A ^{13}C -n.m.r. investigation of the ionizations within an inhibitor- α -chymotrypsin complex

Evidence that both α -chymotrypsin and trypsin stabilize a hemiketal oxyanion by similar mechanisms

Michael D. FINUCANE, E. Anne HUDSON and J. Paul G. MALTHOUSE*

Department of Biochemistry, University College Dublin, Belfield, Dublin 4, Ireland

^{13}C -n.m.r. was used to investigate the structure of the inhibitor enzyme complex formed when α -chymotrypsin is alkylated by L-1-chloro-4-phenyl-3-tosylamido-[2- ^{13}C]butan-2-one. Two signals are detected. The one at 204.82 ± 0.11 p.p.m. does not titrate from pH 3 to 9 and is assigned to alkylated methionine-192. The second signal titrates from 99.08 p.p.m. to 103.44 p.p.m. with $\text{p}K_a$ 8.67. This signal is assigned to a tetrahedral adduct formed between the hydroxy group of serine-195 and the inhibitor. The titration shift of the tetrahedral adduct is ascribed to the ionization of the hemiketal hydroxy group. It is proposed that the resulting oxyanion is stabilized by interaction with the imidazolium ion of histidine-57. It is argued that this interaction must raise the $\text{p}K_a$ of at least 70 % of histidine-57 to > 11 . On denaturation/autolysis of the inhibitor-enzyme complex neither of the signals associated with the intact complex is detected, but a new signal is observed that titrates from 203.52 p.p.m. to 206.08 p.p.m. with $\text{p}K_a = 5.27$. This titration shift is assigned to the ionization of the imidazolium ion of alkylated histidine, confirming that the inhibitor has alkylated histidine-57. The significance of these results for the catalytic mechanism of the serine proteinases is discussed.

INTRODUCTION

It is generally accepted that in reactions catalysed by the serine proteinases the formation and breakdown of the acyl-enzyme intermediate proceeds via a tetrahedral intermediate. For natural peptide substrates it is tetrahedral-intermediate formation or breakdown that is thought to be rate-limiting. Kinetic studies with thiono substrates imply that transition-state stabilization of the oxyanion of the tetrahedral intermediate is essential for efficient catalysis (Asbóth & Polgár, 1983). Consequently there is considerable interest in determining how such transition-state stabilization is achieved. However, for optimal catalytic efficiency, intermediates such as tetrahedral intermediates should not accumulate, and so transition-state analogues have been used to investigate their stabilization by the serine proteinases (Wyeth *et al.*, 1980; Lowe & Nurse, 1977; Chen *et al.*, 1979; Malthouse *et al.*, 1983, 1985; Shah *et al.*, 1984; Primrose *et al.*, 1985; McMurray & Dyckes, 1986).

Komiyama & Bender (1979) have argued that catalysis by the serine proteinases could not proceed via a tetrahedral intermediate if the histidine $\text{p}K_a$ remains at 7.0 on tetrahedral-intermediate formation. However, the results obtained by Asbóth & Polgár (1983) suggested that transition-state stabilization of the oxyanion was essential for efficient catalysis in the serine proteinases. Kosiakoff & Spencer (1981) suggested that if an ion-pair interaction occurred between the oxyanion and the imidazolium ion of histidine-57, this could raise the $\text{p}K_a$ of histidine-57 and allow it to act as a general acid catalyst for tetrahedral intermediate breakdown.

Shaw and co-workers have devised substrate-derived chloromethyl ketone inhibitors that specifically inhibit trypsin (Shaw & Springhorn, 1967; Coggins *et al.*, 1974) and chymotrypsin (Ong *et al.*, 1965; Schoellmann & Shaw, 1963) by alkylating N-3 of histidine-57. ^{13}C n.m.r. has been utilized to demonstrate that for trypsin such inhibitors, in addition to alkylating N-3 of histidine-57, also form tetrahedral adducts as a result of addition of the hydroxy group of serine-195 to the inhibitor chloromethyl ketone (Malthouse *et al.*, 1983, 1985; Scott *et al.*, 1986). By examining the pH-dependence of the ^{13}C -n.m.r. signals from Z-[2- ^{13}C]Lys-CH₂Cl and Z-[1- ^{13}C]Lys-CH₂Cl it was deduced (Malthouse *et al.*, 1985; Primrose *et al.*, 1985) that trypsin could stabilize the oxyanion of the tetrahedral adduct by an ion-pair interaction with the imidazolium ion of histidine-57. As a consequence of this ion-pair interaction the $\text{p}K_a$ of at least 50 % of the oxyanion was lowered from ≥ 13.0 to approx. 8 and the $\text{p}K_a$ of at least 50 % of the imidazolium ion of histidine-57 is raised to > 11 .

