Thyroglobulin Type-1 Domains in Equistatin Inhibit Both Papain-like Cysteine Proteinases and Cathepsin D*

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Equistatin from sea anemone is a protein composed of three thyroglobulin-type 1 domains known to inhibit papain-like cysteine proteinases, papain, and cathepsins B and L. Limited proteolysis was used to dissect equistatin into a first domain, eq d-1, and a combined second and third domain, eq d-2,3. Only the N-terminal domain inhibits papain ($K_i = 0.61$ nM). Remarkably, equistatin also strongly inhibits cathepsin D with $K_i = 0.3$ nM but not other aspartic proteinases such as pepsin, chymosin, and HIV-PR. This activity resides on the eq d-2,3 domains ($K_i = 0.4$ nM). Papain and cathepsin D can be bound and inhibited simultaneously by equistatin at pH 4.5, confirming the physical separation of the two binding sites. Equistatin is the first inhibitor of animal origin known to inhibit cathepsin D. The obtained results demonstrate that the widely distributed thyroglobulin type-1 domains can support a variety of functions.

The recently discovered thyroglobulin type-1 domain inhibitors, thyropins, are a group of proteins that have the ability to inhibit both cysteine (1) and a group of as yet uncharacterized cation-dependent proteinases (2). Thyroglobulin type-1 domain is a structural element first found in thyroglobulin, a molecule that serves as the precursor of the thyroid hormone and in which three different types of cysteine-rich domains are present (3). This domain is exclusively present on the N-terminal section and is repeated 11 times (4). Similar type-1 domains, recognizable by the sequence motif of Cys-Trp-Cys-Val, have been found in many other proteins including saxiphilin (5, 6), pancreatic carcinoma marker proteins (GA-733) (3), testicin (10), major histocompatibility complex class II-associated p41 invariant chain (11), chum salmon egg cysteine protease inhibitor (ECI) (12), and equistatin (13, 14). In these proteins (except ECI and equistatin) the type-1 domain represents only part of the molecule. The function of these repetitive sequences is unknown, although there is evidence that the type-1 domain itself can act as an inhibitor of cysteine proteinases, as shown in equistatin, ECI, and p41 fragment (11–15).

Equistatin is a protein isolated from sea anemone Actinia equina. It is a reversible and tightly binding competitive inhibitor of papain-like cysteine proteinases. Sequence data (SWISS-PROT accession number P8149) have shown that the inhibitor has an $M_r$ of 21,755 and is composed of three repeated thyroglobulin type-1 domains (13, 14). The first domain (eq d-1) has 43% sequence identity with the second domain (eq d-2) and 49% with the third domain (eq d-3), whereas eq d-2 and eq d-3 show 32% identity. In the present study we have examined the inhibitory activity of the thyroglobulin type-1 domains present in equistatin. This has been done by dissecting the molecule by limited proteolysis into folded domain structures, isolating the domains and investigating their inhibitory activities against the cysteine proteinase: papain; a number of aspartic proteinases: cathepsin D, pepsin, chymosin, and HIV-PR; and serine proteinase: trypsin.

EXPERIMENTAL PROCEDURES

Materials—Equistatin from A. equina and β-trypsin were prepared as described (Refs. 13 and 16, respectively). Cathepsin D was purified from human and porcine liver using a procedure slightly modified from that previously described (17). Papain (crystallized twice) was obtained from Sigma and further purified by affinity chromatography (18). Pepstatin (a specific inhibitor of aspartic proteinases), pepson, and calf chymosin were also purchased from Sigma, and chromogenic substrate H-Pro-Thr-Glu-Phe-NeO$_2$-Arg-Leu-Oh was from Novabiochem. The HIV-PR and its substrate Ala-Thr-His-Glu-Val-Tyr-Phe(No$_2$)$_2$-Val-Arg-Lys-Ala were generously provided by Dr. Bruce Korant.

Preparation of Equistatin Domain-1 (eq d-1) and Domain-2,3 (eq d-2,3)—Equistatin was subjected to limited proteolysis with β-trypsin, 500 μg being incubated with 5 μg of β-trypsin in 0.5 ml of 0.1 m Tris/HCl buffer, pH 8.0, for 40 min at 37 °C. The reaction was stopped by the addition of trifluoroacetic acid.

