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Inhibition of cysteine proteinases and dipeptidyl peptidase I by egg-white cystatin

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The interactions between egg-white cystatin and the cysteine proteinases papain, human cathepsin B and bovine dipeptidyl peptidase I were studied. Cystatin was shown to be a competitive reversible inhibitor of cathepsin B (K_i 1.7nM, k_{-1} about $2.3 \times 10^{-3} s^{-1}$). The inhibition of dipeptidyl peptidase I was shown to be reversible ($K_{i(app.)}$ 0.22nM, k_{-1} about $2.2 \times 10^{-3} s^{-1}$). Cystatin bound papain too tightly for K_i to be determined, but an upper limit of 5pM was estimated. The association was a second-order process, with k_{+1} $1.0 \times 10^7 M^{-1} \cdot s^{-1}$. Papain was shown to form equimolar complexes with cystatin. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of complexes formed between papain or cathepsin B and an excess of cystatin showed no peptide bond cleavage after incubation for 72h. The reaction of the active-site thiol group of papain with 5,5'-dithiobis-(2-nitrobenzoic acid) at pH8 and 2,2'-dithiobispyridine at pH4 was blocked by complex-formation. Dipeptidyl peptidase I and papain were found to compete for binding to cystatin, contrary to a previous report. The two major isoelectric forms of cystatin were found to have similar specific inhibitory activities for papain, and similar affinities for papain, cathepsin B and dipeptidyl peptidase I. This, together with specific oxidation of the *N*-terminal serine residue with periodate, showed the *N*-terminal amino group of cystatin 1 to be unimportant for inhibition. General citraconylation of amino groups resulted in a large decrease in the affinity of cystatin for dipeptidyl peptidase I. It is concluded that the interaction of cystatin with cysteine proteinases has many characteristics similar to those of an inhibitor such as aprotinin with serine proteinases.

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]. *C*-Terminal groups were: NMec, 7-(4-methyl)coumarylamide; NNap, 2-naphthylamide. Other abbreviations are: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; E-64, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(4-guanidino)butane; Ep-475, L-3-carboxy-*trans*-2,3-epoxypropyl-leucylamido-(3-methyl)butane; *F*, fluorescence (arbitrary units); Nbs_2, 5,5'-dithiobis-(2-nitrobenzoic acid); 2-Pys_2, 2,2'-dithiobispyridine; SDS, sodium dodecyl sulphate. The abbreviations used for enzyme kinetic parameters are: [E], concentration of enzyme not bound by inhibitor; $[E]_t$, total enzyme concentration; [I], concentration of free inhibitor; $[I]_t$, total inhibitor concentration; $k_{+1(app.)}$, apparent rate constant of association in the presence of substrate; $k_{-1(app.)}$, apparent rate constant of dissociation in the presence of substrate; $k_{cat.}$, catalytic constant; K_i, inhibition constant; $K_{i(app.)}$, apparent inhibition constant in the presence of substrate; K_m, Michaelis constant; [P], product concentration after addition of inhibitor; [S], substrate concentration; *t*, time elapsed after addition of inhibitor; v_i, rate of reaction at steady state in the presence of inhibitor; v_0, rate of reaction in the absence of inhibitor; τ, defined in eqn. (5).

Cystatin isolated from chicken egg-white (Anastasi *et al.*, 1983) is a protein of M_r 13143 (Turk *et al.*, 1983; Schwabe *et al.*, 1984). Egg-white cystatin is a tight-binding inhibitor of ficin, papain (Fossum & Whitaker, 1968), cathepsin B (Keilová & Tomášek, 1974), cathepsin H and cathepsin L (Anastasi *et al.*, 1983) and also dipeptidyl peptidase I (Keilová & Tomášek, 1975). Other cystatins have been found in mammalian tissues and body fluids: their inhibitory properties are very similar

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to those of egg-white cystatin, and their amino acid sequences show evolutionary homology (Barrett *et al.*, 1984). For the sake of brevity, the term 'cystatin' is used in the present paper to refer to chicken egg-white cystatin.

Little is known about the mechanism of inhibition of proteases (i.e. proteinases and peptidases) by cystatins, although it has been reported to have some unusual characteristics. Thus the fact that cystatins bind cysteine proteinases in which the active-site thiol group is blocked (reviewed by Anastasi *et al.*, 1983) has raised the possibility that binding is not at the active site of the enzyme. Also, it has been suggested that egg-white cystatin has separate inhibitory sites for cathepsin B and dipeptidyl peptidase I (Keilová & Tomášek, 1975). In the present paper we re-examine the interactions in an attempt to relate them to those of better-characterized low-M_r protein inhibitors of proteinases such as aprotinin and the soya-bean Kunitz inhibitor, which interact with the enzymes in a substrate-like way, competing with substrate to form tight reversible complexes at the active site.

