Isolation and Characterization of Human Plasma α_1 -Proteinase Inhibitor and a Conformational Study of its Interaction with Proteinases

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(Received 8 April 1976)

1. α_1 -Proteinase inhibitor was isolated from human plasma by a five-step procedure. Isoelectric focusing showed that six components focused between pH 4.85 and 4.95. 2. The mol.wt. of the inhibitor was 52000 by sedimentation equilibrium and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The amino acid and carbohydrate compositions of the inhibitor were also determined. 3. The far-u.v. c.d. (circular-dichroism) spectrum indicated that the inhibitor had about 36% α -helical content. 4. The loss of proteinase-inhibitory activity when the inhibitor was exposed to pH values less than 5.0 or greater than 10.5 was accompanied by small changes in the far-u.v. c.d. spectrum and large changes in the near-u.v. c.d. spectrum. The change at alkaline pH was associated with ionization of tyrosine residues. 5. Interaction of inhibitor with chymotrypsin caused perturbation of the c.d. spectrum and this was used to follow the interaction and show a 1:1 stoichiometry. 6. C.d., electrophoresis and isoelectric focusing showed that the inhibitor-enzyme complex is degraded by free enzyme. 7. Parallel studies with trypsin indicated that it too forms a 1:1 complex with inhibitor and is degraded by excess of enzyme.

Human plasma contains a number of proteinase inhibitors (Heimburger *et al.*, 1971), but most of the anti-tryptic activity is due to a protein, which migrates electrophoretically in the α_1 -globulin region (the α_1 -proteinase inhibitor or α_1 -anti-trypsin).

Isolation of the α_1 -proteinase inhibitor was first described by Bundy & Mehl (1959) and Schultze *et al.* (1962). These purifications employed steps at acid pH values that destroyed much of the inhibitory activity of the protein. Purifications of the protein by using milder procedures have been reported by several authors: Myerowitz *et al.* (1972) [who found two α_1 -anti-trypsins (electrophoretically fast and slow)]; Crawford (1973); Chan *et al.* (1973); Murthy & Hercx (1973); Johnson *et al.* (1974); Laurell *et al.* (1975). There is, however, considerable disagreement about the composition and physical characteristics of the protein probably due, at least in part, to contamination of some of the preparations by albumin and α_1 -acid glycoprotein. In the present paper we describe the isolation of α_1 -proteinase inhibitor demonstrably free of albumin, α_1 -acid glycoprotein or other contaminants, and it is hoped that the composition and characterization data presented will help to resolve the discrepancies in the literature. We also describe some of the properties of the molecule related to its conformation by c.d. (circular dichroism).

There is disagreement about the stoichiometry of interaction of human plasma α_1 -proteinase inhibitor (α_1 -anti-trypsin) with proteinases and little is known of the nature of the interactions. From enzyme-inhibition experiments Crawford (1973) and Chan *et al.* (1973) concluded that combination of the inhibitor with trypsin was equimolar, although Crawford (1973) found the inhibitor combined with 18% more trypsin than would have been expected if the reaction was a 1:1 molar combination. Moroi & Yamasaki (1974) electrophoresed mixtures of trypsin and inhibitor on polyacrylamide gels containing sodium dodecyl sulphate. A band in a position corresponding to mol.wt. 74000 was attributed to a covalently linked 1:1 complex of enzyme and inhibitor. However, Johnson *et al.* (1974) and Pannell *et al.* (1974) concluded from enzyme-inhibition and electrophoresis experiments that one molecule of inhibitor could combine with two molecules of trypsin.

Measurement of enzyme inhibition is an indirect way of studying complex-formation. Addition of substrate solution to assay the free enzyme may perturb the enzyme-inhibitor equilibrium, and the presence of excess of enzyme in the equilibrium mixtures may result in proteolytic degradation of enzyme-inhibitor complex, so that complexes of inhibitor with more than one molecule of enzyme might not be observed.

The use of separation methods such as electrophoresis to investigate the composition of mixtures of enzyme and inhibitor is also subject to the criticism that physical separation of the components of an equilibrium mixture may perturb the equilibrium. These disadvantages are avoided by observing some property of one or both reactants that changes on interaction and can be measured without perturbing the equilibrium between them, i.e. by a spectroscopic or electrometric method. The reaction can then be followed directly, and be performed in the presence of an excess of inhibitor, so that proteolytic degradation of the complex is less likely than in enzyme inhibition studies where enzyme is in excess.

We have used c.d. spectroscopy as a direct method of observing the interaction between the inhibitor and proteinases, and have supplemented this by the use of electrophoresis and isoelectric focusing.

Parts of this work have been communicated briefly elsewhere (Saklatvala & Wood, 1975; Wood *et al.*, 1975).

Materials

Bovine trypsin (EC 3.4.21.4; type II), twice crystallized, bovine α -chymotrypsin (EC 3.4.21.1; type II), crystallized three times from four-times crystallized chymotrypsinogen, Bz-Arg-OEt (benzoyl-L-arginine ethyl ester), Bz-Tyr-OEt (benzoyl-L-tyrosine ethyl ester), dithiothreitol, *p*-chloromercuribenzenesulphonic acid, 5,5'-dithiobis-(2-nitrobenzoic acid) and Triton X-100 were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Hide-powder azure (B grade) was from Calbiochem, Hereford HR4 9BO, U.K. D-10-Camphorsulphonic acid was from Eastman Kodak Co., Rochester, NY, U.S.A. DEAE-cellulose (DE-52) was from W. and R. Balston, Maidstone, Kent, U.K. Concanavalin A covalently bound to Sepharose 4B was from Pharmacia (GB), London W5 5SS, U.K. Ampholine was from LKB Instruments, London SE20, U.K. Cellulose acetate was from Sartorius Membrane filter, Gottingen, West Germany. Rabbit anti-human serum was from Hoechst U.K., Hounslow, U.K. All other chemicals were A.R. Grade, where available, and were from BDH Chemicals, Poole, Dorset, U.K. A Diaflo apparatus was used for ultrafiltration (Amicon, High Wycombe, Bucks., U.K.).

