_2 \ll k_3$ when amide substrates are employed, Eqn. 1 becomes Eqn. 2. In this formulation E represents enzyme, S, substrate, and H protons;

$$\begin{array}{ccccc} H_2E & & H_2ES & & \\ \updownarrow K_e & k_1 & \updownarrow K_{es} & k_2 & \\ S + HE & \rightleftarrows & HES & \to & HE + P_1 + P_2 \\ \updownarrow K_e' & k_{-1} & \updownarrow K'_{es} & & \\ E & & ES & & \end{array} \qquad (2)$$

HES represents the productive form of the enzyme–substrate complex; k_1, k_{-1}, and k_2 are the specific rate constants for each step, and the dissociation constants have their usual meaning, $K_e = [H] \cdot [HE]/[H_2E]$, etc.

The steady-state rate equation for determining k_{cat} and K_m(app) is Eqn. 3:

$$\frac{v}{[E_0]} = k_{obs} = \frac{k_{cat}\,[S]}{[S] + K_m\,(app)} \qquad (3)$$

However, k_{cat} and K_m(app) are functions of pH as follows[18]:

$$k_{cat} = k_c\,(1 + [H]/K_{es} + K'_{es}/_1H])^{-1} \qquad (4)$$

where k_c is the pH-independent maximum value of k_{cat}, and

$$K_m\,(app) = K_m\,(1 + [H]/K_e + K_e'/[H])\,(1 + [H]/K_{es} + K_{es}'/[H])^{-1} \qquad (5)$$

where K_m is the pH-independent Michaelis constant, and dividing Eqn. 4 by Eqn. 5

$$k_{cat}/K_m\,(app) = (k_c/K_m)\,(1 + [H]/K_e + K_e'/[H])^{-1} \qquad (6)$$

From plots of log $[k_{cat}/K_m(app)]$ *vs.* pH, pK_e and pK'_e can be evaluated[19] while the analogous constants for the ionization of the enzyme–substrate complexes, pK_{es} and pK'_{es}, can be estimated from plots of log k_{cat} *vs.* pH, following the rationale of DIXON[20], *i.e.* (1) the graphs consist of straight-line sections of integral slopes joined

by curved portions, (2) each bend indicates the pK of an ionizing group, and lines drawn through the straight portions intersect at a pH corresponding to the pK, and (3) the curvature at the bends is such that the point of intersection of the two straight lines misses the curved line drawn through the experimental points by a vertical distance of 0.3 unit.

The pK values listed in Table II were established by (1) drawing the line of zero slope through the experimental points in the intermediate pH region, (2) making a preliminary estimate of each pK from the points 0.3 unit below this line on the experimental curves, and (3) constructing theoretical curves based on Eqns. 4 and 6, varying the pK values by increments of 0.1 to obtain optimal fit to the experimental points.

RESULTS

Table I shows the calculated values of K_m(app) and k_{cat} at each pH for the trypsin- and Ac-trypsin$_{Im}$-catalyzed hydrolysis of BAA. It is apparent that the

TABLE I

STEADY STATE KINETIC CONSTANTS FOR THE TRYPSIN- AND AC-TRYPSIN$_{Im}$-CATALYZED HYDROLYSIS OF BAA AS A FUNCTION OF pH

0.6–6.0 mM BAA, 0.1–20 μM enzyme, 50 mM buffer, 5 mM KCl, I = 0.19 established by addition of NaCl, 25.0°. Data represent mean ± S.D.

