Stable monomeric form of an originally dimeric serine proteinase inhibitor, ecotin, was constructed via site directed mutagenesis

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Abstract **Ecotin, a homodimer protein of *E. coli*, is a unique member of canonical serine proteinase inhibitors, since it is a potent agent against a variety of serine proteinases having different substrate specificity. Monomers of ecotin are held together mostly by their long C-terminal strands that are arranged as a two-stranded antiparallel β-sheet in the functional dimer. One ecotin dimer can chelate two proteinase molecules, each of them bound to both subunits of ecotin at two different sites, namely the specific primary and the non-specific secondary binding sites. In this study the genes of wild type ecotin and its Met^{84}Arg P1 site mutant were truncated resulting in new forms of ecotin that lack 10 amino acid residues at their C-terminus. These mutants do not dimerize spontaneously, though in combination with trypsin they assemble into the familiar heterotetramer. Our data suggest that this heterotetramer exists even in extremely diluted solutions, and the interaction, which is responsible for the dimerization of ecotin, contributes to the stability of the heterotetrameric complex.**

Key words: Ecotin; Serine proteinase inhibitor; Site directed mutagenesis

1. Introduction

Specificity of the canonical serine proteinase inhibitors operating on a substrate-like manner is determined mainly by their P1 residue, the side chain which fits well into the binding pocket of the targeted enzyme [1-4]. This residue is usually Lys or Arg in trypsin inhibitors, Tyr, Phe, Leu or Met in chymotrypsin inhibitors, and Leu, Val, Met or Ala in elastase inhibitors. These inhibitors are quite selective: potent trypsin inhibitors are, at best, poor blockers of chymotrypsin or elastase, and conversely, strong chymotrypsin and elastase inhibitors hardly affect trypsin. By contrast, ecotin, a periplasmic protein of *Escherichia coli*, is remarkably promiscuous. It is a potent agent against a series of serine proteinases despite their wide range of substrate specificity. Specifically, ecotin strongly inhibits all the pancreatic serine proteinases, trypsin, chymotrypsin and elastase, and other serine proteinases like factor Xa and XIIa, plasma kallikrein, human leukocyte elastase and fiddler crab collagenase. It does not inhibit subtilisin and members of the aspartyl-, sulfhydryl- and metallo-proteinase classes [5-9]. The gene for ecotin has been cloned and the reactive site P1 residue has been determined to be Met^{84} [6]. The presence of methionine at this position was originally believed to underlie ecotin's broad specificity [6]. However, in a subsequent study we replaced this Met P1 residue by Leu, Lys and Arg and measured K_I values on trypsin, chymotrypsin and elastase. Surprisingly, ecotin retains much of its activity in spite of the change at the P1 site. Consequently, we suggested that residues away from the P1 site are intimately involved in the binding of the different proteinases [7]. A high-resolution X-ray structure of the ecotin-trypsin complex shows this to be the case [10]. Wild-type ecotin acts as a homodimer, forming tetramers with the inhibited proteinases. It was found that the manner of ecotin-trypsin interaction is unique. Due to its special dimeric form ecotin can chelate two trypsin molecules, each of them bound by two different sites, the specific primary and the non-specific secondary binding site of the dimer ecotin. The same mechanism of action was proposed against chymotrypsin, elastase and fiddler crab collagenase [10,11]. Monomers of ecotin are held together by their long C-terminal strands that are arranged as a two-stranded antiparallel β-sheet in the functional dimer [10]. In this recent study a part of the gene of wild-type ecotin and its Met^{84}Arg P1 site mutant [7] was deleted by PCR resulting in new forms of ecotin that are truncated at their C-terminus. These new mutants fail to dimerize, form stable monomers, but constitute tetramers with trypsin as do their dimeric forms. Equilibrium kinetic constants of these monomeric forms were determined on trypsin. Our data suggest that wild-type ecotin can form a heterotetramer with trypsin even in very diluted solutions, and that the region which is responsible for the dimerization of the wild-type ecotin interferes with the stability of the tetrameric ecotin-proteinase complex.

2. Materials and methods

2.1. Bacterial strains, vectors, enzymes and chemicals

Bovine trypsin (TPCK) was purchased from Worthington. Fractogel TSK-DEAE 650(S) and Affi-Gel 10 were obtained from Merck and from Bio-Rad, respectively. Reagents for oligonucleotide synthesis were provided by Applied Biosystems. Ampli-Taq polymerase was purchased from Perkin-Elmer Cetus, the T7 sequencing kit from Pharmacia, and other DNA modifying enzymes from Amersham, Boehringer and Fermentas. The FlexiPrep kit and the Qiaex Gel Extraction Kit were purchased from Pharmacia and Qiagen, respectively. *E. coli* strains BL21(DE3) and XL1 Blue were purchased from Novagen and Stratagene, respectively. Expression vector pT7-7 was provided by Dr. Stan Tabor of Harvard Medical School, Boston, MA. BlueScript vector was obtained from Stratagene. IPTG, *N*-benzoyl-L-arginine-*p*-nitroanilide, MUB and the fluorescent burst titrant MUGB [12] were purchased from Sigma. The fluorescent turnover substrates *N*-succinyl-Ala-Ala-Pro-Arg-AMC and *N*-succinyl-Ala-Ala-Pro-Trp-AMC were synthesized as described previously [13]. All other chemicals were either reagent grade or the best commercially available.

