Pig pancreatic anhydro-elastase

Role of the serine-195 hydroxy group in the binding of inhibitors and substrate

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The binding constants of a number of ligands were measured for pancreatic elastase (PE) and anhydro-elastase (AE) in order to assess the contribution of Ser-195 to substrate and inhibitor binding by PE. AE was purified by affinity chromatography on a column containing immobilized turkey ovomucoid inhibitor. The AE had $0.1 \pm 0.1\%$ of the activity of the native enzyme and contained 0.8 ± 0.06 residue of dehydroalanine per molecule. A difference electron-density map, derived from an X-ray crystallographic analysis of AE, showed that the modified residue was Ser-195. The complexing of 3-carboxypropionyl-Ala-Ala-Ala-*p*-nitroanilide (SAN) to the active site of AE was also demonstrated by X-ray-diffraction analysis of an AE crystal soaked overnight with substrate. The nitroanilide moiety was not observed in the difference map. AE was shown to bind turkey ovomucoid inhibitor with a dissociation constant (K_d) of $0.3 \pm 0.06 \mu\text{M}$ compared with $0.10 \mu\text{M}$ for PE. The K_d of the AE-SAN complex (0.2 mM) was comparable with the Michaelis constant for SAN with PE (1.0 mM). A number of inhibitors, such as elastatinal, which forms a hemiketal adduct with PE, while others such as the β -lactams, which function as acylators of the active-site serine residue, bound AE with a lower affinity than to PE. The binding of a peptidylchloromethane (acetyl-Ala-Ala-Pro-Ala-CH₂Cl) to AE occurs without evidence for alkylation of histidine. The binding constants for benzoisothiazolinone and 3,4-dichloroisocoumarin to PE differed from their binding constants to AE by less than a factor of 4.0-fold. The contribution of the hydroxy group of Ser-195 to the binding of these inhibitors to PE in their non-covalent complexes is relatively small, even though they inactivate PE by an acylation mechanism. These results suggest that the hydroxy group on Ser-195 in PE is of secondary importance in the energetics of ligand binding, in contrast with its essential role in the catalytic properties of the enzyme.

INTRODUCTION

Human polymorphonuclear leucocytes contain an endopeptidase capable of degrading elastin as well as a variety of other connective-tissue proteins (Stein *et al.*, 1985). This enzyme, commonly referred to as human leucocyte elastase (EC 3.4.21.37), has long been considered a potential therapeutic drug target because of its involvement in inflammatory tissue injury (Janoff, 1972). A number of investigators (Powers & Tuhy, 1973; Doherty *et al.*, 1986; Hassal *et al.*, 1979) have synthesized inhibitors of human leucocyte elastase that may have potential usefulness for treatment of emphysema, adult respiratory-distress syndrome and other related diseases (Stein *et al.*, 1985). Pig pancreatic elastase (PE, EC 3.4.21.36), the best characterized of all elastases, shares a number of properties with human leucocyte elastase and has often been used as a model of that enzyme for the study of the active-site binding by human leucocyte elastase (e.g. see Hassal *et al.*, 1979; Yoshimura *et al.*, 1982; Meyer *et al.*, 1985). In the mechanism of action of the serine proteinases, Ser-195 plays a double role as part of the catalytic machinery of the enzyme and as a component of the binding site for substrate and inhibitors. The relative importance of the latter is difficult to analyse in the framework of the intact enzyme where catalysis is taking place. We have prepared a chemically

modified PE, anhydro-elastase (AE), in which Ser-195 has been converted into dehydroalanine, in an effort to clarify this issue.

