Equistatin, a New Inhibitor of Cysteine Proteinases from Actinia equina, Is Structurally Related to Thryoglobulin Type-1 Domain

(Received for publication, November 19, 1996, and in revised form, March 23, 1997)

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It is well known that the activities of the lysosomal cysteine proteinases are tightly regulated by their endogenous inhibitors, cystatins. Here we report a new inhibitor of cysteine proteinases isolated from sea anemone Actinia equina. The inhibitor, equistatin, is an acidic protein with pI 4.7 and molecular weight of 14,129. It binds tightly and rapidly to cathepsin L (K<sub>i</sub> = 5.7 × 10<sup>5</sup> M<sup>−1</sup> s<sup>−1</sup>, K<sub>a</sub> = 0.051 mM) and papain (K<sub>a</sub> = 1.2 × 10<sup>2</sup> M<sup>−1</sup> s<sup>−1</sup>, K<sub>a</sub> = 0.57 mM). The lower affinity for cathepsin B (K<sub>i</sub> = 1.4 nM) was shown to be due mainly to a lower second order association rate constant (K<sub>a</sub> = 0.04 × 10<sup>8</sup> M<sup>−1</sup> s<sup>−1</sup>). The inhibitor is composed of 128 amino acids forming two repeated domains with 48% identity. Neither of the domains shows any sequence homology to cystatins, but they do show a significant homology to thyroglobulin type-1 domains. A highly conserved consensus sequence motif of Cys-Trp-Cys-Val together with conserved Cys, Pro, and Gly residues is present in major cystatins, but they do show a significant homology to thyroglobulin type-1 domains. This superfamily currently includes equistatin, major histocompatibility complex class II-associated p41 invariant chain, nidogen, insulin-like growth factor protein, saxiphilin domain α, pancreatic carcinoma marker proteins (GA733), and chum salmon egg cysteine proteinase inhibitor. In each of the domains of the equistatin, the three residues are similarly conserved, and the sequences Val-Trp-Cys-Val and Cys-Trp-Cys-Val are present in domains a and b, respectively. We suggest that equistatin belongs to a new superfamily of protein inhibitors of cysteine proteinases named thyroglobulin type-1 domain inhibitors. This superfamily currently includes equistatin, major histocompatibility complex class II-associated p41 invariant chain fragment, and chum salmon egg cysteine proteinase inhibitor.

Sea anemones are known to be a rich source of variety of polypeptide neurotoxins (1, 2) and neuropeptides (3), but little is known about the presence of proteolytic enzymes and their regulation. Papain-like cysteine proteinases are tightly regulated by their endogenous inhibitors, cystatins. Here we report a new inhibitor of cysteine proteinases isolated from sea anemone Actinia equina. The inhibitor, equistatin, is an acidic protein with pI 4.7 and molecular weight of 14,129. It binds tightly and rapidly to cathepsin L (K<sub>i</sub> = 5.7 × 10<sup>5</sup> M<sup>−1</sup> s<sup>−1</sup>, K<sub>a</sub> = 0.051 mM) and papain (K<sub>a</sub> = 1.2 × 10<sup>2</sup> M<sup>−1</sup> s<sup>−1</sup>, K<sub>a</sub> = 0.57 mM). The lower affinity for cathepsin B (K<sub>i</sub> = 1.4 nM) was shown to be due mainly to a lower second order association rate constant (K<sub>a</sub> = 0.04 × 10<sup>8</sup> M<sup>−1</sup> s<sup>−1</sup>). The inhibitor is composed of 128 amino acids forming two repeated domains with 48% identity. Neither of the domains shows any sequence homology to cystatins, but they do show a significant homology to thyroglobulin type-1 domains. A highly conserved consensus sequence motif of Cys-Trp-Cys-Val together with conserved Cys, Pro, and Gly residues is present in major cystatins, but they do show a significant homology to thyroglobulin type-1 domains. This superfamily currently includes equistatin, major histocompatibility complex class II-associated p41 invariant chain, nidogen, insulin-like growth factor protein, saxiphilin domain α, pancreatic carcinoma marker proteins (GA733), and chum salmon egg cysteine proteinase inhibitor. In each of the domains of the equistatin, the three residues are similarly conserved, and the sequences Val-Trp-Cys-Val and Cys-Trp-Cys-Val are present in domains a and b, respectively. We suggest that equistatin belongs to a new superfamily of protein inhibitors of cysteine proteinases named thyroglobulin type-1 domain inhibitors. This superfamily currently includes equistatin, major histocompatibility complex class II-associated p41 invariant chain fragment, and chum salmon egg cysteine proteinase inhibitor.