Experimental

Buffers

Buffer A was 0.10M-sodium/potassium phosphate buffer, pH 5.85, containing 1mM-disodium EDTA and 25mM-NaCl.

Enzymes

Papain. Papain was prepared from spray-dried papaya latex (from Powell and Scholefield, Liverpool, U.K.) in accordance with the method of Baines & Brocklehurst (1979), and further purified by the method of Stuchbury *et al.* (1975). We were not able to obtain 100% activatable papain by this method, however. Typically, the material eluted from thiol-Sepharose was 80% active in titration with E-64 by the method of Barrett *et al.* (1982).

Cathepsin B. Cathepsin B was purified from human liver essentially as described by Schwartz & Barrett (1980). The molar concentration of active enzyme was determined by titration with E-64 (Barrett *et al.*, 1982).

Dipeptidyl peptidase I. This was prepared from bovine spleen essentially as described by McDonald *et al.* (1972). It was stored fully activated in a buffer containing 10mM-2-mercaptoethanol and 10mM-NaCl.

The active-site molarity of dipeptidyl peptidase I was determined by titration with cystatin. It was necessary to work with enzyme and inhibitor at high concentrations (40–50nM), because K_i is high, and this in turn necessitated the use of short (30s) assays at low temperature (15°C). The procedure has been described in detail elsewhere (Nicklin, 1984). Gly-Phe-NNap (50μM) was the substrate used in the assay in buffer A, and the turnover rate was found to be $5.3s^{-1}$ under these conditions.

Concentrations of papain, cathepsin B and dipeptidyl peptidase I given in the text represent molarity of active enzyme.

Cystatin

Cystatin was purified from chicken egg-white as forms 1 and 2 separated by chromatofocusing (Anastasi *et al.*, 1983), but on the Mono P column of the f.p.l.c. apparatus. The purity of the separated forms was examined by analytical isoelectric focusing in polyacrylamide gels (pH range 5–8) (Barrett, 1970). Visually, each component was at least 90% homogeneous. The active-site molarity of the solutions was determined by titration with papain (Barrett, 1981). This showed that the material was 50–55% active, on the basis of the $A^{1\%}_{280} = 8.7$ (Anastasi *et al.*, 1983), $M_r = 13143$ (Schwabe *et al.*, 1984), and the assumption of a 1:1 binding ratio for papain (see the Results section).

Experiments were made with cystatin 1 except where otherwise stated.

Substrates

Z-Phe-Arg-NMec, Z-Arg-Arg-NNap,2HCl and Gly-Phe-NNap were obtained from Bachem Feinchemikalien, Bubendorf, Switzerland. Stock solutions of Z-Phe-Arg-NMec and Gly-Phe-NNap were in dimethyl sulphoxide, whereas Z-Arg-Arg-NNap,2HCl was dissolved in water.

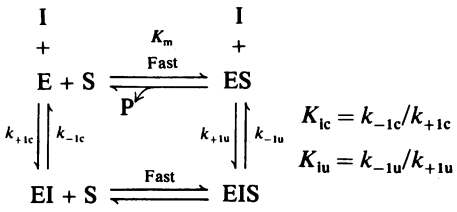
Fluorimetry

A Perkin–Elmer LS-3 spectrofluorimeter fitted with a temperature-controlled cell holder and chart recorder was used for the fluorimetric assays. The instrument was standardized with a solution of product (0.5μM-2-naphthylamine or 0.1μM-7-amino-4-methylcoumarin) containing the appropriate substrate at the concentration used in the assays. The maximum amount of hydrolysis never exceeded 1%. The fluorescence of 2-naphthylamine was monitored with excitation at 335nm and emission at 414nm, and 7-amino-4-methylcoumarin with excitation at 360nm and emission at 460nm.

For stopped assays (Barrett, 1980), reference samples also contained iodoacetate at appropriate concentrations, and ΔF values were calculated by subtraction of appropriate blanks.

Determination of $K_{i(app.)}$ and $k_{+1(app.)}$ for cathepsin B and dipeptidyl peptidase I

Principle. The method developed for the determination of K_i has the advantage over conventional methods (e.g. Cha, 1976; Baici & Gyger-Marazzi, 1982) that it deals with relative rates of



Scheme 1 (after Baici & Gyger-Marazzi, 1982)

the uninhibited to the inhibitied enzymes at steady state, measured under conditions where the formation of enzyme–inhibitor complex does not significantly deplete the free inhibitor, and therefore eliminates the exact concentration of the enzyme from consideration.