Since it is the oxyanion and not its conjugate acid that is the catalytically relevant species (Malthouse *et al.*, 1985), then these results demonstrate a mechanism of tetrahedral-adduct stabilization that would allow productive and efficient transition-state stabilization of tetrahedral intermediates by trypsin. Consequently it is important that we establish whether this method of tetrahedral-adduct stabilization is unique to trypsin or whether it is adopted by other serine proteinases. This question is investigated in the present paper by using a specific chloromethyl ketone inhibitor, Tos-[2- ^{13}C]Phe-CH₂Cl, to inhibit α -chymotrypsin.

Abbreviations used: Tos-Phe-CH₂Cl, L-1-chloro-4-phenyl-3-tosylamidobutan-2-one; Z-Lys-CH₂Cl, L-7-amino-3-

* To whom correspondence and requests for reprints should be addressed.

MATERIALS AND METHODS

Materials

α -Chymotrypsin (type II; 3 $\times$ crystallized; salt-free; from bovine pancreas), *N-trans*-cinnamoylimidazole, *N*-3-carboxypropionyl-L-phenylalanine *p*-nitroanilide, phenylmethanesulphonyl fluoride and Tos-Phe-CH₂Cl were obtained from Sigma Chemical Co., Poole, Dorset, U.K. L-[1-¹³C]Phenylalanine (99 atom %) was obtained from MSD Isotopes (U.K. agents: Cambrian Gases, Croydon, Surrey, U.K.). All other chemicals used were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Methods

Synthesis of Tos-[2-¹³C]Phe-CH₂Cl. L-[1-¹³C]Phenylalanine was converted into Tos-[2-¹³C]Phe-CH₂Cl as described by Schoellmann & Shaw (1963). The ¹H-n.m.r. spectrum of Tos-Phe-CH₂Cl synthesized in this way was identical with that reported by Kézdy *et al.* (1967), with the chloromethylene group giving a characteristic singlet at 4.08 p.p.m. in [²H]chloroform. This singlet was split into a doublet ($J = 3.91$ Hz) with Tos-[2-¹³C]Phe-CH₂Cl. The ¹³C-n.m.r. spectrum of Tos-[2-¹³C]Phe-CH₂Cl in [²H]chloroform gave a single signal at 201.64 p.p.m.

Enzyme solutions. Protein concentrations for both native and inhibited α -chymotrypsin were determined spectrophotometrically by using $\epsilon_{280} = 50\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ (Dixon & Neurath, 1957). The amount of active enzyme (73–76%) was determined by titration with *N-trans*-cinnamoylimidazole at pH 5.0 (Schonbaum *et al.*, 1961).

Inhibition of α -chymotrypsin by Tos-[2-¹³C]Phe-CH₂Cl. Tos-[2-¹³C]Phe-CH₂Cl (10 to 45 mM) was dissolved in methanol and portions were added to 100 ml of 0.5 mM active α -chymotrypsin in 0.12 M-sodium phosphate buffer, pH 7.0, to give a final concentration of 1 mM-Tos-[2-¹³C]Phe-CH₂Cl. The reaction mixture was gently stirred. The extent of inhibition was determined by removing samples and assaying with *N*-3-carboxypropionyl-L-phenylalanine *p*-nitroanilide (1 mM) in 0.1 M-sodium phosphate buffer, pH 6.9, at 25 °C. The initial rate of *p*-nitroaniline production was determined at 410 nm by using $\epsilon_{410} = 8800\text{ M}^{-1}\cdot\text{cm}^{-1}$. When the activity of the α -chymotrypsin was diminished to 6% of its original value (Kézdy *et al.*, 1967), 0.17 ml of 100 mM-phenylmethanesulphonyl fluoride in propan-2-ol was added and the solution was concentrated by ultrafiltration on an Amicon PM10 membrane. This concentrated solution (approx. 15 ml) was then dialysed against 3 $\times$ 1 litre of 1 mM-HCl to remove low- M_r materials. The inhibited chymotrypsin retained 0.66% of its activity towards *N*-3-carboxypropionyl-L-phenylalanine *p*-nitroanilide after this procedure.

pH titrations and analysis of pH-dependent changes in chemical shift. These were carried out as described by Malthouse *et al.* (1985).