Weiner *et al.* (1966) first demonstrated that catalytically inactive anhydro-chymotrypsin retained its ability to bind both the substrate *N*-benzoyl-L-phenylalanine and the inhibitor proflavin. Subsequent studies by Feinstein & Feeney (1966) and Ako *et al.* (1974) showed that anhydro-chymotrypsin, as well as anhydro-trypsin, could bind naturally occurring inhibitors such as lima-bean and soya-bean trypsin inhibitors with affinities similar to those of the native enzymes. Anhydro-chymotrypsin and chymotrypsin were shown to bind these reversible protein inhibitors rapidly and stoichiometrically with a dynamic exchange, indicating that Ser-195 did not significantly contribute to the free energy of complex-formation (Ako *et al.*, 1974). However, Nishikata (1983) using anhydro-chymotrypsin and Tsai & Bender (1984) using anhydro-elastase demonstrated a large decrease in affinities for small synthetic inhibitors such as chymostatin analogues and arylboronic acids respectively.

In the present studies we have examined the binding and inhibition of several ligands to AE and PE. In addition, we have used X-ray crystallography to show the binding of a PE substrate to the active site of AE. The data suggest that the contribution of the hydroxy group

Abbreviations used: PE, pancreatic elastase; AE, anhydro-elastase; SAN, 3-carboxypropionyl-Ala-Ala-Ala-*p*-nitroanilide; CK, acetyl-Ala-Ala-Pro-Ala-CH₂Cl.

of Ser-195 in ligand binding depends on the nature of the ligands as well as the subsequent reactions with the enzyme.

MATERIALS AND METHODS

Materials

'Trypsin 1:300' was obtained from United States Biochemical Corp., Cleveland, OH, U.S.A.; SAN, phenylmethanesulphonyl fluoride, di-isopropyl phosphorofluoridate and α_1 proteinase inhibitor were obtained from Sigma Chemical Co.; turkey ovomucoid inhibitor was from Worthington Biochemical Corp.; CK was from Enzyme Systems Products, Livermore, CA, U.S.A.; elastatinal was from Peninsula Laboratories, Belmont, CA, U.S.A. Elastase inactivators 2-(2-ethylbutyryl)-1,2-benzisothiazolin-3-one and 3-acetoxymethyl-7 α -methoxy-3-cephem-4-carboxylate 1,1-dioxide t-butyl ester were obtained from Dr. M. Zimmerman and Dr. J. B. Doherty of this Institute. 3,4-Dichloroisocoumarin was generously given by Dr. James C. Powers of the Georgia Institute of Technology, Atlanta, GA, U.S.A. All other reagents were of analytical grade.

Preparation of AE

Pig PE was purified from 'Trypsin 1:300' according to the procedure described by Shotton (1970). AE was prepared by alkali treatment of phenylmethanesulphonyl fluoride-treated PE as described by Ako *et al.* (1972a,b), and isolated by affinity chromatography on a turkey ovomucoid inhibitor-Sepharose column essentially as described by Murphy *et al.* (1982) except that elution was with an acetic acid gradient from 0 to 0.1 M. Protein fractions bound to and eluted from the affinity column that had negligible enzyme activity and high dehydroalanine content were used as AE. Dehydroalanine content of the protein were determined by the method of Weiner *et al.* (1966). Elastase activity was assayed by the spectrophotometric method of Bieth *et al.* (1974), with SAN as substrate.

Crystallization of PE and AE

Diffraction-quality crystals of native PE and AE measuring approx. 0.2 mm³ were grown at room temperature by the hanging-drop vapour-diffusion method described by McPherson (1982). The conditions used were similar to those reported by Shotton *et al.* (1971), except that we used 6.0 mM-Na₂SO₄ in 0.1 M-sodium acetate buffer, pH 5.0, in 10 μ l droplets of enzyme solution at a concentration of 15.0 mg/ml. The plastic coverslips containing enzyme drops were suspended over wells containing 0.1 M-Na₂SO₄ in 0.1 M-acetate buffer at pH 5. For X-ray-diffraction analysis of the AE complexes, SAN dissolved in dimethyl sulphoxide was added directly to hanging drops containing crystals to a final concentration of 1.0 mM. After overnight soaking, crystals were mounted in sealed glass capillaries for data collection.