Methods

Assay of proteolytic enzymes

Stock solutions of chymotrypsin in 1 mM-HCl were assayed spectrophotometrically at 25°C by using Bz-Tyr-OEt as substrate in 0.1 M-Tris/HCl, pH 7.8, containing 20 mM CaCl₂, as described by Hummel (1959). The specific activity (45–48 μ mol of Bz-Tyr-

OEt hydrolysed/min per mg of protein) was comparable with the highest values cited in the literature and commercial preparations of chymotrypsin generally contain only about 10% (w/w) catalytically inactive material (Wilcox, 1970). The concentration of chymotrypsin solutions was determined spectrophotometrically by using $E_{1\text{cm}}^{1\%} = 20.5$ at 282 nm (Wilcox, 1970) and a mol.wt. of 25000.

Stock solutions of bovine trypsin in 1 mM-HCl were dialysed against a large volume of 1 mM-HCl and assayed potentiometrically with Bz-Arg-OEt as substrate at pH 7.8 and 25°C, as described by Robinson *et al.* (1971). The specific activity was 42 μ mol of Bz-Arg-OEt hydrolysed/min per mg of protein. Robinson *et al.* (1971) calculated the maximum rate at which Bz-Arg-OEt was hydrolysed under the above conditions by preparations of α - and β -trypsins, which had 0.96–0.91 active sites per molecule. By using the data of Robinson *et al.* (1971) it is estimated that the trypsin we used contained about 61% (w/w) catalytically active trypsin. The concentration of trypsin solutions was determined spectrophotometrically by using $E_{1\text{cm}}^{1\%} = 15.4$ at 280 nm.

Assay of trypsin inhibition to monitor purification procedures

The insoluble substrate hide-powder azure, described by Rinderknecht *et al.* (1970), was used. A solution (1 ml) of enzyme (0.4 μ g/ml) in 0.1 M-Tris/HCl, pH 7.5, containing 2 mM-CaCl₂ and 0.1% Triton X-100 was mixed with the same buffer (1 ml) or inhibitor solution (1 ml) in buffer, and incubated at 37°C for 20 min. A portion (1 ml) of the mixture was added to substrate (3 mg) in a siliconized conical flask (25 ml) and incubated, with shaking, at 37°C for 20 min. Undigested substrate was removed by rapid filtration and E_{595} of the filtrate was determined. The same assay system was used to measure inhibition of leucocyte extracts from rheumatoid synovial fluid prepared as described by Pryce-Jones *et al.* (1974). For this the assay buffer contained 1 M-KCl.

Investigation of the inhibition of proteinases by purified inhibitor

Inhibition of trypsin was measured with Bz-Arg-OEt as substrate by using a modification of the method of Schwert & Takenaka (1955). The reaction was carried out in 0.1 M-Tris/HCl, pH 8.0, containing 0.2 mM CaCl₂. Trypsin (1 ml; 7.2 μ g/ml) was mixed with 2 ml of inhibitor solution and incubated at 25°C for 15 min. Substrate solution (0.1 ml; 5.6 mg/ml of water) was added and rapidly mixed. E_{253} was recorded at 25°C in a Unicam SP.1700 recording spectrophotometer.

Chymotrypsin inhibition was measured by using Bz-Tyr-OEt as substrate. The procedure used was the same as for trypsin, except that the substrate was dis-

solved (5.2 mg/ml) in a mixture of 3 vol. of methanol (spectroscopic grade) and 2 vol. of water, and E_{256} was recorded.

Purification of α_1 -proteinase inhibitor

Step 1: precipitation with $(\text{NH}_4)_2\text{SO}_4$. Human plasma (400 ml) from two individuals was diluted with water (400 ml), and solid $(\text{NH}_4)_2\text{SO}_4$ added at 4°C to 60% saturation (i.e. 361 g/litre added). After 15 min the precipitate was removed by centrifuging and $(\text{NH}_4)_2\text{SO}_4$ added to the supernatant solution to 80% saturation (i.e. 129 g/litre added). The precipitate was collected by centrifuging and dissolved in 10 mM-Tris/HCl, pH 8.6 (100 ml). The solution was dialysed exhaustively against the same buffer.

Step 2: chromatography on DEAE-cellulose. The dialysed solution was applied to a column (9 cm $\times$ 15 cm) of DEAE-cellulose equilibrated with 10 mM-Tris/HCl, pH 8.6. The column was eluted with a linear gradient of NaCl (starting eluent 5 litres of equilibration buffer containing 0.05 M-NaCl; limiting eluent 5 litres of the same buffer containing 0.08 M-NaCl), which removed most of the α_2 - and β -globulins. The remaining proteins were then eluted with equilibration buffer containing 0.5 M-NaCl. This solution was concentrated to about 50 ml in a Diaflo ultrafiltration cell with a PM30 membrane.

Step 3: affinity chromatography on concanavalin A-Sepharose. The solution was applied to a column (4.4 cm $\times$ 23 cm) of concanavalin A-Sepharose, which had been equilibrated with 0.1 M-Tris/HCl, pH 7.5, containing 0.5 M-NaCl, 1 mM-MgCl₂, 1 mM-MnCl₂ and 1 mM-CaCl₂. The column was washed with 5 column vol. of equilibrating buffer, which removed most of the albumin. Protein was then eluted with α -D-methyl glucopyranoside (0.1 M in equilibration buffer). The eluate was concentrated to 5–10 ml by ultrafiltration and the solvent exchanged for 10 mM-Tris/HCl, pH 8.6, by means of the ultrafiltration cell.