We define, for Scheme 1, $K_{i(app.)} = ([S]+K_m)/([S]/K_{iu}+K_m/K_{ic})$, so that for a purely competitive inhibitor, where $K_i = K_{ic}$ and K_{iu} approaches infinity:

$$K_{i(app.)} = (1+[S]/K_m)K_i \quad (1)$$

We may also define, for Scheme 1:

$$k_{-1(app.)} = k_{iu}[S]K_{ic} + \frac{k_{ic}K_mK_{iu}}{[S]k_{ic} + K_mK_{iu}} \quad (2)$$

whence for a purely competitive inhibitor $k_{-1} = k_{-1(app.)} = k_{-1c}$.

To calculate $K_{i(app.)}$, we followed the progress of the hydrolysis of a substrate by an enzyme in the absence of inhibitor (v_0) and then added inhibitor in a negligibly small volume, and allowed the rate of reaction to fall to a new steady state (v_i). The total concentration of enzyme needed to be at least 10-fold less than the $K_{i(app.)}$ to be determined. Substrate concentration needed to be effectively constant, i.e. no more than 2% hydrolysis was allowed.

It is easily shown that:

$$v_0/v_i = 1+[I]/K_{i(app.)} \quad (3)$$

thus $(v_0/v_i - 1)$ was plotted against i. The line passed through the origin and had a gradient of $1/K_{i(app.)}$.

To measure $k_{-i(app.)}$ we adopted the following procedure. The full equation for the curve of product release in the above experiment is:

$$[P] = v_it + \frac{v_i}{k_{-1(app.)}}\left(\frac{[I]}{K_{i(app.)}+[I]}\right)\left[1-\exp\left(\frac{-k_{-1(app.)}1+[I]}{K_{i(app.)}t}\right)\right] \quad (4)$$

which approaches the asymptote:

$$[P] = v_it + v_i\tau \quad (5)$$

Extrapolating the asymptote to [P] = 0, τ was measured exactly as described by Baici & Gyger-Marazzi (1982), and the value $k_{-1(app.)}$ was determined by finding the mean of

$$k_{-1(app.)} = (1/\tau)(1 - v_i/v_0) \quad (6)$$

for a number of points at different concentrations of inhibitor.

Method. Cathepsin B or dipeptidyl peptidase I in buffer A (30μl) was added to 30μl of 100mM-dithiothreitol in the fluorimeter cuvette and preincubated for 2min. Buffer A at 25°C was added to give a total volume of 2.97ml. Then 30μl of substrate solution was added to the cuvette with mixing. The fluorescence of the mixture was monitored on a chart recorder. Inhibitor was added with rapid mixing by inversion of the cuvette, and the further progress of the fluorescence change was monitored until the steady-state rate of hydrolysis (v_i) could be accurately determined.

Stoppered assays (Barrett, 1980) were used with the method of Wilkinson (1961) to calculate K_m (0.34mM) and $k_{cat.}$ (144s^{-1}) for cathepsin B with Z-Arg-Arg-NNap. McDonald *et al.* (1969) have shown the K_m of Gly-Phe-NNap for dipeptidyl peptidase I to be 170μM, at pH6.0 and 37°C.

Determination of $k_{+1(app.)}$ of papain and cystatin

Principle. The interaction of papain and cystatin was found to be effectively irreversible under the conditions used. However, a simple first-order analysis of inhibition was not possible, because the time course of the inhibition of papain by cystatin was too rapid if the papain was at the lowest concentration commensurate with the accurate determination of rate, and cystatin was at a 10-fold higher concentration. The method adopted is appropriate to the analysis of the second-order interaction of any irreversible inhibitor in small excess over the enzyme. The uninhibited rate of hydrolysis of substrate by papain (v_0) was determined, and then the reaction was followed after the addition of cystatin in a negligible volume. The progress curve was analysed by iterative least-squares fitting of 10–15 ([P],t) pairs to eqn. (7). This equation is derived from the textbook equation for the second-order interaction where

$$[P] = \frac{v_0}{[E]_t}\left(\frac{1}{k_{+1(app.)}}\ln\left\{\frac{\exp\{k_{+1(app.)}t([I]_t-[E]_t)\}-[E]_t/[I]_t}{1-[E]_t/[I]_t}\right\}-t([I]_t-[E]_t)\right) \quad (7)$$

the reactants (E and I) are not present in equal concentrations. The standard equation was substituted and rearranged to yield $e = f(t)$, whence $dp/dt = f(t)$, which was integrated to yield eqn. (7).

