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THE PLACE OF HUMAN γ-TRACE (CYSTATIN C) AMONGST THE CYSTEINE PROTEINASE INHIBITORS

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Native γ-trace, a small basic protein present in high concentration in cerebrospinal fluid, semen and neuroendocrine cells, but of unknown biological function, is shown to be a potent inhibitor of the cysteine proteinases papain, ficin, and human cathepsins B, H and L. It proves to be the tightest-binding protein inhibitor of cathepsin B so far discovered. The name cystatin C is proposed for γ-trace to reflect the many similarities in activity and structure to chicken egg-white cystatin and mammalian cystatins A and B. The inhibition constants of cystatin C, taken together with its widespread distribution in human tissues and extracellular fluids, suggest that a physiological function could well be the regulation of cysteine proteinase activity.

Human γ-trace, also called post-γ-globulin, is a protein of low M_r (13,260) first described in 1961 as a constituent of normal cerebrospinal fluid, and urine from patients with renal failure (1-3). The protein is particularly abundant in neuroendocrine cells (4-8), and high extracellular concentrations have also been detected in cerebrospinal fluid (9) and semen (10). Although the primary structure of γ-trace has been determined (7), its biological role is unknown.

Very recently, a cysteine proteinase inhibitor from pathological human sera was shown to have an N-terminal amino acid sequence matching that of γ-trace, and it was suggested that the proteins are probably identical (11). In the present study we confirm and extend that report, suggesting that the physiological function of the protein may well be the local regulation of cysteine proteinase activity. We propose the name (human) cystatin C for the protein that has been called γ-trace or human cystatin (11), and emphasize its relationship to egg-white cystatin (12) and cystatins A and B (13,14).

MATERIALS AND METHODS

Human γ-trace (cystatin C). The protein was isolated from urine of patients with renal failure as described (8). No contamination of the preparation could be detected by agarose gel electrophoresis, SDS/poly-

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acrylamide gel electrophoresis or immunoelectrophoresis developed with polyvalent antisera against human plasma and urinary proteins. The N-terminal amino acid sequence was that of the undegraded protein, Ser-Ser-Pro-Gly-Lys-Pro-Pro-Arg-Leu-Val-Gly-Gly- (15).

Other cystatins. Chicken egg-white cystatin and human cystatins A and B were isolated as previously described (12,13).

Enzymes. Human cathepsins B, H and L, papain, ficin, bromelain and dipeptidyl peptidase I were obtained as described previously (13). The calcium-dependent cysteine proteinase, calpain II, was purified from chicken gizzard smooth muscle by a method similar to that of Hathaway et al. (16).

Enzyme assays, active site titration, determination of kinetic constants, and stability. These were as reported previously (13). Values of k_1 and k_{-1} were determined as described (17,18).

Immunological methods. Antisera were prepared in our laboratories (8,12-14). Immunodiffusion and immunoelectrophoresis were as described (19).

RESULTS

Stoichiometry of interaction with papain. Titration of papain with cystatin C, as described for egg-white cystatin (20), showed that enzymic activity decreased as a linear function of the amount of cystatin C. On the basis of M_r 13,260 and protein concentration (Lowry), the freeze-dried protein was 57% active. Purified egg-white cystatin also shows only 50 - 60% activity in such titrations (12), but it is known that complex formation has equimolar stoichiometry (18).

Kinetics of inhibition of proteases. K_i values for papain, three human lysosomal cysteine proteinases and the exopeptidase dipeptidyl peptidase I are given in Table 1, together with values for other cystatins that have been reported earlier. Ficin also showed tight-binding inhibition, but bromelain was not inhibited by 3 uM, or calpain II by 1.8 uM, cystatin C.

Values of k_1 and k_{-1} were determined as $3.1 \times 10^6 M^{-1}s^{-1}$ and $8.1 \times 10^{-4}s^{-1}$ for cathepsin B, and $2.4 \times 10^7 M^{-1}s^{-1}$ and $6.8 \times 10^{-3}s^{-1}$ for cathepsin H.

Stability to heat and pH. Cystatin C was completely stable to 80°C for 10 min (pH 6.5), as had been found previously for the serum inhibitor (11), and was stable to pH 2.0 at 25°C for 10 min in 0.10 M glycine/HCl buffer.

