Macromolecular chelation as an improved mechanism of protease inhibition: structure of the ecotin – trypsin complex

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The 2.4 Å crystal structure ($R = 0.180$) of the serine protease inhibitor ecotin was determined in a complex with trypsin. Ecotin's dimer structure provides a second discrete and distal binding site for trypsin and, as shown by modelling experiments, other serine proteases. The second site is ~45 Å from the reactive/active site of the complex and features 13 hydrogen bonds, including six that involve carbonyl oxygen atoms and four bridged by water molecules. Contacts ecotin makes with trypsin's active site are similar to, though more extensive than, those found between trypsin and basic pancreatic trypsin inhibitor. The side chain of ecotin Met84 is found in the substrate binding pocket of trypsin where it makes few contacts, but also does not disrupt the solvent structure or cause misalignment of the scissile bond. This first case of protein dimerization being used to augment binding energy and allow chelation of a target protein provides a new model for protein–protein interactions and for protease inhibition.

Key words: allostery/chelation/crystallography/protease inhibition/protein–protein interactions

Introduction

Modulation of serine protease activity is the goal of numerous research efforts spanning various technologies and addressing a panoply of clinical indications. Consequently, naturally occurring proteinaceous inhibitors are of great interest as templates for the alteration of natural control mechanisms and as a source of basic design principles. Ecotin is a dimeric serine protease inhibitor found between the inner and outer membranes of *Escherichia coli*, where each 142 amino acid monomer can inhibit one protease molecule (Chung *et al.*, 1983; McGrath *et al.*, 1991b). Ecotin is a highly effective inhibitor of chymotrypsin, elastase and trypsin with K_i s in the range 50 pM to 1 nM (Chung *et al.*, 1983; unpublished data). This unusual ability to strongly inhibit three proteases with different specificities was found to be mediated by the presence of Met84 (McGrath *et al.*, 1991b) at the inhibitor

reactive site, also known as P1 (Schechter and Berger, 1967). Recently, ecotin has been found to be a picomolar inhibitor of human neutrophil elastase and of various blood coagulation proteases, including Factor Xa (Seymour *et al.*, 1994). Tick anticoagulant protein (TAP) (Waxman *et al.*, 1990) is the current paradigm for Factor Xa inhibitor design, and ecotin was found to be as efficacious as TAP using an amide substrate (Lauwereys *et al.*, 1993) and assayed in the prothrombinase complex (U. Sinha, personal communication). Ecotin's therapeutic potential in the areas of emphysema, thrombosis, pancreatitis and sepsis have heightened the interest in the structural basis for its exceptional potency.

Just as serine proteases are the most intensely studied of the four protease classes, their inhibitors are also the most numerous and well characterized to date. Small molecule inhibitors which have proved useful include reversible transition state mimics such as peptide aldehydes and boronates, and irreversible reagents such as peptidyl chloromethanes and sulfonyl fluoride derivatives (Powers and Harper, 1986). Protein inhibitors are classified as the non-specific macroglobulins, the serpins, hirudin (Rydel *et al.*, 1990) and the 'substrate-like' inhibitor class (Laskowski and Kato, 1980), which includes ecotin. This last inhibitor category is composed of hundreds of molecules which fall into at least 10 different families according to primary and tertiary structural similarities (Bode and Huber, 1992). While they display a variety of different structural motifs, they all share substrate-like binding at a single site located on a surface loop, in which the side chain of P1 binds in the substrate binding pocket of the protease (S1 site) (Bode and Huber, 1992). This primary interaction provides the majority of the binding energy, with the remainder supplied by neighboring hydrogen bonds and van der Waals contacts along the P3–P3' stretch on the inhibitor loop (Laskowski and Kato, 1980). The 2.4 Å X-ray crystal structure of ecotin was determined in a complex with trypsin to investigate whether the affinity of ecotin for disparate serine proteases is achieved solely by the reactive site loop featuring the non-optimal (in the case of trypsin) ecotin Met84–protease interaction, or if additional contacts between protease and inhibitor allow the observed combination of tight binding and pan-specificity.

