Cystatin-like cysteine proteinase inhibitors from human liver

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Cysteine proteinase inhibitor (CPI) forms from human liver were purified from the tissue homogenate by alkaline denaturation of cysteine proteinases with which they are complexed, acetone fractionation, affinity chromatography on *S*-carboxymethyl-papain–Sepharese and chromatofocusing. The multiple forms of CPI were shown immunologically to be forms of two proteins, referred to as CPI-A (comprising the forms of relatively acidic pI) and CPI-B (comprising the more basic forms). CPI-A and CPI-B are similar in their M_r of about 12400, considerable stability to pH 2, pH 11 and 80°C, and tight-binding inhibition of papain, several related cysteine proteinases and dipeptidyl peptidase I. K_i values were determined for papain, human cathepsins B, H and L, and dipeptidyl peptidase I. The affinity of CPI-A for cathepsin B was about 10-fold greater than that of CPI-B, whereas CPI-B showed about 100-fold stronger inhibition of dipeptidyl peptidase I. For all the cysteine proteinases the liver inhibitors were somewhat less tight binding than cystatin. The resemblance of both CPI-A and CPI-B in several respects to egg-white cystatin is discussed. CPI-A seems to correspond to the epithelial inhibitor described previously, and CPI-B to the inhibitor from other cell types [Järvinen & Rinne (1982) *Biochim. Biophys. Acta* 708, 210–217].

Relatively few protein inhibitors specific for cysteine proteinases are known, and for none of them has the inhibitory mechanism been elucidated. Since the cysteine proteinases cathepsins B, H and L are responsible for most of the proteolytic activity of lysosomes, and are therefore likely to make a major contribution to intracellular proteolysis, the intracellular mechanisms for their regulation are clearly of interest.

It is now well recognized that mammalian cells contain cysteine proteinase inhibitors of M_r about

Abbreviations used: the abbreviations used for amino acid derivatives and *N*-terminal groups are based on the standard conventions [Biochem. J. (1972) 126, 773–780]; the *C*-terminal group NMec was 7-(4-methyl)coumarylamide. Other abbreviations are: Bistris, 2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CPI, cysteine proteinase inhibitor; Tos, tosyl; Cm-papain, *S*-carboxymethyl-papain; compound E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl leucylamido-(4-guanidino)butane; f.p.l.c., fast protein liquid chromatography (Pharmacia system); [E], total concentration of enzyme; [I], total inhibitor concentration; K_i , dissociation constant for inhibitor; v_i , velocity in the presence of inhibitor; v_0 , velocity without inhibitor.

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13000. These have been detected in rabbit, rat and human skin (Udaka & Hayashi, 1965; Järvinen, 1978; Järvinen *et al.*, 1978; Hibino *et al.*, 1980a,b), human squamous-cell carcinomata (Rinne *et al.*, 1980), rat liver (Kominami *et al.*, 1981, 1982), human spleen (Järvinen & Rinne, 1982), bovine spleen (Brzin *et al.*, 1982), pig leucocytes (Kopitar *et al.*, 1981), and other tissues and plasma (Roughley *et al.*, 1978; Lenney *et al.*, 1979; Gauthier *et al.*, 1983). In molecular mass and several other properties the inhibitors resemble cystatin, the inhibitor present in chicken egg-white and serum (Anastasi *et al.*, 1983).

We describe here the purification of two immunologically distinct inhibitors from human liver, each in several molecular forms, and compare them with egg-white cystatin.

Experimental

Materials

Cm-papain–Sepharese and egg-white cystatin were prepared as described by Anastasi *et al.* (1983).