Enzymic activity of crystalline PE and AE

Evaluation of PE and AE crystals for enzymic activity was performed by incubating crystals at room temperature in 0.5 ml of 0.1 M-sodium acetate buffer, pH 5.0, containing 0.2 mM-SAN (125 nmol). Under these conditions the crystals remained intact. A PE crystal

completely hydrolysed 125 nmol of SAN within 24 h. An AE crystal of similar size hydrolysed 6.3 nmol of SAN after 72 h, but hydrolysed only 0.3 nmol of SAN after 72 h when 5 mM-di-isopropyl phosphorofluoridate was present.

Equilibrium binding assay

In order to measure AE binding by turkey ovomucoid inhibitor, α_1 proteinase inhibitor, phenylmethanesulphonyl fluoride, 2-(2-ethylbutyryl)-1,2-benzisothiazol-3-one and 3,4-dichloroisocoumarin, an indirect kinetic estimate of the dissociation constant was made. Each of these inhibitors was preincubated at 25 °C in 0.5 ml of 0.1 M-Tris/HCl buffer, pH 8.0, containing 90 mM-NaCl for 5 min with different amounts of AE, then further incubated for 3 min with 2.5 μ g of PE (0.1 nmol). Substrate was then added and the initial rates of substrate cleavage were measured. The rates of cleavage without PE or with and without AE were negligible. Since most of these inhibitors were tightly bound to PE (see Table 1), the concentration of free ligand [L] could be calculated from the expression:

$$[L] = [PE]_0[1 - (V/V_0)]$$

where $[PE]_0$ is the concentration of PE (100 nM here), V is the rate of SAN cleavage with ligand and V_0 is the rate of SAN cleavage in the absence of ligand. The dissociation constant for the AE-ligand complex was calculated for each concentration of AE from the expression:

$$K_{AE} = [L]\{[AE]_0 - ([L]_0 - [L])\}/([L]_0 - [L])$$

where K_{AE} is the dissociation constant for L, $[L]_0$ is the total ligand concentration and $[AE]_0$ is the total AE concentration. This calculation assumed (1) that 1 mol of inhibitor bound to 1 mol of AE, with a single dissociation constant K_{AE} , (2) that the binding of L to PE did not perturb the ratio of ligand-free and ligand-bound AE, and (3) that the binding of L to AE was unchanged over the PE assay period. The reproducibility of K_{AE} , typically $\pm 25\%$ for a 5-fold range in AE, suggested that these assumptions are valid.

Measurement of SAN binding by fluorescence quenching and u.v. difference spectra

The fluorescence spectra of AE and AE complexed with SAN were measured at an excitation wavelength of 280 nm and emission spectra from 290 nm to 450 nm according to the method of Lehrer & Fasman (1966).

All difference u.v. absorption spectra were measured at 25 °C by the method of Dahlquist *et al.* (1966) with a pair of split cells in a Beckman 35K spectrophotometer attached to a Bascom-Turner 8210 electronic recorder (Newton, MA, U.S.A.). A base-line was first recorded in a memory by placing samples of protein and ligand respectively in the protein and the ligand compartments of the sample cell and the reference cell. Measurement of difference spectra commenced after mixing the contents of the two compartments in the sample cell. The base-line was then subtracted from the difference spectra. Dissociation constants for binding of ligands to AE were determined from u.v. difference spectra according to the following equation:

$$\log K_d = \log([P]/[PL]) + \log[L]$$

where K_d is the dissociation constant, concentration of

free ligands $[L]$ is $[L_t] - [PL]$, $[L_t]$ is total ligand concentration, $[PL]$ is the concentration of the protein-ligand complex and $[P]$ is the concentration of free protein. The ratio of the peak height ($\Delta A_{\max}$) in the difference spectra at saturation of ligand to AE to the peak height (ΔA) at some other ligand concentration is a measure of $[PL]$ at that ligand concentration:

$$\Delta A / \Delta A_{\max} = [PL] / [P_t]$$

with $[P_t]$ being the total protein concentration. A plot of $\log[L]$ versus $\log([PL]/[P])$ gives a line with an intercept of $-\log K_d$. The AE concentration used was $14.6 \mu\text{M}$. $[L_t]$ values used were from $55.0 \mu\text{M}$ to $247.5 \mu\text{M}$, in 0.1 M -sodium phosphate buffer, pH 7.2, containing 50 mM -NaCl.