Results and discussion

We had previously produced large quantities of recombinant ecotin in an *E. coli* expression system and co-crystallized the inhibitor with rat trypsin mutant Asp102Asn (McGrath *et al.*, 1991a). A novel addition to the molecular replacement method allowed structure determination. The search model was the coordinates for the 2.3 Å crystal structure of rat trypsin mutant Asp102Asn (Sprang *et al.*, 1987). Since this model describes only 60% of the scattering matter in the cell, and provides no information for the inhibitor, the initial

Fig. 1. (A) Monomeric ecotin is a seven-stranded flattened barrel which is missing strand B, the first strand of the first sheet (prepared using MolScript; Kraulis, 1991). (B) Schematic representation of the polypeptide chain arrangement for the ecotin monomer showing main chain hydrogen bonds and the location of the disulfide bond.

electron density map was ambiguous in the vicinity of ecotin. The model was built after improving the electron density for ecotin using PRISM (Bystroff *et al.*, 1993), which provides for the application of connectivity constraints in phase calculation (Baker *et al.*, 1993). The final model contains an ecotin dimer and two bound trypsin molecules in the asymmetric unit (E_2-T_2), where each ecotin–trypsin complex ($E-T$) was refined independently. There are 360 putative water molecules per asymmetric unit, and $R_{\text{cryst}} = 0.180$ for the 6–2.4 Å data with r.m.s. errors on bond lengths and angles at 0.010 Å and 2.85°, respectively. The refined coordinates describe all but the first four amino acids and surface residues 90 and 91 of ecotin, which are highly disordered.

Overall structure

The ecotin monomer is arranged in a single domain best described as a mostly antiparallel seven-stranded β -barrel (Figure 1A). The topology is similar to the jelly-roll motif with one significant anomaly: two strands are interchanged resulting in a parallel arrangement of two strands. A deconstructed jelly-roll protein features a sheet of strands BIDG, a hinge region, then a sheet of strands FEHC. In ecotin, strand E rather than strand F of the second sheet lies closest to the hinge region (Figure 1B). The position switch for the two strands places strand E parallel to strand H, contrary to the all antiparallel nature of jelly-roll fold proteins. The possibility that the strands were mistraced was explored by examination of electron density maps calculated after the entire loop, comprising strands E and F, was deleted

from the coordinate set and the remainder of the model subjected to simulated annealing. The resultant electron density for the omitted region was unambiguous and indicated that it had been correctly built. Examination of the primary structure of the two strands also suggests that arrangement as a typical jelly-roll would be precluded in this structure. Strand E is an edge strand and solvent accessible. It is characterized by an abundance of polar and charged residues. Strand F is mostly buried in the interior of the protein and features many hydrophobic residues including three tyrosines (Tyr69, Tyr71 and Tyr72), a valine (Val73) and a phenylalanine (Phe74). Reversing the position of these strands to comply with a typical jelly-roll would thrust hydrophobic strand F into solvent. Rearrangement of these two strands would also necessitate a strand switch at some other place in the molecule and there is no ambiguity in the ecotin trace.

The functional dimer of ecotin resembles a butterfly, with approximate dimensions of $70 \times 50 \times 20$ Å. The structure is curiously non-globular, with a surface area that far exceeds what is expected for a molecule of its size (Sprang *et al.*, 1979): a clue, perhaps, to its unusual protease binding characteristics. Most of the contacts between ecotin monomers occur between the long C-terminal strands (Figure 2). In the dimer, these strands are arranged as a two-stranded antiparallel β -sheet, with 10 main chain hydrogen bonds between residues 125 and 142 of each monomer. The surface area which is buried (Lee and Richards, 1971) upon dimerization is >1400 Å² per monomer. Neither ecotin's monomer structure nor its mode of dimerization resembles

Fig. 2. The C-terminal strands (residues 125–142) of each monomer form a two-stranded antiparallel β -sheet with 10 main chain hydrogen bonds, several of which are visible in this view (green dashed lines). Additional dimer stabilization is provided by several side chain and main chain hydrogen bonds between Arg22, Val24 and Gln26 and the C-terminal Val140 and Arg142 of the cognate monomer. A salt bridge is found between Arg22 and the C-terminal carboxyl group of ecotin (prepared using Insight II, Biosym, Inc.).