N.m.r. spectra. Spectra were recorded by using a Bruker WP80 wide-bore spectrometer at 20.115 MHz for ¹³C nuclei. Sample size was 7–8 ml with 20 mm-diameter sample tubes. The pulse sequence of Belton *et al.* (1985) was used to minimize acoustic ringing. Spectral condi-

tions were: 4000 time-domain data points; 0.46 s acquisition time; spectral width 220 p.p.m.; 2–5 Hz exponential weighting factor; 0.5 s relaxation delay time; approx. 1 W broadband decoupling. ²H₂O (20%, v/v) was incorporated into all samples in order to obtain a deuterium lock signal. Samples also routinely contained 0.4 M-KCl to minimize the dimerization of α -chymotrypsin (see the Results section), as well as 18 mM-Tris and 24 mM-glycine to minimize pH changes at alkaline pH values.

Referencing of n.m.r. signals. All ¹³C chemical shifts in aqueous media are quoted relative to tetramethylsilane at 0 p.p.m. with 1,4-dioxan as a secondary standard. Internal dioxan (1–5%, v/v) was at 67.40 p.p.m. and external dioxan (100%, v/v) in a coaxial insert was at 67.16 p.p.m. if no correction for magnetic susceptibility was made. [2-¹³C]Glycine at a concentration of 2 mM was also used as an internal standard [δ_1 (low pH) = 42.44 ± 0.01 p.p.m., δ_2 (high pH) = 45.54 ± 0.09 p.p.m., $pK_a = 9.86 \pm 0.04$].

RESULTS

Tos-[2-¹³C]Phe-CH₂Cl gave a single resonance at 201.64 p.p.m. in [²H]chloroform and at 200.13 p.p.m. in [²H]₆dimethyl sulphoxide. Because of the low solubility of Tos-Phe-CH₂Cl, spectra could not be obtained in fully aqueous media. However, in 50% (v/v) [²H]₆-dimethyl sulphoxide two signals were detected, one at 203.65 p.p.m. and the other at 96.62 p.p.m., which were assigned to the ketone and its hydrate respectively. Similar signals have been observed for Z-[2-¹³C]-Lys-CH₂Cl (205.7 p.p.m.) and its hydrate (96.4 p.p.m.) in aqueous solutions (Malthouse *et al.*, 1983).

Alkylation of α -chymotrypsin with Tos-[2-¹³C]Phe-CH₂Cl

α -Chymotrypsin was alkylated with Tos[2-¹³C]Phe-CH₂Cl and separated from low- M_r materials by dialysis as described in the Materials and methods section. The ¹³C-n.m.r. spectrum of Tos-[2-¹³C]Phe-CH₂Cl-inhibited α -chymotrypsin contained two new signals at pH 4.1, one at 204.8 p.p.m. and the other at 99.0 p.p.m. (Fig. 1b), which are not present in the spectrum of α -chymotrypsin (Fig. 1a). The spin-lattice relaxation time (T_1) of the signal at 99.0 p.p.m. and the protein carbonyl carbon atoms (170–180 p.p.m.) was much shorter ($T_1 = 0.59 \pm 0.02$ s) than that for Tos-[2-¹³C]Phe-CH₂Cl in 50% (v/v) [²H]₆dimethyl sulphoxide ($T_1 = 4.8 \pm 0.02$ s), as expected for ¹³C nuclei rigidly attached to a protein (Malthouse, 1986).

