Human plasma α -cysteine proteinase inhibitor

Purification by affinity chromatography, characterization and isolation of an active fragment

Anne D. GOUNARIS,* Molly A. BROWN and Alan J. BARRETT

Department of Biochemistry, Strangeways Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K.

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Human plasma α -cysteine proteinase inhibitor (α CPI) was purified by a two-stage method: affinity chromatography on *S*-carboxymethyl-papain-Sepharose, and high-resolution anion-exchange chromatography. The protein was obtained as a form of M_r about 64000 and material of higher M_r (about 100000). In sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with reduction, both forms showed a major component of M_r 64000. An antiserum was raised against α CPI, and 'rocket' immunoassays showed the mean concentration in sera from 19 individuals to be 35.9 mg/dl. Both low- M_r and high- M_r forms of α CPI were confirmed to be sialoglycoproteins by the decrease in electrophoretic mobility after treatment with neuraminidase. α CPI was shown immunologically to be distinct from antithrombin III and α_1 -antichymotrypsin, two serine proteinase inhibitors from plasma with somewhat similar M_r values. α CPI was also distinct from cystatins A and B, the two intracellular low- M_r cysteine proteinase inhibitors from human liver. Complexes of α CPI with papain were detectable in immunoelectrophoresis, but dissociated to free enzyme and intact inhibitor in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The stoichiometry of binding of papain was close to 1:1 for both low- M_r and high- M_r forms. α CPI was found to be a tight-binding inhibitor of papain and human cathepsins H and L (K_i 34 pM, 1.1 nM and 62 pM respectively). By contrast, inhibition of cathepsin B was much weaker, K_i being about 35 μ M. Dipeptidyl peptidase I also was weakly inhibited. Digestion of α CPI with bromelain gave rise to an inhibitory fragment of M_r about 22000, which was isolated.

The best known of the cysteine proteinases (once called 'thiol' proteinases) is papain, and most of the

known cysteine proteinases fall into the superfamily of proteins homologous with papain (Barrett *et al.*, 1984). Since this group contains the major lysosomal proteinases cathepsins B, H and L, as well as chymopapain, which is used medically, the physiological inhibitors of the enzymes are of interest. Of the small number of proteins known to act as tight-binding inhibitors of these cysteine proteinases, all are small proteins, M_r about 13000, except the plasma protein, α CPI.

Human α CPI was first described by Sasaki *et al.* (1977), and further work by Ryley (1979), Sasaki's group (Sasaki *et al.*, 1981; Taniguchi *et al.*, 1981) and Järvinen (1979) showed that the protein occurs in relatively low- M_r and high- M_r forms in plasma, with α_2 and α_1 electrophoretic mobilities respectively. Järvinen (1979) described the purification of the two forms by use of affinity chromatography on a form of immobilized papain.

Abbreviations used: the abbreviations used for names of amino acid derivatives and *N*-terminal groups are based on the standard conventions [Biochem. J. (1984) 219, 345–373]. The *C*-terminal groups are: CH₂Cl, chloromethane, NPhNO₂, *p*-nitroanilide, and NMec, 7-(4-methyl)coumarylamide. Other abbreviations used are: Cm-, *S*-carboxymethyl; α CPI, plasma α -cysteine proteinase inhibitor; E-64, L-3-carboxy-2,3-epoxypropyl-leucylamido-(4-guanidino)butane; f.p.l.c., fast protein liquid chromatography (Pharmacia system); [I]_t, total inhibitor concentration; K_i , dissociation constant for inhibitor; $K_{i(\text{app})}$, apparent dissociation constant for inhibitor uncorrected for competition by substrate; [S], substrate concentration; SDS, sodium dodecyl sulphate; v_0 , rate; v_i , rate in the presence of inhibitor.

* Present address: Department of Chemistry, Vassar College, Poughkeepsie, NY 12601, U.S.A.

Experimental

Materials

Plasma was from blood supplied by a local blood-transfusion centre, and contained acid citrate/dextrose anticoagulant as described previously (Barrett *et al.*, 1979). Volumes of plasma given in the text have been corrected for dilution by the anticoagulant.