Streptomyces subtilisin inhibitor, another dimeric bacterial serine protease inhibitor (Takeuchi *et al.*, 1991).

Interactions with trypsin

Primary. Met84 of ecotin binds at the S1 site of trypsin, as determined biochemically (McGrath *et al.*, 1991b). Trypsin favors Arg or Lys side chains N-terminal to the scissile bond and is disinclined to cleave after Met; the resultant unpaired charged Asp189 in the trypsin substrate binding pocket alone is estimated to weaken binding by ~ 3 kcal/mol (Fersht *et al.*, 1985). Perhaps due to its flexibility and van der Waals contact capability, Met seems to be the best compromise reactive site residue (McGrath *et al.*, 1991b) and is found in several inhibitors which demonstrate varying degrees of broad specificity. In this complex with trypsin (Figure 3A), the Met84 side chain is found as the fairly usual '–t' rotamer (Ponder and Richards, 1987), and the lie of the side chain is strikingly similar to Lys15, the P1 amino acid in basic pancreatic trypsin inhibitor (BPTI) as it interacts with rat trypsin (Perona *et al.*, 1993). Methionine side chains infrequently form hydrogen bonds in proteins (Gregoret *et al.*, 1991), and in this structure ecotin Met84–S δ accepts one hydrogen bond (3.3 Å) from a water molecule. The arrangement of atoms in the region of the scissile bond indicates that ecotin and trypsin have formed a distorted Michaelis complex, as typically seen in serine protease–inhibitor complexes (Figure 3B) (Marquart *et al.*, 1983).

Met84 of ecotin is found on an extended surface loop, similar to the reactive site residues of other small serine protease inhibitors such as BPTI (Ruhlmann *et al.*, 1973), STI (Sweet *et al.*, 1974) and turkey ovomucoid third domain (Fujinaga *et al.*, 1987). Residues flanking P1 on this loop

also contribute to the binding energy (Bode and Huber, 1991). In the ecotin–trypsin complex these subsite interactions are equivalent to those found in the BPTI–trypsin complex (Figure 2). Ecotin has the somewhat unusual Met at position P1', which forms numerous hydrophobic contacts with trypsin. BPTI and ecotin are dissimilar in their size, topology and approach to the trypsin active site. However, when the ecotin–rat trypsin structure is superimposed with a BPTI–rat trypsin structure (Perona *et al.*, 1993) using C α s of the trypsin catalytic triad residues, the two inhibitors are essentially isostructural for the critical residues P2–P1'. Comparable with many other small serine protease inhibitors, a disulfide bond proximal to the reactive site in ecotin stabilizes the reactive site loop by covalently anchoring it to an inner supporting loop (Bode and Huber, 1992) [Cys50–Cys87 (P3') in ecotin], while numerous hydrogen bonds between the inner and outer loops provide additional support. In ecotin, residues 89–92 of the outer loop extend away from the inner loop, making few contacts. Not surprisingly, the electron density is poor for these four amino acids. Residues 90 and 91 have been excluded from the final model because of insufficient density.

A two to four residue stretch of the support loop often contacts the protease, and it is this region that probably precludes binding of the small substrate-like inhibitors to proteases such as thrombin, which have extended loops in this vicinity (Bode *et al.*, 1989). Ecotin also interacts with trypsin through this site (residues 51–54), with Arg54 occupying an equivalent role to Arg39 of BPTI. In all, the primary contact area (Lee and Richards, 1971) between trypsin and ecotin is 1900 Å², whereas it is 1400 Å² for the BPTI–trypsin complex (Janin and Chothia, 1976). Thus, the primary ecotin–trypsin recognition site is standard for