Method. Papain was diluted to 4nM and activated for 15min at 40°C with 1mM-dithiothreitol in buffer A. Then 30μl of the solution (final 40pM) was added to buffer A containing Z-Phe-Arg-NMec (to give 20μM), dimethyl sulphoxide (1%) and Brij 35 (0.1%) in the cuvette, and v_0 was recorded. Cystatin (15–45μl of a 32nM solution) was added and the progress curve of the inhibition was monitored for 30min, and analysed as described above.

Polyacrylamide-gel electrophoresis

The Ammediol (2-amino-2-methylpropane-1,3-diol)/glycine/HCl buffer system (Bury, 1981) was used with slab gels of 12.5% total acrylamide concentration for electrophoresis of protein–SDS complexes with and without reduction, and also for electrophoresis of native proteins. M_r standards in the reduced SDS/polyacrylamide-gel system were cytochrome *c* (M_r 13000), soya-bean trypsin inhibitor (M_r 22000), carbonic anhydrase (M_r 29000) and immunoglobulin G heavy chain (M_r 50000).

Cross-linking of papain-cystatin complexes

Papain and cystatin, separately and mixed, were treated with dimethyl suberimidate (Thomas, 1978). Mixtures were prepared by taking 1.0ml of 10μM-papain in 20mM-sodium/potassium phosphate buffer, pH6.8, containing 1mM-EDTA, or buffer alone, adding 0.1ml of 100mM-dithiothreitol, and then adding 0.3ml or 0.6ml of 40μM-cystatin in the same buffer, or buffer alone, after 5min pre-activation time. Each mixture was transferred by gel chromatography on Sephadex G-25 into 165mM-boric acid/NaOH buffer, pH9.0, that had been degassed for 30min on a water pump. Dimethyl suberimidate dihydrochloride was weighed and dissolved rapidly to 200mM concentration in the borate buffer, to which 2 molar equivalents of NaOH had been added. The solution was dispensed into the reaction mixture in a 1:10 volume ratio within 30s of starting to dissolve the material. The tubes, now containing 18.2mM-dimethyl suberimidate, were rapidly mixed and left for 16h at 25°C. Protein was precipitated from 20% (w/v) trichloroacetic acid, centrifuged and redissolved in sample buffer for SDS/polyacrylamide-gel electrophoresis with reduction.

Reactivity of the active-site thiol group in papain–cystatin complexes

Reaction with Nbs_2 at pH8. Papain was prepared active and free of low-M_r thiol by reduction of 67%-activatable papain (5mg/ml, 2ml) with 0.1M-cysteine for 30min in a 20mM-acetic acid/NaOH buffer, pH5.0, containing 1mM-EDTA, followed by gel chromatography on Sephadex G-25, into 20mM-acetic acid/NaOH buffer, pH4.2, containing 1mM-EDTA, that had been gassed with N_2. The protein was collected and stored at 0°C.

Reaction mixtures (1ml) were set up containing 5.1μM active papain alone, or with 39μM-Ep-475 or 8.4μM-cystatin, or both, the cystatin being added first. The mixtures were initially in 0.6ml of 50mM-triethylamine/CO_2 buffer, pH8.5, but were made up to 1.0ml, pH8.0, by addition of 0.5M-Tris/HCl buffer. The reaction was started by addition of 0.01ml of 10mM-Nbs_2, and allowed to proceed for 5min.

Reaction with 2-Pys_2 at pH4. Solutions of activated papain, cystatin and 2-Pys_2 in 20mM-formic acid/NaOH buffer, pH4.0, containing 1mM-EDTA, were mixed in the spectrophotometer cuvette to give active papain (5.0μM in the final 1ml volume), cystatin (a range of concentrations up to 7.5μM final), and 2-Pys_2 (167μM). A control mixture without papain was placed in the reference beam of the double-beam spectrophotometer, and the change in A_{343} of the sample was recorded after introduction of the 2-Pys_2.

Re-activation of dipeptidyl peptidase I–cystatin complexes by papain

Dipeptidyl peptidase I was diluted to 19.4nM in 20ml of buffer A containing 1mM-dithiothreitol. Cystatin was added to 20.4nM. After 5min, a zero-time sample was withdrawn for the assay of dipeptidyl peptidase I, and activated papain (to 20.0nM) was added to start the reaction. Samples were withdrawn at intervals for the assay of dipeptidyl peptidase I and papain.

Assays were made with short incubation times to minimize the effect of dissociation of inhibitor complexes during the assay. Thus, for papain, 0.25ml samples from the reaction mixture were made up to 1.0ml containing 10μM-Z-Phe-Arg-NMec at 25°C, and 'stopped' after 15s by addition of 9ml of buffer A containing 2mM-sodium iodoacetate for measurement of the fluorescence of liberated aminomethylcoumarin. For dipeptidyl peptidase I, 0.99ml samples were made 50μM with respect to Gly-Phe-NNap in 1ml, and stopped after 15s by dilution of a 30μl sample into 3ml of

buffer A containing 2mM-sodium iodoacetate for fluorimetric determination of 2-naphthylamine.