Effect of pH on the ¹³C signals from the Tos-[2-¹³C]Phe-CH₂- α -chymotrypsin complex

When the pH was raised from 4 to 7.6, there was a progressive reversible decrease in signal intensity, resulting in no signals being detected at pH > 7.6. Neet & Brydon (1970) have shown that although the aggregation of α -chymotrypsin is prevented when it is alkylated with Tos-Phe-CH₂Cl there is a progressive increase in aggregation of the alkylated enzyme as the pH is raised. Therefore 0.4 M-KCl was incorporated into samples to prevent aggregation during pH studies.

The chemical shift of the signal at 204.82 ± 0.11 p.p.m. (Fig. 1b) did not titrate over the pH range 3–9.0 (Fig. 3). The chemical shift of the signal at 99.0 p.p.m. (Fig. 1b), however, increased with increasing pH. This increase in

Fig. 1. ^{13}C -n.m.r. spectra of α -chymotrypsin and Tos-[2- ^{13}C]Phe- CH_2Cl -inhibited chymotrypsin before and after alkaline denaturation/autolysis

Acquisition parameters were as described in the Materials and methods section except that 49 152, 51 201 and 13 472 transients were recorded for spectra (a), (b) and (c) respectively. Sample conditions were: (a) 2.2 mM- α -chymotrypsin, 0.4 M-KCl, pH 4.0; (b) 1.9 mM-Tos-[2- ^{13}C]Phe- CH_2Cl -chymotrypsin complex, pH 4.1; (c) denatured/autolysed Tos-[2- ^{13}C]Phe- CH_2Cl -chymotrypsin complex, same sample as (b) except it had been incubated for 5 h at pH 10.8 before being adjusted to pH 4.0. All samples contained 20% (v/v) $^2\text{H}_2\text{O}$. Sample temperature was 26 $^\circ\text{C}$.

Fig. 2. pH titration of the signal at 99.0 p.p.m. to 103.4 p.p.m. in the intact Tos-[2- ^{13}C]Phe- CH_2Cl -chymotrypsin complex

Acquisition parameters were as described in the Materials and methods section with 4096 transients being recorded per spectrum. Sample conditions were: 1.8 mM-Tos-[2- ^{13}C]Phe- CH_2Cl -chymotrypsin complex, volume 8 ml, 0.4 M-KCl, 18 mM-Tris, 24 mM-glycine, 2 mM-[2- ^{13}C]glycine, 20% (v/v) $^2\text{H}_2\text{O}$ and sample temperature 26 $^\circ\text{C}$. The continuous line was calculated by using the equation:

$$\delta_{\text{obs.}} = (S_1 + S_2 \cdot K_a/[H]) / (1 + K_a/[H])$$

and the fitted parameters $\text{p}K_a = 8.67 \pm 0.02$, $S_1 = 99.08 \pm 0.01$ p.p.m. and $S_2 = 103.44 \pm 0.02$ p.p.m.

Fig. 3. pH titration of the signals at 203.5 p.p.m. to 206.1 p.p.m. in the intact and denatured/autolysed Tos-[2- ^{13}C]Phe- CH_2Cl -chymotrypsin complex

●, Denatured/autolysed complex. Acquisition parameters were as described in the Materials and methods section except that a relaxation delay of 2 s was used at pH values above 7.0 and 4096 to 65 500 transients were recorded per spectrum. The sample conditions were as described in the legend to Fig. 1(c). The continuous line was calculated as described in the legend to Fig. 2, by using the fitted parameters $\text{p}K_a = 5.27 \pm 0.07$, $S_1 = 203.52 \pm 0.07$ p.p.m. and $S_2 = 206.08 \pm 0.07$ p.p.m. ■, Intact complex. Sample conditions and acquisition parameters were as described in the legend to Fig. 2. The continuous line is the mean of the data points, which was 204.82 ± 0.11 p.p.m.

chemical shift was due to a single ionizing group of $\text{p}K_a = 8.67 \pm 0.02$ (Fig. 2). The sample retained 0.66% catalytic activity and after an hour at pH 10.2, alkaline-denaturation/autolysis resulted in a 50% decrease in signal intensity. The remaining signal, however, titrated as before at lower pH values. A sample of this denatured/autolysed inhibitor-enzyme complex was prepared by adjusting the pH to 10.8 and leaving the sample at this pH for 5 h. After this treatment, no signal was detected in the region 99.0–103.4 p.p.m. even when the pH was lowered to 4.0 (Fig. 1c). The signal at 204.82 ± 0.11 p.p.m. was not present in the denatured/autolysed sample; instead a new signal was detected that had a chemical shift of 203.8 p.p.m. at pH 4.0 (Fig. 1c). This signal did titrate over the pH range 3–9.0 (Fig. 3) with $\text{p}K_a = 5.27 \pm 0.07$.

