Reinvestigation of the Reaction of Chymotrypsin with *N*-Furylacryloyltryptophan Derivatives at Acidic pH

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The reaction of α -chymotrypsin with *N*^α-3-(2-furyl)acryloyl-L-tryptophan methyl ester (FA-Trp-OMe) and amide has been investigated in aqueous and dimethylsulphoxide cryosolvent solutions from pH 2 to 7 and over a wide temperature range. Previous reports have suggested that an intermediate preceding the acyl-enzyme can be detected spectrophotometrically in the reaction with methyl esters of FA-Trp and FA-Tyr at low pH [Yu & Viswanatha (1969) *Eur. J. Biochem.* **11**, 347–352], and that this intermediate is an oxazolinone [Coletti-Previero *et al.* (1970) *FEBS Lett.* **11**, 213–217]. We show that the previous interpretations of the time-dependent spectral changes were incorrect, and that the only detected intermediate is the acyl-enzyme. This may be isolated by gel filtration at pH < 2.5, 1°C, owing to its relative stability. The pH-dependence of the rates of acylation and deacylation from pH 8.5 to 2.0 are consistent with a single ionization of $pK \approx 7.0$ in both aqueous and cryosolvent solutions.

In connection with a detailed investigation of the mechanism of serine-proteinase catalysis by cryoenzymology (Fink, 1976*a,b*, 1977; Douzou, 1977), we have had occasion to re-examine the reaction of α -chymotrypsin with FA-Trp substrates. Previous reports based on the reaction of esters of FA-Tyr and FA-Trp with α -chymotrypsin at low pH indicated the presence of an intermediate before the acyl-enzyme (Yu & Viswanatha, 1969). The intermediate was identified as an oxazolinone by Coletti-Previero *et al.* (1970). Our investigation indicates, contrary to these proposals, that the intermediate is an artefact, and that steady-state experiments at low pH are consistent solely with the formation and breakdown of the acyl-enzyme. Our observations and interpretations are also in accord with a recent stopped-flow study by Kunugi *et al.* (1978). From the spectral changes and kinetics in the reaction of chymotrypsin with FA-Trp-OMe, they concluded that in the region pH 9–5.5 the system is consistent with a simple Michaelis-complex acyl-enzyme pathway, with no other intermediate accumulating.

The incorporation of a chromophoric reporter group in a particular region of the substrate potentially allows one to detect transformations of enzyme-substrate intermediates, and to determine if that part of the substrate, or its environment, is involved in a particular elementary step. Such knowledge may be very useful in providing a link between solution and crystallographic studies. The *N*^α-3-(2-furyl)acry-

loyl moiety is particularly well-suited as such a probe for the acyl-amino locus of chymotrypsin substrates (Bernhard *et al.*, 1965), and has previously been so used (Barman & Gutfreund, 1966; Hess *et al.*, 1970; Miller & Bender, 1968; Brot & Bender, 1969; Kunugi *et al.*, 1978). The esters and amides of FA-Trp and FA-Tyr were of especial interest for our cryoenzymological studies, since not only are they specific substrates, but intermediates additional to the Michaelis complex and acyl-enzyme have been reported (Yu & Viswanatha, 1969; Hess *et al.*, 1970; Coletti-Previero *et al.*, 1970).

Materials and Methods

α -Chymotrypsin (three-times-crystallized) was obtained from Worthington (Freehold, NJ, U.S.A.) or Sigma; chymotrypsinogen was from Worthington. Stock solutions were prepared in 1 mM-HCl. FA-Trp-OMe and FA-Trp-NH₂ were products of Cyclo Chemical Co. (Los Angeles, CA, U.S.A.), and were recrystallized from methanol. Enzyme activity was determined by active-site titration (Schonbaum *et al.*, 1961).

Cryosolvents, aq. 65% (v/v) dimethyl sulphoxide were prepared as previously described (Fink, 1976*a*; Fink & Geeves, 1979). Low-temperature experiments were carried out by using our standard procedures (Fink & Geeves, 1979). T.l.c. analysis of the reaction products was carried out with silica-gel plates and a solvent system composed of chloroform/methanol (9:1, v/v). Spectrophotometric experiments were carried out with a Cary 118 spectrophotometer, and

Abbreviations used: FA-Trp, *N*^α-3-(2-furyl)acryloyl-L-tryptophan; OMe, methyl ester; OEt, ethyl ester; pH*, apparent pH in cryosolvent.

the pH-stat experiments utilized a Radiometer apparatus. Proton-release experiments were performed spectrophotometrically, with 2,4-dinitrophenol as indicator, the A_{400} being monitored. Gel-filtration experiments were carried out on a column (60cm $\times$ 0.9cm) of Sephadex G-25.