Vydac C18 Chromatography—The β-trypsin digest of equistatin was separated by high performance liquid chromatography (Milton Roy Co.) using a reverse-phase Vydac C18 column equilibrated with 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Elution was performed using a linear gradient of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Absorbance was monitored at 215 nm. Peptide fractions were collected and assayed for inhibitory activities toward papain and cathepsin D. The same HPLC conditions were used when the mixture of equistatin and cathepsin D was applied. Prior to application the inhibitor and the enzyme were incubated in 0.1 m sodium acetate buffer, pH 3.7, at 37 °C. The incubation time was 2 h, and the concentration of the proteinase was 30% (w/w).

N-Terminal Sequence Determination—An Applied Biosystems liquid pulse sequencer 475A connected on line to a phenylthiohydantoin analyzer 120A from the same manufacturer was used for automated amino acid sequence analyses.

Polyacrylamide Gel Electrophoresis and Microsequencing—SDS-PAGE was performed using PhastSystem apparatus (Pharmacia Bio- tech Inc.). Samples and molecular weight markers ranging from 14,000–94,000 (Pharmacia) were run in the presence of 5% SDS on an 8–25% gradient polyacrylamide gel.

Electrophoretic separation under native conditions was carried out using the same apparatus. The 12.5% homogeneous polyacrylamide gel and buffer strips having a pH of approximately 4.1 were used. The complex was made by mixing 10 μl porcine cathepsin D and 40 μl equistatin in 0.2 m sodium acetate buffer, pH 4.0. Proteins were stained with Coomassie Blue R-250. To identify proteins migrating on native PAGE, microsequencing of the separated proteins was performed. After electrophoresis, the gel was soaked into transfer buffer (25 mm Tris, 100 mm glycine, and 20% methanol, pH 8.6) for 10 min. During this time a PVDF difluoride membrane (Bio-Rad) was rinsed with 100% methanol and stored in transfer buffer. The gel, sandwiched between a sheet of

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‡ The abbreviations used are: ECI, salmon egg cysteine protease inhibitor; HIV-PR, human immunodeficiency virus protease; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

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polypepivindene difluoride membrane and several sheets of blotting paper, was assembled into a blotting apparatus (Biometra) and electroeluted for 1 h at 65 mV in transfer buffer. The PVDF membrane was washed in deionized water for 5 min. Proteins-electroeluted onto PVDF membranes were stained with 0.05% Coomassie Blue R-250 in 50% methanol and 10% acetic acid for 1 min and cut out. Membranes were destained with 50% methanol, air-dried, and placed in the cartridge block of the sequenator.

**Determination of Protein Concentration**—Protein concentrations were determined from absorbance at 280 nm using molar absorption coefficients calculated from the amino acid sequences (19). A molar absorption coefficient of 37,700 m$^{-1}$ cm$^{-1}$ was calculated for equistatin, 13,355 m$^{-1}$ cm$^{-1}$ for eq d-1, 20,345 m$^{-1}$ cm$^{-1}$ for eq d-2,3, 49,320 m$^{-1}$ cm$^{-1}$ for cathepsin D, 52,185 m$^{-1}$ cm$^{-1}$ for pepsin, and 54,090 m$^{-1}$ cm$^{-1}$ for chymosin. The concentration of papain was determined spectrophotometrically using a molar absorption coefficient of 56,200 m$^{-1}$ cm$^{-1}$ (20).

**Inhibition Kinetics of Cathepsin D—Pepstatin, a tightly binding inhibitor of cathepsin D, was used to titrate human cathepsin D, pepsin, and chymosin. The active site titrated cathepsin D was then used to determine the active concentrations of equistatin, eq d-1, and eq d-2,3 as follows.**

Cathepsin D (final concentration, 0.1 μM) was incubated with increasing amounts of pepstatin (final concentration, 0–1.5 μM) in 390 μl of 0.1 M sodium acetate buffer, pH 4.1. After 50 min of incubation, 10 μl of 100 nM Z-Phe-Arg-Pro-Thr-Glu-Phe(NO$_2$)-Arg-Leu-OH was added. The initial cleavage rates were monitored as a function of decreasing absorbance at 300 nm with Perkin-Elmer Lambda 18 Spectrophotometer (21).