Table 1. Comparison of inhibition constants for cystatin C with published values for other cystatins (13). The figures are K_i values (nanomolar)

	Egg-white cystatin	Human cystatin A	Human cystatin B	Human cystatin C
Papain	<0.005	0.019	0.12	<0.005
Cathepsin B	1.7	8.2	73	0.25
Cathepsin H	0.064	0.31	0.58	0.28
Cathepsin L	0.019	1.3	0.23	<0.005
Dipeptidyl peptidase I	0.35	33	0.23	3.5

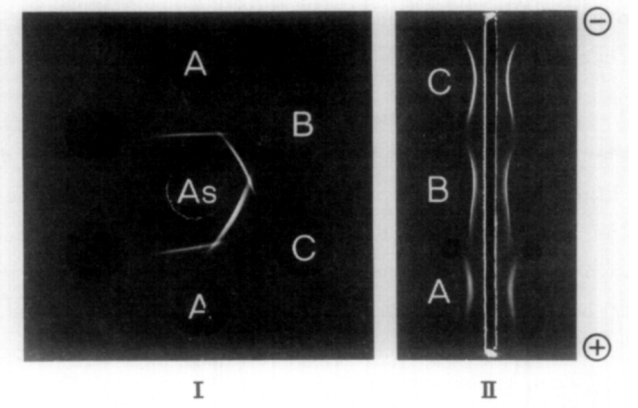

Fig. 1. Immunodiffusion and immunoelectrophoresis of human cystatins. I. Non-identity of human cystatins A, B and C in double immunodiffusion. The centre well contained a mixture of antisera to the three proteins, and the outer wells contained cystatins A, B and C, as marked. It can be seen that all of the precipitin lines crossed in reactions of non-identity. II. Relative mobilities of cystatins A, B and C in immunoelectrophoresis. The well contained a mixture of human cystatins A, B and C, and the trough contained the mixed antisera (as in I). The three arcs (identified in separate preliminary runs) show that the proteins decreased in anodal mobility in the order A, B, C.

Immunological comparison with the other cystatins. Cystatin C showed no cross-reaction with human cystatins A or B in immunodiffusion or immunoelectrophoresis (Fig. 1). Nor was there any cross-reaction with egg-white cystatin (not shown), despite the similarities in sequence (Fig. 2).

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                5         10        15        20        25        30
(a)       S-E-D-R-S-R-L-L-G-A-P-V-P-V-D-E-N-D-E-G-L-Q-R-A-L-Q-F-A-M-A-
(b)    S-S-P-G-K-P-P-R-L-V-G-G-P-M-D-A-S-V-E-E-E-G-V-R-R-A-L-D-F-A-V-G-
(c)    M-I-P-G-G-L-S-E-A-K-P-A-T-P-E-I-Q-E-I-V-D-K-V-K-P-Q-L-E-
(d) acM-M-C-G-A-P-S-A-T-M-P-A-T-T-E-T-Q-E-I-A-D-K-V-K-S-Q-L-E-

                35        40        45        50        55        60
(a)       E-Y-N-R-A-S-N-D-K-Y-S-S-R-V-V-R-V-I-S-A-K-R-Q-L-V-S-G-I-K-Y-
(b)       E-Y-N-K-A-S-N-D-M-Y-H-S-R-A-L-Q-V-V-R-A-R-K-Q-I-V-A-G-V-N-Y-
(c)          -E-K-T- -N-E-T-Y-G-    -K-L-E-A-V-Q-Y-K-T-Q-V-V-A-G-T-N-Y-
(d)          -E-K-A- -N-Q-K-F-D-    -V-F-K-A-I-S-F-R-R-Q-V-V-A-G-T-N-F-

                65        70        75        80        85        90
(a)       I-L-Q-V-E-I-G-R-T-T-C-P-K-S-S-G-D-L-Q-S-C-E-F-H-D-E-P-E-M-A-
(b)       F-L-D-V-E-L-G-R-T-T-C-T-K-T-Q-P-N-L-D-N-C-P-F-H-D-Q-P-H-L-K-
(c)       Y-I-K-V-R-A-G-D-N-K-Y-M-       -H-L-K-    -V-F-K-S-L-P-G-Q-N-
(d)       F-I-K-V-D-V-G-E-E-K-C-V-       -H-L-R-    -V-F-E-P-L-P-H-E-N-