Assignment of signals

The chemical shift of the signal at 99.0 p.p.m. to 103.4 p.p.m. is characteristic of an sp^3 hybridized carbon atom, and demonstrates that chymotrypsin can stabilize the formation of a tetrahedral adduct with the ^{13}C -enriched carbon atom of Tos-[2- ^{13}C]Phe- CH_2Cl . The fact that this signal is not present in the denatured inhibitor complex (Fig. 1c) demonstrates that only the intact enzyme can stabilize the tetrahedral adduct. We propose that this tetrahedral adduct results from the formation of a hemiketal between the Tos-[2- ^{13}C]Phe- CH_2Cl carbonyl group and the hydroxy group of serine-195 (structures A and B in Scheme 1).

The titration shift of this signal is very similar to that observed for the analogous signal observed in the Z-[2- ^{13}C]Lys- CH_2 -trypsin complex (Table 1), and it is therefore assigned to ionization of the hemiketal

Scheme 1. Structures and chemical shifts of the intact and denatured/autolysed Tos-[2-¹³C]Phe-CH₂-chymotrypsin complexes
R = Tos-NHCH(CH₂C₆H₅). •C, ¹³C.

Table 1. Titration constants obtained by ¹³C-n.m.r. studies of Z-[2-¹³C]Lys-CH₂-trypsin and Tos-[2-¹³C]Phe-CH₂-chymotrypsin complexes

All chemical shifts have been referenced by using glycine as a common internal reference, as described in the Materials and methods section.

Inhibited species	Trypsin inhibited by Z-[2- ¹³ C]Lys-CH ₂ Cl*		α-Chymotrypsin inhibited by Tos-[2- ¹³ C]Phe-CH ₂ Cl	
	Titration shift (p.p.m.)	pK _a	Titration shift (p.p.m.)	pK _a
Intact inhibitor-enzyme complex	98.95 to 103.08	7.88	99.08 to 103.44	8.67
Denatured/autolysed inhibitor-enzyme complex	203.64 to 206.62	5.26	203.52 to 206.08	5.27

* Data from Malthouse *et al.* (1985).

assignment is discussed in detail in the Discussion section.

The chemical-shift values (203.52 p.p.m. to 206.08 p.p.m.) and pK_a value (5.27 ± 0.07) of the signal observed on denaturation (Figs. 1c and 3) are essentially identical with the values obtained on denaturation of the analogous Z-[2-¹³C]Lys-CH₂-trypsin complex (Table 1). As discussed previously (Malthouse *et al.*, 1985), the chemical shift of this signal is characteristic of a ketone and its titration behaviour is that expected for an alkylated imidazole (structures C and D in Scheme 1). This therefore confirms that the inhibitor has alkylated histidine-57.

In addition to alkylating N-3 of histidine-57 in α-chymotrypsin (Ong *et al.*, 1965), Tos-Phe-CH₂Cl has also been shown to alkylate approx. 30% of methionine-192 (Stevenson & Smillie, 1968). The resulting sulphonium ion would not be expected to ionize in the pH range 3–9, as is observed for the signal at 204.82 ± 0.11 p.p.m. in the intact inhibitor-enzyme complex (Fig. 3). Therefore this signal is assigned to the species resulting from alkylation of methionine-192 by

Tos-[2-¹³C]Phe-CH₂Cl (structures A and B in Scheme 1). Naider & Bohak (1972) have shown that at alkaline pH values thiols can be used to regenerate methionine after it has been converted into the corresponding sulphonium ion by alkylation with iodoacetate, iodoacetamide and phenylacetyl bromide. They have also shown that other nucleophiles such as phenol, hydroxylamine and hydrazine can also regenerate methionine from its sulphonium salt, albeit less effectively than with thiols. We therefore propose that the failure to observe the signal at 204.82 p.p.m. in denatured samples is due to breakdown of the sulphonium ion at alkaline pH values catalysed by the autolysis products of α-chymotrypsin. The low solubility of free Tos-Phe-CH₂Cl precludes its detection in aqueous solution.