Fig. 3. (A) The refined coordinates for ecotin Met84 (in yellow) bound at the active site of rat trypsin Asp102Asn (in white) are shown with $2F_o - F_c$ density contoured at 1σ , visualized using FRODO (Jones, 1978). The catalytic triad residues of trypsin are indicated, as are reactive site residues P3–P1' of ecotin. Numerous van der Waals contacts are found between the Met84 side chain and the trypsin binding pocket. The solvent structure in the binding pocket is quite similar whether Met or Lys is bound; six putative waters are found in the pocket in both cases, including an equivalent water molecule to the one that bridges the interaction between Lys15 of BPTI and Asp189 of trypsin (Bode and Schwager, 1975). (Only one of these water molecules, in red, is clearly seen in this view.) (B) The environment of trypsin within 4 Å of Met84 in ecotin. Trypsin is shown in thick lines and ecotin in thin lines. In the region of the scissile bond, Met84-C is found in a sub-van der Waals contact with Ser195-O γ , indicating that catalysis has been halted prior to formation of the acyl enzyme intermediate (Huber *et al.*, 1974); Met84-O forms two hydrogen bonds with the trypsin oxyanion hole (Gly193-N and Ser195-N) (Henderson *et al.*, 1971). Additional contacts (not visible) between the reactive site loop and trypsin are as found for many of the substrate-like inhibitors, including the hydrogen bond between the amide nitrogen of P1 and Ser214-O, and the hydrogen bonds which result from formation of a two-stranded anti-parallel β -sheet between P3 of the inhibitor (Ser82 in ecotin) and Gly216.

Fig. 4. (A) C α trace of the E₂-T₂ heterotetramer shows how the dimeric structure of ecotin allows it to bind trypsin at two different and distal sites. The ecotin monomers are in orange and gold, while the two bound tryptins are blue and green. The side chain atoms of reactive site Met84 of ecotin are shown as solid white spheres bound in the primary trypsin binding site. Trp67 of ecotin is approximately at the center of the secondary binding site for trypsin and is labeled by number for each monomer. (B) Ecotin-trypsin interactions at the secondary site. Trypsin is displayed at the top as a C α trace, with main chain and side chain atoms shown explicitly where hydrogen bonding interactions are made with ecotin. Two segments of trypsin are shown, the C-terminal helix from Y234 to N245 and the type I turn at H91–F94. Two segments of ecotin are shown similarly at the bottom with thin lines. Loops A65–D70 and R108–L113 are perched at the ends of the β -strands and form interactions with trypsin. The 108–113 loop bonds with trypsin 91–94, while ecotin 65–70 interacts with both segments of trypsin.

Table I. Interactions between trypsin and ecotin at the secondary site and modeled putative interactions between ecotin and chymotrypsin or elastase

Ecotin	Trypsin (Å)	Chymotrypsin ^a	Elastase
Ser34-O γ	Arg96-N η 1 3.0	—	—
Gly66-N	—	Asn236-O δ 1	—
Gly66-O	Asp236-N 3.1	—	Ser236-N
	Trp237-N 3.1	—	Trp237-N
Trp67-O	His91-N ϵ 2 2.9	Asn91-N δ 2	His91-N ϵ 2
Trp67-N ϵ 1	—	Gln240-O ϵ 1	Ser236-O γ
Gly68-O	Asn93-N δ 2 3.2	Asn101-N δ 2	—
Tyr69-O η	—	Trp237-N ϵ 1	Trp237-N ϵ 1
Asp70-N	—	—	Tyr93-O η
Asp70-O δ 2	Asn93-N δ 2 3.0	Lys93-N ζ	—
Arg108-N ϵ	—	Asn95-N δ 2 ^b	—
Arg108-N η 1	Asn93-O δ 1 2.7	Lys93-O ^b	Tyr93-O
Arg108-N η 2	Phe94-O 2.7	Tyr94-O ^b	Trp94-O ^b
Asn110-O δ 1	—	—	Pro93-O
Asn110-N δ 2	Pro92-O 2.9	Ser92-O ^b	Tyr93-O ^b

^aProtein data bank coordinates used were 6cha for chymotrypsin and 3est for pancreatic elastase.

^bMore than one protease atom could form a hydrogen bond at this site.