Cathepsins B and H from human liver were prepared 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 ion-exchange chromatography (G. D. J. Green, unpublished work). Rat cathepsin L was kindly given by Dr. H. Kirschke, Physiological Chemistry Institute, Halle (Saale), German Democratic Republic. Human cathepsin G was purified as described by Saklatvala & Barrett (1980). Papain and papaya peptidase were isolated from *Carica papaya* latex essentially as described by Baines & Brocklehurst (1979) and Robinson (1975) respectively. Clostripain, chymotrypsin (type VII), ficin (crystalline), thermolysin (proteinase type X) and trypsin (bovine, 2 $\times$ crystallized, type XII) were from Sigma Chemical Co. Bromelain (crystalline) was from Boehringer Corp., Lewes, East Sussex, U.K. Pig pancreatic elastase was kindly given by Dr. J. Travis, Department of Biochemistry, Boyde Graduate Studies Research Center, Athens, GA, U.S.A. Streptococcal cysteine proteinase (Elliott & Liu, 1970) was kindly given by Dr. S. Elliott, Department of Pathology, University of Cambridge, Cambridge, U.K., and bovine spleen dipeptidyl peptidase I by Dr. J. K. McDonald, Medical University of South Carolina, Charleston, SC, U.S.A.

Aminomethylcoumarin substrates were purchased from Bachem, Bubendorf, Switzerland, Cambridge Research Biochemicals, Harston, Cambridgeshire, U.K., or Universal Biologicals, Cambridge, U.K.

Antisera to the acid and neutral forms of human spleen cysteine proteinase inhibitor (Järvinen & Rinne, 1982) were kindly given by Dr. M. Järvinen, Department of Anatomy, University of Oulu, Oulu, Finland. Antisera to liver CPI forms were raised as described below. Antiserum against human plasma α -cysteine proteinase inhibitor was prepared by Dr. A. Gounaris in this laboratory (unpublished work).

Electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was done in the 2-amino-2-methylpropane-1,3-diol (Ammediol)/glycine/HCl discontinuous buffer system described by Bury (1981) in slab gels of 12.5% total acrylamide concentration.

Analytical isoelectric focusing

Cylindrical gels were run as described by Barrett (1970) with Pharmalyte (Pharmacia) ampholytes of appropriate range. Often, the gels were sliced longitudinally, and one half was stained for protein (Bürk *et al.*, 1983) and the other was cut transversely into 3 mm segments. Each segment was immersed in 0.5 ml of water and stored overnight before the supernatant fluid was subjected to pH

determination and assay of inhibitory activity for papain.

Enzyme assays

Papain, papaya peptidase A, bromelain, ficin, cathepsin B and cathepsin L were assayed fluorimetrically with Z-Phe-Arg-NMec as substrate at pH 6.0; clostripain was assayed with Z-Phe-Arg-NMec at pH 7.5 in the presence of 10 mM-CaCl₂, and cathepsin H was assayed with Arg-NMec as substrate at pH 6.5 (Barrett & Kirschke, 1981; Barrett *et al.*, 1982). Trypsin, chymotrypsin, pancreatic elastase, cathepsin G and thermolysin were assayed with azocasein in 0.10 M-Tris/HCl buffer, pH 8.1, containing 20 mM-CaCl₂ (Anastasi *et al.*, 1983). Dipeptidyl peptidase I was assayed fluorimetrically 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.

Molar concentrations of active papain and cathepsins B, H and L were determined by titration with compound E-64 (Barrett *et al.*, 1982), and a maximal estimate of the concentration of dipeptidyl peptidase I was calculated from the protein concentration of the solution.

Assay of cysteine proteinase inhibitors by inhibition of papain

Cysteine proteinase inhibitors were assayed by a micro-scale titration of papain essentially as described by Anastasi *et al.* (1983). Sometimes the titration was done with Z-Arg-NMec as substrate at a final concentration of 5 μ M, instead of Z-Phe-Arg-NMec. In such cases the concentration of papain (itself standardized by titration with compound E-64) was 2.5 μ M during the preincubation. Assays were completed in the usual way (Barrett & Kirschke, 1981). By the nature of the assay, the result was obtained in terms of molar concentration of inhibitory binding sites.

Ultrafiltration

CPI was transferred from solution in one buffer to another and concentrated by ultrafiltration under pressure of N₂ over a YM-5 membrane (Amicon).