DISCUSSION

The pK_a of 8.67 obtained from the titration shift of the signal at 99.0 p.p.m. to 103.4 p.p.m. (Fig. 2) is very similar to the pK_a of approx. 8.5 associated with the conformational change that inactivates chymotrypsin

(Oppenheimer *et al.*, 1966; Himoe *et al.*, 1967; McConn *et al.*, 1969; Ghélis *et al.*, 1970; Fersht & Requena, 1971). The conformational change results from the ionization of the isoleucine-16/aspartate-194 ion-pair that inactivates chymotrypsin as the carboxy group of aspartate-194 moves and protrudes into the active site, preventing substrate binding (Sigler *et al.*, 1968).

The binding of ligands such as indole raises the pK_a of the isoleucine-16/aspartate-194 ion-pair to approx. 10.5 (Garel & Labouesse, 1970), whereas chemical modification of the hydroxy group of serine-195 by groups such as di-isopropyl phosphorofluoridate prevents the conformational change by raising the pK_a of the ion-pair to > 11 (Oppenheimer *et al.*, 1966; Sigler *et al.*, 1968; McConn *et al.*, 1969). Therefore we would expect Tos-Phe-CH₂Cl-inhibited α -chymotrypsin to be locked in the 'active conformation' at least up to pH 11. This prediction is supported by the results reported by Hess *et al.* (1970), who studied the intrinsic tryptophan fluorescence of chymotrypsin. They found that the fluorescence of chymotrypsin depended on two groups, one of pK_a approx. 6.0 and the other of pK_a approx. 8.5. The pK_a of 8.5 reflects the conformational change of chymotrypsin and the pK_a of approx. 6.0 is due to histidine-40 (Hess *et al.*, 1970; Vandlen & Tulinsky, 1973). With Tos-Phe-CH₂Cl-inhibited chymotrypsin the pK_a of approx. 6.0 was unchanged but the second pK_a was not detected up to pH 11. Therefore the pK_a of the isoleucine-16/aspartate-194 ion-pair is greater than 11 in Tos-Phe-CH₂Cl-inhibited chymotrypsin and the pK_a of 8.67 cannot be assigned to the conformational change resulting from the ionization of this ion-pair. Since ionization of the imidazolium ion of histidine-57 should produce a titration shift of no more than 0.6 p.p.m. (Malthouse *et al.*, 1985), we conclude that the titration shift of 4.36 p.p.m. cannot be solely ascribed to the ionization of this imidazolium ion (pK_1 ; Scheme 2), but must also reflect oxyanion formation (pK_2 ; Scheme 2).

Robillard & Shulman (1974*a,b*) have examined the effect of pH on the signal due to the N-1 hydrogen-bonded proton of histidine-57 in chymotrypsin. With negatively charged boronic acid inhibitors, they found that the pK_a was raised to > 9.5 . However, when chymotrypsin was alkylated with Z-Gly-Leu-Phe-CH₂Cl the signal titrated with a pK_a of 8.4, but the titration shift was only one-third of that observed with the free enzyme. (N.B. They also reported that similar results were obtained with Tos-Phe-CH₂Cl-inhibited chymotrypsin.) The diminished titration shift for the hydrogen-bonded proton (Robillard & Shulman, 1974*a*) could result from (a) approx. 30% of histidine-57 having pK_a 8.4 (pK_1 ; Scheme 2), (b) the effect of oxyanion formation (pK_2 ; Scheme 2) on the chemical shift of the hydrogen-bonded proton, or (c) a combination of (a) and (b). Thus we may conclude that no more than 30% histidine-57 has pK_a 8.4. Robillard & Shulman (1974*a*) observed no additional titration shift at pH values above 9.8, suggesting that at least 70% of histidine-57 has $pK_a > 9.8$ in the Tos-Phe-CH₂-chymotrypsin complex. Assuming that the ionization of histidine-57 produces a 'normal' titration shift (0.6 p.p.m.) at the hemiketal carbon atom, we could deduce that no more than 14% of the observed oxyanion titration shift (4.36 p.p.m.) is due to the ionization of histidine-57 (pK_1 ; Scheme 1). However, this assumption is not valid, as the observed titration shifts within a system of two or more interacting groups are not