The equilibrium dissociation constants ($K_i$) for the interaction between the equistatin, eq d-1, or eq d-2,3 and cathepsin D were determined by the equilibrium method. The procedure (see previous paragraph) was repeated with lower concentrations of cathepsin D (final concentration, 6.4 nm) and inhibitors (final concentration, 0–10 nm). $K_i$ was obtained from the dependence of ($v_o$/$v$) on $I$ according to the equation $v_o/v = (E_0/IE - 1 - K_i) + (K_i + 1 - E_0^2 - 4KE_0^2)$, where $v_o$ and $v$ are velocities with and without inhibitor, respectively, $I$ is the inhibitor concentration, and $E$ is the enzyme concentration (22). The potential inhibitory properties of equistatin against pepsin (12.5 nm), chymosin (12 nm), and HIV-PR (30 nm) were tested in a same way as against cathepsin D.

**Inhibition Kinetics of Papain—Papain purified by affinity chromatography (18) had a thiol content of 0.92 ± 0.05 mol/mol of enzyme as determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid).** Active site titrated papain was then used to determine the active concentrations of papain as described previously (23) and that of equistatin as follows. Papain (final concentration, 0.1 μM) mixed with cathepsin D (final concentration, 0.115 μM) was incubated with increasing amounts of equistatin (final concentration, 0–2 μM) in 500 μl of 50 mM sodium acetate buffer, pH 4.5, containing 0.1 M NaCl, 5 mM dithiothreitol, and 1 mM EDTA. After 45 min of incubation at 25 °C, 500 μl of 100 mM Z-Phe-Arg-p-nitroanilide was added, and the residual activity of papain was measured as described previously (23). The kinetics of inhibition of papain were analyzed according to Ref. 13. All experiments were done under pseudo first order conditions with at least 10-fold molar excess of inhibitor. Papain activity was assayed in the presence of fluorogenic substrate Z-Phe-Arg 4-methyl-7-coumarylamide, and the release of product was monitored continuously at excitation and emission wavelengths of 370 and 460 nm, respectively, using a Perkin-Elmer LS50B fluorometer. All progress curves were fitted by non-linear regression analysis to the integrated rate equation (24): $[P] = v_o + v_e + v_i(t - e^{-kt}$), where $[P]$ presents the product concentration, $v_o$ and $v_e$ are the initial and the steady-state velocities, respectively, $t$ is time, and $k$ is the observed pseudo-first order rate constant. The second order rate constant $k_o$ was calculated from the slope of the plot $k$ versus $[I]$ (slope = $k_o/([I] + 1/S(K_{in}))$) + $k_o$. The dissociation rate constants, $k_o$, were obtained from individual measurements $k_o = k/e/v_o$, and the equilibrium inhibition constant, $K_{in}$, was calculated from $K_{in} = k_o/k_e$. $K_{in}$ values of 65 μM were used for papain (25), 2 μM for cathepsin L (26), and 150 μM for cathepsin B (27).

**RESULTS AND DISCUSSION**

Equistatin was tested for inhibitory activity against papain, the aspartic proteinases cathepsin D, pepsin, chymosin, and HIV-PR and against serine proteinase, trypsin. It inhibits papain as previously shown (13) and also cathepsin D but not other proteinases. In addition, it has been reported that p41 fragment does not have any inhibitory effect on cathepsin D (15). To check whether equistatin acts as a substrate for cathepsin D, we incubated both in different molar ratios for different periods of time and subjected the mixtures to reverse-phase HPLC system. No degradation products were observed (Fig. 1A). In contrast, equistatin was found to be a good substrate for trypsin, and this fact was used for the separation of the thyroglobulin type-1 domains by a limited proteolysis with β-trypsin. Two major peaks were obtained on reverse-phase HPLC (Fig. 1B). The molecular weights, estimated by the SDS-PAGE under non-reducing conditions, were about 7,000 and 14,000 (Fig. 2A). N-terminal sequences of the fragments locate them in the sequence of the equistatin molecule as shown in Fig. 3. Their sizes, determined by SDS-PAGE under nonreducing conditions (Fig. 2A), are consistent with their being domains. The smaller fragment starts with the N terminus of the equistatin and therefore corresponds to the first domain (eq d-1). Every larger fragment revealed two sequences, starting with Ala$^{68}$ and Val$^{152}$2. The Lys$^{67}$-Ala$^{68}$ bond is positioned at the beginning of the second domain (Fig. 3). Equistatin as isolated is substantially nicked between Arg$^{151}$ and Val$^{152}$ but the fragments are linked by a disulphide bond.2 A narrow double band, visible on SDS-PAGE (Fig. 2A), suggests the presence of cleavage by trypsin very near to the C terminus. The fragment with N-terminal Ala$^{68}$ thus represents the combined second and third domains, eq d-2,3 (Fig. 3).