Sea anemones are known to be a rich source of variety of polypeptide neurotoxins (1, 2) and neuropeptides (3), but little is known about the presence of proteolytic enzymes and their inhibitors. A chymotrypsin-like protease was first isolated from the Caribbean sea anemone Metridium senile (13). Recombinant human cathepsin B and human cathepsin L were prepared as described previously (14, 15).

Inhibitor Purification—A. equina specimens were collected on the northern coast of the Adriatic sea. The anemones (3 kg) were frozen, partially thawed, cut into small pieces, and homogenized in 4.5 liters of deionized water. Nonsoluble material was removed by centrifugation at 13,000 × g for 45 min. The supernatant was adjusted to pH 10.5 and incubated at room temperature for 1 h. Neutralization to pH 7.0 was followed by additional centrifugation at 13,000 × g for 45 min. The clear supernatant was applied to a carboxymethyl papain-Sepharose column (6 × 10 cm) previously equilibrated with 0.01 M Tris/HCl buffer, pH 8.0, containing 1 M NaCl and 0.1% Brij. After thorough washing of the column, bound proteins were eluted with 0.01 M NaOH. Fractions (20 ml) were collected and assayed for inhibitory activity toward papain using benzoyl-m-Arg-β-naphthylamide as substrate (16). The inhibitory fractions were pooled and concentrated by ultrafiltration (Amicon YM-5). The concentrate was applied to a Sephadex G-50 column (4.5 × 140 cm) equilibrated with 0.01 M Tris/HCl buffer, pH 7.7, containing 0.1 M NaCl, and eluted at a flow rate of 18 ml/h. Inhibitory fractions with molecular weights of about 16,000 were pooled, concentrated (Amicon, YM-5), and dialyzed against 0.01 M Tris/HCl buffer, pH 7.2. The dialyzed sample was then applied to a DEAE-Sephaloc column (2 × 25 cm) equilibrated with the same buffer. The column was washed extensively, and bound proteins were eluted with a linear salt gradient (0–0.1 M NaCl in 0.01 M Tris/HCl buffer, pH 7.2) at a flow rate 18 ml/h. Equistatin eluted at 0.07 M NaCl.

SDS-PAGE and Analytical Isoelectric Focusing—SDS-PAGE and isoelectric focusing were performed on a PhastSystem apparatus (Pharmacia Biotech Inc.) following the manufacturer’s instructions. The inhibitor and molecular weight markers ranging from M<sub>r</sub> 14,400 to 94,000 were run in the presence of 0.5% SDS and 5% 2-mercaptoethanol on an 8–25% gradient polyacrylamide gel. The pI of the inhibitor was determined by calibrating the gel with isoelectric focusing marker proteins with pI values ranging from 3.5 to 8.15.

Protein Sequence Determination—Equistatin was reduced overnight with β-mercaptoethanol at 37 °C and S-pyridylethylated (17). Pyridylethylated equistatin was hydrolyzed with glycyl endopeptidase as de-
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Equistatin was purified from A. equina. 4 nmol of pyridylethylated equistatin were fragmented using 2% (w/v) S. aureus V8 proteinase in 0.5 M sodium lactate buffer, pH 4.0, at 37 °C for 20 h. Both enzyme hydrolyses were performed in a final volume of 500 μL. Reactions were stopped by the addition of trifluoroacetic acid. The resulting peptide mixtures were separated by high-performance liquid chromatography (Milton Roy Co.) using a reverse phase ChromSpher C18 column equilibrated with 0.1% (v/v) trifluoroacetic acid in water. Elutions was performed using various linear gradients of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The absorbance was monitored at 215 nm. Protein samples were hydrolyzed in 6.0 M HCl at 110 °C for 24 h. Analyses of the peptide hydrolysates were performed on an Applied Biosystems 421A amino acid analyzer with precolumn phenylisothiocyanate derivatization. An applied Biosystems liquid pulse sequencer 475A, connected on line to a phenylthiohydantoin analyser 120A from the same manufacturer, was used for automated amino acid sequence analyses.

Determination of Protein Concentration—Protein concentration of equistatin was determined by absorption measurements at 280 nm using a molar absorption coefficient of 28,800 M⁻¹ cm⁻¹ determined by the method of Pace et al. (19) from the amino acid sequence or by the method of Lowry et al. (20) using bovine serum albumin as standard. The concentration of papain was determined spectrophotometrically using a molar absorption coefficient of 65,200 M⁻¹ cm⁻¹ (21).

