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Low-Temperature Reactions of Trypsin with *p*-Nitroanilide Substrates: Tetrahedral Intermediate Formation or Enzyme Isomerization†

Paul D. Compton and Anthony L. Fink*

ABSTRACT: The reactions of trypsin with the *p*-nitroanilides of N^{α}-carbobenzoxy-L-lysine, N^{α}-carbobenzoxy-L-arginine, and N^{α}-benzoyl-L-arginine have been studied in the 0 to −30 °C temperature region, over a range of pH* values, using 65% (v/v) aqueous dimethyl sulfoxide cryosolvent. At alkaline pH*, −30 °C, the catalytic reaction appears as a slow "burst" of product (or intermediate) followed by turnover. For all three substrates, the *rate* of the burst phase is identical. Preincubation of the enzyme at −30 °C abolishes the burst. On addition of trypsin to the cryosolvent at −30 °C, a time-dependent decrease in fluorescence emission is observed with the same rate as that of the burst with the anilides. The burst phase is thus interpreted as reflecting a temperature/solvent-induced isomerization of trypsin to a less catalytically efficient form, rather than the previously suggested formation of a tetrahedral intermediate [Compton, P. D., & Fink, A. L. (1980) *Biochem. Biophys. Res. Commun. 93*, 427–431]. The isomerization is not observed at high temperature (≥0 °C) or at neutral pH*. The burst phase was not observed with aqueous methanol cryosolvent, indicating that it is sensitive to both cosolvent and temperature.

There is considerable interest in the question of whether tetrahedral adducts exist as discrete intermediates or as transition states in protease catalysis. We have investigated the trypsin-catalyzed hydrolysis of *p*-nitroanilide substrates by using cryoenzymology in order to detect such putative intermediates. In an earlier study (Compton & Fink, 1980), we reported that a "burst" of absorbance in the 350–410-nm region could be observed prior to turnover in the reaction with N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide (ZLyspNA)[1] and attributed the reaction to the formation of a tetrahedral intermediate. Previous investigations (Fink, 1974) have shown that neither the catalytic nor the structural properties of trypsin are perturbed by exposure at 0 °C to 65% (v/v) dimethyl sulfoxide. However, the present structural (protein fluorescence emission) and catalytic (anilide hydrolysis) studies indicate that at lower temperatures in this cryosolvent a structural transition occurs resulting in reduced reactivity toward anilide substrates. Trypsin has a long history of temperature- and ligand-induced transitions (Otero et al., 1980; Mares-Guia et al., 1981) to which the present isomerization must be added.

Experimental Procedures

Materials. N^{α}-Carbobenzoxy-L-lysine *p*-nitroanilide, lot F877, purified by preparative TLC, and the arginine analogue, lot 63797P, were obtained from Vega Biochemicals. The former was also obtained as a generous gift from Prof. Tesser, Organic Chemistry Department, Nijmegen, Holland. N^{α}-Benzoyl-L-arginine *p*-nitroanilide, lot 18C0224, was from Sigma; 3 times crystallized trypsin from Worthington, lot TRL 3 30D727, was used without further purification. Stock solutions of enzyme were prepared in 1 mM HCl and stored at 4 °C. The activity was checked daily by burst titration with

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[1] Abbreviations: TI, tetrahedral intermediate; ZLyspNA, N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide; ZArgpNA, N^{α}-carbobenzoxy-L-arginine *p*-nitroanilide; ZLyspNP, N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester; BzArgpNA, N^{α}-benzoyl-L-arginine *p*-nitroanilide; TLC, thin-layer chromatography.

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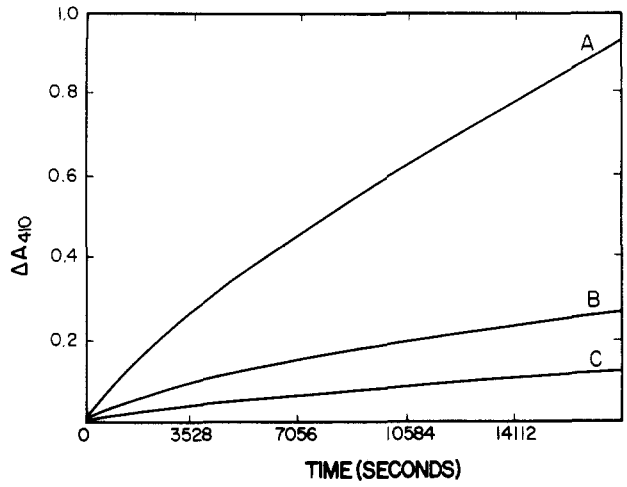