Miscellaneous analyses

Analytical polyacrylamide-disc-gel electrophoresis was done at pH 4.5 in 7.5 % polyacrylamide gels by the procedure of Reisfeld *et al.* (1962). Amino acid analysis of elastase was performed before and after incubation with a 5-fold molar excess of acetyl-Ala-Ala-Pro-Ala-CH₂Cl at 25°C in 0.1 M -sodium phosphate buffer, pH 7.2. Samples were dialysed extensively against water at 4°C and hydrolysed for 22 h in 6 M -HCl at 105°C . Quantitative amino acid analysis was performed on a Beckman 121 MB amino acid analyser.

X-ray data collection

X-ray diffraction data to 0.25 nm ($2.5 \text{ \AA}$) resolution were collected in shells from one large crystal by using a Syntex/Nicolet P2, diffractometer fitted with an extended (65 mm) arm and helium beam tunnel. A modified peak-top omega scan (Sparks, 1982) of 0.25° was used to measure reflexion intensities at 0.5 to $2.0^\circ/\text{min}$, depending on the resolution. Background intensity was measured on both sides of the peak, at 0.75° from the centre, for one-quarter the time of the peak scan. Ten check reflexions scattered throughout reciprocal space were routinely monitored during data collection. Empirical absorption (North *et al.*, 1967), linear radiation decay and Lorentz polarization corrections were applied to the data (Saper, 1983). All reflexions with intensity $I > \sigma(I)$ were included. Scaling, structure factor and electron-density calculations were done with the PROTEIN (Steigemann, 1974) structure-analysis package of programs. Starting phases for this study were calculated from the 0.25 nm pig PE co-ordinates reported by Sawyer *et al.* (1978) as deposited in the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977). Substrate models were fit to the experimental density by using the program FRODO (Jones, 1978, 1982) as modified by Bush (1984), on an Evans and Sutherland Multi-Picture System attached to a VAX 11/780 computer.

RESULTS

Properties of AE

An elution profile of porcine PE, inactivated with phenylmethanesulphonyl fluoride, followed by treatment with 0.1 M -NaOH, from a turkey ovomucoid inhibitor-Sepharose column is shown in Fig. 1. Fraction III, which showed less than 0.1% of native activity with SAN and contained $0.8 \pm 0.06 \text{ mol}$ of dehydroalanine per mol of elastase, was used for the present studies. Fraction II

Fig. 1. Affinity chromatography of crude AE (alkali-treated phenylmethanesulphonyl fluoride-treated PE) on a turkey ovomucoid inhibitor-Sepharose column

Crude AE (157 mg) was dissolved in 58 ml of 50 mM -Tris/HCl buffer, pH 8.0, containing 0.2 M -KCl, treated with 10 mM -di-isopropyl phosphorofluoridate and then applied to a $5 \text{ cm} \times 23 \text{ cm}$ column of turkey ovomucoid inhibitor-Sepharose. The column was first eluted with 50 mM -Tris/HCl buffer, pH 8.0, and then at fraction 220 (arrowed) a linear gradient of 0 – 0.1 M -acetic acid was used to elute the column. Fractions (3.3 ml) were collected into tubes each containing 2.0 ml of 2.0 M -sodium acetate at a flow rate of 1.0 ml/min at 4°C . Absorbance at 280 nm was determined for each fraction. Fractions were pooled as indicated in the Figure, concentrated by pressure dialysis and analysed for dehydroalanine content and PE activity.

contained only about 0.2% of native activity, but its elution from turkey ovomucoid inhibitor-Sepharose and its behaviour in polyacrylamide-gel electrophoresis differs from that of fraction III, probably owing to

Fig. 2. Section of the difference electron-density map of AE versus native PE

Negative residual density is shown superimposed on the amino acids participating in substrate catalysis as well as a disulphide located near the active site of the enzyme. Model co-ordinates are from the Brookhaven Protein Data Bank (Bernstein *et al.*, 1977) as described by Sawyer *et al.* (1978).