Cm-papain-Sepharose (0.6 mg of papain/g wet wt. of gel) was prepared as described by Anastasi *et al.* (1983), and Cm-chymopapain-Sepharose was prepared in an analogous manner. Chymopapain was partially purified from latex of *Carica papaya* by a salt fractionation procedure based on that of Brocklehurst *et al.* (1981). Papain was isolated from *Carica papaya* latex by a modification of the method of Baines & Brocklehurst (1979). Ficin (twice-crystallized) was from Sigma (London) Chemical Co. Bromelain (stem) was the crystalline suspension from Boehringer Corp. (London) Ltd. Human cathepsins B and H were isolated as described by Schwartz & Barrett (1980). Human cathepsin L was purified from a homogenate of human liver by acid treatment, acetone fractionation, gel chromatography and f.p.l.c. ion-exchange chromatography (G. D. J. Green & A. J. Barrett, unpublished work). Human plasma kallikrein and antiserum against it (which also reacts with prokallikrein) were available in the laboratory (Nagase & Barrett, 1981), as were the human liver cysteine-proteinase inhibitors cystatin A and cystatin B, and antisera against them (Green *et al.*, 1984). Bovine spleen dipeptidyl peptidase I, kindly given by Dr. J. K. McDonald (Medical University of South Carolina, Charleston, SC, U.S.A.), was further purified by f.p.l.c. Neuraminidase (*Vibrio cholerae*; 500 units/ml) was from Koch-Light Laboratories. Bovine trypsin (type XII) and bovine thrombin were from Sigma.

Samples of purified α CPI (mixed high- M_r and low- M_r forms) and anti-(α CPI) serum for comparisons were kindly given by Dr. H. C. Ryley (Welsh National School of Medicine, Cardiff, U.K.). Antiserum to papain was kindly given by Dr. E. Shapira, The Children's Memorial Hospital, Chicago, IL, U.S.A. Antisera against antithrombin III, α_1 -antichymotrypsin and haptoglobin were purchased from Behringwerke A.G. The non-ionic detergent Brij 35 was purchased from BDH Chemicals, and Ultrogel AcA-44 from LKB Instruments. Di-isopropyl phosphorofluoridate was from Sigma, and Pro-Phe-Arg-CH₂Cl was a gift from Dr. E. N. Shaw, Brookhaven National Laboratory, Upton, NY, U.S.A.

Enzyme assays

Papain, bromelain, cathepsin B and cathepsin L were assayed fluorimetrically with Z-Phe-Arg-

NMec as substrate at pH 6.0, and cathepsin H was assayed with Arg-NMec as substrate at pH 6.5 (Barrett & Kirschke, 1981; Barrett *et al.*, 1982). Dipeptidyl peptidase I was assayed with Gly-Phe-NMec (10 μ M) in 0.10 M-sodium phosphate buffer, pH 6.5, containing 1 mM-EDTA, 50 mM-NaCl and 1 mM-dithiothreitol. Trypsin was assayed with Bz-DL-Arg-NPhNO₂ as substrate, thrombin with Boc-Val-Pro-Arg-NMec, and plasma kallikrein with Z-Phe-Arg-NMec, all as described by Nagase & Barrett (1981).

Quantification of inhibitory activity

The procedure used for titration of α CPI with papain was one previously described for cystatin, in which the substrate was Z-Phe-Arg-NMec (Anastasi *et al.*, 1983).

K_i values were determined by use of continuous fluorimetric assays in a Perkin-Elmer LS-3 spectrofluorimeter standardized with 0.2 μ M-aminomethylcoumarin for readings at λ_{em} 360 nm and λ_{exc} 460 nm. The substrates (as used in assays: see above) were at 10 μ M final concentration, and all measurements were made with less than 2% hydrolysis. For cathepsin B and cathepsin H, K_m was known to be much greater than 10 μ M, with the respective substrates (Barrett & Kirschke, 1981). For human cathepsin L, K_m for Z-Phe-Arg-NMec was found to be 2 μ M by the method of Wilkinson (1961) (A. J. Barrett, unpublished work). For papain and dipeptidyl peptidase I, $v_0/[S]$ was constant in the range 5–20 μ M substrate, so it was concluded that $K_m \gg 10 \mu$ M. The concentrations of the enzymes during the assay were approx. 0.02 nM (cathepsin B), 0.1 nM (human cathepsin L), 0.1 nM (cathepsin H), 0.01 nM (papain) and <0.1 nM (dipeptidyl peptidase I).