FIGURE 1: Time-dependent absorbance changes in the reaction of trypsin with *p*-nitroanilide substrates in 65% dimethyl sulfoxide at −30 °C. Conditions were pH* 9.6, E_0 = 10 μM, and S_0 = 1.5 mM. Curve A is for ZLyspNA, curve B is for ZArgpNA, and curve C is for BzArgpNA.

p-nitrophenyl *p*-guanidinobenzoate (from Nutritional Biochemicals) or by kinetic assay (Bender et al., 1965) with N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester, lot F7713, from Vega Biochemicals. Substrate stock solutions were typically prepared at concentrations of 0.05 M in 90% acetonitrile–water. Concentrations were determined by the amount of *p*-nitroaniline released during trypsin hydrolysis. Reagent-grade materials were used throughout. Cryosolvent buffers were 0.1 M formate, acetate, cacodylate, or borate. Dimethyl sulfoxide (spectral grade from Mallinckrodt) was vacuum distilled after being dried over calcium hydride.

Methods. Standard cryoenzymological procedures are described elsewhere (Fink & Geeves, 1979). Cryosolvents were prepared on a volume per volume basis. The values of the apparent pH (pH*) were measured with a glass electrode and a Radiometer PHM 26 pH meter. Values of pH* below 0 °C were determined by extrapolation from standard plots of pH* vs. temperature. For example, 65% dimethyl sulfoxide–cacodylate of pH* 8.85 at 25 °C has values of 9.2 at 0 °C and 9.6 at −30 °C.

Absorbance measurements were done with Cary 118, Cary 219, or Perkin-Elmer 320 spectrophotometers, equipped with thermostated cell blocks and dry gas purging. Molar extinction coeficients for *p*-nitrophenol and *p*-nitroaniline were measured as a function of temperature in the cryosolvent at various pH* values. Fluorescence studies of trypsin were carried out with a Perkin-Elmer MPF-4 instrument, with excitation at 280 nm and emission at 330 nm unless otherwise noted. Burst amplitudes and rate constants were determined from kinetic analyses for a first-order reaction (the burst) followed by a zero-order reaction.

Results

Reaction of Trypsin with p-Nitroanilide Substrates. As previously reported (Compton & Fink, 1980), when trypsin was reacted with ZLyspNA in 65% dimethyl sulfoxide at −30 °C, at pH* >6.7, a slow burst phase was observed followed by a linear absorbance increase corresponding to turnover (Figure 1). For pH* 9.6, [trypsin] = 10 μM, and [ZLyspNA] = 1.5 mM ($S_0 \sim K_m$), the first-order rate constant for the burst phase was 2.6×10^{-4} s^{-1}. At 410 nm, the amplitude of the burst was 0.18 ODU; the molar extinction coefficient for *p*-nitroaniline is 14.5×10^3 M^{-1} cm^{-1} under these conditions. The initial velocity (v_i) was 6.2×10^{-9} M s^{-1}; the velocity of the linear phase after completion of the burst (v_i') was 3.0×10^{-9} M s^{-1}.

Table I: Kinetic Properties of the Burst Reaction with *p*-Nitroanilide Substrates[a]

substrate	k_{obsd}[b]	v_i[c]	v_i'[c]	v_i/v_i'
ZLyspNA	2.6	6.2	3.0	2.0
ZArgpNA	2.8	1.5	0.69	2.2
BzArgpNA	2.9	0.83	0.36	2.3

[a] Conditions were 65% dimethyl sulfoxide, pH* 9.2, −30 °C, E_0 = 10 μM, and S_0 = 1.5 mM. [b] ×10^{-4} s^{-1}. [c] ×10^{-9} M s^{-1}.

The time-dependent changes in absorbance during the interaction of trypsin with ZArgpNA and BzArgpNA in 65% dimethyl sulfoxide, pH* 9.6, −30 °C, were also investigated. As in the case of the lysine derivative, a burst phase was noted (Figure 1). The most interesting part of these results is that the first-order rate constant for the burst, 2.7×10^{-4} s^{-1}, was identical (±11%) for all three substrates! The initial velocities and the velocities of the linear phase following the burst were different for the different substrates, although their ratios were independent of substrate ($v_i/v_i' = 2.2 \pm 0.15$), and are shown in Table I.

There are a number of possible underlying phenomena which could give rise to the existence of a burst reaction with the same rate for the three different anilide substrates. It is unlikely that formation of a tetrahedral intermediate, however, would have the same rate for the different substrates. Rather, the underlying cause of this kinetic transient is likely to reside with the enzyme. The most plausible explanation of the burst is that it corresponds to a slow isomerization of the enzyme to a less active form. If this is the case, the velocities v_i and v_i' correspond to the initial velocities for the two forms of the enzyme. A variety of experiments were therefore undertaken to determine if indeed this was the correct interpretation and to kinetically characterize the isomerization.