Results and Discussion

When FA-Trp-OMe was mixed with α -chymotrypsin in aqueous solution in the range pH 2–3 and 25–30°C, we observed similar time-dependent changes in A_{320} or A_{330} as previously reported for esters of both FA-Tyr (Yu & Viswanatha, 1969) and FA-Trp (Coletti-Previero *et al.*, 1970) (e.g., Fig. 1a). These experiments were carried out under conditions of $[E]_0 \approx [S]_0 \approx 50$ – $100 \mu\text{M}$, and show an initial increase (phase 1) followed by a decrease (phase 2) and a subsequent increase (phase 3) in A_{320} . These reactions were previously ascribed to formation of an oxazolinone (phase 1), conversion into the acyl-enzyme (phase 2) and deacylation (phase 3) (Yu & Viswanatha, 1969; Coletti-Previero *et al.*, 1970). When the experiments were monitored by repetitive spectral scans (280–450nm), however, it was noted that, although phases 1 and 2 were apparent only in the near-u.v. region, phase 3 was seen at all wave-

lengths, albeit with decreasing intensity at higher wavelengths. These results suggested that phase 3 was due to a light-scattering effect, and not related to the catalytic reaction.

The following observations confirm that phase 3 is an artefact due to aggregation of the enzyme, rather than deacylation as suggested by Yu & Viswanatha (1969), and that phase 2 corresponds to deacylation (turnover). (1) If the enzyme and substrate concentrations were decreased to $\leq 20 \mu\text{M}$, other conditions being maintained constant, only phases 1 and 2 were seen. (2) Occasionally at pH < 3 an actual precipitate or turbidity could be observed at completion of a run in which phase 3 was observed. (3) On increasing the pH to above 3, both phases 1 and 2 soon became too fast to observe without rapid-mixing apparatus, and phase 3 was absent. The rate of phase 2, but not phase 1, could be slowed down by decreasing the enzyme concentration. For example at pH 4.0, $[E]_0 = 5 \mu\text{M}$, $[S]_0 = 50 \mu\text{M}$, phase 2 was complete in 150s; however, there was no evidence of phase 3. In stopped-flow experiments with $[E]_0 \approx [S]_0 \approx 100 \mu\text{M}$ for FA-Tyr-OEt, Barman & Gutfreund (1966) observed transients corresponding to phases 1 and 2 up to pH 6.6. The rate of phase 2 was consistent with that of deacylation, based on steady-state experiments and high pH. More recently Kunugi *et al.* (1978) examined the reaction of chymotrypsin with FA-Trp-OMe as a function of pH, by using stopped-flow spectrophotometry. Above pH 5.5, two transients corresponding to phases 1 and 2 were observed; no evidence for a triphasic reaction was detected. (4) When the reaction was monitored by using the pH-stat or a pH-indicator in the vicinity of pH 4 (i.e., above the pK of FA-Trp) the rate of H^+ release corresponded to that of phase 2 (Table 1). The concentration of acid released in phase 2 was equivalent to the substrate concentration, in experiments with $[S]_0 > [E]_0$. (5) When the reaction was carried out at

Fig. 1. Time-dependent changes in A_{320} in the reaction of α -chymotrypsin with FA-Trp-OMe (a) at pH 2.45, 30°C and (b) at pH 4.05, 1.0°C

(a) $[E]_0 = 80 \mu\text{M}$, $[S]_0 = 30 \mu\text{M}$. The numerals refer to phases 1–3 discussed in the text. (b) $[E]_0 = 80 \mu\text{M}$, $[S]_0 = 30 \mu\text{M}$. No further ΔA was observed.

Table 1. The rate of H^+ release in the reaction of α -chymotrypsin with FA-Trp-OMe

Method	Temperature (°C)	pH	$k_{\text{obs.}} (\text{s}^{-1})$
ΔA_{320}^*	6.3	4.05	1.4×10^{-2}
	6.0†	4.05	1.2×10^{-2}
	2.8	4.01	7.8×10^{-3}
	1.9	3.0	5.4×10^{-4}
pH-stat	4.0	4.05	9.2×10^{-3}
H^+ release‡	2.7	4.0	6.2×10^{-3}

* Phase 2 as detected by ΔA_{320} .

† Control reaction with 2,4-dinitrophenol present.

‡ As monitored by ΔA_{400} in the presence of the indicator 2,4-dinitrophenol; control experiments showed no change in A_{400} in the absence of the indicator.