It was shown previously that the molar binding stoichiometry of equistatin and papain is 1:1 (13). When cathepsin D was titrated with equistatin, 1 mol of the latter was also needed to saturate 1 mol of cathepsin D (Fig. 4A). Even more, by titrating

\[ \text{FIG. 1. HPLC analyses of equistatin. Chromatograms shows the HPLC elution profile of equistatin after incubation with different enzymes. In panel A the equistatin was incubated with cathepsin D in a final molar ratio of 2:1, and in panel B the equistatin was fragmented using 1% (w/w) β-trypsin. Identities of peaks were based on the N-terminal sequences.}\]
Inhibitory Specificity of Thyroglobulin Type-1 Domains

we demonstrated that the two inhibitory activities of equistatin
are structurally separate. The residual activities of both papain and cathepsin D are listed in Table I.

To assign the inhibitory activities to individual domains
of equistatin, a kinetic analysis of the inactivation of papain and cathepsin D was performed. The results are given in Table II. The N-terminal domain, eq d-1, exhibited practically the same inhibitory characteristics against papain as intact equistatin, p41 fragment, and ECI (12, 13, 15). The two-domain C-terminal fragment, eq d-2,3, showed little or no inhibition of papain.

The kinetics of binding of equistatin to cathepsin D was performed using a synthetic substrate that contains a chromophore, the nitrophenylalanine residue, in the P1 position. Using H-Pro-Thr-Glu-Phe-Phe(NO2)2-Arg-Leu-OH as substrate means that 6.4 nM enzyme is the minimum concentration usable, so that the determination of subnanomolar \( K_i \) values cannot be precisely determined. However, the equilibrium dissociation constant for the interaction between cathepsin D and equistatin \( (K_i = 0.3 \text{nM}) \) indicates that equistatin is a strong inhibitor of cathepsin D even at 5 \( \mu \text{M} \) inhibitor concentration.

Equistatin showed practically no influence on enzyme activities at 2 \( \mu \text{M} \) inhibitor concentration.

**TABLE I**

| Enzyme          | Initial velocity \( v_0 \) | Initial velocity \( v_0 \) | Initial velocity \( v_0 \) | Initial velocity \( v_0 \) | Initial velocity \( v_0 \) | Initial velocity \( v_0 \) |
|-----------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| Papain          | 100                        | 115                       | 0                         | 0.141                     | -0.115                    |
| Human cathepsin D | 100                       | 115                       | 20                        | 0.125                     |
| Equistatin      | 100                        | 115                       | 41                        | 0.090                     |
| Human cathepsin D | 100                       | 115                       | 82                        | 0.027                     |
| Equistatin      | 100                        | 115                       | 102.5                     | 0.015                     |
| Human cathepsin D | 100                       | 115                       | 122                       | 0.009                     |
| Equistatin      | 100                        | 115                       | 163.5                     | 0.006                     |

**TABLE II**

| Enzyme          | Inhibitor | \( 10^{-5} \times k_{a} \) | \( 10^{-4} \times k_{d} \) | \( K_i \) |
|-----------------|-----------|----------------------------|---------------------------|-----------|
| Papain          | equistatin' | 12 ± 0.6                  | 65 ± 1.5                  | 0.57 ± 0.04 |
| eq d-1          | 1.8 ± 0.35 | 11 ± 0.3                  | 0.61 ± 0.01               | >1000'    |
| eq d-2,3        | ND         | ND                        | ND                        | >1000'    |
| Human cathepsin D | equistatin | ND                        | ND                        | 0.3 ± 0.16 |
| eq d-1          | ND         | ND                        | ND                        | >1000'    |
| eq d-2,3        | ND         | ND                        | ND                        | 0.4 ± 0.15 |
| Pepsin          | equistatin | ND                        | ND                        | >1000'    |
| Chymosin        | equistatin | ND                        | ND                        | >1000'    |
| HIV-PR          | equistatin | ND                        | ND                        | >1000'    |

* Continuous rate assay was used for kinetic analysis of the interaction of papain with inhibitors. \( K_i \) was calculated from the ratio \( k_{f}/k_{r} \). \( K_i \) error estimates were obtained as follows: both errors of \( k_{f} \) and \( k_{r} \) were summed and divided by the square root of the number of measurements.