Active Site Titration—The following buffers were used in all kinetic and equilibrium studies: 0.1 M phosphate buffer, pH 6.0, containing 5 mM dithiothreitol and 1 mM EDTA (for papain and cathepsin B) or 0.34 M sodium acetate buffer, pH 5.5, containing 5 mM dithiothreitol and 1 mM EDTA (for cathepsin L). Active site titrations of cathepsins B and L were performed using cysteine proteinase inhibitor Ep-475 as described previously (22). Papain, further purified by affinity chromatography (23), had a thiol content of 0.92 ± 0.05 mol/mmol of enzyme as determined by reaction with 5,5'-dithiobis(2-nitrobenzoic acid). Active site-titrated papain was used to titrate equistatin as follows. Papain (0.1 μM final concentration) was incubated with increasing amounts of equistatin (0–0.2 μM final concentration) in 200 μL of 0.1 M phosphate buffer, pH 6.0, containing 5 mM dithiothreitol and 1 mM EDTA at 25 °C. After 15 min of incubation, 1800 μL of 100 mM Z-Phe-Arg p-nitroanilide was added, and the residual activity of papain was monitored continuously at 410 nm with a Perkin-Elmer Lambda 18 spectrophotometer (22). The data were analyzed by computer fitting to the theoretical binding equation (24).

Kinetics of Inhibition of Papain and Cathepsins B and L by Equistatin—The kinetics of the reaction between equistatin and papain, cathepsin B, and cathepsin L were analyzed by continuous measurements of the loss of enzymatic activity in the presence of substrate under pseudo first-order conditions with at least a 10-fold molar excess of the inhibitor. Equistatin in increasing concentrations and the fluorogenic substrate (10 inhibitor. Equistatin in increasing concentrations and the fluorogenic pseudo first-order conditions with at least a 10-fold molar excess of the loss of enzymatic activity in the presence of substrate under

RESULTS AND DISCUSSION

Purification of Equistatin—Equistatin was purified from A. equina by a procedure similar to that used for the isolation of cysteine proteinase inhibitors of human origin (28). Initially, the supernatant was exposed to alkaline pH to dissociate the complexes between the inhibitor and other proteins. The most selective purification step, affinity chromatography on carboxymethyl papain-Sepharose, then allowed separation of papain-inhibiting proteins from the majority of noninhibitory proteins. This was followed by gel filtration on Sephadex G-50 (Fig. 1A), where the low molecular weight inhibitor (equistatin) was separated from high Mr inhibitor(s) of cysteine proteinases, which were not further characterized. Final purification was achieved by DEAE-Sephalac chromatography, from which the inhibitor eluted as a single peak at 0.07 M NaCl (Fig. 1B). About 5 mg of pure equistatin was obtained from 3 kg (fresh weight) of sea anemones.

SDS-PAGE and Analytical Isoelectric Focusing—On SDS-PAGE under reducing conditions, equistatin migrates as a single band with Mr of about 16,000 (Fig. 2A). The molecular weight is higher than the molecular weights of either stefins or cystatins (Mr 11,000 and 13,000, respectively) but lower than those of kininogens (Mr 50,000–100,000) (12). The stefins, the cystatins, and the kininogens are proteins with similar sequences and, until recently, were the only known endogenous inhibitors of papain-like cysteine proteinases. On analytical isoelectric focusing, the inhibitor is shown to be an acidic protein with a pI value of 4.7. Very faint bands with pI values of 4.9 and 4.5, probably corresponding to the isoforms of the inhibitor (see below for explanation), could also be seen (Fig. 2B).

Amino Acid Sequence of Equistatin—The major and minor N-terminal amino acid sequences, labeled NI-1 and NI-2, respectively, are shown in Fig. 3A. Sequence analyses of the peptides derived from glyceryl endopeptidase digestion provided the amino acid sequence of the whole molecule (Fig. 3A). The largest peptide, G-3, spanned the middle part of the inhibitor and overlapped with both NI sequences. The C-terminal sequence was confirmed by peptides G-5 and G-(5+6), which ended with a pyridylethylated Cys residue that is not a glyceryl endopeptidase cleavage site. Additional overlapping peptides, designated as E peptides (Fig. 3A) were obtained by S. aureus V8 proteinase digestion. During protein sequence analysis we
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Amino Acid Sequence Comparison—Alignment of equistatin-related proteins relative to thyroglobulin, saxifilin, and nidogen. The comparison sequences are human thyroglobulin domain 1.1 (Tg), mouse invariant chain (p41), and human insulin-like growth factor binding proteins (IGFBP). The comparison sequences are human thyroglobulin domain 1.1 (Tg), mouse invariant chain (p41), and human insulin-like growth factor binding proteins (IGFBP). The comparison sequences are human thyroglobulin domain 1.1 (Tg), mouse invariant chain (p41), and human insulin-like growth factor binding proteins (IGFBP).