Fig. 3. Difference electron-density map at 0.25 nm (2.5 Å) resolution of an AE crystal soaked in a 1.0 mM solution of SAN versus AE

Stereo view of the active-site region of AE shows positive electron density corresponding to SAN. Several amino acid residues are identified for reference.

additional structural alterations during base-elimination beyond the conversion of Ser-195 into dehydroalanine.

AE crystals were analysed by X-ray diffraction and found to be isomorphous with crystals of native PE. A

difference electron-density map of AE (Fig. 2) revealed the absence of both the hydroxy group of Ser-195 and an SO_4^{2-} ion, designated SU-2, which had been observed in the active site of native PE (Sawyer *et al.*, 1978). Some

Fig. 4. Effects of SAN on intrinsic fluorescence of AE and on difference u.v. absorption of AE

(a) The concentration of AE was $28.7 \mu\text{M}$ in 20 mM-Tris/HCl buffer, pH 7.3, containing 90 mM-NaCl. Excitation was at 280 nm and emission spectra from 290 nm to 450 nm were determined in the buffer at 22 °C. Spectrum 1, AE alone. Spectra 2–6, AE in the presence of the following concentrations of the ligand: $27.7 \mu\text{M}$ (2), $55.4 \mu\text{M}$ (3), $83.1 \mu\text{M}$ (4), $110.9 \mu\text{M}$ (5) and $138.6 \mu\text{M}$ (6). SAN was added as a solution in dimethyl sulphoxide. The effects of solvent and protein absorption on the fluorescence spectra were small and were corrected for by using solvent without ligand. The concentration of the ligand to generate the maximum quenching was derived from extrapolation of a curve of ligand-concentration-dependent quenching. (b) AE ($14.6 \mu\text{M}$) was mixed with SAN in 0.1 M-phosphate buffer, pH 6.8, at 25 °C at the concentrations indicated by using a split cell with the same reactants on opposite sides. (i) $247.6 \mu\text{M}$ -SAN; (ii) $192.5 \mu\text{M}$ -SAN; (iii) $165.0 \mu\text{M}$ -SAN; (iv) $110.0 \mu\text{M}$ -SAN; (v) $55.0 \mu\text{M}$ -SAN; (vi) AE alone. The maximum difference at 275 nm (ΔA_{max}) was used for the calculation of the extent of ligand binding. The ligand concentration that begins to show a constant magnitude of the difference spectra was derived from extrapolation of the concentration-dependent ΔA plot.

negative density in the region of Cys-58 may be due to its conversion into dehydroalanine as a result of alkaline treatment (Jones *et al.*, 1983). No other significant features were observed in this Fourier difference map, suggesting that any other structural alterations in fraction III of AE caused by the chemical modification were negligible.

Difference electron-density maps at 0.25 nm resolution of AE crystals complexed with the substrate SAN show strong density in the active-site region of AE (Fig. 3). The C-terminus of the substrate is found near dehydroalanine-195. The side chain of the P₁ alanine residue of the substrate fits the S₁ specificity pocket. P₂ and P₃ alanine residues follow along the polypeptide backbone of the enzyme at residues Phe-215 and Val-216, all as expected from previous studies of peptidyl inhibitors (see, e.g., James *et al.*, 1980). Finally, the N-terminus of the substrate ends in the vicinity of Trp-172 of the enzyme. This latter observation may explain the quenching of fluorescence in AE that results from binding of substrate (Fig. 4a). Positive electron density in the vicinity of Trp-172 indicates a slight shift from its original position on substrate binding. However, no negative electron density is observed. Also, no electron density is observed corresponding to the *p*-nitroanilide group in the S₁' region of the enzyme. This was unexpected, since experiments with crystals of AE soaked in solutions containing 5 mM-di-isopropyl phosphorofluoridate and 0.2 mM-SAN showed only 0.2% hydrolysis after 72 h.