Direct hydrogen bonds (in Å) between ecotin and trypsin at the secondary binding site are enumerated. Possible hydrogen bonds, as determined by modeling, are shown for ecotin–chymotrypsin and ecotin–elastase interactions.

protein–protein interfaces (Janin and Chothia, 1990), and both the number of inhibitor residues involved (15 residues) and the total number of interacting residues in the complex (34 residues) are in the expected ranges.

Secondary. Examination of the structure of the E₂–T₂ complex shows that there is an unexpected second interaction site between protease and inhibitor (Figure 4A). A loop at the distal end of ecotin, relative to the reactive site, and comprising residues Gly66, Trp67, Gly68, Tyr69 and Asp70 and neighboring residues Ser34, Arg108 and Asn110 makes a total of nine hydrogen bonds (Table I) to residues 91–96 and the C-terminal helical tail of trypsin (residues 233–240) (Figure 4B). An additional four hydrogen bonds are mediated by water molecules. More than 30 van der Waals contacts are also made and the total area of contact is ~950 Å². While the number of hydrogen bonds is average for protein–protein recognition sites, the contact area is approximately half the norm (Janin and Chothia, 1990) (e.g. Fab–lysozyme = 1600 Å²; Sheriff *et al.*, 1987). Thus, the secondary binding locus represents the minimal contact end of a spectrum in protein–protein recognition sites since relatively few non-directional van der Waals contacts are made, and ecotin is essentially tethered to trypsin at this site by hydrogen bonds. As the complexity of a protein–protein binding event increases, such as in rigidification of the interface in standard protease–inhibitor interactions or at the high end of the spectrum, monomer stabilization in oligomeric proteins, the surface area buried and consequently the van der Waals contacts greatly increase.

One ecotin does not contact one trypsin at both sites. Rather, the particular dimer arrangement of ecotin allows one inhibitor subunit to bind trypsin at the active site, while the other contacts the protease using this secondary binding site. The second protease molecule is similarly chelated by the 2-fold symmetric inhibitor. Thus, additional stabilization of the complex is obtained by favorable interactions at a site 45 Å from the inhibitor reactive site. An important aspect of macromolecular chelation is that the binding energy reaped from the second site is not offset by a large entropic deficit,

since the trypsin is already immobilized and correctly aligned on the primary inhibition site of ecotin. Of the hundreds of substrate-like serine protease inhibitors thus far characterized, this is the first case in which a second and distal binding site has been observed. More generally, it is a novel example, in protein–protein interactions, of a dimeric protein providing two distinct sites for recognition and binding of a target protein.

Trypsin is ~35% identical to its pancreatic homologs and provides a structural foundation for assessing ‘double recognition’ of those proteases by ecotin. Known structures of chymotrypsin (Blevins and Tulinsky, 1985) and elastase (Meyer *et al.*, 1988) were docked onto the secondary binding site on ecotin. Although sequences are not conserved at the secondary site on the proteases (residues 91–96 and 233–240), modeling potential ecotin–protease interactions at this site (where the only adjustments made to the coordinates were optimization of the rotamers for hydrogen bond formation) showed that for each of the above proteases, many hydrogen bonds are possible with ecotin (Table I). We propose that ecotin recoups binding energy which is not available with the Met84–protease interaction by forming additional bonds to a distal structural motif common to many serine proteases. Tighter binding through augmentation of contact surfaces is reminiscent of the hirudin–thrombin complex in which non-standard binding at the active site is compensated for by additional interactions at the thrombin anion binding exosite (Rydel *et al.*, 1990). Ecotin and hirudin provide two distinct paradigms for engineering and design of improved protease inhibitors.