pH	*K_m (app) (mM)*		*k_{cat} (sec^{-1})*	
	Trypsin	*Ac-trpsin$_{Im}$*	*Trypsin*	*Ac-trypsin$_{Im}$*
6.0	17.1 ± 1.4	13.6 ± 0.9	0.34 ± 0.02	0.44 ± 0.02
6.5	8.1 ± 0.4	7.9 ± 1.0	0.42 ± 0.02	0.71 ± 0.06
7.0	4.5 ± 0.2	4.4 ± 0.1	0.56 ± 0.01	1.02 ± 0.01
7.5	3.5 ± 0.1	2.6 ± 0.2	0.57 ± 0.01	1.04 ± 0.04
8.0	3.8 ± 0.1	2.2 ± 0.1	0.70 ± 0.02	1.18 ± 0.03
8.4	3.7 ± 0.3	2.3 ± 0.1	0.71 ± 0.03	1.26 ± 0.03
8.7	3.1 ± 0.2	1.9 ± 0.1	0.67 ± 0.02	1.06 ± 0.02
9.4	3.5 ± 0.2	1.9 ± 0.1	0.63 ± 0.02	1.02 ± 0.03
9.8	3.3 ± 0.2	2.5 ± 0.2	0.51 ± 0.01	1.11 ± 0.04
10.2	4.0 ± 0.2	2.5 ± 0.1	0.40 ± 0.01	0.95 ± 0.02
10.6	6.7 ± 0.3	2.6 ± 0.2	0.35 ± 0.01	0.82 ± 0.03
11.0		6.0 ± 0.8		0.77 ± 0.06

enhancement of k_{cat} due to acetylation of the enzymes persists throughout the pH range investigated, while the values of K_m(app) for Ac-trypsin$_{Im}$ are significantly lower than those for trypsin at all pH values greater than 7.0.

These data have been employed for the assessment of apparent pK values of ionizations which affect enzymic activity based upon the kinetic scheme outlined in Eqn. 2.

Fig. 1 illustrates plots of log k_{cat} as a function of pH for both enzymes. Values of pK_{es} and pK'_{es}, corresponding to the apparent ionization constants for the enzyme–substrate complexes consumed in the rate determining step, have been estimated as described above. Values of pK_e and pK'_e, corresponding to apparent

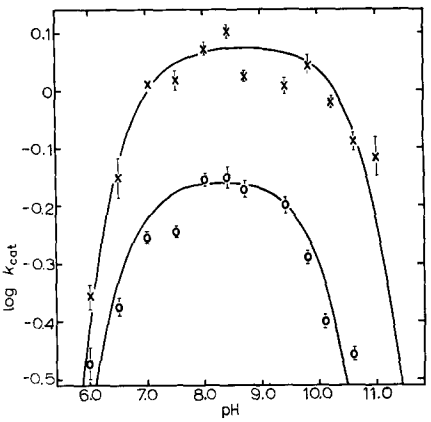

Fig. 1. The pH-dependence of log k_{cat} for the trypsin- (○—○) and Ac-trypsin$_{Im}$- (×—×) catalyzed hydrolysis of α-*N*-benzoyl-L-argininamide in 50 mM buffer, 5 mM KCl, I = 0.19 (25.0°). The solid lines were calculated from Eqn. 4 and the values of k_c, K_{es} and K'_{es}.

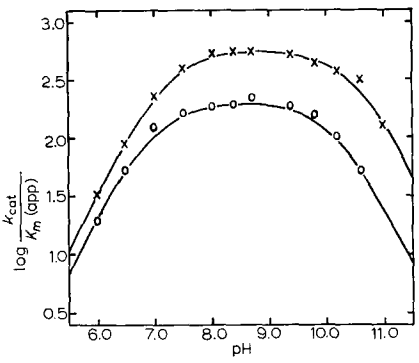

Fig. 2. The pH-dependence of log [k_{cat}/K_m (app)] for the trypsin- (○—○) and Ac-trypsin$_{Im}$- (×—×) catalyzed hydrolysis of α-*N*-benzoyl-L-argininamide in 50 mM buffer, 5 mM KCl, I = 0.19 (25.0°). The solid lines were calculated from Eqn. 6 and the values of k_c, K_m, K_e and K_e'.

ionization constants for the free enzymes, have been deduced in the same fashion from the plots of the pH dependence of log [k_{cat}/K_m(app)] for the two enzymes, which are shown in Fig. 2. Table II includes the pK values thus derived.