                95        100       105       110       115
(a)       K-Y-T-T-C-T-F-V-V-Y-S-I-P-W-L-N-Q-I-K-L-L-E-S-K-C-Q
(b)       R-K-A-F-C-S-F-Q-I-Y-A-V-P-S-Q-G-T-M-T-L-S-K-S-T-C-Q-D-A
(c)       E-   -D-L-V-L-T-G-Y-Q-V-D-K-N-K-D-D-E-L-T-G-F
(d)       K-   -P-L-T-L-S-S-Y-Q-T-D-K-E-K-H-D-E-L-T-Y-F
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Fig. 2. An alignment of sequences of cystatins. The sequences are those of (a) egg-white cystatin (22,23), (b) human cystatin C (γ-trace) (7,11), (c) human cystatin A (24), and (d) rat (liver) cystatin A (25). The locations of the gaps, which were not unequivocal, were suggested to us by inspection of output from the ALIGN program, kindly provided by Dr W.C Barker (National Biomedical Research Foundation, Georgetown University Medical Center, Washington DC, USA). Boxed residues are those that are identical between the other proteins and cystatin C. We use the numbering system of egg-white cystatin, since that was the first reported.

Immunoelectrophoresis showed the very different electrophoretic mobilities of the three human cystatins.

DISCUSSION

Like the other cystatins that have so far been studied in detail, cystatin C is a tight-binding inhibitor of papain, ficin and the homologous lysosomal cysteine proteinases. All three human cystatins bind cathepsin H with K_i about 0.5 nM, but they vary enormously in affinity for cathepsin B, cystatin C having by far the greatest affinity (Table 1). Indeed, cystatin C is most unusual amongst protein inhibitors in inhibiting cathepsin B about as strongly as cathepsin H.

The kinetic constants for inhibition of lysosomal cysteine proteinases by cystatin C allow us to use the approach of Bieth (21) to decide whether this protein has the potential to be a physiologically significant inhibitor. The concentrations of cystatin C in plasma, seminal fluid and cerebrospinal fluid have been found to be 0.08, 3.8 and 0.4 uM, respectively (9,10). Thus, assuming an inhibitor concentration of 1 uM at a site of action, we can calculate that the "delay time" for 97% inhibition of equimolar proteinase is 1.5 s, and the minimum time for 13% dissociation of the complex is 170 s. The corresponding values for cathepsin H would be 0.2 s and 20 s, and the control of cathepsin L would be far more rigorous still. For these reasons, we suggest that cystatin C probably contributes to the physiological inhibition of any cysteine proteinases that pass from the lysosomal system to extracellular fluid, and also to the cytoplasm in some cells. In addition, we have found (A.J. Barrett and G.H. Coombs, unpublished results) that cystatin C is a powerful inhibitor of the cysteine proteinase of the protozoan parasite, Leishmania mexicana, so an additional defensive role is possible.

Our characterization of cystatin C has confirmed that the proteins we know as cystatins have important properties in common. These may be summarized by saying that cystatins inhibit papain, ficin, cathepsins B, H and L, and dipeptidyl peptidase I, but not bromelain or calpain. In addition to active proteinases, inactive derivatives such as S-carboxymethylated papain are bound. All the cystatins are non-glycosylated proteins of M_r about 13,000. They are very stable to extremes of pH and heat, and the amino acid sequences show sufficient similarities to indicate that the proteins are homologous. Fig. 2 shows an alignment of the four available sequences, with insertion of some gaps. From the point of view of looking for a conserved sequence that might represent the active site of the inhibitors, we are particularly struck by the conservation of identity, or at least type, of residues in the segment 51-67.

We consider that the similarities between the cystatins are such that it is best to think of them as a single group of related proteins, despite the

fact that there are marked differences in amino acid composition and sequence, and isoelectric point. The mean pI values range from the acidic for cystatin A through the approximately neutral cystatin B to the basic cystatin C, but each cystatin has multiple forms, so that ion-exchange chromatography seldom affords clean separations.

The difficulties of distinguishing the cystatins have led to confusion in the literature, and it is not possible to attribute the described activities to individual members of the group. Cystatin-like inhibitors have been detected in serum of rat, cattle and man (26-29), but they generally differ from cystatin C in being weak inhibitors of cathepsin B, and one human inhibitor has been reported not to affect papain or ficin (28). Even allowing for species differences, it is likely that cystatin C is not the only protein of this type to be found in extracellular fluids.

The most promising approach to resolving the cystatins is the use of immunological methods, and these have already given information about the intracellular distribution of the human proteins. Thus, cystatin A occurs in epidermal cells (30) and polymorphonuclear leucocytes (31,14), and cystatin B in squamous epithelial cells (32) and lymphocytes (14). Cystatin C is particularly abundant in cells of the adrenal medulla, pancreatic islets, thyroid gland and adenohypophysis (4-7), and has also been localized to certain brain cortical neurons (8). These differences in distribution may well reflect differences in function of the proteins despite their similar properties.

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