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THE DETECTION, ACCUMULATION AND STABILIZATION OF A TETRAHEDRAL INTERMEDIATE IN TRYPSIN CATALYSIS

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Summary The reaction of trypsin with N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide has been studied in the 0 to -60°C temperature region, over a wide range of pH* values, using aqueous-dimethyl sulfoxide cryosolvent. Spectrophotometric and kinetic evidence for the detection and stabilization of a tetrahedral intermediate is presented. The rate of formation of this intermediate, and the concentration accumulated, were pH*-dependent. At pH* $\leq$ 6 (< -40°C) essentially no tetrahedral adduct is formed or accumulated. Instead another intermediate, resembling the substrate in its spectral properties, is detected.

In spite of numerous studies suggesting the existence of discrete tetrahedral intermediates (as opposed to transition state complexes) in the enzyme-catalyzed hydrolysis of esters and amides (1-7), unambiguous evidence for the presence of such species is lacking. However, recent spectral observations in stopped-flow (6, 7) and cryoenzymological (8, 9) investigations support the existence of tetrahedral intermediates.

Most physical techniques capable of revealing the existence of a tetrahedral intermediate (e.g., X-ray crystallography, N.M.R., Raman) require that the intermediate be present in high concentration and possess a relatively long life-time. One means of achieving such conditions is to use cryoenzymology. Previous studies (10) have shown that neither the catalytic nor the structural properties of trypsin are adversely affected by 65% (v/v) dimethyl sulfoxide at subzero temperatures.

MATERIALS AND METHODS

N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide, lot F877 (mp 86-88°), and *p*-nitrophenyl ester, lot F7713 (mp 136-138°) were obtained from Vega Biochemi-

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1. Abbreviations: DMSO = dimethyl sulfoxide; CBZ = N^{α}-carbobenzoxy; pNA = *p*-nitroanilide; TI = tetrahedral intermediate.

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cals. Twice-recrystallized trypsin from Worthington was used without further purification. Stock solutions of enzyme were prepared in 10^{-3} M HCl and stored at 4°C. The activity was checked daily by burst titration or kinetics assay with CBZ-Lys *p*-nitrophenyl ester (11). Reagent grade materials were used throughout. Standard cryoenzymological procedures were used in all experiments (12). A typical experiment involved mixing enzyme and substrate at the desired low temperature and monitoring the reaction by absorbance changes at 410 nm, or by repetitive scanning.

RESULTS

The steady state kinetic parameters for the trypsin-catalyzed hydrolysis of CBZ-LyspNA were determined in aqueous solutions: k_{cat} = 0.3 s; K_m = 1.5 mM, at 25°C, pH 6.4. Comparison with the corresponding values of k_{cat} for esters ($21 s^{-1}$) of CBZ-Lys (10, 13) indicates that the rate-limiting step for anilide hydrolysis is acylation. When the enzyme was mixed with CBZ-LyspNA at temperatures above -25°C, a linear increase in the absorbance at 410 nm was observed (Fig. 1A). This was unambiguously identified as turnover by allowing the reaction to proceed to completion and measuring the amount of *p*-nitroaniline released. At temperatures between -30° and -45°C a "burst" reaction was observed (at pH* > 6)(Fig. 1B,C). This faster reaction was manifested as an increase in absorbance in the 345-420 nm region and is ascribed to formation of the tetrahedral intermediate. Turnover was negligible at temperatures below -45°C, and resulted in the first-order accumulation of the intermediate, with a λ_{max} = 355 ± 2 nm (Fig. 1C). Under such conditions, $E = 5 \times 10^{-6}$ M, $S = 2.8 \times 10^{-4}$ M pH* = 8.4, 65% DMSO, -45°C, approximately 80% of the active enzyme is in the form of the tetrahedral intermediate (assuming an extinction coefficient for the tetrahedral adduct of the same magnitude as that for *p*-nitroaniline under the same conditions (8)).