Materials and methods

Preparation and crystallization of the ecotin–trypsin complex were as previously described (McGrath *et al.*, 1991a), except for a small variation in crystallization conditions: diffraction-quality crystals were obtained at a protein concentration of 15 mg/ml in the presence of 0.15 M sodium cacodylate, pH 6.5, 0.3 M sodium acetate and 15.5% (w/v) polyethylene glycol 4000 (initial concentrations) at 18°C. These conditions yielded a P2₁ unit cell in which the asymmetric unit contained one heterotetramer, E₂–T₂, rather than the two heterotetramers predicted for the previous P2₁

crystal form (McGrath *et al.*, 1991a). Analysis of diffracted crystals by SDS-PAGE showed that ecotin and rat trypsin Asp102Asn were present in stoichiometric amounts. The space group $P2_1$ and unit cell parameters, $a = 80.66$ Å, $b = 83.11$ Å, $c = 62.46$ Å and $\beta = 97.5^\circ$, were determined by precession photographs using $\text{CuK}\alpha$ radiation at 50 kV and 60 mA. A 2.3 Å X-ray data set (95% complete) was collected using a Mar image plate system at UCSD and reduced using MOSFLM; 84 876 measurements of 39 955 reflections where mean $I/\sigma = 6.6$ and R_{symm} to 2.4 Å = 0.087. Non-crystallographic symmetry elements were located using the self-rotation search in X-plor (Brunger, 1990) with a real space Patterson search method (Huber, 1985) performed in spherical polar space (ϕ , ψ , χ). The subsequent cross-rotation search clearly showed the non-crystallographic 2-fold axis at $\phi = 20^\circ$, $\psi = 104^\circ$. Molecular replacement using X-plor (Brunger *et al.*, 1987) commenced with a rotation search in Eulerian space. When the 7–4 Å data were used, the peaks were 8.0 and 5.5 standard deviations above the mean value (σ). Patterson correlation refinement (Brunger, 1990) was then used to optimize the position of the rotated molecule. The translation function was a reciprocal space correlation of E^2 s for the same range of data as used above. One unambiguous peak was present for each rotation solution (9.7 and 4.9 σ). An additional search was required to locate the relative position of the two molecules in the Y direction and the relative choice of origin. Rigid body refinement was used on this two trypsin starting model and the starting R -factor was 44% to 2.8 Å. Electron density was ambiguous for ecotin, and PRISM was used as previously described (Bystroff *et al.*, 1993) and allowed placement of $\text{C}\alpha$ atoms for residues 9–87 and 95–139 of ecotin. Conventional refinement and building using X-plor (Brunger *et al.*, 1987) and FRODO (Jones, 1978), respectively, commenced with $R_{\text{cryst}} = 0.36$. At the end of the refinement process, the non-crystallographic symmetry restraints were removed to allow for conformational differences in the molecules resulting from different crystal packing environments. Only one region in trypsin (residues 75–80) required rebuilding at this juncture. The final refined model has coordinates for all but the first four amino acids and residues 90 and 91 of ecotin (which are apparently highly disordered), a total of 360 putative water molecules for the asymmetric unit, and $R_{\text{cryst}} = 0.180$ for the 6–2.4 Å data with r.m.s. errors on bond lengths and angles at 0.010 Å and 2.85° , respectively.

We evaluated the correctness of our final model using 3-D profiles as described by Luthy *et al.* (1992). This analysis represents an independent test of the correctness of a model whereby the probability that a given amino acid would be found in a particular structural environment is calculated using the statistical preferences of the amino acids (Bowie *et al.*, 1991). Structures which are incorrectly modeled tend to have low scores as determined with this algorithm, and the score will often drop below zero for segments where amino acids reside in especially unsuitable environments. The score for monomeric ecotin was good (48.9), though not quite as high as expected (~ 64). Although the profile window plot never dropped below zero, scores did approach zero in two regions of the protein. One region corresponds to the reactive site loop of ecotin, specifically residues P5–P1'. This surface loop in ecotin, and most small serine protease inhibitors, is quite hydrophobic and much more suited to being buried in a complex with a protease than being exposed in the free inhibitor. The second low-scoring site is found at the C-terminus, where dimerization contacts are made. Thus, the problem areas represent the two sites of extensive protein–protein contacts in ecotin: information which was unknown to the profile program. A Ramachandran plot of the ecotin monomer identified Lys76 as the sole non-glycine deviant from expected ϕ – ψ angles.

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