Binding of inhibitors and substrates to AE

Summarized in Table 1 are binding and kinetic constants for the complexing of seven inhibitors and one substrate to PE and to AE. The dissociation constants

for PE were inferred indirectly from kinetic measurements, and the dissociation constants for AE were measured by spectrophotometric titration or indirectly from kinetic measurements in the equilibrium binding assay as indicated.

For turkey ovomucoid inhibitor there was only a slight difference between the binding constants, being 0.1 μ M with PE and 0.3 μ M with AE. The K_m for SAN cleavage by PE reported by Bieth *et al.* (1974) of 1.0–1.2 mM is near that which we have determined. The binding constant (K_{AE}) for binding of SAN to AE determined by u.v.-difference-absorption-spectral analysis (Fig. 4b) was 0.2 ± 0.15 mM. Thus the binding of turkey ovomucoid inhibitor and SAN to PE depended mainly on interactions other than those associated with the serine hydroxy group. In contrast, the inhibition constant by elastatinal on PE was determined to be 0.2 μ M, whereas K_d for AE–elastatinal complex was estimated to be about 50.0 μ M, representing a 250-fold decrease in binding. Also, the binding of α_1 proteinase inhibitor to PE was shown to be moderately decreased by dehydration of Ser-195; this is an unexpected result, since acylation of PE by α_1 proteinase inhibitor has been considered to be not essential to inhibition (Travis & Salvesen, 1983).

A β -lactam, 3-acetoxymethyl-7 α -methoxy-3-cephem-4-carboxylate 1,1-dioxide t-butyl ester, which was shown to be a mechanism-based inactivator of human leucocyte elastase (Doherty *et al.*, 1986), bound PE with a K_i of 1.2 μ M at pH 5.0. The dissociation constant (K_d) of the cephem–AE complex, measured by u.v. difference absorption spectra, was 100 μ M. This binding occurred without evidence of ring-opening of the β -lactam. The binding of CK and phenylmethanesulphonyl fluoride to PE was noticeably affected by modification of Ser-195. The decrease in binding of CK was accompanied by a loss in the capacity to alkylate the active-site histidine residue. The acid hydrolysate of the CK-treated elastase showed about 1.2 residues of histidine modified per molecule, but all six histidine residues were recovered in the acid hydrolysate of CK-treated AE. In contrast, 2-(2-ethylbutyryl)-1,2-benzisothiazolin-3-one (Zimmerman *et al.*, 1980), as well as 3,4-dichloroisocoumarin (Harper *et al.*, 1985), which have been demonstrated to be potent acylating inactivators of elastase, showed a relatively small decrease in binding as a consequence of serine conversion, suggesting a small contribution of the hydroxy group of Ser-195 to the complex-formation.

DISCUSSION

When PE was inactivated with phenylmethane-sulphonyl fluoride and then treated with base to generate AE, at least four products were obtained (see Fig. 1). This heterogeneity could be caused by the side reactions of the alkaline elimination at disulphide bonds (Jones *et al.*, 1983) and/or by the cleavage of peptide bonds readily sensitive to base. After purification on turkey ovomucoid inhibitor–Sephacrose, one portion (fraction III) of the AE was crystallized. X-ray-diffraction analysis of the crystalline AE preparation revealed no significant alteration of structure relative to the native PE, except dehydration of the Ser-195 residue and loss of a nearby SO_4^{2-} ion. X-ray-crystallographic analysis of crystals of AE soaked with the substrate SAN provided direct evidence for its binding. Unfortunately, the present study of substrate binding was unsuccessful in detecting possible S₁'–P₁'