The amount of TI accumulated, and the turnover rate, were directly proportional to the enzyme concentration under conditions of excess substrate. At pH* < 6, TI formation was negligible at temperatures above -40°C. Under such conditions a linear increase in absorbance at 410 nm occurred. This corresponded to turnover. At pH* 5.1, -40°C, monitoring of the reaction by repetitive scans indicated that neither turnover nor TI formation occurred.

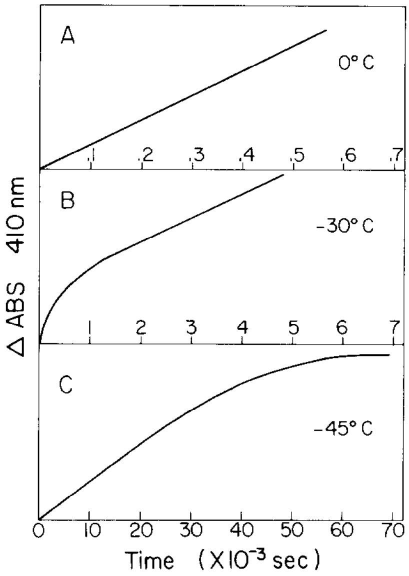

Figure 1. Traces of time vs. absorbance for the reaction of trypsin with CBZ-LyspNA at subzero temperatures in 65% DMSO. A, 0°C, E = 1.0×10^{-5} M, S = 1.7×10^{-3} M, pH* = 8.3; B, -30°C, E = 1.0×10^{-5} M, S = 1.7×10^{-3} M, pH* = 8.3; C, -45°C, E = 5.0×10^{-6} M, S = 2.8×10^{-4} M, pH* = 8.4.

Instead, a new species accumulated with a spectrum having a λ_{max} of approximately 330 nm, red-shifted by 5 nm from the substrate (Compton and Fink, in preparation).

The pH*-rate profile for the formation of the TI was obtained under conditions of excess substrate, at a temperature which showed first-order formation of the intermediate, followed by a linear absorbance increase due to turnover. A bell-shaped pH*-dependence was found, with $pK_1^* = 7.8$ and $pK_2^* = 8.7$ (-30°C). The amount of the TI accumulated increased with increasing pH* and decreasing temperature.

DISCUSSION

The key steps in catalysis by serine proteases involve nucleophilic attack by Ser-195, with base catalysis by the aspartate-imidazole system of pK 7 to form the putative tetrahedral intermediate. Subsequent collapse to the acyl-enzyme involves acid-catalyzed departure of the leaving group. Although some controversy exists about which group in the active site is responsible for the observed pK, there is little question that the formation of the tetrahedral intermediate would exhibit a pK around 7. Furthermore, at high pH the TI would accumulate because the imidazole-aspartate system could not function as a general acid.

The reaction preceding turnover is interpreted as tetrahedral intermediate formation for the same reasons used in the case of elastase (9) and papain (8). Both the kinetic and spectral data are consistent with expected properties of the TI. Predictably, increasing the enzyme concentration increased the rate of the turnover reaction and the magnitude of the absorbance change associated with formation of the TI, whereas increasing the substrate concentration increased the rate of formation of the adduct but not the turnover rate (under conditions of excess, but not saturating, substrate concentration). Similarly, the amplitudes of the observed absorbance changes at 410 nm are appropriate for TI formation and turnover. The data are inconsistent with a rate-limiting conformational change following formation of the enzyme-substrate complex, or substrate distortion (8, 9).

The λ_{max} of 355 nm for the TI is similar to that observed in the case of elastase and papain (8, 9). The pK* of 7.8 in the rate of formation of the TI is attributed to the imidazole-aspartate group. Based on the normal heat of ionization of imidazole, we estimate a pK of around 6 at 25°C for formation of the TI. This implies that the pK of the imidazole is perturbed from its value of 6.9 in the free enzyme to about 5-6 in the ES complex.

The properties of the TI detected in this study are very similar to those we have previously reported for tetrahedral intermediates of papain and elastase, also trapped at subzero temperatures (8, 9). Our results support the existence of a discrete tetrahedral intermediate, and also identify the conditions necessary to trap the intermediate for further study by other methods.

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