Average values for the pH-independent constants, k_c and K_m were calculated by employing Eqns. 4 and 6, the values for k_{cat} and K_m(app) from Table I, and the apparent ionization constants (K_e, K'_e, *etc.*). The values are: for trypsin, $k_c = 0.7 \pm 0.1$ sec^{-1}, and $K_m = 3.4 \pm 0.3$ mM; and for Ac-trypsin$_{Im}$, $k_c = 1.2 \pm 0.1$ sec^{-1}, and $K_m = 2.1 \pm 0.3$ mM.

TABLE II

APPARENT pK VALUES FOR PROTONIC IONIZATIONS AFFECTING THE TRYPSIN- AND AC-TRYPSIN$_{Im}$-CATALYZED HYDROLYSIS OF BAA

Enzyme	pK_e	pK_e'	pK_{es}	pK'_{es}
Trypsin	7.0	10.1	6.2	10.4
Ac-trypsin$_{Im}$	7.2	10.5	6.3	11.0

DISCUSSION

The pK'_e for trypsin is correlated closely with the pK of a conformational equilibrium of the enzyme, which is altered by the binding of substrates or competitive inhibitors[21]. The difference between pK'_e and pK'_{es} is in agreement with the observed proton uptake accompanying inhibitor binding. A similar agreement between kinetic and conformational pK values has been observed for chymotrypsin (EC 3.4.4.5)[10,24]. These pK values have been attributed to the α-amino group of the N-terminal isoleucyl residue[25], which forms a buried ion pair with the aspartyl car-

boxylate ion adjacent to the active center serine[26]. The N-terminal α-amino group has been implicated in maintaining the active conformation of trypsin[22,23]. In both cases the evidence is indirect, since in neither enzyme has the pK of this ionizing group been determined directly, as has that of the analogous group in elastase (EC 3.4.4.7)[27]. The pK of 9.7 for this group is lower than the apparent pK for the enzyme–substrate complex and intermediate between the apparent pK values for chymotrypsin and trypsin. In contrast to the results with chymotrypsin and trypsin, acetylation of the α-amino group of elastase does not destroy catalytic activity[27].

CHEVALLIER AND YON[8] have examined the pH dependence of k_{cat} for the trypsin-catalyzed hydrolysis of BAA. The reported values were $pK_{es} = 6.9$ and $pK'_{es} = 10.1$ at 37°. The value for pK_{es} of 6.2 calculated in the present study at 25° agrees more closely with the value of 6.4 obtained by INAGAMI[28] for the trypsin-catalyzed hydrolysis of α-N-benzyloxycarbonyl-L-arginine p-toluidide at the same temperature. Previous studies of the pH dependence of log $[k_{cat}/K_m(app)]$ were limited to the alkaline range. WANG AND CARPENTER[9] reported a value of 10.4 for pK'_e in the trypsin-catalyzed hydrolysis of BAA at 30°, compared to 10.1 at 25° in the present study.

The pK_e of 7.0, which previously has not been determined for trypsin, presumably reflects the participation of histidine 46 in the reaction mechanism[29]. Proton release in the pH range of 6.0 to 7.5 has been observed to accompany the binding of neutral compounds by chymotrypsin and rationalized on the basis of hydrogen bond formation between histidine 57 and serine 195 (ref. 30).

The differences between pK_e and pK_{es} for trypsin and Ac-trypsin$_{Im}$ could reflect the same phenomenon, although proton release was not observed to accompany the binding of benzamidine by trypsin at neutral pH[21].

The differences between the pH-independent constant k_c and K_m for the trypsin- and Ac-trypsin$_{Im}$-catalyzed hydrolysis of BAA rule out altered protonic equilibria as an explanation for the enhancement of catalytic activity which accompanies acetylation of the enzyme and confirm the previous suggestion that the acetylation of exposed tyrosyl residues increases the magnitude of the acylation rate constant in the trypsin-catalyzed hydrolysis of specific amide substrates[3,4].

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