1°C, pH 4 (see below), only phases 1 and 2 were observed (Fig. 1b). (6) With FA-Trp-NH₂, $[E]_0 \approx [S]_0 \approx 100 \mu\text{M}$, in the range pH 2–3 (25°C), for which the turnover reaction would be expected to be approx. 500 times slower than for the methyl ester (Zerner *et al.*, 1964), a reaction very similar in appearance and rate to phase 3 was found. However, t.l.c. analysis of portions removed during this period showed no evidence of product formation. (7) Extrapolation of the deacylation rate of FA-Trp-chymotrypsin (Miller & Bender, 1968) to the pH 2–3 region, assuming a single catalytically significant group of pK 7.0, predicts values of the order observed for phase 2. Similarly, by using the reported value of $k_{\text{cat.}}/K_m$ for FA-Trp-OMe (Brot & Bender, 1969), assuming a pK of 7.0 and correcting for the effect of temperature, we estimate a value of $k_{\text{obs.}} \approx 2 \times 10^{-3} \text{ s}^{-1}$ for deacylation, in good agreement with the measured value of $k_{\text{obs.}}$ for phase 2 of $4 \times 10^{-3} \text{ s}^{-1}$ at pH 4.0, 1°C (Fig. 1b).

Reactions in aqueous solution at low temperature

By lowering the reaction temperature, phases 1 and 2 could be observed at higher pH (Fig. 1b). The rate of deacylation, phase 2, became very slow at pH < 3 (1°C); for example at pH 2.3 the half-life was approx. 2 h. Phase 1, on the other hand, was some 10-fold faster, hence the intermediate formed in phase 1 is quite long-lived under these conditions, sufficiently so that if it were the acyl-enzyme it would be feasible to separate it from unchanged substrate and product by gel filtration. Such an experiment was performed with Sephadex G-25 at pH 2.3 (1°C) as follows. Excess chymotrypsin (50 μM) was mixed with FA-Trp-OMe at pH 2.3 (1°C), and the reaction monitored by ΔA_{320} . On completion of phase 1, the mixture was placed on the gel column, and eluted with pH 2.3 buffer at 1°C. Monitoring of the eluent at A_{280} revealed a major peak (fraction 1) emerging at the void volume, followed by a much smaller peak (fraction 2) at an eluent volume corresponding to the elution volume of FA-Trp and FA-Trp-OMe. Analysis of the area of the peaks indicated that approx. 80% of the substrate co-eluted with the enzyme. Fraction 1 was transferred to a cuvette, and changes in A_{320} were monitored spectrophotometrically at 25°C. A decrease in A_{320} was observed, analogous to phase 2, and with similar kinetics.

In a control experiment the enzyme was replaced by chymotrypsinogen. In this case, analysis of the elution profile indicated all the substrate eluted in the fraction corresponding to the second peak. These results are entirely consistent with phase 1 corresponding to formation of the acyl-enzyme, and phase 2 to its subsequent hydrolysis (i.e., deacylation). The small amount of material eluting in fraction 2 in the first gel-filtration experiment is of the correct magnitude to correspond to the amount of FA-Trp expected

to arise from deacylation during chromatography. This experiment demonstrates that it is possible to use gel filtration to isolate specific acyl-chymotrypsins in aqueous solution at suitably low pH (e.g., 2–2.5) and temperature (0–5°C) (cf. Fink, 1973a,b).

In connection with determination of suitable conditions for the above gel-filtration experiment, the reaction of FA-Trp-OMe with α -chymotrypsin was monitored at A_{320} at 1°C over the pH range 2.0–4.1. Phase 1 was observed over this whole range. Phase 2 was also detected, but not monitored to completion at the lower pH values, owing to its very low rate. No evidence for a reaction preceding phase 1 was observed.

Comparison with previous studies at low pH

The question now arises as to how our findings can be reconciled with those of Coletti-Previero *et al.* (1970) and Yu & Viswanatha (1969). The former authors reported evidence that the product of phase 1 is the oxazolinone, on the basis of experiments involving quenching of the reaction with acetone at –80°C, followed by extraction with ethyl acetate and subsequent t.l.c. analysis. We suggest the following explanation. At the completion of phase 1 the product is the acyl-enzyme. The quenching with acetone at –80°C prevents deacylation, owing to both the low temperature and the decreased water concentration (Fink, 1973a). Reversion of the acyl-enzyme back to the Michaelis complex will not occur significantly, owing to the low methanol concentration. Consequently, as far as the normal catalytic pathway is concerned, the acyl-enzyme is trapped. However, since oxazolinone formation from the acyl-enzyme would be an intramolecular reaction, it is not unreasonable that it might occur at an appreciable rate under these conditions. The formation of oxazolinones from activated esters of *N*-acylamino acids is well-established (De Jersey *et al.*, 1969), and the acyl-enzyme is clearly an activated ester in which the enzyme is the leaving group.