Immunological methods

Antisera were raised in two rabbits by intramuscular injection of CPI preparations. One animal was immunized with a mixture of CPI forms eluted from the chromatofocusing column at acidic pH (giving antiserum A), and the second was injected with forms eluted at basic pH (giving antiserum B). A total of four injections, each of 100 μ g, was given at 3-weekly intervals. Immunogen for the first injection was emulsified in Freund's complete adjuvant, and subsequently

Freund's incomplete adjuvant was used. Antisera were stored at -20°C .

Double immunodiffusion was in 1% agarose in 20 mM-sodium potassium phosphate buffer, pH 7.2, containing 0.15 M-NaCl, and plates were allowed to develop for 24 h before being washed overnight in 1% NaCl, dried and stained with Brilliant Blue G (Barrett, 1974).

Measurement of K_i values

K_i values were determined by use of continuous fluorimetric assays in a Perkin-Elmer LS-3 spectrofluorimeter standardized with 0.2 μM -aminomethylcoumarin for readings at λ_{em} , 360 nm and λ_{exc} , 460 nm. The substrates (as used in assays: see above) were used at 10 μM 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 μM , with the respective substrates (Barrett & Kirschke, 1981). For human cathepsin L, K_m for Z-Phe-Arg-NMec was found to be 2 μM , 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 μM substrate, so it was concluded that $K_m \gg 10 \mu\text{M}$. The concentrations of the enzymes during the assay were 0.02 nM (cathepsin B), 0.01 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 μl of stock enzyme solution and 40 μl 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 μl 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 μl 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, values were obtained for five inhibitor concentrations. Apparent 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) and, when $[I]_i \gg [E]_i$, $v_0/v_i - 1$ versus $[I]_i$. 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.

Purification of liver cysteine proteinase inhibitors

The inhibitors were purified in five main steps: homogenization of the tissue, alkali treatment,

acetone fractionation, affinity chromatography and chromatofocusing.

Homogenization. Frozen tissue (2–3 kg) was partially thawed, trimmed of fat and cut into small pieces (about 1 cm^3). Portions (200 g) were homogenized in 400 ml of 1% NaCl/2% (v/v) butan-1-ol/3 mM-disodium EDTA at 4°C by use of a Waring blender. Particulate matter was removed by centrifugation (2000 g for 30 min at 4°C) and discarded. The supernatant was filtered through glass-wool.

Alkali treatment. The supernatant was adjusted to pH 11.0 by addition of 3 M-NaOH with stirring. After 2 h at 4°C the pH was lowered to pH 6.5 by the gradual addition of 2 M-HCl with stirring. Precipitated protein was removed by centrifugation (2000 g for 30 min at 4°C) and discarded.

Acetone fractionation. The supernatant (at 4°C) was placed in an ice/NaCl bath. An equal volume of acetone was run in with stirring, over a period of 10 min. The precipitate was removed by centrifugation (2000 g for 30 min at 4°C), and discarded. A further portion of acetone equal in volume to the first was added to the supernatant as before. The precipitate was collected by centrifugation (2000 g for 30 min at 4°C) and the supernatant discarded.

The pellet was extracted overnight at 4°C by stirring in 300 ml of 50 mM-sodium phosphate buffer, pH 6.5, containing 0.5 M-NaCl and 0.1% Brij 35. Undissolved material was removed by centrifugation (10000 g for 30 min at 4°C) and discarded.

Affinity chromatography. The supernatant was applied to a Cm-papain-Sepharose column (50 mm diameter, 300 ml bed volume) that had been equilibrated with 50 mM-sodium phosphate buffer, pH 6.5, containing 0.5 M-NaCl and 0.1% Brij 35. The column was washed with the same buffer until the A_{280} approached zero. The bound protein was eluted with 50 mM-tripotassium phosphate, pH 11.5, containing 0.5 M-NaCl and 0.1% Brij 35. The fractions containing the single peak of protein eluted by the alkaline buffer were combined and the pH was adjusted to 6.5 with 1.0 M-HCl.