Reaction of Trypsin with N^{α}-Carbobenzoxylysine p-Nitroanilide and Its Derivatives. The possibility that ZLys or LyspNA might be inhibitors of the reaction of trypsin with ZLyspNA and responsible for the burst was investigated by repeating the above experiment in the presence of these compounds at concentrations of 7.7×10^{-5} and 1.9×10^{-4} M, respectively. The initial velocities (v_i) were independent of the adduct concentration, indicating no inhibitory effects at these concentrations.

The effect of Ca^{2+} on the catalytic reaction in cryosolvent was also investigated. When trypsin (10 μM) was reacted with ZLyspNA (1.5 mM) in 65% dimethyl sulfoxide, pH* 9.6, −30 °C, in the presence of 0.01 M Ca^{2+}, the kinetic parameters obtained were the following: initial velocity (v_i) = 6.3×10^{-9} M s^{-1}; k_{obsd} for the burst phase = 1.8×10^{-4} s^{-1}; and v_i' = 3.2×10^{-9} M s^{-1}. The initial velocities before and after the burst are therefore unaffected by the presence of Ca^{2+}, whereas the rate for the burst phase is decreased somewhat (30%). Altogether 12 experiments were done without Ca^{2+} under these experimental conditions, and this value lies within 1 standard deviation of the mean for the first-order rate constant for the burst phase.

Some of the N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide supplied by Vega was found to be the racemic mixture. Comparison of the reaction of trypsin with the D–L mixture of ZLyspNA with that of the homogeneous L isomer revealed little effect of the D isomer on the reaction. The values obtained for all the kinetic parameters were within ±10% except for the first-order rate constant for the burst which was 17% slower for the racemic mixture.

Table II: Effect of Enzyme and Substrate Concentration on the Burst Reaction with ZLyspNA

[ZLys-pNA]	[trypsin]	k_{obsd}[e]	Π[a]	v_i[f]	v_i'[f]
3.1[b]	5.8	5.5	0.03	16.0	5.0
3.0[b]	10.0	4.8	0.06	29.1	9.7
3.1[b]	23.0	5.5	0.12	52.0	17.3
0.7[c]	10.5	1.1	0.30	4.3	1.9
1.5[d]	10.2	2.6	0.18	6.2	3.0
2.7[c]	10.5	1.9	0.32	7.3	3.2

[a] OD at 410 nm. [b] 65% dimethyl sulfoxide, pH* 9.2, −30 °C. [c] 65% dimethyl sulfoxide, pH* 9.8, −30°C. [d] 65% dimethyl sulfoxide, pH* 9.6, −30 °C. [e] $\times 10^{-4}$ s^{-1}. [f] $\times 10^{-9}$ M s^{-1}.

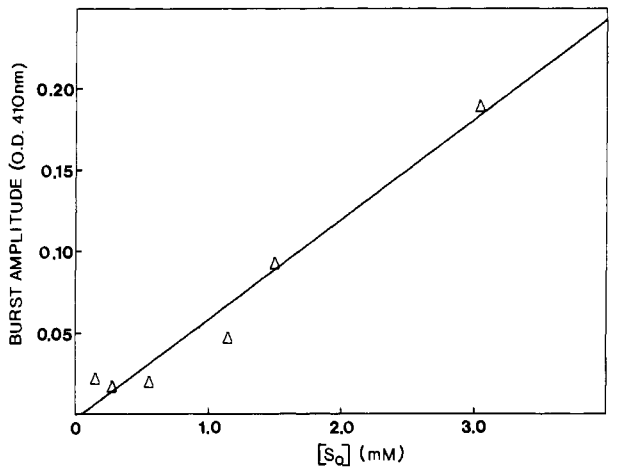

FIGURE 2: Effect of substrate concentration on the amplitude of the burst for the trypsin-catalyzed hydrolysis of ZArgpNA. Conditions were E_0 = 10.8 μM, 65% dimethyl sulfoxide, pH* 9.2, −30 °C. The molar extinction coefficient for *p*-nitroaniline under these conditions is 14.5 × 10^3 M^{-1} cm^{-1}. The line drawn is a least-mean-squares fit to the data.

Factors Affecting the Burst. In order to permit resolution of some of the possible models to account for the experimental observations, a variety of additional experiments were carried out. For example, solvent-mediated inhibition of the enzyme should be independent of the enzyme concentration, whereas contaminant-mediated inhibition should be dependent on the trypsin concentration. In the micromolar range, the value of k_{obsd} for the burst phase was independent of trypsin concentration, the average value being 2.9 (±12%) × 10^{-4} s^{-1} at −30 °C, pH* 9.6, for [ZLyspNA] = 1.5 mM.