Step 4: electrophoresis on polyacrylamide gel. A Buchler Poly-Prep apparatus (Nuclear-Chicago Corp., Des Plaines, IL, U.S.A.) was used essentially as described by Jovin *et al.* (1974). The resolving gel (300 ml) contained 7.5% (w/v) polyacrylamide and 0.25% *NN'*-methylenebisacrylamide in 0.35 M-Tris/HCl, pH 8.9, and was polymerized in the presence of 0.05% $(\text{NH}_4)_2\text{S}_2\text{O}_8$ and 0.05% *NNN'*-tetramethylethylenediamine. The stacking gel (20 ml) containing 3% (w/v) polyacrylamide and 0.25% *NN'*-methylenebisacrylamide was prepared similarly. The upper (cathode) reservoir buffer was 52 mM-Tris/glycine, pH 8.9, and the lower reservoir buffer was 0.1 M-Tris/HCl, pH 8.1. The gel assembly was cooled by running tap water (<10°C) and was subjected to pre-electrophoresis (5 h; 40 mA, constant current); otherwise subsequent recovery of inhibitor activity was low. Electrophoresis was carried out at 40 mA.

Step 5: chromatography on DEAE-cellulose. A concentrated solution of inhibitor from step 4 was applied to a column (2.5 cm $\times$ 10 cm) of DEAE-cellulose equilibrated with 10 mM-Tris/HCl, pH 8.6, and was eluted with a linear NaCl gradient (0–0.2 M). The inhibitor-containing fractions (50–100 mg of protein) were pooled and concentrated to 5 ml.

Analytical electrophoresis

Electrophoresis in polyacrylamide-gel cylinders was done with the same gel and buffer solutions as for preparative polyacrylamide-gel electrophoresis. Gels were stained with Amido Black.

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate was carried out as described by Weber *et al.* (1972).

Electrophoresis on cellulose acetate membranes was carried out in barbiturate buffer, pH 8.6 ($I = 0.05$). Membrane strips were stained with Ponceau S or Amido Black.

Analytical isoelectric focusing

This was done in cylindrical polyacrylamide gels containing 7.5% (w/v) acrylamide monomer and 1% (w/v) Ampholine carrier ampholytes as described by Wrigley (1971). The anode solution was 0.2% H_2SO_4 and the cathode solution 0.4% ethanolamine.

Analytical ultracentrifugation

A Beckman model E ultracentrifuge fitted with a photoelectric scanner and Multiplex accessories was used.

Sedimentation velocity was investigated by the band-centrifugation method (Vinograd *et al.*, 1963). Protein solution (15 μ l, 2 mg/ml) was layered on to 0.5 M-NaCl and centrifuged at 60000 rev./min, at 20°C in an An-D rotor. The sedimentation coefficient was calculated from movement of the peak and corrected to standard conditions ($s_{20,w}$) as described by Studier (1965).

For sedimentation-equilibrium experiments samples of protein solution (0.64 mg/ml in 10 mM-Tris/HCl, pH 8.6, containing 0.1 M-NaCl) were centrifuged at 13000 rev./min, at 20°C, in an An-GTi rotor. The radial distribution of E_{280} was recorded. The molecular weight of the protein (M_2) was calculated from the relationship for a multi-component system at sedimentation equilibrium.

$$\frac{d \ln c_2}{dr^2} = \frac{M_2 \omega^2}{2RT} \left(\frac{\partial \rho}{\partial c_2} \right)_\mu^0 \quad (1)$$

where c_2 is the concentration of protein (g/ml), ρ is the density of the system and $(\partial \rho / \partial c_2)$ is the density increment (Reisler & Eisenberg, 1969).

The density increment was determined by the mechanical oscillator method of Kratky *et al.* (1973) by using a Digital Densimeter (Anton Paar, Graz, Austria). Protein samples were dialysed against the solvent buffer before density measurements were made.

Amino acid analysis

Protein samples were hydrolysed in 6M-HCl in sealed tubes at 115°C for 24, 48 and 72h, and were analysed on a Jeol GLC5AH amino acid analyser (Jeol, Tokyo, Japan) by using standard techniques. Two samples of protein were oxidized with performic acid by the method of Hirs (1967) and cysteine was measured chromatographically as cysteic acid.

Determination of thiol groups

Free thiol groups were measured spectrophotometrically by reaction of the protein with 5,5'-dithio-bis(2-nitrobenzoic acid) (Robyt *et al.*, 1971) or by spectrophotometric titration with *p*-chloromercuribenzenesulphonic acid (Tilander *et al.*, 1965). The latter method was also used to measure thiol groups in a solution of the inhibitor that had been denatured with 6M-urea [1ml of protein solution (2.2mg/ml) + 1.3ml of 10M-urea, 3h at room temperature (20°C)]. Samples of a further inhibitor preparation were either (a) treated with Na₂SO₃ [2ml of protein (4.5mg/ml) in Tris/HCl, pH 8.0, + 2ml of 25μM-Na₂SO₃, 1h at room temperature] (McArdle, 1967) and free thiol groups then measured with *p*-chloromercuribenzenesulphonic acid, or (b) treated with dithiothreitol (Cleland, 1964); the solution was then separated from low-molecular-weight products by gel filtration on Sephadex G-25, and free thiol groups were measured with 5,5'-dithio-bis(2-nitrobenzoic acid).

Carbohydrate analysis

Hexoses were determined by the orcinol/H₂SO₄ method of Hartley & Jevons (1962) and by the phenol/H₂SO₄ method of Ashwell (1966) with mannose as standard. Hexosamines were determined by the method of Cessi & Piliego (1960) after hydrolysis of protein samples for 3h in 4M-HCl at 100°C. Sialic acid was determined as described by Aminoff (1961) after hydrolysis for 1h in 0.05M-H₂SO₄ at 80°C.