The protein solution was transferred into 75 mM-Tris/acetic acid, pH 9.3, and concentrated to 10 ml by ultrafiltration.

Chromatofocusing. Chromatofocusing was carried out by use of an f.p.l.c. system (Pharmacia), essentially as described by the manufacturers. The sample was run on the Mono P column (20 cm $\times$ 0.5 cm), which had been equilibrated with 75 mM-Tris/acetic acid, pH 9.3. Polybuffer 96 (diluted 12-fold and adjusted to pH 6.0 with 1.0 M-HCl) was used as eluent at a flow rate of 1 ml/min. On completion of the pH gradient, protein still bound to the column was eluted with 1.0 M-NaCl, transferred into 25 mM-Bis-Tris/HCl buffer, pH 6.3, by ultrafiltration, and re-run on the Mono P

column, which had been equilibrated with the same buffer. The protein was eluted with Polybuffer 74 (diluted 10-fold and adjusted to pH 4.0 with 1.0M-HCl) at a flow rate of 1 ml/min.

Pools representing the separated forms of CPI were made from both chromatofocusing runs.

Results

Purification of CPI-A and CPI-B

The progress of a typical purification is summarized in Table 1.

The initial homogenate contained free cathepsins B and H, so presumably the inhibitors were entirely complexed. The alkali treatment destroyed the lysosomal cysteine proteinases, liberating the inhibitors in assayable form. A large amount of inactive protein was incidentally precipitated during the re-adjustment to pH 6.5, and could be removed by centrifugation.

Fractionation of the soluble material with acetone decreased the total amount of protein to about 10g, and also eliminated α -cysteine proteinase inhibitor (as shown by double immunodiffusion against antiserum to that protein).

Affinity chromatography on Cm-papain-Sepharose yielded a single sharp peak of protein (5–10mg) that proved to consist almost entirely of the two CPI forms. The Cm-papain-Sepharose was used repeatedly without noticeable deterioration.

All of the material from the affinity column was loaded on to the Mono P chromatofocusing column of the f.p.l.c. apparatus, and multiple peaks, all inhibitory to papain, were resolved in the range pH 9.0–6.0 (Fig. 1a). Material not eluted in this range was re-run in a second gradient, pH 6.0–4.0, and a further series of peaks was obtained (Fig. 1b). Again all the peaks showed inhibitory activity.

The pH values at which the peaks of CPI were eluted during the chromatofocusing steps were

reproducible between preparations, and allowed the major forms to be named according to the pH values, but the relative amounts of the individual forms varied from one preparation to another. It was found that the method of making two completely separate chromatofocusing runs (see the

Fig. 1. Separation of multiple forms of human liver CPI by f.p.l.c. chromatofocusing on the Mono P column in the pH ranges (a) 9–6 and (b) 6–4

Run (a) was continued to pH 6.0 (39 ml), but without protein eluted beyond that shown. Similarly, earlier and later parts of the pH 6–4 run (b) were devoid of protein. The four major forms of CPI collected for further study are marked with the pH values of elution, '8.3', '8.1', '7.7' and '5.2'.

Table 1. Results of a typical purification of human liver CPI forms, from 2.4 kg of tissue

The weights of CPI are calculated from the results of titration with papain, on the basis of an M_r of 12400. Amounts of protein are estimated approximately as $A_{280} \times \text{ml}$. The specific activities are expressed as mg of CPI/ A_{280} unit of protein. Yields of the individual forms of CPI separated by chromatofocusing varied so much between preparations that no values could usefully be included here, but an impression of the relative amounts in one preparation can be gained from Fig. 1.