Scheme 2. Ionizations of the intact Tos-[2-¹³C]Phe-CH₂-chymotrypsin complex

necessarily the same as for either group in isolation. This is exemplified by the titration shifts of the α -methylene carbon atom in Z-[1-¹³C]Lys-CH₂-trypsin complexes (Primrose *et al.*, 1985). Liang & Abeles (1987) have measured titration shifts of 4.3 and 4.4 p.p.m. for oxyanion formation in model hemiketals. These titration shifts are very similar to the oxyanion titration shifts of 4.36 p.p.m. observed in the present study with chymotrypsin and to that of 4.13 p.p.m. observed with trypsin (Malthouse *et al.*, 1985). However, a full oxyanion titration shift should only occur if there is perfect tetrahedral symmetry, which can only be assumed if the quaternary carbon atom has four identical ligands (Batchelor *et al.*, 1975). Consequently, a full oxyanion titration shift for a quaternary hemiketal carbon atom with perfect tetrahedral symmetry may be > 4.4 . Also, a 'normal' oxyanion titration shift at the hemiketal carbon atom cannot be assumed if the oxyanion interacts with the imidazolium ion of histidine-57. Therefore we conclude that at least 70% of the oxyanion has pK_a 8.67 and at least 70% of histidine-57 has a $pK_a > 9.8$ in the Tos-Phe-CH₂-chymotrypsin complex.

Therefore both α -chymotrypsin and trypsin are able to stabilize oxyanion formation by means of ion-pair formation with the imidazolium ion of histidine-57, which results in the pK_a of at least 50% of histidine-57 being raised to > 11 . The fact that both enzymes have this capability is consistent with this being a general feature of the catalytic mechanism of the serine proteinases that provides stabilization of the tetrahedral intermediate as well as facilitating its breakdown to products by enabling histidine-57 to act as a general acid catalyst for leaving-group expulsion.

Liang & Abeles (1987) have observed by ¹³C n.m.r. a tetrahedral adduct at 104 p.p.m. when Ac-Leu-[1-¹³C]-Phe-CF₃ is bound by chymotrypsin. The signal at 104 p.p.m. was assigned to a zwitterionic tetrahedral adduct analogous to that observed in chloromethyl ketone inhibitor-enzyme complexes except that histidine-57 was not alkylated. The chemical shift of this signal did not change from pH 4.8 to 9.9, although there was a decrease in intensity of the ¹⁹F signal of the trifluoromethyl group with pK_a 4.9. They argue that the signal at 104 p.p.m. should be assigned to the oxyanion of the ionized hemiketal on the basis of its chemical shift and because of the fact that a deuterium isotope shift of 0.065 p.p.m., due to a hemiketal hydroxy group, was not

observed when solutions of enzyme in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ were compared. It is difficult to dismiss the possibility that such a small difference in chemical shift could be masked by small changes in chemical shift resulting from, for example, small conformational changes occurring when the enzyme is in $^2\text{H}_2\text{O}$ as opposed to $^1\text{H}_2\text{O}$. This, coupled with the failure to detect any change in chemical shift consistent with oxyanion formation, leads us to conclude that the evidence for this species being due to an ionized hemiketal is not definitive. However, since in the present work we have demonstrated that chymotrypsin can stabilize oxyanion formation, and since the chemical shift of the ionized hemiketal carbon atom is very similar (103.44 p.p.m.) to that observed by Liang & Abeles (1987), we conclude that they were observing an ionized hemiketal with $\text{p}K_a \leq 4.8$ and a chemical shift of 104 p.p.m. Liang & Abeles (1987) estimated that the oxyanion in their inhibitor–enzyme complex should have $\text{p}K_a$ 6.1 if it is stabilized to the same extent as the oxyanion in the chloromethyl ketone inhibitor–trypsin complex. Since a titration shift with $\text{p}K_a$ 6.1 was not observed, they concluded that this may result from the interaction of the oxyanion and the oxyanion hole not being optimal in chloromethyl ketone inhibitor–enzyme complexes as a result of the alkylation of histidine-57. As we have now measured the $\text{p}K_a$ of the oxyanion in the Tos-[1- ^{13}C]Phe-CH₂-chymotrypsin complex, we can estimate the effect of replacing the -CH₂ImH⁺ moiety by -CF₃ by using eqn. (1) (De Tar, 1982)