The method was as follows. The fluorimeter cuvette and all solutions were pre-warmed to 30°C (20°C for cathepsin L). Into the cuvette was placed 40 μ l of stock enzyme solution and 40 μ l of 100 mM-dithiothreitol. A 1–2 min period was allowed for activation, and then 3.84 ml of the appropriate assay buffer and 40 μ l of 1 mM substrate solution in dimethyl sulphoxide were thoroughly mixed in. Once a stable reaction rate had been recorded, the inhibitor was added in 40 μ l and the whole mixed well. The reaction rate was followed for up to 60 min as it relaxed progressively to the new linear rate corresponding to the concentration of enzyme in equilibrium with the enzyme-inhibitor complex. Control experiments were made to detect spontaneous decay of enzymic activity during the period of the experiment. Typically, data were obtained for five inhibitor concentrations. K_i values were determined from re-plots of the form $[I_i]/(1-v_i/v_0)$ versus v_0/v_i (Henderson, 1972). When experiments were made with $[S] \ll K_m$, corrections

were made by use of the relationship $K_i = K_{i(\text{app})}/(1 + [S]/K_m)$ for simple competition.

Raising of antiserum

A sheep was injected on three occasions at 2-week intervals; on each occasion, 1 mg of purified low- M_r α CPI in 1 ml was emulsified with an equal volume of Freund's adjuvant, and injected intramuscularly in the thigh. On the first occasion, complete adjuvant was used, and subsequently incomplete adjuvant. The animal was bled 2 weeks after the third injection.

Immunoelectrophoresis, immunodiffusion and 'rocket' electroimmunoassay

These techniques were performed as described by Barrett (1974) and Barrett *et al.* (1979). The 'rocket' immunoassay was standardized with low- M_r α CPI, assuming $A_{280}^{1\%} = 5.9$ (Ryley, 1979), and it was also assumed that all forms of α CPI reacted equally in the assay.

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was done by the method of Bury (1981) in gels of 7% or 12.5% polyacrylamide. Samples were either reduced with 2-mercaptoethanol, or run unreduced after treatment with iodoacetate, as described by Barrett *et al.* (1979). M_r calibration was with phosphorylase *b* (M_r 94000), transferrin (M_r 78000), bovine serum albumin (M_r 68000), immunoglobulin G heavy chain (M_r 50000), carbonic anhydrase (M_r 29000), immunoglobulin G light chain (M_r 25000), soya-bean trypsin inhibitor (M_r 21000), cytochrome (M_r 12750) and aprotinin (M_r 6500). M_r values for unknown components were calculated by use of linear plots of $\log M_r$ versus migration distance.

Pore-limit gradient gel electrophoresis of native proteins was done at pH 9.1 in gels of 4–26% polyacrylamide as described by Barrett *et al.* (1979).

Ultrafiltration

This was done in an Amicon cell fitted with a PM-10 or PM-30 membrane, under pressure of N_2 .

Results

Purification of α CPI

α CPI was purified from plasma in two steps, affinity chromatography on Cm-papain-Sepharose and anion-exchange chromatography.

Affinity chromatography. Plasma (400 ml) was run at 300 ml/h on to a column (12 cm $\times$ 4.5 cm, 190 cm³) of Cm-papain-Sepharose equilibrated with 0.05 M-sodium citrate buffer, pH 6.5, contain-

ing 0.5 M-NaCl and 0.1% Brij 35. The column was washed with 500 ml of the citrate buffer followed by about 2 litres of 0.05 M-potassium phosphate buffer, pH 8.5, also containing the NaCl and Brij 35, to lower the A_{280} of the effluent to less than 0.05. The inhibitor was eluted with 0.05 M-potassium phosphate/NaOH, pH 11.5, also containing the NaCl and Brij 35. Effluent fractions (5 ml) were collected into tubes containing 1 ml of 0.25 M- KH_2PO_4 to effect rapid neutralization of the solution. Fractions containing the peak of protein (A_{280}) eluted with alkali were combined, concentrated to 10 ml by ultrafiltration, and transferred into 50 mM-Tris/25 mM-HCl buffer, pH 8.0.

On some occasions, a smaller (60 cm³) column of Cm-chymopapain-Sepharose was used in the same way, but with smaller volumes of the wash solutions.