	Volume (ml)	CPI (mg)	Protein (A_{280} units)	Purification factor	Specific activity (mg/ A_{280} unit)
Alkali-treated homogenate	4760	16.3	146600	1	0.000111
Acetone fraction	282	9.85	9620	9.2	0.00102
CM-papain-Sepharose	86.5	3.78	5.62	6050	0.67
Sample for chromatofocusing	15	3.65	4.52	7268	0.808

Fig. 2. Double immunodiffusion of separated forms of liver CPI against antisera A and B

The central row of wells contained samples from peaks of CPI eluted from the Mono P chromatofocusing column at the indicated pH values. All the upper wells contained antiserum against CPI-B, whereas the lower wells contained antiserum against CPI-A.

Experimental section) gave better results than simply attempting to re-equilibrate the column to the Bistris buffer with the sample still adsorbed.

In a preparation from 2–3 kg of human liver we typically obtained a total of 4–8 mg of CPI in the various forms. The final purification factor of 7300 was comparable with the value of 6600 obtained by Järvinen & Rinne (1982) for human spleen.

Immunochemical discrimination between forms A and B of CPI. The individual forms of CPI from chromatofocusing were run in immunodiffusion against the two antisera raised against the acidic (antiserum A) and basic (antiserum B) forms of CPI (see the Experimental section).

The multiple forms of CPI that were eluted from the first chromatofocusing column above pH 7.7 all gave immunoprecipitation reactions only with antiserum B. Material comprising the peak eluted at pH 7.7, and all the forms eluted in the second run, gave reactions with antiserum A, although the pH 7.7 material also gave a weak reaction with antiserum B. The relationship between the antigens reacting with antisera A and B respectively was one of complete immunological non-identity (results not shown). We conclude that the several forms of CPI that were resolved represent multiple forms of two immunologically distinct proteins, which we designated provisionally as 'CPI-A' and 'CPI-B' in accordance with the reactions with the antisera, and relatively more acidic and basic elution pH values. The peak '7.7' behaved as if it was predominantly a CPI-A form contaminated with a small amount of a CPI-B form.

We found that the liver CPI-A forms reacted with Järvinen's antiserum against the 'acidic' form of CPI from human spleen, whereas the CPI-B forms reacted with his antiserum against the 'neutral' inhibitor. These were reactions of complete identity with those given by our antisera.

Properties of CPI-A and CPI-B

The properties of four of the major forms of CPI, CPI-A forms '5.2' and '7.7' and CPI-B forms '8.1' and '8.3' were studied further.

In sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (with reduction) all four forms showed very similar mobilities to that of cytochrome *c* (M_r 12400) (results not shown). Each of the CPI-A forms ran as a single band, whereas the two CPI-B forms ran as doublets. Järvinen & Rinne (1982) commented on similar behaviour of the 'neutral' but not the 'acidic' forms of human spleen CPI, and Kominami *et al.* (1981) found the same with rat liver CPI.

When some preparations of the CPI-B forms were run in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis without reduction, a minor component of apparent M_r 25000 appeared. One such preparation was subjected to gel chromatography on Sephadex G-75, and it was indeed found that about 20% of the protein and inhibitory activity ran as a component of higher M_r than the main fraction. This disulphide-dependent dimerization was never seen with CPI-A, and is reminiscent of that mentioned by Järvinen & Rinne (1982) for the spleen inhibitor.

Isoelectric focusing. The CPI-B forms were subjected to isoelectric focusing in polyacrylamide gel. The stained bands of protein in one half of each gel corresponded to zones of inhibitory activity in the other. The pH values of the gel segments containing the two major forms were 6.25 and 6.35, as compared with the pH values of 8.1 and 8.3 for elution from the chromatofocusing column. Similarly, the '5.2' form of CPI-A focused at pH 4.50.

Järvinen & Rinne (1982) reported the isoelectric points of the two forms of the 'neutral' variant of CPI from human spleen to be to 6.0 and 6.5.

Stability of CPI forms to low pH and high temperature. The stability of the small- M_r protein inhibitors of cysteine proteinases to heat and extremes of pH has been commented on repeatedly in the literature.