Spectroscopy

Absorption spectra were recorded with a Unicam SP.1700 spectrophotometer.

C.d. spectra were determined with a Cary model 60 spectropolarimeter with model 6003 c.d. attachment (Cary Instruments, Monrovia, CA, U.S.A.). The cell compartment was at 27 ± 1°C and the instrument was calibrated with D-10-camphorsulphonic acid (Cassim

& Yang, 1969). The slit widths were programmed to give a constant spectral band width of 1.5nm. The light-path was chosen so that $E \leq 0.8$.

To investigate the effect of pH on the c.d. spectrum of α_1 -proteinase inhibitor, protein solution was dialysed exhaustively against 0.1M-KCl and the pH adjusted by stepwise addition of either 0.1M-HCl or NaOH (0.1M and 1.0M). To determine the change of c.d. when α_1 -proteinase inhibitor was titrated with chymotrypsin, successive volumes (12μl) of chymotrypsin solution (10mg/ml; 0.4mM) were added to 2.9ml of inhibitor solution (0.52mg/ml; 10⁻³M). Titration with trypsin was performed similarly except that the inhibitor concentration was 0.96mg/ml (12.5μM).

Protein concentration

In protein fractionation procedures protein concentration was determined by using Folin and Ciocalteu's reagent as described by Miller (1969) with bovine serum albumin as standard. The concentration of purified inhibitor solutions was determined from E_{280} by using $E_{1\text{cm}}^{1\%} = 4.7$. This value was obtained after dialysing three preparations against 1mM-Tris/HCl, pH 8.0, and drying them to constant weight *in vacuo* over P₂O₅ at 90°C. The weight of buffer salts was corrected for and $E_{1\text{cm}}^{1\%}$ at 280nm was found to be 4.75, 4.72 and 4.71 for the three samples respectively [cf. values of 4.3 (Crawford, 1973) and 6.7 (Johnson *et al.*, 1974)].

Results and Discussion

Purification of α_1 -proteinase inhibitor

Plate 1 shows electrophoresis of the products from each step of the purification on cellulose acetate and on polyacrylamide gel; albumin and the α_1 -proteinase inhibitor run in virtually the same position on the latter. Albumin was removed by means of affinity chromatography on concanavalin A-Sepharose (step 3). Preparative polyacrylamide electrophoresis (step 4 shown in Fig. 1), removed α_1 -acid glycoprotein (the first peak eluted) as well as slower running material. Electrophoresis on polyacrylamide gel and cellulose acetate indicated that the final product was homogeneous, and on immunoelectrophoresis in agarose at pH 8.6 the protein gave a single precipitin line against anti-human serum.

In a typical purification, 17% of the inhibitor activity of plasma was recovered with about 50-fold increase in specific activity (Table 1). The pure protein (1mg) inhibited 0.57mg of trypsin or 0.48mg of chymotrypsin. The protein totally inhibited the proteolytic activity of the rheumatoid synovial-fluid leucocyte extracts. Apart from α_2 -macroglobulin no other leucocyte proteinase inhibitors were found on fractionation of plasma proteins.

EXPLANATION OF PLATE I

Electrophoresis of samples from successive steps in the purification of α_1 -proteinase inhibitor

(a) Cellulose acetate; (b) polyacrylamide. (1) Human plasma; (2)–(6) the products of steps 1–5 of the purification. See the text for details.

EXPLANATION OF PLATE 2

Isoelectric focusing of α_1 -proteinase inhibitor in a pH4-6 gradient

(a) Purified inhibitor (100 μ g), the individual bands of inhibitor are labelled 1-6; (b) 100 μ g of inhibitor+100 μ g of trypsin; (c) 100 μ g of inhibitor contaminated with albumin; (d) 20 μ g of pure human albumin.

EXPLANATION OF PLATE 3

Cellulose acetate electrophoresis of mixtures of α_1 -proteinase inhibitor and chymotrypsin

(a) Chymotrypsin; (b) α_1 -proteinase inhibitor; (c-i) mixtures of chymotrypsin and inhibitor. Molar ratio of chymotrypsin to inhibitor in the mixtures: (c) 0.25; (d) 0.5; (e) 0.6; (f) 1.0; (g) 1.3; (h) 1.6; (i) 2.0. E, Chymotrypsin; I, inhibitor; C₁, enzyme-inhibitor complex; C₂, degraded enzyme-inhibitor complex. The arrow indicates the point of application of the samples.

EXPLANATION OF PLATE 4

Isoelectric focusing in polyacrylamide gel of mixtures of α_1 -proteinase inhibitor and chymotrypsin: pH3–10 gradient

(a) Inhibitor (100 μ g); (b) 100 μ g of inhibitor+25 μ g of chymotrypsin; (c) 100 μ g of inhibitor+50 μ g of chymotrypsin; (d) 100 μ g of inhibitor+100 μ g of chymotrypsin. Molar ratio of chymotrypsin to inhibitor: (a) 0.52; (b) 1.08; (c) 2.08. I , Inhibitor bands; F_1 , bands of enzyme–inhibitor complex; F_2 , bands arising from degradation of enzyme–inhibitor complex.

EXPLANATION OF PLATE 5

Cellulose acetate electrophoresis of mixtures of α_1 -proteinase inhibitor and trypsin

(a) Trypsin; (b) inhibitor; (c-j) mixtures of trypsin and inhibitor. Molar ratio of trypsin to inhibitor in the mixtures: (c) 0.2; (d) 0.4; (e) 0.6; (f) 0.8; (g) 1.0; (h) 1.25; (i) 1.4; (j) 2.0. E, Trypsin; I, inhibitor; C₁', trypsin-inhibitor complex; C₂', material arising from degradation of trypsin-inhibitor complex. The arrow indicates the point of application of the samples.

