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DETECTION OF A TETRAHEDRAL INTERMEDIATE IN THE TRYPSIN-CATALYSED HYDROLYSIS OF SPECIFIC RING-ACTIVATED ANILIDES

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Summary

The substituent dependence for k_{cat}/K_m of trypsin anilide hydrolysis is consistent with a rate-limiting general acid-base catalysed breakdown of a tetrahedral intermediate. The formation and disappearance of this intermediate during the hydrolysis of α-*N*-acetyl-L-lysine *p*-nitroanilide is observed in stopped-flow experiments.

The formation of an initial enzyme·substrate complex and an acyl enzyme is well documented for serine proteases [1,2]. By analogy with nonenzymatic reactions, an intermediate with a tetrahedral configuration of the carbonyl carbon is expected to occur prior to acylation of the enzyme. The occurrence of this intermediate is consistent with a number of indirect kinetic observations [3—7] and has been observed in the crystal structure of complexes formed by trypsin and trypsin protein inhibitors [8,9]. Recent studies of the hydrolysis of α-*N*-benzoyl-DL-arginine *p*-nitroanilide by plasmin [10, 11] and of the hydrolysis of *N*-acetyl-L-Ala-L-Pro-L-Ala *p*-nitroanilide by elastase [12] have been interpreted in terms of a tetrahedral intermediate. We report here spectral and kinetic data consistent with the formation and disappearance of a tetrahedral intermediate in the trypsin catalysed hydrolysis of α-*N*-acetyl-L-lysine ring-activated anilides.

Bovine trypsin was obtained from Worthington, and the normality of the enzyme stock solutions was determined by titration with *p*-nitro-*p'*-guanidinobenzoate [13]. The substrates α-*N*-acetyl-L-lysine *p*-nitro-, *p*-methylsulfonyl- and *p*-acetylanilides were prepared as previously described [5,14].

Enzyme-substrate binding constants were calculated from the differences in the absorbance at 469 nm of the enzyme·proflavin complex in the presence

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and absence of substrates [15]. The trypsin·proflavin binding constant was determined by spectrophotometric titration of trypsin with proflavin at 469 nm.

The kinetic studies of the hydrolysis of anilides were carried out by monitoring the release of anilines spectrophotometrically. The wavelengths used were 410 nm for *p*-nitroanilide, 294 nm for *p*-methylsulfonylanilide and 335 nm for *p*-acetylanilide. The specificity constant k_{cat}/K_m was determined from first-order plots of the kinetic data when the substrate concentration was much less than the Michaelis constant.

Spectral measurements were made on a Cary 118 Spectrophotometer and stopped-flow measurements on a Durram D-10 Stopped-Flow.

All measurements were carried out in 20 mM $CaCl_2$, 50 mM Tris·HCl, pH 8.3 at 25°C.

TABLE I

KINETIC AND BINDING PARAMETERS FOR THE TRYPSIN-CATALYSED HYDROLYSIS OF α-*N*-ACETYL-L-LYSINE ANILIDES (Ac-L-Lys-X)

Conditions:
20 mM $CaCl_2$, 50 mM Tris·HCl, pH 8.3 at 25° C. K_s was determined from proflavin displacement. σ^-, see ref. 19.

X	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	K_s (mM)	σ^-
p-$NO_2C_6H_4NH$	1430 ± 40	0.66 ± 0.12	1,27
p-$CH_3SO_2C_6H_4NH$	2850 ± 210	1.69 ± 0.18	1.05
p-$CH_3COC_6H_4NH$	1240 ± 80	1.57 ± 0.15	0.87

The specificity constants and substrate-binding constants obtained from proflavin displacement are summarized in Table I. The trypsin·proflavin dissociation constant was found to be $1.33 \cdot 10^{-4}$ M. In order to consider the substituent effect on k_{cat}/K_m, the substrate-binding constants should be the same, as in the case for *p*-methylsulfonyl- and *p*-acetylanilides. For these two substrates a positive Hammet ρ value of about 2.0 can be calculated for k_{cat}/K_m. This ρ value is consistent with the mechanism suggested by model studies [5], namely rate-limiting general acid-base catalysed breakdown of a tetrahedral intermediate. The enhanced binding of the *p*-nitroanilide suggests an interaction of the hydrophobic leaving group with the protein and is consistent with the presence of a hydrophobic leaving-group-binding pocket in trypsin [16].

The general mechanism of hydrolysis of the anilides (S) by trypsin (E) can be written as:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k'_{-1}}{\overset{k'_{+1}}{\rightleftharpoons}} ET \xrightarrow{k'_{+2}} EA + P_1 \xrightarrow{k_{+3}} E + P_2 \qquad (1)$$

where ET is the tetrahedral intermediate, EA is the acyl enzyme, and P_1 and P_2 are products. If the hydrolysis of the *p*-nitroanilide is monitored at the isosbestic point (341 nm) of the substrate and first product in a stopped-flow experiment, an increase in absorbance is observed followed by a decrease