For ZLyspNA, the observed rate of the burst phase varied with substrate concentration, and the amplitude of the burst was proportional to the enzyme concentration and relatively insensitive to the substrate concentration. Some typical data for ZLyspNA are given in Table II. However, for ZArgpNA, the rate of the burst phase was independent of substrate concentration, whereas the amplitude increased in a linear fashion with increasing substrate concentration (Figure 2). The values for v_i/S [9.8 (±22%) × 10^{-7} s^{-1}] and v_i'/S [3.4 (±25%) × 10^{-7} s^{-1}] were essentially independent of substrate concentration in the region S = 0.16–4.1 mM (E_0 = 10.8 μM). This means that $K_m >> S_0$ for ZArgpNA, in contrast to the case with ZLyspNA. Using the data for v_i, we calculated values of k_{cat} = 6 × 10^{-4} s^{-1} and K_m = 6.6 mM for ZArgpNA at −30 °C, pH* 9.2.

For ZArgpNA, the pH* dependence of the burst amplitude was sigmoidal, with pK^* = 7.8 (at −30 °C). The burst amplitude increased with pH* to a limiting value of 0.1 ODU for E = 10 μM and S = 1.5 mM, corresponding to 0.70 equiv of enzyme. The pH dependences of v_i and k_{obsd} for the burst were very similar, with a pK^* = 8.0 ± 0.3 at −30 °C and the rate beginning to decrease above pH* 9. The limiting value of v_i' was 7.2 × 10^{-10} M s^{-1}. The pH* dependence of these rate parameters for ZLyspNA was similar.

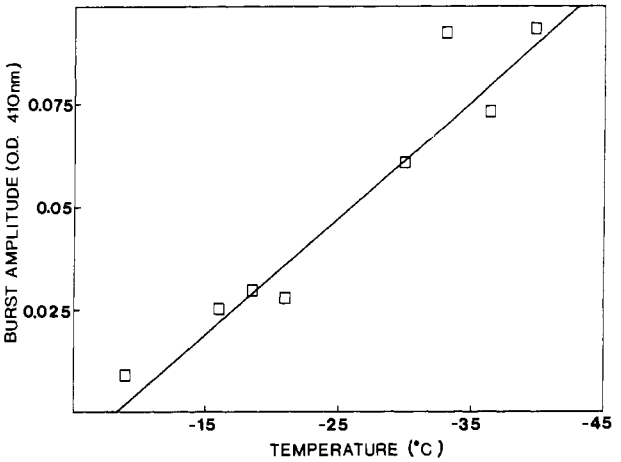

FIGURE 3: Effect of temperature on the amplitude of the burst in the reaction of trypsin with ZArgpNA. Conditions were 65% dimethyl sulfoxide, pH* 9.2, E_0 = 10.8 μM, and S_0 = 1.5 mM.

The reaction of trypsin with ZArgpNA in 65% dimethyl sulfoxide, pH* 9.2, was studied over the 0 to −40 °C range. The amplitude of the burst increased linearly with decreasing temperature (Figure 3). Arrhenius plots of v_i and v_i' were almost parallel, with E_a = 13.3 ± 3 and 15.0 ± 3 kcal mol^{-1}, respectively. In order to emphasize the differential effect of temperature on v_i and v_i', the data were plotted in the form of an Arrhenius plot by using the ratio of v_i/v_i'. The resulting plot was linear and indicated that the energy of activation of v_i' was 2.8 kcal mol^{-1} higher than that of v_i. An Arrhenius plot for the burst-phase reaction of trypsin (10 μM) with ZArgpNA (1.5 mM) in 65% dimethyl sulfoxide, pH* 9.2, over the range −40 to −10 °C, was linear, yielding an energy of activation of 20.9 ± 2 kcal mol^{-1}.

The effect of 65% dimethyl sulfoxide on the trypsin-catalyzed hydrolysis of ZArgpNA was determined by comparing k_{cat}/K_m for 0 and 65% dimethyl sulfoxide, pH* 6.5, 0 °C. The values obtained were 4.1 (±16%) × 10^2 and 0.15 (±7%) M^{-1} s^{-1}, respectively, indicating a decrease of 2745 induced by the cosolvent. This may be compared to a decrease of 740 for ZLyspNP under comparable conditions (Fink, 1974).