EXPLANATION OF PLATE 6

Isoelectric focusing in polyacrylamide gel of mixtures of α_1 -proteinase inhibitor and chymotrypsin: pH4–6 gradient

(a) Inhibitor (25 μ g); (b) 100 μ g of inhibitor+15 μ g of trypsin; (c) 100 μ g of inhibitor+50 μ g of trypsin; (d) 100 μ g of inhibitor+100 μ g of trypsin. Molar ratios of trypsin to inhibitor in the mixtures: (a) 0.54; (b) 1.08; (c) 2.16. I, Inhibitor; F₁', enzyme-inhibitor complex; F₂' and F₃', bands arising from degradation of enzyme-inhibitor complex.

Molecular weight

In sedimentation-equilibrium experiments graphs of $\ln c_2$ versus r^2 were linear. The mean value of the mol.wt. for five samples of one inhibitor preparation was 52000 ± 1400 (s.d.). A single value determined on a second preparation was within this range.

The density increment used in the computation of M , was 0.287, which corresponds to a partial specific volume ($\bar{v}$) of 0.713 ml/g (Reisler & Eisenberg, 1969). Band-centrifugation traces showed a single peak with $s_{20,w} = 3.75$ S.

Electrophoresis of inhibitor on polyacrylamide gels containing sodium dodecyl sulphate showed a single band whose position corresponded to $M = 53000$. An identical pattern was obtained with inhibitor that had been treated with mercaptoethanol and 4M-urea.

Isoelectric focusing

When the inhibitor was subjected to isoelectric focusing on polyacrylamide gels, the protein focused into three densely staining bands (bands 3, 4 and 5 in Plate 2) and three faint bands (bands 1, 2 and 6) lying between pH 4.85 and 4.95. A similar pattern was observed by Crawford (1973) and Pannell *et al.* (1974).

Plate 2 also shows the appearance of a preparation of inhibitor contaminated with albumin; a gel containing purified human albumin alone is shown for reference, and it can be seen that albumin focuses as two bands just to the cathode side of bands 1 and 2.

Isoelectric focusing of a mixture of inhibitor with an excess of trypsin (Plate 2) shows that all of the inhibitor protein is displaced towards the cathode, presumably by forming complexes with the more positively charged enzyme. Thus all of the bands of the inhibitor preparation are active proteinase inhibitor, and none is contaminating protein.

Amino acid and carbohydrate analysis

The composition shown in Table 2 accounts for 93% of the material. It is closely similar to the

analyses reported by Heimbürger *et al.* (1971) and Crawford (1973). Compared to Crawford (1973) there are small differences with amino acids, particularly aspartic acid, glutamic acid and leucine, and this material contained 10% more hexosamine and 20% less hexose. Pannell *et al.* (1974) found only two residues of sialic acid per inhibitor molecule, whereas we found six residues per molecule, which is in agreement with Crawford (1973) and Heimbürger *et al.* (1971).

Rather variable results for the cysteine content of this protein exist in the literature. Heimbürger *et al.* (1971) found no cysteine, Crawford (1973) found one residue per molecule and Pannell *et al.* (1974) four residues per molecule. It is possible that this variation of cysteine content was due to contamination of the purified inhibitor by small amounts of albumin. In the inhibitor preparation described in Table 2, 0.4 residue of cysteine per molecule was found, but another preparation contained one residue per molecule. In several preparations there were no detectable

Fig. 1. Preparative electrophoresis: step 4 in the purification of α_1 -proteinase inhibitor

Protein from step 3 (150 mg) was applied to the polyacrylamide gel. See the text for details. (a) Inhibition of trypsin (Δ) and leucocyte proteinase ($\bullet$) by the eluate, and (b) E_{280} of the eluate.

Table 1. Purification of α_1 -proteinase inhibitor

Protein concentrations were determined by using the Folin-Ciocalteu reagent. See the text for details.

	Protein (mg)	Inhibitor specific activity (mg of trypsin inhibited per g of protein)	Purification (fold)	Inhibitor recovery (%)
Plasma	27800	14.5	1.0	
Step 1; $(\text{NH}_4)_2\text{SO}_4$ precipitation	11190	22	1.6	61
Step 2; DEAE-cellulose chromatography	5740	33	2.3	47
Step 3; affinity chromatography	370	435	30.0	40
Step 4; Electrophoresis	152	665	46.0	25
Step 5; DEAE-cellulose chromatography	95	720	50.0	17

Table 2. *Amino acid and carbohydrate composition of α_1 -proteinase inhibitor*

The results are the means ($\pm$ S.D.) of 24, 48 and 72 h hydrolyses in 6M-HCl, except where noted.

Component	Composition (nmol/mg of protein)	Residue wt. (μ g/mg of protein)	Residues/ molecule
Lysine	619 $\pm$ 3	79.4	32
Histidine	246 $\pm$ 4	33.8	13
Arginine	125 $\pm$ 2	19.6	7
Aspartic acid	774 $\pm$ 12	96.9	40
Threonine*	530	53.6	28
Serine*	392	34.2	20
Glutamic acid	891 $\pm$ 27	115.1	46
Proline	311 $\pm$ 6	30.2	16
Glycine	399 $\pm$ 4	22.8	21
Alanine	427 $\pm$ 9	33.4	22
$\frac{1}{2}$ -Cystine†	7.6 $\pm$ 0.7	0.78	0.4
Valine	465 $\pm$ 7	46.1	24
Methionine	115 $\pm$ 1	15.1	6
Isoleucine	341 $\pm$ 5	38.7	17
Leucine	796 $\pm$ 17	90.1	41
Tyrosine	106 $\pm$ 3	17.3	6
Phenylalanine	492 $\pm$ 6	72.5	26
Tryptophan‡	54	10.1	3
Hexose	291	47.2	15
Hexosamine	177	35.9	9
Sialic acid	116	33.6	6
Total		923.1	398

* By extrapolation to zero hydrolysis time.