Samples of each of the four major forms of CPI were diluted to approx. 1 μ M concentration in (a) 0.10 M-glycine/HCl buffer, pH 2.0, at 20°C, or (b) 0.10 M-sodium/potassium phosphate buffer, pH 6.0, at 80°C. After 10 min, samples from each treatment were diluted once more (at least 10-fold) into the pH 6.0 buffer at 0°C. Controls were kept in the pH 6.0 buffer at 0°C. The residual activities

Table 2. *Stability of CPI forms to low pH and high temperature*

See the text for experimental details.

		Recovery (%)	
		pH 2.0	80°C
CPI-A	'5.2' form	77	67
	'7.7' form	70	90
CPI-B	'8.1' form	100	87
	'8.3' form	100	95

were determined by titration with papain and expressed as percentages of the control values (Table 2).

Specificity of the CPI forms as proteinase inhibitors. Screening assays to survey the inhibitory specificity of the four major forms of CPI were made with the inhibitors at 20 µg/ml (1.6 µM), and with 15 min preincubation. For some, parallel assays were made with egg-white cystatin under identical conditions. Where amounts of enzyme are given by weight, the percentages of active enzyme were unknown.

None of the forms of CPI showed any inhibition of the non-cysteine proteinases trypsin (1 µg/ml), chymotrypsin (2 µg/ml), pancreatic elastase (3 µg/ml), cathepsin G (4 µg/ml) or thermolysin (0.3 µg/ml).

Cathepsins B, H and L, papain and dipeptidyl peptidase I were strongly inhibited, and K_i values were determined (see below).

Ficin (3 nM) also was completely inhibited by a slight molar excess of either form of CPI, or of egg-white cystatin. Järvinen & Rinne (1982) similarly found strong inhibition of ficin by the human spleen inhibitors.

Papaya peptidase (0.20 µg) was completely inhibited by 2 µg (the smallest amount used) of all four forms of CPI, or egg-white cystatin.

Clostripain, the bacterial cysteine proteinase specific for arginyl bonds (20 ng/ml), was inhibited 40–53% by CPI-A forms and 6–13% by CPI-B forms, whereas egg-white cystatin gave 46–51% inhibition (all at the standard concentration of 1.6 µM). Previously, we failed to detect the inhibition of clostripain by cystatin (Anastasi *et al.*, 1983), for reasons that are unclear.

Bromelain (2 µg/ml) was inhibited 66–82% by CPI-A and CPI-B forms, but much smaller amounts of these inhibitors (1 and 0.1 µg) and egg-white cystatin gave similar amounts of inhibition, also. This behaviour of bromelain has been mentioned by Järvinen & Rinne (1982), and is apparently due to heterogeneity of the commercial enzyme preparation: it contains a major fraction of

Table 3. K_i values for CPI-A and CPI-B from human liver, and egg-white cystatin

The CPI-A was the '5.2' form, and the CPI-B the '8.1' form (see the text). Egg-white cystatin was mixed forms 1 and 2, since previous work has shown no difference in inhibitory activities.

	K_i (nM)		
	CPI-A	CPI-B	Cystatin
Papain	0.019	0.12	<0.005
Cathepsin B (human)	8.2	73	1.7
Cathepsin H (human)	0.31	0.58	0.064
Cathepsin L (human)	1.3	0.23	0.019
Dipeptidyl peptidase I (bovine)	33	0.23	0.35

activity that is strongly inhibited, and a minor component that is scarcely affected at all by these inhibitors.

The activity of the streptococcal cysteine proteinase (15 µg/ml) was not affected by 20 µg/ml of the four forms of CPI, or egg-white cystatin.

K_i values for forms of human liver CPI with cysteine proteinases and dipeptidyl peptidase I. In assays with the enzymes and inhibitors at high dilution, it was readily apparent that the degree of inhibition varied with absolute concentrations, as would be expected for tight-binding reversible inhibition, and the procedure of Henderson (1972) was therefore used to analyse the data. The interaction of papain with cystatin was too tight to be measured accurately. Cathepsin L was unstable under assay conditions (even at 20°C), so that determination of inhibited reaction rates could proceed for only 30 min. It is possible that maximal inhibition had not been attained, and that true K_i values are lower than those found. The results are summarized in Table 3.