Preincubation Experiments. Trypsin (10 μM) was incubated in 65% dimethyl sulfoxide, pH* 9.6 at −30 °C for 4 h (5.6 half-lives of the burst reaction), prior to the addition of ZLyspNA (1.5 mM). A linear absorbance increase was observed at 410 nm; i.e., the burst phase was no longer apparent! The initial velocity was 1.8 (±6%) × 10^{-9} M s^{-1} compared to 3.2 × 10^{-9} M s^{-1} for v_i' under conditions with enzyme preincubation. The apparently lower activity in the control without incubation probably reflects the lack of some stabilization of the enzyme by the presence of the substrate. The enzyme was also incubated for 20 h under similar experimental conditions during which the absorbance spectrum was scanned from 500 to 350 nm at frequent intervals. If aggregation of the enzyme occurred, it would show up as a wavelength-dependent increase in absorbance due to light scattering. No changes were noted, signifying the absence of aggregation. At the end of the 20-h period, the pH* was adjusted to 6.8, ZLyspNP was added (8.4 μM), and the first-order rate constant was measured for the catalyzed hydrolysis of the ester. A value of 21.2 M^{-1} s^{-1} was calculated for k_{cat}/K_m (see below).

Reaction in Methanol Cryosolvent. The reaction of trypsin with N^{α}-carbobenzoxy-L-arginine *p*-nitroanilide in 70%

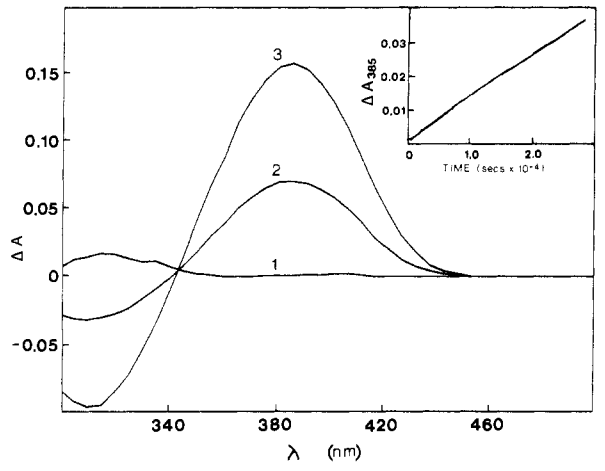

FIGURE 4: Reaction of trypsin with ZArgpNA in 70% methanol. Conditions were pH* 9.6, −30 °C, E_0 = 1.0 μM, and S_0 = 45 μM. Difference spectra are shown as the reaction proceeded. The reference sample contained ZArgpNA (45 μM) under the same conditions of pH* and temperature. The difference spectra have been corrected for the contribution of the enzyme. Scan 1 was taken immediately after mixing enzyme and substrate. Scan 2 corresponds to 5.2×10^4 s, and scan 3 was taken after 1.4×10^5 s. The inset shows the increase in absorbance at 410 nm for the first 30 000 s, twice the time period over which the burst is completed when the reaction is carried out in dimethyl sulfoxide.

methanol, pH* 9.6, was investigated at subzero temperatures by monitoring time-dependent changes in the spectrum of the reaction mixture over the 300–500-nm range. At −30 °C, turnover was observed (a decrease in absorbance around 310 nm due to depletion of substrate and an increase around 385 nm due to formation of product) (Figure 4). A first-order rate constant of 1.8×10^{-6} s^{-1} was obtained by using the Guggenheim method of analysis for E_0 = 1.1 μM and S_0 = 46 μM, for both the increase at 385 nm and the decrease at 310 nm. The estimated amount of product released was in excellent agreement with that expected, based on the substrate concentration. No sign of a significant amount of any preceding intermediate species was noted, nor was there any indication of deviation from the first-order kinetics. At −55 °C, the turnover reaction was negligible, and no reaction was observed.

Reactions with N^{α}-Carbobenzoxy-L-lysine p-Nitrophenyl Ester. Previous investigations (Fink, 1974) had shown that incubation of trypsin in 65% dimethyl sulfoxide at 0 °C resulted in no loss of activity when assayed with ZLyspNP. However, these experiments were carried out at neutral pH, and as noted above, the putative solvent-mediated isomerization of the enzyme is temperature and pH dependent. However, the large spontaneous rate of hydrolysis of ZLyspNP at high pH* precluded investigations of the reaction under conditions of high pH*. The reaction of trypsin with ZLyspNP in 65% dimethyl sulfoxide was thus studied at pH* 6.8 and −30 °C. The rate of release of *p*-nitrophenol was monitored either at 375 nm ($\Delta\epsilon$ = 1260 M^{-1} cm^{-1}) or at the isosbestic point, 350 nm ($\Delta\epsilon$ = 6596 M^{-1} cm^{-1}). With [ZLyspNP] = 2.8 mM, a zero-order reaction was observed over a 20-h period ([trypsin] = 10 μM), from which a k_{cat} value of 1.7×10^{-3} s^{-1} was calculated. When substrate concentrations of 5.5 or 7.7 μM ($S_0 << K_m$) were used, first-order kinetics were observed, which yielded a value of k_{cat}/K_m = 25.3 M^{-1} s^{-1}. From this value and that of k_{cat} obtained from the above-mentioned zero-order reaction, we calculate a value of $K_m = 6.6 \times 10^{-5}$ M. To confirm that these last two experiments reflected turnover, and not formation of an acyl-enzyme, an additional aliquot of substrate (60 μM) was added at the completion of the reaction. The ensuing rate of product release was inconsistent with the previous reaction having stopped at the acyl-enzyme stage.