† Result of five analyses, two of which involved oxidation to cysteic acid.

‡ Determined after alkaline hydrolysis.

free thiol groups in either native or urea-denatured protein, but free thiol groups (0.7–1.0 per molecule) were found after reducing the protein with either Na_2SO_3 or dithiothreitol. These observations are in agreement with those of Laurell & Thulin (1975) and Laurell *et al.* (1975), who also found that the inhibitor had one thiol group per molecule only after reduction. It is likely that the inhibitor has only one intrachain cysteine residue, which in the native protein forms a disulphide with a small molecule such as glutathione or cysteine.

Circular dichroism

In the c.d. spectra in the far u.v. (Fig. 2), the c.d. has been presented as mean residue ellipticity $[\theta]$ (calculated by using a mean residue weight of 115); the optical activity of the inhibitor has been attributed entirely to the polypeptide part of the molecule and any contribution from the carbohydrate portion has been ignored. The work of Stone (1971) and Dickinson & Bush (1975) indicates that the carbohydrate acetamido and carboxylate groups might give rise to weak c.d. bands near 210 and 225 nm respectively, and to stronger bands near 200 nm. The magnitude of any such contributions to the c.d. of α_1 -proteinase inhibitor cannot be assessed precisely,

but the data of Stone (1971) and Dickinson & Bush (1975) suggest that it will not amount to more than 5% of the observed ellipticity at 210 nm; it will be considerably less at higher wavelengths and probably more significant below 210 nm.

The general shape of the c.d. spectrum at pH 8.0 suggests that the protein has appreciable α -helical content. Chen *et al.* (1974) have recently presented reference c.d. spectra for α -helix, β -pleated sheet and unordered conformations of polypeptide chain. From these spectra the β -form and unordered structure are isodichroic at 224.5 nm. The α -helical content of the inhibitor molecule, calculated by the method of Chen & Yang (1971) from $[\theta]_{242.5}$ for the inhibitor and for the reference structures, was about 36%. The reference spectra were used to calculate the spectra expected for combinations of 36% α -helix and various proportions of β -structure and unordered structure. That shown in Fig. 2 (36% α -helix; 8% β -pleated sheet; 56% unordered) was closest to the observed spectrum. The difference between the observed and calculated spectra probably reflect the uncertain carbohydrate contribution in addition to the well known limitations of this approach to protein structure determination.

The c.d. spectrum in the near u.v. at pH 7.5 (Fig. 3) shows molecular ellipticity $[\theta]$ (calculated by using

Fig. 2. Far-u.v. c.d. spectra of α_1 -proteinase inhibitor

—, pH 8; ·····, pH 11.5; ····, pH 4.5; ----, calculated c.d. spectrum for 36% α -helix + 8% β -pleated sheet + 56% unordered, $[\theta]$ is the mean residue ellipticity. See the text for details.

$M = 52000$), between 250 and 310 nm. In accordance with the extensive compilation of data by Strickland (1974) the positive peaks at 258 and 264 nm and probably the negative peak at 269 nm are assigned to phenylalanine (26 residues/molecule); the positive band at 295 nm is due to tryptophan (three residues/molecule); the large negative band from 275 to 290 nm contains unresolved contributions from tryptophan and tyrosine (six residues/molecule).

Effect of pH on the stability and conformation of α_1 -proteinase inhibitor

The inhibitor underwent irreversible loss of activity against trypsin (Fig. 4a) when exposed to pH values greater than 10.5 or less than 5.0. The loss of activity below pH 5.0 correlates with striking changes in the c.d. spectrum. There was a progressive disappearance of the negative peak (275–290 nm) as the pH was changed from 5.0 to 3.5 (Fig. 4a). The magnitude of the tryptophan band (295 nm) also decreased, but the

phenylalanine peaks (260–270 nm) were not changed significantly. These effects were irreversible. The fall of $[\theta]_{281}$ as the pH was lowered parallels the fall of inhibitor activity (Fig. 4a); the mid-point of the change was at approx. pH 4.2. The acid-pH-dependence of the far u.v. c.d. spectrum was relatively small (Fig. 2), most of the change having occurred between pH 5.0 and pH 4.5. The loss of inhibitory activity is thus associated with minor conformational changes affecting the optical activity of tyrosine and tryptophan side chains and associated with protonation of groups with $pK_a \approx 4.2$, probably carboxylate groups.

Irreversible changes also occurred in the near-u.v. c.d. spectrum at pH > 10.4 (Fig. 3b). These were similar to those in acid solution, but were time-dependent; the change in the c.d. spectrum between pH 10.4 and 11.0 took about 40 min to complete. The fall of $[\theta]_{281}$ as the pH was increased parallels the fall of inhibitor activity (Fig. 4a); the mid-point of the transition was at approx. pH 11.0. Changes in the far u.v. c.d. spectrum above pH 10.4 were also time-dependent, and consistent with the loss of about 10% of the α -helical content of the native protein at pH 11.5. The loss of inhibitory activity in alkaline solution is thus accompanied by conformational changes associated with the titration of groups with $pK_a \approx 11$.

Insight into the nature of the ionizing groups was gained from spectrophotometric titration of the protein to alkaline pH. The change in the absorption spectrum due to tyrosine ionization was maximal at 300 nm with the same time-dependence as the c.d. changes at alkaline pH. The plots of E_{300} and $[\theta]_{281}$ versus pH were parallel (Fig. 4b). Calculation of the tyrosine and tryptophan content from the absorption spectra by the method of Goodwin & Morton (1946) gives 6.0 residues of tyrosine and 2.2 residues of tryptophan per molecule (cf. Table 1). We conclude that the biological activity of the inhibitor is highly dependent on its conformation and that the loss of activity at alkaline pH is due to a conformational change associated with tyrosine ionization.