Possible cleavage of cystatin by cysteine proteinases

Cysteine proteinase (10μM) was incubated with 20μM-cystatin under conditions described in the Results section. The mixture was then treated with a sufficient excess of iodoacetate to inhibit the enzyme rapidly, lest it became free and active during denaturation, and also to block the low-M_r thiol activator. Samples were run in SDS/polyacrylamide-gel electrophoresis both with and without reduction.

Modification of the N-terminus of cystatin

Cystatin 1 in 10mM-sodium/potassium phosphate buffer, pH6.9, was mixed with sodium periodate solution to give 0.18mM-cystatin 1 and 0.49mM-periodate. The uptake of periodate was monitored by determining the residual concentration as described by Fields & Dixon (1968), and was found to occur in at least two phases, the first being very rapid ($t_{\frac{1}{2}}$ approx. 100s) and corresponding to roughly 0.2mM uptake. After 3h, samples were separated from periodate by chromatography on Sephadex G-25.

Samples of the periodate-oxidized material were treated to convert the *N*-terminal glyoxylic acid group into glycine by the method of Dixon & Fields (1972). A 0.5ml portion of each sample (to give a final concentration of 0.10mM oxidized cystatin) was adjusted to 2M-sodium acetate/0.2M acetic acid/0.2M-glutamic acid hydrochloride, final pH5.2. Then 10μl of 1M-$CuSO_4$ was added to give 10mM final concentration. The reaction mixture was maintained at 20°C for 30min before separation of the modified cystatin from the reagents on Sephadex G-25.

Samples of the proteins were freeze-dried and the *N*-termini of 10nmol of untreated cystatin 1, periodate-oxidized cystatin 1 and the transaminated protein were identified by dansylation, hydrolysis and t.l.c. (Gray, 1972; Croft, 1972). Untreated cystatin 1 yielded dansyl-serine; no *N*-terminal residue could be detected in the periodate-treated sample, and dansyl-glycine was identified after the transamination reaction.

Citraconylation of cystatin

Citraconic anhydride was used to modify amino groups of cystatin essentially by the method of Atassi & Habeeb (1972). Cystatin (mixed forms, freeze-dried) was dissolved in 20mM-sodium/potassium phosphate buffer, pH6.8, to 2mg/ml. NaOH (2M) was added with stirring to raise the pH to 8.5. Then 80μl of citraconic anhydride was added in 5μl portions over 4h, and the pH of the reaction mixture was maintained in the range pH8.2–8.8 by the addition of NaOH. The protein was equilibrated with 0.05M-Tris/HCl, pH8.2, by chromatography on Sephadex G-25.

Samples of the citraconylated protein were run in polyacrylamide-gel electrophoresis. The protein appeared to be homogeneously modified, running much faster than the untreated protein.

Results and discussion

Inhibition kinetics

Cathepsin B. Values of $K_{i(app.)}$ and $k_{-1(app.)}$ for cathepsin B and cystatin were determined as described in the Experimental section. The values of $K_{i(app.)}$ in the presence of 0.05mM- ($0.15K_m$) and 0.39mM- ($1.14K_m$) Z-Arg-Arg-NNap were found to be 1.85 and 3.68nM respectively. The ratio of 1.99 between these values was in good agreement with the value of 1.86 predicted on the basis of fully competitive inhibition, so that the actual K_i value could be calculated to be 1.7nM.

k_{-1} was found to be $2.3 \times 10^{-3} s^{-1}$ at 25°C. The individual values of $k_{-1(app.)}$, determined as described in the Experimental section from single experiments, showed no systematic variation as a function of inhibitor concentration, which is consistent with a second-order association reaction.

Dipeptidyl peptidase I. $K_{i(app.)}$ of cystatin 1 with dipeptidyl peptidase I was determined as described in the Experimental section to be 0.22nM, and k_{-1} about $2.2 \times 10^{-3} s^{-1}$.

Papain. Papain binds very tightly to cystatin, and attempts to measure K_i were confounded by the instability of papain at high dilution over very long periods.