Experiments at 0 °C. The K_m for the reaction with ZLyspNP in 65% dimethyl sulfoxide, pH* 6.5, is 1.5 mM at 0 °C (Fink, 1974). The first-order kinetics obtained when trypsin (0.8–10 μM) reacted with ZLyspNP (1.5×10^{-4} M) under these conditions gave values of k_{cat}/K_m = 3.4 (±15%) $\times 10^2$ M^{-1} s^{-1}. The addition of Ca^{2+} at 0.01 M had no effect, k_{cat}/K_m = 3.2 (±8%) $\times 10^2$ M^{-1} s^{-1}.

Similar experiments were carried out with ZLyspNA: for [trypsin] = 10 μM, [ZLyspNA] = 71 μM, and 65% dimethyl sulfoxide, pH* 9.2, 0 °C, k_{cat}/K_m = 3.0 (±10%) M^{-1} s^{-1}. The reaction was cleanly monoexponential. Preincubation of the enzyme in the cryosolvent for 6 h prior to addition of the substrate had no effect on k_{cat}/K_m.

Fluorescence Studies. If the observed burst phase in the low-temperature kinetics of trypsin in 65% dimethyl sulfoxide cryosolvents is due to a temperature-induced isomerization, it is possible that the isomerization might be monitored directly by using the intrinsic fluorescence of the enzyme. Trp-215, for example, is close to the active site and could be a sensitive probe for small structural perturbations in the active-site vicinity. In aqueous solution, pH 6.5, 0 °C, the fluorescence emission maximum for trypsin is 330 nm. The presence of 65% dimethyl sulfoxide (at 0 °C) results in no change in the λ_{max} for emission, but an increase in intensity. At −30 °C, λ_{max} changes to 328 nm. No significant time-dependent changes were noted on incubation for 17 h at −30 °C, pH* 6.5.

The fluorescence emission maximum of trypsin was shifted from 333 nm at pH 9.6, 0 °C, in 0% dimethyl sulfoxide to 328 nm at pH* 9.6, −30 °C, in 65% dimethyl sulfoxide, with an intensity increase of 2. A first-order quenching process was observed on mixing trypsin with the cryosolvent at −30 °C, pH* 9.6. The rate of 2.8×10^{-4} s^{-1} for this process is within 4% of the value obtained for the burst reaction observed during anilide hydrolysis. Experiments at 0 °C in 65% dimethyl sulfoxide show no change in the enzyme fluorescence emission intensity as a function of time.

The absorbance spectrum of trypsin in 65% dimethyl sulfoxide, pH* 9.6, −30 °C, was scanned from 320 to 250 nm over a period of 3.6 h. A 2% decrease in the intensity of the absorbance at the λ_{max} of 280 nm was noted. The small amplitude of the change precluded accurate kinetic measurements.

Discussion

The rate-limiting step in trypsin-catalyzed hydrolysis of *p*-nitroanilides is acylation (Fastrez & Fersht, 1973). The first-order burst phase preceding turnover in the reaction of trypsin with ZLyspNA was previously interpreted (Compton & Fink, 1980) as tetrahedral intermediate (TI) formation, predominantly on the basis of the effects of variation in enzyme and substrate concentration and pH on its kinetic properties.

Taken alone, most of the kinetic data observed for the burst phase in the present investigation for each of the *p*-nitroanilide substrates is consistent with the expected properties of TI formation. Predictably, increasing the enzyme concentration increased the turnover rate and the magnitude of the absorbance change attributed to TI formation. Similarly, the amplitudes of the observed absorbance changes at 410 nm were reasonable for TI formation and turnover, assuming an extinction coefficient for the intermediate similar to that for *p*-nitroaniline. The pK^* around 8 for the rate and amplitude of the burst reaction is certainly consistent with what one would expect for a TI. On the basis of the normal heat of ionization

Scheme I

$$\begin{array}{ccccccc} E + S & \xleftrightarrow{K_s} & ES & \xrightarrow{k_2} & EA + P_1 & \xrightarrow{k_3} & E + P_2 \\ \Big\| k_{-i} \; k_i & & & & & & \\ E^* + S & \xleftrightarrow{K_s^*} & E^*S & \xrightarrow{k_2^*} & E^*A + P_1 & \xrightarrow{k_3^*} & E^* + P_2 \end{array}$$

for imidazole, a pK of close to 6 at 25 °C may be extrapolated from the −30 °C data.