Anion-exchange chromatography. The material eluted from the Cm-papain-Sepharose column at pH 11.5 comprised the low- M_r form of α CPI, high- M_r forms of α CPI, and minor contaminants. These were separated by anion-exchange chromatography. The Mono Q anion-exchange column (5 cm long) was used in a Pharmacia f.p.l.c. system. The linear gradient increased from 0.05 M-Tris/0.025 M-HCl to 0.50 M-Tris/0.25 M-HCl, pH about 8.0, at 22°C, after the sample had been applied in the starting buffer. Some protein passed unadsorbed through the column, and a second minor peak (peak I) was eluted at 0.10 M- Cl^- , followed by three complex major peaks (II, III and IV) (Fig. 1).

Unadsorbed material, and that in peak I, lacked inhibitory activity, but contained an enzyme that hydrolysed Z-Phe-Arg-NMec at neutral pH; this was inhibited by di-isopropyl phosphorofluoridate and Pro-Phe-Arg- CH_2Cl , and was identified as plasma kallikrein by double immunodiffusion against anti-kallikrein serum in comparison with an authentic sample. We have no explanation for the affinity of kallikrein (or perhaps prokallikrein) for Cm-papain-Sepharose.

Inhibitory activity was detected in effluent fractions comprising peaks II and IV, but not peak III. Samples from fractions 32 (peak II) and 46 (peak IV) were run in SDS/polyacrylamide-gel electrophoresis (Fig. 2; see below), and the two peaks of inhibitory activity were found to correspond to the low- M_r and high- M_r forms respectively of α CPI. In a typical purification we obtained 27.8 mg of low- M_r α CPI and 13.5 mg of high- M_r α CPI from 400 ml of plasma.

Immunochemical tests

Tests were made to establish the concentration of α CPI in serum, to discover whether any other plasma proteins that might be inhibitors of cysteine proteinases have affinity for Cm-papain-

Fig. 1. *Mono Q f.p.l.c. of αCPI*

The inhibitory material (5 ml) from the Cm-papain-Sepharose affinity column was run on the Mono Q column equilibrated with 0.05 M-Tris/0.025 M-HCl buffer, pH 8.0, and eluted with a linear gradient to 0.50 M-Tris/0.25 M-HCl buffer, pH 8.0, at 22°C. The bars indicate points at which samples were taken for SDS/polyacrylamide-gel electrophoresis (Fig. 2). —, A₂₈₀; — —, Cl⁻ gradient.

Sepharose, and whether the purified αCPI fractions have immunological reactivities of any other proteins.

Concentration of αCPI in human serum. Nineteen samples of serum from hospital patients were run in 'rocket' immunoelectrophoresis in agarose gel containing anti-αCPI serum, together with standard samples of αCPI. It was found that the concentration was 35.9 mg/dl (range 24.0–48.8 mg/dl).

Test of αCPI preparations for other proteins. A solution of crude αCPI from the Cm-papain-Sepharose column (1 mg/ml), plasma that had passed through the column, and purified αCPI (1 mg/ml) were run in double immunodiffusion against antisera to haptoglobin, antithrombin III, human liver cystatins A and B and total human serum proteins. Of these antigens, only αCPI and plasma kallikrein were detected in the fraction eluted from the affinity column, and only αCPI in the purified preparation. The plasma that had passed through the affinity column was conspicuously depleted of αCPI and kallikrein (or prokallikrein), but not of haptoglobin, antithrombin III or α₁-antichymotrypsin.

Rather surprisingly, the commercial antiserum to whole human serum did not react with αCPI. Immunoelectrophoresis of the high-*M_r* and low-*M_r* forms of αCPI showed no precipitin arcs (suggesting the absence of gross contamination by other serum proteins), whereas plates developed with the anti-αCPI serum produced arcs confirming the α₂- and α₁-mobilities respectively of the low-*M_r* and high-*M_r* forms reported by Sasaki *et al.* (1981).