Interaction of the inhibitor with chymotrypsin studied by c.d.

The c.d. spectrum of an equimolar mixture (10 μ M, pH 8.0) of enzyme and inhibitor differs significantly from the sum of the c.d. spectra of the two proteins (Fig. 5), particularly in the near-u.v. region. The positive ellipticity between 287 and 300 nm, due primarily to tryptophan side-chains, is very much decreased by the interaction of the proteins. Interaction also has a pronounced effect on the c.d. spectrum between 250 and 280 nm, where there are contributions from phenylalanine, tyrosine and cystine residues.

Whatever the nature of the molecular interactions that cause perturbation of the c.d. spectrum, the latter

Fig. 3. Near-u.v. c.d. spectra of α_1 -proteinase inhibitor

(a) —, pH 7.5 and pH 5.0; ----, pH 4.5; ····, pH 4.0; - · - · - ·, pH 3.5. (b) —, pH 7.7 and pH 9.6; ----, pH 11.0; - · - · - ·, pH 11.6. $[\theta]$ is the molecular ellipticity. See the text for details.

does afford a means of studying the stoichiometry of the enzyme-inhibitor interaction. A solution of inhibitor was titrated with a solution of chymotrypsin and 10 min after each addition the ellipticity was determined at 290 nm as described; at this wavelength the ellipticity of the inhibitor is zero, that of the enzyme is positive and that of the enzyme-inhibitor mixture is negative (Fig. 5). As the titration proceeded ψ_{290} changed linearly with enzyme concentration

(Fig. 6) until the molar ratio of the reactants was 1:1. Addition of more chymotrypsin caused an abrupt change of the slope of the titration curve. It is concluded that enzyme and inhibitor form a 1:1 complex and that the spectrum of the equimolar mixture of the reactants (Fig. 5b) is in fact the c.d. spectrum of this complex.

The increase in noise level during the titration, due to increase in the absorbance, was such that the

Fig. 4. (a) pH-dependence of stability of α_1 -proteinase inhibitor and (b) effect of alkaline pH on u.v. absorption and c.d. of α_1 -proteinase inhibitor

(a) Inhibitor solutions, initially at pH 7.5, were exposed to the indicated pH for 1 h and then assayed at pH 7.5. Inhibitor activity (●) is expressed as percentage of the activity of a solution of inhibitor kept at pH 7.5. Also shown is the variation of c.d., $[\theta]_{281}$ (○) with pH. See the text for details. (b) Change of E_{300} (○) and $[\theta]_{281}$ (●) with pH.

values of ψ_{290} after the end point are imprecise. Nevertheless it appeared that they are not as expected for an additive effect of excess of chymotrypsin (broken line in Fig. 6). The titration was repeated with ellipticity being determined at 280 nm. This was not expected to give such a sharp end point as ψ_{290} , because at 280 nm the complex and the inhibitor both have negative ellipticity. Formation of a 1:1 complex was still observed, and addition of excess of enzyme caused a discontinuity in the titration curve; the departure from the straight line expected for an additive effect of excess chymotrypsin (broken line in Fig. 6) being even more marked than at 290 nm. In a further experiment inhibitor solution (containing 29 nmol of inhibitor) was mixed with 35 nmol of enzyme and ψ_{280} was recorded continuously for 20 min, instead of after 15 min equilibration. The ini-

tial value of ψ_{280} was approximately that expected for 29 nmol of complex plus 6 nmol of enzyme, but it increased over the ensuing 10 min as shown in Fig. 6 (inset). It seemed likely that after rapid formation of the 1:1 complex the excess of chymotrypsin catalysed hydrolysis of the complex.

In order to test this the c.d. experiments were repeated at pH 5.4, since the enzyme is then expected to be less active than at pH 8.0. Titration curves at pH 5.4 (Fig. 7) were similar to those at pH 8.0, except that the time-dependent discontinuity in ψ_{280} at the end point was no longer observed. Thus the enzyme and inhibitor interact in essentially the same manner at pH 5.4 and 8.0 and the results are consistent with the suggestion that at pH 8.0 the anomalous c.d. observed in the presence of excess of enzyme is due to proteolysis of the complex.

Interaction of the inhibitor with trypsin studied by c.d.

The c.d. spectra of the trypsin preparation and of an equimolar mixture of trypsin and inhibitor are shown in Fig. 8(a). The latter differs markedly from the sum of the c.d. spectra of enzyme and inhibitor, showing that with this enzyme too, interaction causes some change in the conformation of one or both proteins. A titration curve in which ψ_{292} was determined after adding successive amounts of enzyme to an inhibitor solution at pH 8.0 was linear up to 0.57 mg of trypsin per mg of inhibitor (Fig. 8b). The slope of the titration curve after this end point coincides with that expected for an additive effect of trypsin in excess of that required to complex with the inhibitor. The end point observed in the c.d. titration curve (0.57 mg of trypsin per mg of inhibitor) is almost identical with that observed in the inhibition titration of trypsin with inhibitor, i.e. 0.56 mg of trypsin per mg of inhibitor. Since only 65% (w/w) of the sample of trypsin used was catalytically active, the stoichiometry of the reaction was 1 mol of inhibitor combined with 0.8 mol of trypsin.

Further insight into the changes that occur in the enzyme-inhibitor systems when enzyme is in excess was gained from experiments using electrophoresis and isoelectric focusing.