In order to establish an upper limit for K_i, 0.05nM (final concentration) activated papain was added to a continuous-rate assay mixture at 25°C in buffer A containing 0.1% Brij-35, and Z-Phe-Arg-NMec (10μM final concentration) was added. Once the linear initial rate of substrate hydrolysis had become apparent, 0.30nM- (final concentration) cystatin 1 was added. After 30min the activity of the enzyme had fallen to less than 2% of the initial rate of reaction, showing that $K_{i(app.)}$ was less than 2% of the free inhibitor concentration (0.25nM) under these conditions (eqn. 2). K_i is therefore less than 5pM.

Assuming that the dissociation of the complexes would not be significant over a period of 30min, $k_{+1(app.)}$ was determined under conditions in which the second-order nature of the reaction could be tested (see the Experimental section). With the iterative computer analysis of data, the second-order rate constant for association was determined at three inhibitor concentrations, with 20μM substrate. The mean value of the rate constant was

found to be $1.04 \times 10^7 M^{-1} \cdot s^{-1}$ ($n = 12$, range 0.90×10^7–1.17×10^7), in experiments with 0.16–0.48nM-cystatin, at 40°C.

Taken together, the kinetic results indicate that inhibition by cystatins is reversible and competitive. Thus the inhibition of cathepsin B by cystatin is shown to be reversible by the fact that a steady state is reached in the presence of a large excess of inhibitor. By the same criterion, the inhibition of cathepsins H and L also is reversible (Green *et al.*, 1984). The inhibition of cathepsin B is competitive with substrate, and it can be assumed that inhibition of the other members of the papain superfamily is similar.

Papain, cathepsin B and dipeptidyl peptidase I all interact rapidly with cystatin. The k_{+1} value for cathepsin B and the $k_{+1(app.)}$ value for dipeptidyl peptidase I can be calculated from our results as $1.4 \times 10^6 M^{-1} \cdot s^{-1}$ and $1.0 \times 10^7 M^{-1} \cdot s^{-1}$ respectively, and $k_{+1(app.)}$ of papain was determined directly as $1.0 \times 10^7 M^{-1} \cdot s^{-1}$, when $[S] \ll K_m$. Values of k_{+1} reported for the low-M_r protein inhibitors of serine proteinases are similar to these or lower, the highest, reported by Means *et al.* (1974), being $8.2 \times 10^6 M^{-1} \cdot s^{-1}$ for soya-bean trypsin inhibitor with trypsin.

Comparison of the major forms of cystatin

The determinations of K_i described above were made with cystatin 1. Comparative studies with cystatin 2 gave results identical within experimental error. Since cystatin forms 1 and 2 differ in that one lacks the *N*-terminal eight or nine residues (Turk *et al.*, 1983; C. Schwabe & A. J. Barrett, unpublished work), this shows that the *N*-terminus is not essential for inhibitory activity.

As is described in the Experimental section, each of the cystatin 1 and cystatin 2 preparations showed a high degree of homogeneity in pI, and yet was only about 50% active. It was tentatively concluded that active and inactive cystatin molecules differ only slightly in structure, and that no ionizable group is involved.

Stoichiometry of the papain–cystatin interaction

The stoichiometry of complex formation between papain and cystatin was investigated by covalent cross-linking of the complex, and then estimating its M_r by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, as is described in the Experimental section.

The electrophoresis gel (Fig. 1) showed broad bands for the cross-linked proteins, as is usually found (Thomas, 1978), but also an intense new double band (C) in lanes (*b*) and (*c*), which correspond to the mixtures of papain and cystatin. This was the only major new band not visible in the controls [lanes (*c*) and (*d*)], and its intensity was not enhanced on increasing the excess of cystatin. The M_r of the new band was 33000 (range 31600–37600), and it was interpreted as a complex of cystatin and papain in 1:1 molar ratio (theoretical M_r 37500).

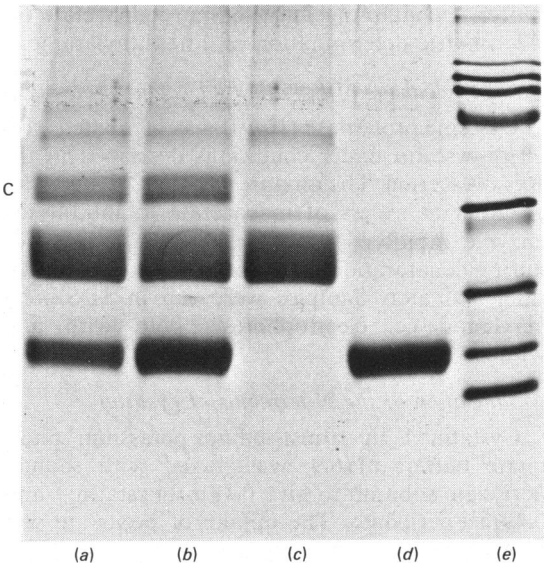


Fig. 1. *Cross-linking of papain–cystatin complexes by dimethyl suberimidate*

Papain, cystatin and mixtures of the two were cross-linked with dimethyl suberimidate as described in the text. They were then run in SDS/polyacrylamide-gel electrophoresis with reduction, alongside M_r standards. (*a*) Cystatin/papain (1.2:1 molar ratio); (*b*) cystatin/papain (2.4:1 molar ratio); (*c*) papain only; (*d*) cystatin only; (*e*) standards. C, complex.