The absence of reactivity for antithrombin III in the αCPI samples shows that αCPI is quite distinct from the thrombin inhibitor, although the latter has been reported to be an inhibitor of papain (Valeri *et al.*, 1980). In view of the fact that it is very unusual for a protein inhibitor to be active against proteinases of more than one catalytic class (Laskowski & Kato, 1980), it may be necessary to consider whether the inhibition of papain by some preparations of antithrombin is due to contamination with αCPI. αCPI also was clearly distinct from α₁-antichymotrypsin. The immunological dissimilarity of αCPI from the low-*M_r* inhibitors from liver was further confirmed by the finding that antiserum to αCPI did not react with the human cystatins in double immunodiffusion.

Characteristics of the molecular forms of αCPI

Experiments were made in an attempt to clarify the structural relationship between the low-*M_r* form of αCPI and the two forms that have greater

M_r and more negative molecular charge.

SDS/polyacrylamide-gel electrophoresis. Fractions from the Mono Q anion-exchange column (Fig. 1) were run in SDS/polyacrylamide-gel electrophoresis with and without reduction (Fig. 2). Without reduction, material from pool II ran with *M_r* 64000, whereas pool IV showed two components of *M_r* about 100000. With reduction, both pools gave a major component of *M_r* 64000, with minor components of lower *M_r*. These are formed by the degradation of αCPI by a proteinase

Fig. 2. SDS/polyacrylamide-gel electrophoresis of samples from Mono Q f.p.l.c.

Samples were M_r markers (see the Experimental section) run reduced (a), fraction 32 run unreduced (b) and reduced (c), and fraction 46 run unreduced (d) and reduced (e). It can be seen that the major bands in (b), (c) and (e) run between immunoglobulin G heavy chain and serum albumin, with M_r about 64000.

associated with it in some preparations (G. S. Salvesen & A. J. Barrett, unpublished work).

Gel chromatography. Partially purified α CPI from the Cm-papain-Sepharose column was run on a column (85cm $\times$ 1.5cm, 150cm³) of Ultrogel AcA-44 in 0.05M-sodium citrate buffer, pH6.5, containing 0.5M-NaCl and 0.1% Brij 35. Three peaks of protein were obtained, the second and third of which inhibited papain, and gave a precipitin reaction with anti- α CPI serum. When run in SDS/polyacrylamide-gel electrophoresis with and without reduction, peak-2 material ran exactly like the high- M_r , more-retarded, material from Mono Q, whereas peak III was shown to correspond to the low- M_r , less-retarded, fraction. These results suggested that the relative M_r values indicated by SDS/polyacrylamide-gel electrophoresis of the forms do correspond to those of the native proteins.

Effect of neuraminidase on α CPI. The greater negative charge of the high- M_r forms of α CPI relative to the lower- M_r form (seen in immunoelectrophoresis and anion-exchange chromatography) is not directly attributable to the differences in M_r , but might have been due to differences in sialylation.

Samples (50 μ g) of the low- M_r and high- M_r forms of α CPI were treated with neuraminidase (10 Koch-Light units) in 0.1 ml of sodium acetate buffer, pH5.5, containing 6mM-CaCl₂ for 90min at 37°C. Samples were run in immunoelectro-

phoresis, and the plates developed with antiserum against α CPI. The mobilities of both forms of the inhibitor were decreased by the action of neuraminidase, and we concluded that α CPI is a sialoglycoprotein, in agreement with the report by Ryley (1979). Desialylation did not cause the forms to acquire the same electrophoretic mobility, however.

Interaction of α CPI with proteinases

Detection of the α CPI-papain complex. Papain, Cm-papain, α CPI (low- M_r form), and two mixtures, one α CPI with papain, the other α CPI with Cm-papain, were run in immunoelectrophoresis. The plate was developed with antisera against papain and α CPI (Fig. 3a). The precipitin arcs for papain and α CPI against their respective antisera showed their widely different electrophoretic mobilities. Each mixture showed a new precipitin arc of intermediate mobility that was antigenic for both antisera, and was thus identified as an α CPI-papain complex or as an α CPI-Cm-papain complex respectively.

The complex between α CPI and (carboxymethylated) papain was also demonstrated in pore-limit electrophoresis (Fig. 3b).