Table 1. Binding of substrate and inhibitors to PE and AE

Ligand	K_{PE} (μ M)	K_{AE} (μ M)	K_{PE}/K_{AE} ratio
SAN	1200*	200†	0.2
Turkey ovomucoid inhibitor	0.09§	0.3‡	3.3
α_1 Proteinase inhibitor	0.008§	0.25‡	30.0
Phenylmethanesulphonyl fluoride	100§	29000‡	290
2-(2-Ethylbutyryl)-1,2-benzisothiazolin-3-one	0.6§	2.3‡	3.8
3,4-Dichloroisocoumarin	4.8§	16.8‡	3.5
3-Acetoxymethyl-7 α -methoxy-3-cephem-4-carboxylate 1,1-dioxide t-butyl ester	1.2§	100†	83.3
CK	1.2§	100†‡	83.3
Elastatinal	0.2§	50†	250

* Michaelis constant for SAN cleavage (K_s).

† Determined by u.v.-difference-spectral analysis.

‡ Determined by competitive PE inhibition assay.

§ These inhibition constants were derived from inhibition of PE-catalysed amidolysis of SAN, by fitting the initial steady-state rate of SAN cleavage immediately after inhibitor addition to the equation $v = V[S]/([S] + K_s(1 + [I])/K_{PE})$, where v is the reaction rate at inhibitor concentration $[I]$, V is the maximal enzyme rate (at infinite substrate concentration $[S]$) and K_s is the Michaelis constant for SAN (1 mM).

interaction, since the *p*-nitroanilide group of the bound ligand was absent. Even when extreme steps were taken to eliminate residual PE activity by soaking AE crystals with 5 mM-di-isopropyl phosphorofluoridate, some elastase activity could subsequently be detected upon prolonged incubation at 25 °C. It is feasible that enzyme regeneration occurred by rehydration reactions similar to those described by Gold & Fahrney (1964). Since the peptidyl portion of SAN was found in the active site and the *p*-nitroanilide group was absent, it is difficult to determine if the serine-modified enzyme or residual active enzyme is responsible for hydrolysing the substrate. Answers to such questions may eventually be provided by site-specific mutagenesis such as that reported for subtilisin by Bryan *et al.* (1986). Murphy *et al.* (1982) have reported the preparation and crystallization of AE, but they did not observe binding of Ala-Ala-Ala-Lys-Phe by AE crystals soaked in the substrate by electron-density difference maps.

Comparative studies of anhydroserine enzymes with native enzymes of a number of serine proteinases have been reported (Ako *et al.*, 1974; Vincent *et al.*, 1974; Huber *et al.*, 1975; Poulos *et al.*, 1976; Murphy *et al.*, 1982; Nishikata, 1983; Tsai & Bender, 1984; Williams *et al.*, 1984). The remarkable feature of all anhydro-enzymes studied is their capacity to combine with some of their ligands that normally serve as inhibitors or substrates with their affinities for those ligands only slightly altered (Ako *et al.*, 1972*a,b*; Andersson & Wolfenden, 1980; Weiner *et al.*, 1966). However, it is apparent from the present study, as well as the results reported by Tsai & Bender (1984) on their binding of arylboronic acids to AE and the work of Andersson & Wolfenden (1980) on anhydro-chymotrypsin, that compounds that form stable adducts with PE through nucleophilic addition of the Ser-195 hydroxy group to the carbonyl carbon atom (or boron atom) of these ligands showed significantly poorer binding constants to AE than to PE. These results suggested that Ser-195 may be directly involved with the affinity of these ligands to interact with enzyme before the formation of covalent adducts. It must be noted, however, that measurement of inhibition constants for these compounds on PE could have been affected by the reaction step of covalent modification, so that the direct contribution of Ser-195 to the binding of substrates/inhibitors may have been smaller than the ratio indicated in Table 1.