Effect of cystatin binding on the reactivity of the active-site thiol group of papain

Experiments were made to test whether cystatin blocks the chemical reactivity of the active-site thiol group of papain. The reagents chosen were the aromatic disulphides Nbs_2, used at pH8.0, and 2-Pys_2, used at pH4.0 (see the Experimental section). The active-site-directed covalently reacting inhibitor Ep-475 was used to distinguish active-site from other thiol groups.

With Nbs_2 at pH8, it was found that a solution of papain that gave a reaction corresponding to 6.4μM-thiol showed only 1.3μM-thiol after reaction with cystatin or Ep-475 and 1.1μM with both together. The thiol-group reactivity not blocked by the inhibitors (about 20%) probably represented the free thiol group of unactivatable denatured papain in the preparation used. We conclude that the binding of cystatin causes loss of reactivity of

the active-site thiol group of papain towards Nbs_2 at pH 8.

The reaction of 2-Pys_2 at pH 4.0 was studied by monitoring the rapid and delayed phases of the change in A_{343}. The initial very rapid reaction was complete within 1 min, and corresponded to the reaction of 5.0 μM-thiol with the papain solution used. A subsequent slow increase in absorbance was complete after 15 min and was equivalent to 0.5 μM-thiol. The reaction of the active-site thiol group, represented by the rapid phase of the reaction (Baines & Brocklehurst, 1979), was suppressed by the binding of cystatin, and the slow phase of the reaction, which was not affected by cystatin, was too small in amplitude to represent reaction of the active-site thiol group. Rather, it was probably due to the contaminating thiol detected in reaction of papain with Nbs_2 (above).

We conclude from these experiments that cystatin binds sufficiently closely to the active-site thiol group of papain to prevent access of the active-site-directed reagents, and does not merely alter the protonation state of the active site. (See also Note added in proof.)

Does cystatin have a single binding site for endopeptidases and dipeptidyl peptidase I?

Experiments were done to determine whether cysteine proteinases compete with the exopeptidase, dipeptidyl peptidase I, for binding to cystatin, or whether the inhibitor has independent sites for the two types of enzyme, as has been suggested (Keilová & Tomášek, 1975).

An experiment was performed as described in the Experimental section to determine whether papain would compete with dipeptidyl peptidase I for binding of cystatin. Pre-formed complexes of dipeptidyl peptidase I and cystatin were mixed with papain, and the activities of both enzymes were followed. The results of the experiment are shown in Fig. 2. Assays with control incubation mixtures containing only dipeptidyl peptidase I or papain showed that both enzymes were stable over 90 min under the conditions of the incubation. Moreover, under the conditions of the assay, dipeptidyl peptidase I did not cause measurable hydrolysis of Z-Phe-Arg-NMec, and papain did not measurably hydrolyse Gly-Phe-NNap. A control mixture containing cystatin and dipeptidyl peptidase I, but no papain, was 13% active after 90 min, which is in reasonable agreement with 10% observed for the zero-time sample, showing that spontaneous reactivation of cystatin-inhibited dipeptidyl peptidase I did not occur.

Fig. 2 shows that the papain was slowly inhibited, and that there was a parallel reactivation of the dipeptidyl peptidase I. The rate of inhibition of papain was much lower than that

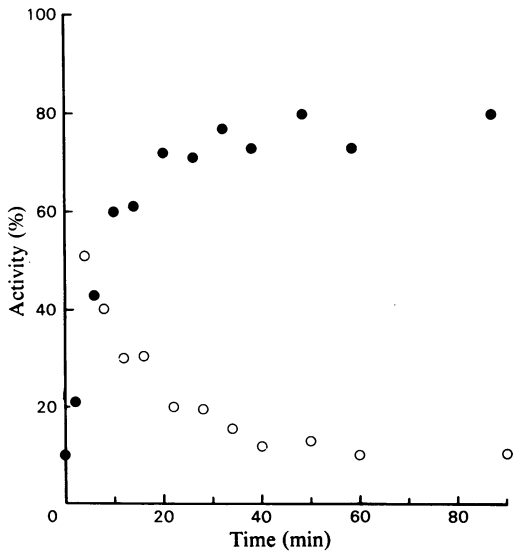


Fig. 2. *Re-activation of dipeptidyl peptidase I inhibited by cystatin 1 by a stoichiometric quantity of papain*