Additional measurements at only one or two inhibitor concentrations were made with the '7.7' and '8.3' forms of CPI. The results suggested that the two forms of CPI-A had similar inhibitory characteristics, as did the two forms of CPI-B. A few assays were also made with rat cathepsin L, and these showed K_i values close to those for human cathepsin L.

Discussion

In agreement with the work of Järvinen's group (see the introduction), our results indicate that human tissues contain two small- M_r proteins that inhibit cysteine proteinases. These are generally similar to each other, but differ in antigenicity, in isoelectric points and in K_i values for some

Fig. 3. Comparison of sequences of (a) egg-white cystatin, (b) rat liver CPI and (c) human leucocyte CPI

Boxes indicate residues that are identical between egg-white cystatin and one of the mammalian proteins (when the gap corresponding to residues 41–42 of egg-white cystatin has been arbitrarily inserted in the mammalian sequences). Over half of the residues are identical between the two mammalian inhibitors. The similarities suggest to us that all three proteins fall into a single superfamily in the terminology of Dayhoff *et al.* (1983). The single-letter code for amino acids is that given in *Biochem. J.* (1969) 113, 1–4, and ac indicates an *N*-terminal acetyl blocking group.

enzymes. By analogy with the work of Hirado *et al.* (1981) and Kominami *et al.* (1981) on a rat liver CPI, the human CPI proteins probably derive from the cytosolic fraction of the tissues. From immunofluorescence studies of Järvinen's group and preliminary work of our own (M. E. Davies, unpublished work) it is clear that the relative amounts of the inhibitors that we know as CPI-A and CPI-B vary greatly in different cell types. Both proteins occur in multiple isoelectric forms.

CPI-A, CPI-B and cystatin all inhibit cathepsins H and L more strongly by at least an order of magnitude than cathepsin B, and this is also true of the small- M_r inhibitor detected in human plasma by Gauthier *et al.* (1983). The CPI-B forms showed an affinity for cathepsin B approx. 10-fold less than the CPI-A forms. Järvinen & Rinne (1982) found the 'neutral' forms of the spleen inhibitor less potent against cathepsin B than were the 'acidic' forms. CPI-A proved to be a weaker inhibitor by about two orders of magnitude than CPI-B or cystatin for dipeptidyl peptidase I. Nevertheless, the inhibition of dipeptidyl peptidase I by all three cysteine proteinase inhibitors, taken in conjunction with their similar M_r values, non-glycoprotein nature and stability to heat and extremes of pH, raises the possibility that they are homologous proteins.

Little can be said about the physiological functions of the cytosolic CPI proteins, at present. There may be a requirement for inhibition of lysosomal enzymes that escape into the cytoplasm, and there are indications that a form of CPI in rabbit skin controls inflammatory reactions (Udaka & Hayashi, 1965). The inhibitors may contribute to

the body's defences against foreign organisms that use cysteine proteinases, including many viruses, the intracellular parasite *Leishmania* (Coombs, 1982) and larger parasites (Dresden *et al.*, 1982).

Note added in proof (received 28 October 1983)

The protein referred to as 'CPI-A' in the present paper has now been shown by amino acid analysis (A. J. Barrett, G. S. Salvesen and N. D. Rawlings, unpublished work), cellular distribution (M. E. Davies & A. J. Barrett, unpublished work) and immunological reactivity (V. Turk & A. J. Barrett, unpublished work) to be identical with an inhibitor from human leucocytes that has recently been sequenced (Machleidt *et al.*, 1983). As has been suggested to us by Dr. Werner Machleidt (personal communication), the sequence of the human inhibitor has sufficient similarity to that of egg-white cystatin (Schwabe *et al.*, 1984) to show that the two inhibitors are homologous (Fig. 3). We therefore propose that CPI-A be called 'human cystatin A' in future. The sequence reported for an inhibitor from rat liver (Takio *et al.*, 1983) is also closely related (Fig. 3).

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