3B). This indicates that equistatin derives from a single ancestral gene that was duplicated and modified during evolution. However, neither domain shows any sequence homology with the members of the cystatin superfamily. The results of isoelectric focusing reveals the presence of at least 2 closely related isoforms. The observed sequence polymorphism mainly in the middle part of the molecule (Fig. 3A). However, the yield of these residues was lower than 20% when compared with the main sequence. The observed sequence heterogeneity together with the results of isoelectric focusing reveals the presence of at least 2 closely related isoforms. As the isolation procedure involves the use of many anemone specimens, the difference in amino acid composition could arise from allelic polymorphism.

The inhibitor comprises 128 amino acid residues including 11 cysteines and has a molecular weight of 14,129. The inhibitor has no potential glycosylation sites of the Asn-X-Ser/Thr type.

Fig. 2. Electrophoretic analyses of equistatin. A, SDS-PAGE of purified inhibitor. Lane 1, equistatin; lane 2, molecular weight standards. Before electrophoresis, the sample was reduced with β-mercaptoethanol for 10 min at 100 °C. The gel was stained with Coomassie Blue. B, isoelectric focusing of equistatin. Lane 1, pI standards; lane 2, equistatin.

Fig. 3. Amino acid sequence of equistatin. A, amino acid sequence and strategy of sequence determination of equistatin. The N-terminal amino acid sequences of native inhibitor (NI-1 and NI-2) and peptides were determined by automated Edman degradation. The peptides were generated by the action of glycyll endopeptidase (G peptides) and S. aureus V8 (E peptides) proteinases. The amino acid residues shown under the major sequence were determined as sequence polymorphism. B, alignment of the amino acid sequences of the two equistatin domains. Alignment of fragments 1–64 and 65–128 was determined by using Genetic Computer Group, Inc. program software package version 7.0 (University of Wisconsin, Madison, WI). Identical amino acids are indicated with *, conservative replacements are indicated by :, and less similar residues are indicated by .

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Amino Acid Sequence Comparison—Alignment of equistatin residues 1–64 with 65–128 shows that the inhibitor consists of a tandem repeat with 48% identity and 60% similarity (Fig. 3B).

The comparison sequences are human thyroglobulin domain 1.1 (Tg), mouse invariant chain (p41), and human insulin-like growth factor binding proteins (IGFBP). The comparison sequences are human thyroglobulin domain 1.1 (Tg), mouse invariant chain (p41), and human insulin-like growth factor binding proteins (IGFBP). The comparison sequences are human thyroglobulin domain 1.1 (Tg), mouse invariant chain (p41), and human insulin-like growth factor binding proteins (IGFBP). The comparison sequences are human thyroglobulin domain 1.1 (Tg), mouse invariant chain (p41), and human insulin-like growth factor binding proteins (IGFBP). The comparison sequences are human thyroglobulin domain 1.1 (Tg), mouse invariant chain (p41), and human insulin-like growth factor binding proteins (IGFBP). The comparison sequences are human thyroglobulin domain 1.1 (Tg), mouse invariant chain (p41), and human insulin-like growth factor binding proteins (IGFBP).
and those in all the other proteins listed in Fig. 4B are approximately 40% for the 49 C-terminal amino acids of both domains. The cysteine-rich sequence motif Cys-Trp-Cys-Val and the positions of some other amino acids (Cys-24, Cys-60, Pro-22, Gln-34, Gly-28, and Gly-49; equista in Fig. 4B) were found to be highly conserved among all related repeats, indicating that the proteins are probably evolutionarily related.

**Active Site Titration and Kinetics of Inhibition**—The sequence data suggest that the two sequentially homologous parts of equistatin (Fig. 3B) may form two potential proteinase binding sites. The binding stoichiometry of papain (active concentration ≈ 95%) and equistatin was therefore determined by titration monitored by the loss of enzymatic activity. 0.95 ± 0.04 mol of equistatin was needed to saturate 1 mol of papain, indicating that the two proteins formed an equimolar complex (Fig. 5). It could be suggested that binding of one proteinase molecule to equistatin prevents binding of the second proteinase molecule, possibly by steric hindrance. However, there are a number of other possibilities. (i) One of the domains is not inhibitory at all, as observed in the kininogens (41). (ii) One of the domains has substantially lower affinity for proteinases, as found for the mucus proteinase inhibitor interaction with various serine proteinases (42). (iii) Both domains bind to the same proteinase molecule but only one of them binds to the active site; the other binds to another site distant from the active site, as reported for rhodin binding to thrombin (43). Additional spectroscopic and structural studies involving mutant proteins will therefore be needed to clarify which of the above hypotheses is correct.