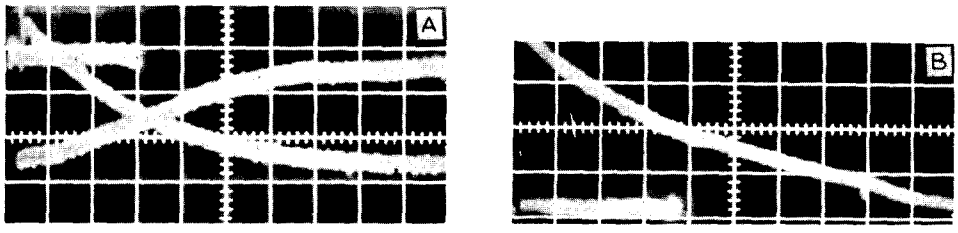

Fig. 1. A. Oscilloscope trace of the trypsin-catalyzed hydrolysis of Ac-L-Lys *p*-nitroanilide monitored at the isosbestic point (341 nm). Vertical scale, 5 mV/division; horizontal scale, 1 s/division for the top trace and 5 s/division for the bottom trace; $[E]_0 = 0.7 \cdot 10^{-4}$ M; $[S]_0 = 1.0 \cdot 10^{-4}$ M. B. Oscilloscope trace of the trypsin-catalyzed hydrolysis of Ac-L-Lys *p*-nitroanilide monitored at 480 nm. Vertical scale, 10 mV/division; horizontal scale, 5 s/division; $[E]_0 = 5.0 \cdot 10^{-5}$ M; $[S]_0 = 5.0 \cdot 10^{-4}$ M.

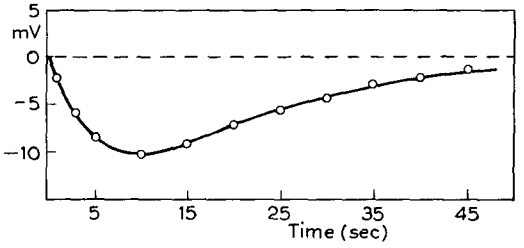

Fig. 2. Time course of the trypsin-catalyzed hydrolysis of Ac-L-Lys *p*-nitroanilide. ○, experimental data taken from Fig. 1A; ——, calculated with Eqn. 2 and $k'_{+1} = 0.13\ s^{-1}$, $k'_{+2} = 0.076\ s^{-1}$.

(Figs. 1A and 2). This corresponds to the formation and disappearance of an intermediate. If the first step of Eqn. 1 is assumed to be rapid compared to the formation and decomposition of ET and if $k'_{+1} \gg k'_{-1}$ and $k_{+3} \gg k'_{+2}$, then

$$[ET] = [ES]_0 \frac{k'_{+1}}{k'_{+2} - k'_{+1}} \left(e^{-k'_{+1}t} - e^{-k'_{+2}t} \right) \quad (2)$$

where $[ES]_0$ is the initial concentration of ES. The assumptions made simply indicate that the formation and breakdown of ET are rate-limiting. The intermediate goes through a maximum at the time.

$$t_{max} = \frac{\ln(k'_{+1}/k'_{+2})}{k'_{+1} - k'_{+2}} \quad (3)$$

The observed value of t_{max} is about 10 s and is independent of the initial enzyme and substrate concentrations as predicted. If the stopped-flow data are fit to Eqn. 2 by nonlinear least squares analysis, it is found that $k'_{+1} = 0.13\ s^{-1}$ and $k'_{+2} = 0.076\ s^{-1}$. The experimental curve and the curve calculated with these constants and Eqn. 2 are shown in Fig. 2. According to this mechanism,

$$\frac{k_{cat}}{K_m} = \frac{k'_{+2}}{K_s K_{ET}}; \quad (4)$$

using the values of k_{cat}/K_m and K_s in Table I, and the rate constants derived from the stopped-flow measurements, $K_{ET} = k'_{-1}/k'_{+1} = 0.1$ which justifies the previous assumption that $k'_{+1} \gg k'_{-1}$.

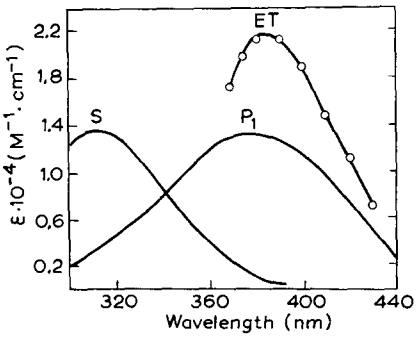

Fig. 3. Absorption spectra of Ac-L-Lys *p*-nitroanilide(S), *p*-nitroaniline(P_1) and ET. The extinction coefficients of the intermediate were calculated from extrapolation of the linear part of kinetic traces similar to Fig. 1B to zero time with the concentration of the intermediate calculated with Eqn. 2 using $k'_{+1} = 0.13\ s^{-1}$, $k'_{+2} = 0.076\ s^{-1}$ and $[ES]_0 \sim [E]_0$. The spectrum of ES was assumed to be identical with that of the substrate.

Kinetics similar to those shown in Fig. 1A have been interpreted in terms of a second enzyme·substrate complex [17]. The hydrolysis, however, can also be monitored in the stopped-flow apparatus at a wavelength of 480 nm which is far from the substrate absorbance maximum of 312 nm. The biphasic nature of the time course (Fig. 1B) suggests the occurrence of two consecutive reactions, the first being complete within about 10 s and the second (linear portion) being the steady-state reaction. The first reaction corresponds to the formation of ET. An approximate spectrum of ET was determined by linear extrapolation of the steady-state portion of the stopped-flow traces to zero time at various wavelengths, and the results are shown in Fig. 3. The large red shift of the spectrum of ET relative to that of the substrate indicates ET is not another noncovalent enzyme·substrate complex [11,12]. The similarity of the spectrum of ET with that of the first product, *p*-nitroaniline, would be expected for a tetrahedral intermediate [11,18]. Therefore, the stopped-flow results provide direct evidence that the breakdown of a tetrahedral intermediate is rate-determining in the hydrolysis of *p*-nitroanilide by trypsin.

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