However, the invariance of the burst reaction rate with different substrates would not be expected if the reaction were formation of the TI. These substrates show a range of reactivity toward trypsin which varies over a factor of 10 (this study; Somorin et al., 1978a,b). One would expect the rate of TI formation to be affected by the same factors which control the acylation step. Thus, it is unlikely that the burst phase represents TI formation.

A number of other possible sources which could be responsible for the presence of the burst phase can be ruled out on the basis of the reported results. These include such factors as product inhibition and contamination of the substrate by the D isomer or LyspNA as well as a structural effect due to the presence or absence of calcium. Similarly, the absence of light scattering eliminates aggregation of the enzyme as a source of the burst. It should be noted that such aggregation is observed at much higher enzyme concentrations and is readily manifested as a wavelength-dependent increase in absorbance of the base line. The data are also inconsistent with nonproductive binding.

Several lines of evidence indicate that the source of the observed burst resides in the enzyme itself and that the physical basis of the phenomenon is an isomerization of the enzyme. These include (1) the abolishment of the burst by preincubation of the enzyme prior to the addition of the substrate, (2) the quenching of the intrinsic fluorescence of the enzyme on incubation at −30 °C in 65% dimethyl sulfoxide with the same rate as that of the burst, and (3) the fact that the rate of the burst is independent of substrate structure. Consequently, the burst phase may be viewed as a first-order loss in the concentration of "fully" active enzyme (E) due to isomerization into a less active form (E*). The observed kinetics thus reflect a change from initial velocity v_i due to E to initial velocity v_i' due to E*.

The proposed cosolvent/temperature-induced isomerization can be represented by Scheme I. There are two versions of Scheme I which we wish to consider. The first case is that in which E* is catalytically inactive and does not bind substrate. In this instance, it can be shown that the rate constant for the burst is given by

$$k_{obsd} = k_i/(1 + S/K_s) + k_{-i} \quad (1)$$

and the burst amplitude (Π) by

$$\Pi = \frac{k_2 k_i E_0/(1 + S/K_s)}{[k_{-i} + k_i/(1 + S/K_s)]^2(1 + K_s/S)} \quad (2)$$

For the case where $S_0 << K_s$, this reduces to

$$k_{obsd} = k_i + k_{-i} \quad (3)$$

and

$$\Pi = k_2 k_i E_0 S/[(k_i + k_{-i})^2 K_s] \quad (4)$$

This model predicts that k_{obsd} for the burst will either be independent of substrate concentration or be inversely proportional to it, whereas Π will increase with increasing substrate concentration. Thus, the model fits the data for ZArgpNA well but not that for ZLyspNA. In addition, the observed increase in v_i' relative to v_i as the temperature is decreased is inconsistent with a model in which the species E* is inactive. The fluorescence and absorbance changes associated with the isomerization do not reflect a gross structural transition. Thus, it is likely that E* is active.

The second case, therefore, is that in which E* is catalytically active and binds substrate. In this instance, the isomerization may proceed with 100% conversion to E*, with a concomitant 2–3-fold decrease (v_i/v_i') in reactivity toward anilide substrates, or an equilibrium between E and E* may exist, with E* of undetermined activity. This model is consistent with the observed dependence of v_i/v_i' on temperature.

For the full Scheme I, it can be shown that

$$k_{obsd} = [k_i(1 + S/K_s^*)]/(1 + S/K_s) + k_{-i}$$

The expression for Π is complex and does not readily reduce to a simple form. The observed difference in the effect of substrate concentration on k_{obsd} and Π for ZLyspNA and ZArgpNA can be explained with this model by differences in K_s and K_s^* relative to S_0 for the two substrates. Thus, for ZArgpNA, $S_0 << K_s$ and K_s^*, and consequently, $k_{obsd} = k_i + k_{-i}$. For ZLyspNA, $K_s^* < S_0 < K_s$, and k_{obsd} increases as S increases since $k_{obsd} \simeq k_i(1 + S/K_s^*) + k_{-i}$. Thus, Scheme I as shown adequately accounts for the results.

Since the enzyme isomerization is slow relative to catalysis, the steady-state assumption may be applied to the system, and at early and late times relative to the burst, the following relationships hold:

$$v_i = k_{cat}E_0S/(K_m + S)$$

and

$$v_i' = k_{cat}^*E_0^*S/(K_m^* + S)$$

Analysis of the observed kinetics is simplified by the fact that only the first 1–4 % of the total reaction was monitored; therefore, $S_0 \simeq S_0 - P$. The amplitude of the apparent burst can be obtained from the integrated version of the Michaelis–Menten equation:

$$Vt = P + K_m \ln [S_0/(S_0 - P)]$$

from which

$$P_{obsd} = v_i t e^{-k_{iso}t} + v_i' t(1 - e^{-k_{iso}t})$$

Simulation of the above scheme gives a good fit to the observed absorbance vs. time curves. Formally the observations reported (e.g., Figure 1), and the model of Scheme I, are similar to those observed in cases of enzymes exhibiting hysteretic behavior (Frieden, 1979; Neet & Ainslie, 1980).