The kinetics of binding of equistatin to papain and cathepsins B and L were studied under pseudo first-order conditions assuming 1:1 binding stoichiometry (see above). The pseudo first-order rate constants were found to increase linearly with increasing concentrations of inhibitor [I], in agreement with 1:1 binding stoichiometry (25). Values of the second-order rate constants ($k_a$), the dissociation rate constants ($k_d$), and the equilibrium constants ($K_i$) are presented in Table I. Rapid binding of equistatin to cathepsin L and papain was observed, but the complexes with papain were 10-fold less stable, with a 5-fold lower association rate constant and a 2-fold higher dissociation rate constant. The rate of complex formation between equistatin and cathepsin B was substantially slower. Its $k_a$ value is >30-fold lower than those for cathepsin L and papain, also reflected in the increased $K_i$ value although the overall effect is partially compensated by a lower $k_d$ value. Cathepsin B (44) differs from papain (45) and its homologue cathepsin L (46) by having an additional loop of about 20 amino acids, which partially occludes the active site, thus interfering with inhibitor binding (47).

The kinetic and equilibrium constants for the interaction of equistatin with cathepsins L and B and papain are similar to those reported for the interactions of these enzymes with cystatins (12, 22, 47). The $K_i$ values are also in reasonable agreement with those obtained for various forms of chum salmon egg cysteine proteinase inhibitor (34, 48) although they differ significantly for the values from the p41 form of invariant chain fragment. The latter was found to be a stronger inhibitor of cathepsin L (~10-fold) and a weaker inhibitor of papain (~3-fold) but did not inhibit cathepsin B at all (33).

In conclusion, a new protein inhibitor of papain-like cysteine proteinases was isolated from sea anemone *A. equina*. The inhibitor, equistatin, is distinct from cystatins but shares significant sequence homology with two other chum salmon egg cysteine proteinase inhibitors, p41 invariant chain fragment and cysteine proteinase inhibitor. The three inhibitors were therefore suggested to form a new superfamily of cysteine proteinase inhibitors. The thyroglobulin type-1 domain motif, common to all three inhibitors, has been identified in a variety of other proteins. Whether this highly conserved thyroglobulin type-1 element indeed acts as an inhibitor of cysteine proteinases in these proteins remains to be established as well as the mechanism of binding to cysteine proteinases.

**Acknowledgments**—We thank Dr. Aleksander Lucu for providing us with sea anemones and Dr. Izotok Dolenc and Robert Kuhelj for their gifts of cathepsins L and B, respectively. We also thank Dr. Roger H. Pain for critical reading of the manuscript.

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J. Biol. Chem. 1997, 272:13899-13903.
doi: 10.1074/jbc.272.21.13899

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Vol. 272 (1997) 13899–13903

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Page 13901: In the course of studies that involved resequencing of equistatin molecule, an error in the published sequence was discovered. Consequently, Figs. 3 and 4 should be replaced by the following figures. These lead to the conclusion that equistatin is not a two-domain protein as previously described, but a three-domain protein with molecular weight of 21,755. The inhibition properties and other characteristics are unchanged.

![Amino acid sequence of equistatin](image)

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
FIG. 4. Alignment of thyroglobulin type-1 repeats. A, schematic diagram of thyroglobulin type-1 repeats as found in thyroglobulin (Tg, 10 repeats), ascidian nidogen (Nido asc, 3 repeats) (49), nidogen (Nido), saxiphilin (Sax, 2 repeats), pancreatic carcinoma marker protein (GA733), p41 invariant chain (p41), insulin-like growth factor binding proteins (IGFBP), chum salmon egg cysteine protease inhibitor (ECI), and equistatin (3 repeats). B, alignment of the equistatin-related proteins relative to the 48 amino acids of each of the three domains of equistatin (equista, equistb, and equistc). The compared sequences are human thyroglobulin domain 1.1 (Tg) (32), human invariant chain (p41) (50), chum salmon egg cysteine protease inhibitor (ECI) (34), mouse nidogen (nido) (36), two domains of bullfrog saxifilin (saxa) (39), human pancreatic carcinoma marker protein (GA733-2) (40), and human insulin-like growth factor binding protein (IGFBP-3) (37). Residues in boldface type are present in at least 6 of 11 sequences. The conserved cysteine residues are indicated with an arrowhead.