As is described fully in the text, 19.4 nM-dipeptidyl peptidase I was pre-incubated with 20.4 nM-cystatin 1, and 20.0 nM-papain was added to start the reaction. The mixture was assayed for papain (○) and dipeptidyl peptidase I (●) activity over a period of 90 min. The activity of the enzymes is expressed as a percentage of the uninhibited controls.

resulting from simple mixing of papain and inhibitor, for which the calculated half-time would have been 5 s on the basis of the second-order rate constant. If the inhibition of papain resulted from its interaction with cystatin liberated by dissociation of complexes with dipeptidyl peptidase I, the rate-determining step for the initial part of the reactivation reaction would have been the dissociation of cystatin from dipeptidyl peptidase I. The expected half-time for this would have been 6 min according to the steady-state measurements, which is in agreement with the observations. As the concentration of free papain fell, it would be expected that more of the free inhibitor would tend to re-associate with dipeptidyl peptidase I, because the association rate constants of dipeptidyl peptidase I and papain are similar. This would give a slow late phase like that shown in Fig. 2.

In separate experiments, it was shown that 1 μM-cathepsin B would reverse the inhibition of 5 pM-dipeptidyl peptidase I by 4.5 nM-cystatin, and that the rate of reaction was compatible with the dissociation constant of the complex of dipeptidyl peptidase I and cystatin. We also found that if the order of addition of the components was reversed, i.e. papain or cathepsin B was added to cystatin

first, dipeptidyl peptidase I was not measurably inhibited.

We conclude from these experiments that papain, cathepsin B and dipeptidyl peptidase I bind competitively to the same region of the cystatin molecule.

Is cystatin cleaved by the endopeptidases it inhibits?

Since cystatin may well be bound in a substrate-like way by the enzymes it inhibits, we attempted to detect cleavage of a peptide bond in cystatin. Such experiments have previously been important in locating the active sites of protein inhibitors of proteinases.

Mixtures of papain and cathepsin B with an excess of cystatin were incubated as described in the Experimental section under a number of different conditions. The SDS/polyacrylamide-gel electrophoretograms showed no cleavage by cathepsin B over 72h at 20°C, or during 4h at 40°C, at pH3.7, 4.0 or 6.0, all in the presence of 1mM-dithiothreitol. A negative result was also obtained with papain under these conditions, and at pH9 during 4h at 40°C in the presence of 10mM-cysteine, and at pH6.8 at 40°C or 55°C during 4h in the presence of 6M-urea and 10mM-cysteine.

Possible role of the N-terminal amino group of cystatin in the inhibition of dipeptidyl peptidase I

We performed experiments to test whether modification of the *N*-terminus of cystatin would affect the inhibitory activity for dipeptidyl peptidase I.

Derivatives of cystatin in which the *N*-terminal serine residue was converted into glyoxylic acid or glycine were prepared as described in the Experimental section. The derivatives were used to titrate papain, but no significant change from the specific activity of control cystatin was detected. The samples were also added to assay mixtures containing dipeptidyl peptidase I to obtain an estimate of $K_{i(app.)}$, but again no significant difference was found between the modified and the control cystatin preparations.

We conclude that the *N*-terminus of cystatin makes no appreciable contribution to the inhibition of dipeptidyl peptidase I.

Effect of general modification of amino groups of cystatin

A solution of *N*-citraconylated cystatin was adjusted to $A_{280} = 0.200$ and used to titrate 3.8μM-papain. The titration mixtures were preincubated for only 5min in order to minimize the reversal of the citraconylation. The results indicated that the modified inhibitor was 28% active, compared with 35% for the starting material.

The modified inhibitor was added to a dipeptidyl peptidase I continuous-rate assay mixture in a final concentration of 0.2μM. The enzyme was slowly inhibited ($t_{\frac{1}{2}}$ approx. 150s) by 67%. This could either imply that the affinity of the citraconylated protein is less than 1/500 of that of the untreated protein and that the rate of interaction is similarly decreased, or that the modified protein had no inhibitory activity at all, but 0.2% was not modified at the requisite residue(s). We favour the latter explanation.

Note added in proof (received 1 August 1984)

Recent experiments have shown that the active-site thiol group of papain is unreactive with iodo[^{14}C]acetate, in papain–cystatin complexes, at pH6.5. This indicates that the steric inhibition of the reaction of the catalytic group with the relatively large molecules of Nbs_2 and 2-Pys_2 also applies to much smaller molecules.

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