SDS/polyacrylamide-gel electrophoresis of α CPI-papain complexes. α CPI (100 μ g) and papain (100 μ g) were mixed in 100 μ l of 0.05M-sodium citrate buffer, pH6.5, containing 0.5M-NaCl, 0.1% Brij 35 and 2mM-cysteine. Iodoacetate was then added in excess over the cysteine, to inactivate the proteinase, before the mixture was run in SDS/polyacrylamide-gel electrophoresis with reduction, in a 12.5%-polyacrylamide gel. Similar experiments were made with ficin and cathepsin B in place of papain. Always, the bands on the gel corresponded to those of the free inhibitor and the free enzyme. We concluded that the α CPI-cysteine proteinase reaction does not involve the formation of a covalent SDS-stable complex, or cleavage of the inhibitor molecule, both of which have been seen with plasma inhibitors of serine proteinases such as α_1 -proteinase inhibitor and antithrombin III (Travis & Salvesen, 1983).

Stoichiometry of the α CPI-papain interaction

This was estimated in two ways: by direct titration of α CPI with papain, and by examination of the molecular size of the complexes.

Titration of α CPI with papain. Solutions of purified low- M_r and high- M_r forms of α CPI were titrated with papain that had itself been standardized with E-64 (Barrett *et al.*, 1982). On the basis of $A_{280}^{1\%} = 5.7$ (Ryley, 1979), it was calculated that the preparations contained one inhibitory site per 73600 and 92400 daltons of protein respectively.

Fig. 3. Electrophoretic detection of α CPI-papain complexes

(a) Immunoelectrophoresis. Well (i), Cm-papain (4 μ g); well (ii), Cm-papain (4 μ g) with α CPI (2 μ g); well (iii), α CPI (4 μ g); well (iv), papain (3.5 μ g) with α CPI (2 μ g); well (v), papain (3.5 μ g). Troughs A contained anti-papain serum, and troughs B contained anti- α CPI serum. (b) Pore-limit electrophoresis. Samples run in the gradient of increasing polyacrylamide concentration (from top down) were (i) standards human transferrin (Tfn) and bovine serum albumin (Bsa), as indicated, (ii) α CPI (low- M_r form), and (iii) α CPI mixed with excess Cm-papain. It can be seen that all of the α CPI was converted into less-mobile complex with the enzyme derivative.

at 25°C for 1 h before removal of samples for gel electrophoresis.

Two protein bands were observed for α CPI incubated with glutaraldehyde in the absence of papain, with apparent M_r values of $54\,700 \pm 1300$ (S.E.M., $n = 7$) and $97\,000$ ($n = 2$). These were interpreted as representing α CPI monomer in which intrachain cross-linking had produced a more compact molecule than the unfolded normal protein, and an interchain-cross-linked α CPI dimer respectively. The M_r of native α CPI was $64\,000 \pm 2\,700$ (S.E.M., $n = 3$). Similarly, monomer and dimer bands were observed for papain cross-linked with glutaraldehyde.

One new band was observed in all incubations of α CPI with papain or Cm-papain in the presence of glutaraldehyde. The apparent M_r of this was $74\,800 \pm 2\,200$ (S.E.M., $n = 3$). The sum of the M_r values observed for glutaraldehyde-treated papain, $24\,200 \pm 1\,200$ (S.E.M., $n = 3$), and glutaraldehyde-treated α CPI, $54\,700 \pm 1\,300$ (S.E.M., $n = 7$) was $78\,900$. This new component was interpreted as a glutaraldehyde-cross-linked complex of α CPI and papain in a 1:1 molar ratio.

Affinity of α CPI for proteinases

α CPI proved to be a tight-binding inhibitor of papain, cathepsin H and cathepsin L, the respective K_i values being 34 pM, 1.1 nM and 62 pM. By contrast, the inhibition of cathepsin B was much weaker, the slight inhibition obtained at the highest inhibitor concentration used (13 nM) being compatible with a K_i of 0.35 μ M. Similarly, dipeptidyl peptidase I was weakly inhibited, with K_i approx. 0.13 μ M.

Low concentrations of α CPI partially inhibited commercial bromelain, but very high concentrations were required to complete the inhibition. This was found to be due to heterogeneity of the commercial bromelain, the major protein component being a cysteine proteinase that was very weakly inhibited by α CPI, with $K_{i(\text{app.})}$ 0.21 μ M (A. J. Barrett, unpublished work).

No inhibition of the serine proteinases trypsin and thrombin by α CPI was detected. The activity of plasma kallikrein was directly detectable in crude α CPI preparations, showing that this serine proteinase also was not inhibited.