The increase in burst amplitude as the temperature decreases implies either relatively less activity of E* (consistent with the v_i/v_i' vs. temperature data) or a shift of the isomerization equilibrium to favor less E* at lower temperatures. The absence of a detectable burst or fluorescence change at 0 °C, at high pH*, is consistent with the isomerization occurring at too fast a rate to be observed under these conditions. Extrapolation of the Arrhenius plot indicates that the rate of the burst would be 0.33 s^{-1} at 25 °C, pH* 9.2.

A number of previous investigations have revealed several thermal transitions of trypsin over the −1.5 to 56.5 °C range in aqueous solution (Otero et al., 1980). The isomerization reported here appears to be unrelated to those previously reported.

The pH dependence of the burst reaction suggests the involvement of a group of pK^* around 8 at −30 °C, most likely an imidazole. The observed phenomenon of decreased activity at high pH bears some resemblance to the high pH induced inactivation of chymotrypsin due to the loss of the Ile-16–

Asp-194 salt bridge (Fersht, 1972; Fersht & Requena, 1971). A similar salt bridge is found in trypsin on activation from the zymogen and is essential for catalytyic activity (Huber & Bode, 1978). In chymotrypsin, the pK of Ile-16 is approximately 9 at 25 °C (Fersht & Requena, 1971). Given the high heat of ionization of ammonium groups, a pK close to 11 would be expected at −30 °C. In view of the pK^* of 8 for the isomerization observed in 65% dimethyl sulfoxide, and the apparent activity of E*, it would appear that the isomerization reported here does not involve the Ile-16–Asp-194 salt bridge.

The lack of a burst in the reaction of trypsin with ZArgpNA when 70% methanol was used as solvent, at −30 °C, indicates that the factor(s) responsible for the enzyme isomerization is (are) not temperature alone but also involve(s) a contribution from the cosolvent. It is possible that the isomerization is specific to the dimethyl sulfoxide system. Alternatively, the isomerization may be much faster in 70% methanol and too rapid to have been detected. The catalytic activity of trypsin has long been known to be sensitive to the presence of various ligands (including substrates which cause substrate-induced activation). Several studies have suggested the possibility of an allosteric site for ligand binding which could affect the activity of the enzyme (Mares-Guia et al., 1981). It is quite possible that dimethyl sulfoxide binds to such a site to bring about the observed isomerization. It would be interesting to examine the X-ray crystallographic structure of trypsin in the presence of dimethyl sulfoxide to see if such a site can be identified.

A recent stopped-flow kinetics investigation of the reaction of trypsin with BzArgpNA in 50% dimethyl sulfoxide in the 0 to −28 °C range revealed a rapid increase in ΔA_{410} prior to linear steady-state turnover. The amplitude of the rapid absorbance increase increased at lower temperatures but was not proportional to the enzyme concentration (Markley et al., 1981). The underlying process responsible for the absorbance increase is not known at present. On the basis of the magnitude of the rates, it is unlikely to be the isomerization reported here.

Markley et al. (1981) also questioned previous reports of tetrahedral intermediates in *elastase* catalysis (Hunkapiller et al., 1976; Fink & Meehan, 1979) and suggested that in substrates containing proline configurational isomerization could lead to two forms of substrate with different kinetic properties. In the low-temperature studies with elastase (Fink & Meehan, 1979), the pH dependence of the observed reaction, the spectra of the intermediate, and the observation of the reaction with succinyl-Ala_3pNA all suggest that the observed reaction was not due to proline isomerization but was consistent with tetrahedral intermediate formation. The data reported in Fink & Meehan (1979), as well as unpublished observations, indicate that much lower temperatures are necessary to observe the reaction then those used by Markley et al. (1981). We believe this is a consequence of the fact that the energies of activation for tetrahedral intermediate formation and breakdown are such that the latter only becomes rate limiting at very low temperatures (approximately −50 °C).

The observation of a temperature/cosolvent-induced isomerization of trypsin in 65% dimethyl sulfoxide at subzero temperatures which results in modification of the enzyme's catalytic properties illustrates the necessity of very careful examination of the effects of cryosolvents on the properties of enzymes. The similarity observed in the kinetic properties of the isomerization compared with those anticipated for the putative tetrahedral intermediate demonstrates the desirability of independent structural evidence to confirm the existence of enzyme–substrate catalytic intermediates.

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