Available evidence indicates that ligands that bind tightly to PE, such as those reported in the present paper, can be placed into at least three categories: (a) inhibitory ligands that depend largely on formation of an adduct to the Ser-195 hydroxy group, as exemplified by simple boronic acids (Tsai & Bender, 1984) or β -lactam inhibitors (Doherty *et al.*, 1986); (b) those that in addition to the active-site serine residue are also significantly governed by binding of subsite regions (Schechter & Berger, 1967) such as the peptidylhalomethanes (Powers & Tuhy, 1973; Poulos *et al.*, 1976) and the peptidylboronic acids described by Kettner & Shenvi (1984); (c) those whose binding is mainly due to the interaction of large domains by hydrogen-bonding and are hydrophobic in nature, with only a small contribution from serine adduct formation, as in the case of most of the naturally occurring protein inhibitors.

In this last case, the binding of turkey ovomucoid inhibitor to PE and AE is largely independent of Ser-195,

and dissociation can only be achieved at low pH. The inhibition of proteinases by α_1 proteinase inhibitor can occur without requirement for formation of the acyl intermediate (Travis & Salvesen, 1983). However, the cleavage of the Met-Ser bond of α_1 proteinase inhibitor by elastase is important to the association rate for the enzyme-inhibitor complex (Travis & Salvesen, 1983; Vincent & Lazdunski, 1972; Drechsel *et al.*, 1984). We have found that α_1 proteinase inhibitor can also form a stable complex with AE as analysed by polyacrylamide-gel electrophoresis (H. R. Williams & T.-Y. Lin, unpublished work), even though the apparent binding was diminished, with a K_{PE}/K_{AE} ratio of about 30:1 (see Table 1). This suggests that the contribution of Ser-195 to the binding of α_1 proteinase inhibitor may be more significant than previously assumed.

The binding of the CK to AE was much weaker than that to PE (see Table 1). This was expected, since previous studies of the interaction of oligopeptidylhalomethanes with a number of serine proteinases as well as anhydro-chymotrypsin have suggested that formation of a tetrahedral hemiketal is a prerequisite to alkylation of the catalytic histidine residue. The formation of the tetrahedral hemiketal has been proposed to involve the participation of the hydroxy group of Ser-195 (Poulos *et al.*, 1976; James *et al.*, 1980). Amino acid analyses of AE treated with CK showed that the CK did not modify any histidine residues, in agreement with the observation by Weiner *et al.* (1966) that tosylphenylalanylchloromethane did not alkylate any histidine residues in anhydro-trypsin. Orientation of the chloromethyl group to the histidine residue appears to be governed by the formation of the tetrahedral hemiketal with Ser-195 at P_1 , even if the correct alignment of P_1 - P_4 in the subsites has occurred. Alternatively, these results may offer support for an inactivation scheme of CK in which a Ser-195-mediated enzyme-bound epoxide intermediate is involved (Powers, 1977).

In contrast with the large decrease in binding to AE observed with the β -lactam and phenylmethanesulphonyl fluoride, it was surprising to find that the acylating inhibitors such as 3,4-dichloroisocoumarin and 2-(2-ethylbutyryl)-1,2-benzisothiazolin-3-one showed only a small decrease in binding of the enzyme as a consequence of serine dehydration. The heterocyclic structure of these compounds may contribute to the strong affinity for the oxy anion hole in the active site, which appears unaltered in AE. This is an example of such cases where the formation of a tetrahedral enzyme-ligand complex and the expulsion of a leaving group are strongly influenced by the interactions of the structural moieties near the carbonyl group to be attacked by Ser-195. Such observations also support the relevance of AE as a model for exploration of structural factors critical to being a good substrate or a potent inhibitor.

Thanks are due to Dr. Martin Poe for helpful discussions during the preparation of this manuscript and Mrs. S. Fischbach for typing of the manuscript.

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Received 7 August 1986; accepted 27 October 1986