* Values of \( K_i \) were determined from the effect of inhibitor on the steady state velocity for human cathepsin D-catalysed hydrolysis of chromophoric substrate.

* Ref. 12.

* ND, not determined.

* eq d-2,3 did not significantly inhibit papain or eq d-1 human cathepsin D even at 5 \( \mu \text{M} \) inhibitor concentration.

**Fig. 2.** Electrophoretic analyses of equistatin. A, SDS-PAGE under nonreducing conditions of dissected equistatin. Lane 2, the combined second and third domains of equistatin (eq d-2,3); lane 3, first domain of equistatin (eq d-1); lane 4, molecular weight standards. B, native PAGE of the formation of the equistatin–cathepsin D complex. Lane 1, porcine cathepsin D; lane 2, equistatin and porcine cathepsin D mixed 30 min before electrophoresis; lane 3, equistatin. The gels were stained with Coomassie Blue, and bands were identified by microsequence analysis.

**Fig. 3.** Schematic diagram of the function of equistatin fragments used in this study. Sites of proteolytic cleavage are indicated by gaps and located by residue numbers. Cleavage sites obtained by the action of \( \beta \)-trypsin are indicated by arrows. The pairing of cysteine residues in the disulfide bond is indicated by a horizontal line connecting cysteine residues.

**Fig. 4.** Inhibition of human cathepsin D by equistatin. A, active site titration of 77 nM human cathepsin D with increasing concentrations of native equistatin. Residual activity is expressed as a percentage of control activity in samples containing no inhibitor. B, the rate of inhibition of cathepsin D (6.4 nM) by equistatin. Experimental conditions for determination of equilibrium dissociation constants are under “Experimental Procedures.” Experimental data were fitted according to Ref. 22.
slightly in covalent structure, e.g., glycosylation, amide content, etc. Equistatin showed no inhibitory activity against other aspartic proteinases, pepsin, chymosin, and HIV-PR, even at 2 μM concentrations of inhibitor, thus showing a high degree of specificity for cathepsin D.

Multiplication of inhibitory reactive sites has also occurred in several families of serine proteinase inhibitors (28–30). In the case of kininogen, two (D2 and D3) of three cystatin-like proteinases (31, 32). The subtle differences between both domains have significant implications for protein interactions. The D2 domain is able to inhibit calpain, and this feature is unique among cystatins. The region responsible for the calpain inhibition is distinct from the inhibitory region for papain-like cysteine proteinases (33). Another example is the IAP (inhibitor of apoptosis proteins) family of proteins where two or three copies of the BIR (baculovirus IAP repeat) domain are present. Not all BIRs are equivalent in their ability to inhibit caspasases (34).

Our results show clearly that the different thyroglobulin type-1 domains present in equistatin, despite their amino acid sequence similarity, can inhibit proteinases of different classes. The first domain inhibits the cysteine proteinases very strongly, and the second and/or third domains inhibit cathepsin D equally strongly. This is particularly clear in the case of equistatin, because this protein is composed only of thyroglobulin type-1 domains. One molecule of cathepsin D binds somewhere on eq d-2,3. The cleavage characterized above may result in a change in conformation and hence function. It is therefore not possible to say, from the properties of equistatin as isolated, whether the uncleaved C-terminal domain has inhibitory activity or not. Alternatively, if the cleavage has no effect on the structure of domain 3, it could be that domain 3 binds cathepsin D, in which case the results show that domain 2 would not. The resolution of the problem will require the production of individual domains by recombinant technology. Efforts to identify the targets of each of the individual thyroglobulin type-1 domains within thyropins and to understand the structural basis of their differential inhibition of proteinases will provide additional insights into the mechanisms by which thyropins regulate the proteolytic activity.

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