Electrophoresis and isoelectric studies of focusing-interaction of the inhibitor with chymotrypsin

Mixtures containing inhibitor (0.2 mM) and different concentrations of enzyme were subjected to electrophoresis at pH 8.6 on cellulose acetate (Plate 3) or isoelectric focusing on polyacrylamide gels with a pH 4-6 gradient (Plate 4). Addition of increasing amounts of enzyme to inhibitor up to a molar ratio of enzyme to inhibitor of 0.6 produces a band, C_1 , in the electrophoresis patterns. A similar effect is

Fig. 5. *C.d. spectra of α_1 -proteinase inhibitor and chymotrypsin*

(a) —, α_1 -Proteinase inhibitor ($10\mu\text{M}$), pH 8.0; ----, $10\mu\text{M}$ -chymotrypsin, pH 8.0. (b) —, Mixture of $10\mu\text{M}$ - α_1 -proteinase inhibitor and $10\mu\text{M}$ -chymotrypsin at pH 8.0; ----, the expected c.d. spectrum of the mixture, calculated by addition of the spectra of the components shown in (a); - · - · -, the difference spectrum between the calculated and observed spectra in the 200–250 nm range.

observed on isoelectric focusing, where interaction of enzyme and inhibitor produces a set of bands, F_1 , which replicate the pattern of the inhibitor. C_1 and F_1 presumably represent the 1:1 complex of enzyme and inhibitor whose formation was deduced from the c.d. results. When E/I (molar ratio of enzyme to inhibitor) > 1 , band C_1 in the electrophoresis pattern disappears and another band, C_2 , appears between the positions of C_1 and the inhibitor; at the same time free enzyme is visible and a band occupying the inhibitor position persists. Isoelectric-focusing patterns of the mixtures of chymotrypsin and inhibitor at $E/I > 1$ (Plate 4) show that all of the free inhibitor has disappeared, the F_1 bands persist and a new set of bands, F_2 , have appeared between the F_1 bands and the free inhibitor position. These F_2 bands must be

material arising from proteolysis of the complex by excess of enzyme, and may correspond to degradation products, which on electrophoresis run in the inhibitor position (Plate 3).

The appearance of new bands in the electrophoresis and isoelectric-focusing patterns when enzyme is in excess is consistent with the evidence from the c.d. experiments that free enzyme causes proteolysis of the complex.

Interaction of the inhibitor with trypsin

The products of the interaction of trypsin and inhibitor were studied by electrophoresis and isoelectric focusing as described for chymotrypsin. On electrophoresis (Plate 5), addition of trypsin resulted

Fig. 6. C.d. titration of α_1 -proteinase inhibitor with chymotrypsin at pH 8.0

Samples of chymotrypsin were added to 29 nmol of α_1 -proteinase inhibitor ($10 \mu\text{M}$) at pH 8.0. The ellipticity was recorded at 280 nm (●) and 290 nm (○). ---- Indicates the expected ellipticity for a simple addition of chymotrypsin after the end point. See the text for details. The inset shows the time-dependence of the change of ellipticity when 29 nmol of inhibitor were mixed with 35 nmol of chymotrypsin.

Fig. 7. C.d. titration of α_1 -proteinase inhibitor with chymotrypsin at pH 5.4

The titration was carried out exactly as in Fig. 2 except that the inhibitor was in 0.1M-sodium acetate buffer, pH 5.4. ---- indicates the expected ellipticity for a simple addition of chymotrypsin after the end point.

in the appearance of a band C_1' , which corresponds to the C_1 band seen when chymotrypsin was used (Plate 3). This is the 1:1 complex and is seen as band F_1' on isoelectric focusing in a pH 3–10 gradient (Plate 6). The C_1' band persists when $E/I > 1$ and is not converted into a faster running form, as was seen with chymotrypsin. However, a faster running band, C_2' , appears as E/I increases, and a band persists in the free inhibitor position even when $E/I > 1$. On the isoelectric-focusing pattern (Plate 6) the bands marked F_2' and F_3' correspond respectively to bands C_2' and the band in the free inhibitor position when $E/I > 1$. Travis *et al.* (1974) performed polyacrylamide electrophoresis on mixtures of the inhibitor with human trypsin and also noted that the complex was degraded by excess of enzyme.

Taken together the results of the c.d. titration and the analysis of the reaction products suggest that the α_1 -proteinase inhibitor forms only a 1:1 complex with trypsin or chymotrypsin.

By using c.d. the reaction can be followed directly (so minimizing the risk of proteolysis of the complex). The 1:1 complex, with either enzyme, may be de-

Fig. 8. C.d. spectra of α_1 -proteinase inhibitor, trypsin and mixtures of the two proteins

(a) —, C.d. spectrum of: α_1 -proteinase inhibitor (1.04 mg/ml; 29 μ M); ---, trypsin (0.48 mg/ml); ·····, a mixture of α_1 -proteinase inhibitor (1.04 mg/ml) and trypsin (0.48 mg/ml); - · - · - ·, the expected spectrum of the mixture calculated by addition of the two components. (b) Titration at pH 8.0 of 2.9 mg of α_1 -proteinase inhibitor (18.5 μ M) with trypsin. Ellipticity recorded at 292 nm. See the text for details.

graded by free enzyme, and the degradation products cause the complicated patterns seen on electrophoresis and isoelectric focusing.

We thank the following members of staff of the Department of Biochemistry, University of Glasgow: Dr. J. G. Beeley for the carbohydrate analyses, Dr. G. Leaf for the amino acid analyses, Dr. R. Eason for the ultracentrifugal analyses. We are grateful to Mr. R. T. Dick of the Blood Transfusion Department, Glasgow Royal Infirmary for supplying us with plasma. The technical assistance of Mr. A. Clark is gratefully acknowledged. The work was done during the tenure of a CIBA Research Fellowship by J. S. The work was supported by grants from the Arthritis

and Rheumatism Council for Research, Organon Laboratories Ltd., and the Wellcome Trust.

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