Coletti-Previero *et al.* (1970) also found that the addition of the oxazolinone to α -chymotrypsin leads to a rapid decrease in A_{320} ; however, it is known that oxazolinones are excellent substrates for α -chymotrypsin, and that cleavage of the oxazolinone ring would lead to a decrease in A_{320} (De Jersey *et al.*, 1969; De Jersey & Zerner, 1969). There is also a kinetic inconsistency in the oxazolinone intermediate hypothesis. The data of Coletti-Previero *et al.* (1970), shown in their Fig. 1, indicate that formation of the acyl-enzyme is faster, starting with the oxazolinone as substrate, than with the corresponding methyl ester. Consequently, if the oxazolinone was normally an intermediate on the pathway from FA-Trp-OMe to the acyl-enzyme, it would be the acyl-enzyme, not the oxazolinone, that would accumulate. We therefore conclude that the oxazolinone detected by

Coletti-Previero *et al.* (1970) is, in fact, an artefact of their analytical procedure, and not normally present on the productive catalytic reaction pathway.

Yu & Viswanatha (1969) reported that acetonitrile extraction at the completions of phases 1–3 yielded FA-Trp-OMe only at the completion of phase 1, and that methanol extraction yielded FA-Trp-OMe at the completion of phase 2 only, with decreasing amounts as phase 3 progressed. These results are incompatible with phase 1 being acylation and phase 2 deacylation, and we are unable to provide an explanation of their basis.

Low-temperature investigations in cryosolvent

We have previously shown that aq. 65% dimethyl sulphoxide is a suitable cryosolvent for chymotrypsin at subzero temperatures (Fink, 1973a, 1974). In the cryosolvent the rate of reaction of chymotrypsin with FA-Trp-OMe in 65% dimethyl sulphoxide at 0°C, at non-saturating substrate concentrations, would be anticipated to be very much decreased compared with the reaction in aqueous solution, due to a hydrophobic effect on substrate binding (Maurel, 1978). This was indeed found to be the case, e.g., for $[S]_0 = 65 \mu\text{M}$, $[E]_0 = 70 \mu\text{M}$, $\text{pH}^* 4.1$, -1.2°C , k_{obs} for phase 1 was $2.3 \times 10^{-4} \text{ s}^{-1}$, and for phase 2 was $5.3 \times 10^{-7} \text{ s}^{-1}$. (The latter value was extrapolated from experiments at $\text{pH}^* 5.5$ – 6.5 , by using $\text{p}K^* = 7.0$.) Comparison with the rate of phase 2 in aqueous solution (Table 1) indicates a 1000-fold decrease in the presence of 65% dimethyl sulphoxide, a decrease somewhat larger than that found with *N*-acetyl-L-tryptophan *p*-nitrophenyl ester (Fink, 1973a, 1974). At 0°C the rates of both phases 1 and 2 increased with increasing pH^* to at least $\text{pH}^* 7$. At lower temperatures, e.g., -30°C , phase 2 was still observable at $\text{pH}^* \geq 6$. No evidence for an additional reaction before phase 1 was detected in experiments at $\text{pH}^* \geq 5.5$ at -30°C (when the overall reaction was estimated to be 10000 times slower than at 25°C , 0% dimethyl sulphoxide).

Conclusions

(1) The second absorbance increase (phase 3) observed in the reaction of chymotrypsin with FA-Trp-OMe at low pH is due to aggregation of the enzyme. (2) Acylation appears as an increase, and deacylation as a decrease, in $A_{320-330}$ at all pH values between 2.0 and 8.5 (cf. Kunugi *et al.*, 1978). (3) The

rates of acylation and deacylation over this pH range are consistent with a single, catalytically significant, ionization of $\text{p}K \approx 7.0$. 4. At $\text{pH} \leq 2.5$, 1°C , the acyl-enzyme is sufficiently stable to be isolated by gel filtration. (5) The spectral changes corresponding to acylation and deacylation were identified when the reaction of FA-Trp-OMe and chymotrypsin was carried out in 65% dimethylsulphoxide at $\leq 0^\circ\text{C}$. (6) No evidence for an intermediate preceding the acyl-enzyme was observed.

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