Preparation of inhibitory fragment from α CPI

Glutaraldehyde-cross-linking of α CPI-papain complexes. Low- M_r α CPI (115 μ g/ml), papain (30 μ g/ml), and a mixture of the two, all in 30 mM-sodium citrate buffer, pH 6.5, containing 0.30 M-NaCl and 0.03% Brij 35, were incubated with glutaraldehyde (10 mM) for 10 min at 40°C and left

α CPI (50 mg) partially purified by affinity chromatography on Cm-chymopapain-Sepharose was incubated with 1 mg of commercial bromelain in a total volume of 5 ml of 0.10 M-sodium phosphate buffer, pH 6.5, containing 1 mM-EDTA. The bromelain had been pre-activated in 1 mM-dithiothreitol, and the concentration of dithiothreitol

Fig. 4. SDS/polyacrylamide-gel (12.5%, reduced) electrophoresis of bromelain digest of α CPI

Lane (a), α CPI (a somewhat aged preparation); lane (b), bromelain digest; lane (c), Ultrogel AcA-44 chromatography peak 4; lane (d), f.p.l.c. Mono Q fraction; lane (e), standards (see the Experimental section).

during the digestion was 0.01 mM. The digestion was for 30 min at 30°C, and the solution was then made 10% (w/v) with respect to sucrose, and applied to a column (1.5 cm $\times$ 84 cm) of Ultrogel AcA-44 equilibrated with 0.1 M-sodium phosphate buffer, pH 6.5. Four major peaks of protein (as A_{280}) were eluted, the third of which comprised material of M_r about 20 000 that was inhibitory for papain.

Fractions representing the inhibitory peak were combined, dialysed against 0.02 M-Tris/HCl buffer, pH 8.0, and run on the Mono Q column of the Pharmacia f.p.l.c. apparatus in a gradient of the same buffer from 0.02 M- to 0.50 M-Tris/HCl. The A_{280} profile of the effluent presented a complex pattern of peaks, including about five major ones, the fourth of which was a doublet. The material comprising this fourth peak was re-run on the Mono Q column under the same conditions; again the peak was double-headed, but the material in the later portion had the higher specific inhibitory activity, and was found to consist primarily of a single component of M_r about 20 000 when run in SDS/polyacrylamide-gel electrophoresis (Fig. 4).

Discussion

The two-step procedure described for the purification of α CPI from human plasma is simpler than those of Sasaki *et al.* (1977) and Ryley (1979). F.p.l.c. on the Mono Q anion-exchange column

separated the high- M_r and low- M_r forms of α CPI, as well as removing contaminants. The first peak of α CPI eluted from the Mono Q was the species of M_r 64 000 that has the lesser anodal mobility in gel electrophoresis, and has been called ' α_2 -thiol proteinase inhibitor'. The α CPI eluted later from Mono Q corresponded to the more-anodal and higher- M_r form that has been called ' α_1 -thiol proteinase inhibitor'; we found this to be heterogeneous, consisting of about two species, with M_r 80 000–100 000, in SDS/polyacrylamide-gel electrophoresis under non-reducing conditions.

The inhibition of cathepsin B by serum was attributed largely to α_2 -macroglobulin in the study by Starkey & Barrett (1973), although there was apparent inhibition by a component of similar M_r to immunoglobulin G. This may have been the result of proteins acting as competing substrates in the assays. Our present results show that the K_i of α CPI for cathepsin B is only about an order of magnitude below its plasma concentration, so that assays with diluted column fractions and a substrate competing for cathepsin B would not have shown distinct inhibition by α CPI.

The physiological function of α CPI remains to be established. The molar concentrations of α_2 -macroglobulin and α CPI in plasma are approx. 3.4 μ M and 5.5 μ M respectively, but α_2 -macroglobulin binds two molecules of papain per molecule (Howell *et al.*, 1983). Sasaki *et al.* (1983) have suggested that inhibition of calpain in the blood may be mediated predominantly by α CPI. This could be important in view of the marked effects of calpain on platelet function reported by Yoshida *et al.* (1983). Unlike α_2 -macroglobulin, α CPI is almost certainly of small enough molecular size to escape from the blood into the tissues, where it could regulate extracellular activities of the lysosomal proteinases.

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