$$\text{p}K_a = -7.39 \sum \sigma_i + 15.472 \quad (1)$$

and the difference in the substituent constants for these groups [-CF₃, $\sigma_i = 0.42$ (Exner, 1978), and -CH₂ImH⁺, $\sigma_i = 0.24$ (Malthouse *et al.*, 1985)]. This calculation predicts that replacing -CF₃ by -CH₂ImH⁺ should raise the $\text{p}K_a$ of their model compound from 9.1 to 10.4, which is very similar to the $\text{p}K_a$ of 10.7 calculated for the oxyanion $\text{p}K_a$ in an analogous Z-Lys-CH₂-imidazole model compound (Malthouse *et al.*, 1985). We therefore estimate that in the Tos-Phe-CH₂-chymotrypsin complex the oxyanion $\text{p}K_a$ is only lowered by approx. 1.7 pH units (10.4–8.67 pH units), and so it would appear that alkylation of histidine-57 does result in non-optimal oxyanion stabilization in chymotrypsin, though this effect is less in the chloromethyl ketone inhibitor–trypsin complex, where the oxyanion $\text{p}K_a$ is lowered by approx. 3 pH units. Liang & Abeles (1987) have shown that even when the oxyanion $\text{p}K_a$ is lowered to 4.8 histidine-57 remains protonated up to pH 9.5. If a similar effect applies with catalytic tetrahedral intermediates, this would allow general acid catalysis of leaving-group expulsion even when the oxyanion $\text{p}K_a$ is lowered by at least 4 pH units.

The substituent constants for -CH₂ImH⁺ ($\sigma_i = 0.24$), -OR [$\sigma_i = 0.27$ (Exner, 1978)] and -NHR [$\sigma_i = 0.17$ to 0.26 (Exner, 1978)] are similar, hence the oxyanions of tetrahedral intermediates formed from amide or ester model compounds should have similar $\text{p}K_a$ values (10.2 to 10.9) to those of alkylated imidazoles. However, with natural peptide substrates, once a tetrahedral intermediate is fully formed the $\text{p}K_a$ of the nitrogen atom of the peptide bond should be similar to that of the parent amine (Satterthwait & Jencks, 1974; Komiyama & Bender, 1979), and so it should be protonated at pH < 10. Protonation should lower the $\text{p}K_a$ of the oxyanion

by 2.6 pH units [-NHR, $\sigma_i = 0.6$ (Exner, 1978); $\Delta\sigma_i = 0.6 - 0.25$] to a value of approx. 8 in model compounds. As we have already seen (owing presumably to differences in solvation and effective dielectric constants), the oxyanion $\text{p}K_a$ may be lowered even further for an enzyme tetrahedral intermediate. Therefore the oxyanion $\text{p}K_a$ is expected to be lowered even if the leaving-group protonation has resulted in deprotonation of histidine-57.

It is widely accepted (Kraut, 1977; Polgár & Halász, 1982) that the oxyanion of a tetrahedral intermediate is stabilized by hydrogen-bonding to the backbone NH of glycine-193 and serine-195 in chymotrypsin, as originally suggested by Henderson (1970). Site-directed mutagenesis experiments with subtilisin (Wells *et al.*, 1986; Bryan *et al.*, 1986; Wells & Estell, 1988) have shown the importance of hydrogen-bonding to the oxyanion, as loss of one hydrogen bond destabilizes oxyanion formation by as much as 20 kJ·mol⁻¹. Since water is an excellent hydrogen-bond donor–acceptor, it is unlikely that oxyanion stabilization is more effective in the oxyanion hole than in fully aqueous media. Though, if the conjugate acid of the oxyanion was less stabilized by hydrogen-bonding than the oxyanion, then this could provide a mechanism for lowering the $\text{p}K_a$ of the oxyanion. However, we suggest that it is more likely that the electrostatic interaction between the imidazolium ion and the oxyanion is the major factor in raising the $\text{p}K_a$ of histidine-57 and lowering the $\text{p}K_a$ of the oxyanion.

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