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Abbreviations: PCR, polymerase chain reaction; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; IPTG, isopropyl-β-D-thiogalactopyranoside; MUB, 4-methylumbelliferone; MUGB, 4-methylumbelliferyl-*p*-guanidinobenzoate; AMC, 7-amino-4-methylcoumarin; Δ133–142 ecotin, ecotin mutant lacking its last 10 C-terminal amino acid residues

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2.2. Mutation of the C-terminus of ecotin

The standard techniques of molecular cloning were performed essentially as described [14,15]. Wild-type and $Met^{84}Arg$ mutant genes of ecotin inserted into the pT7-7 plasmid [7] were used as templates for the polymerase chain reaction. PCR primer oligonucleotides were designed using the sequence of ecotin gene [6]. Primer sequences were: 5′-GCGAGAATTCTACCTGCAGTATTGTTTGCC-3′, corresponding to bases 76–99, and 5′-ACGAGGATCCTTATTACGCCTTCC-AGACGCGG-3′, corresponding to bases 507–522. The first primer had been used previously to clone the ecotin gene [7]. The second primer anneals to the template towards the end of the coding sequence. Consequently, we could amplify a portion of the ecotin gene encoding a truncated polypeptide missing the last 10 amino acids from its C-terminus. The new C-terminus is Ala^{132}. The two oligonucleotides have *Eco*RI and *Bam*HI restriction sites, respectively, indicated by underlining. Primers at the 5′ ends were extended by four additional nucleotides for efficient cutting by the restriction enzymes. Picograms of template DNA, 100 pmol of each primer, 100 nmol of dNTPs and 1 U of Taq polymerase were in 50 μl final volume. 25 PCR cycles were carried out as follows: denaturation for 50 s at 94°C, annealing for 50 s at 65°C and extension for 60 s at 72°C. Amplified DNA was purified by phenol/chloroform extraction and ethanol precipitation, then digested with *Eco*RI and *Bam*HI. Such restriction fragments were gel-purified using Qiaex Gel Extraction Kit and ligated into the precut pT7-7 vector. The products were sequenced by T7 sequencing kit. IPTG induced over-expression was verified by electrophoresis on SDS-PAGE [16].

2.3. Protein purification

E. coli BL21(DE3) culture carrying pT7-7 vector was grown in LB medium containing ampicillin. When the A_{600} reached 0.7, expression was induced with 0.5 mM IPTG. Cells were harvested 8–10 h after induction. Short forms of wild-type and $Met^{84}Arg$ mutant ecotins were purified from *E. coli* as described previously [7] with the following modifications. After isolation of the periplasmic protein fraction the pH was adjusted to 7.5 with Tris base at room temperature. The sample was centrifuged (20000×*g*, 15 min), the supernatant was loaded onto a TSK-DEAE 650 (S) column (1.0 cm×15 cm) equilibrated with 10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$ buffer. The flow-through from this ion-exchange column was collected and the pH was adjusted to 4.5 with 1 N HCl. This sample was loaded onto an Affi-Gel 10 trypsin affinity column, then eluted with 50 mM HCl. Ecotin prepared in this manner proved to be homogeneous as judged by SDS-PAGE.

2.4. Analysis of subunit structure of ecotin and ecotin-trypsin complex by gel permeation chromatography

Analytical gel filtration experiments were performed on a computer-operated Pharmacia FPLC system equipped with an LC550 Plus controller and a UV MII monitor. Wild-type and mutant ecotin were run on Superdex 75 and Superdex 200 while their complexes with trypsin were analyzed on Superdex 75, Superdex 200 and Superose 12 columns at room temperature. Injection volume was always 25 μl; protein concentration varied between 500 μM and 80 nM. The flow rate was 0.5 ml/min. The buffer used in most experiments, when the detection was performed at 280 nm, was 0.15 M NaCl, 20 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$, 0.005% Triton X-100 (buffer 1). When detection at 215 nm was required we used 0.2 M NaCl, 50 mM Na-phosphate, pH 7.5 (buffer 2) as elution buffer. Molecular weight determination was performed on Superdex 75 column in buffer 1. The column was calibrated by the following proteins (obtained from Sigma): BSA dimer (132000) and monomer (66000), carbonic anhydrase dimer (58000), ovalbumin (45000), carbonic anhydrase monomer (29000), soybean trypsin inhibitor (20000) and cytochrome *c* (12400). The molecular weights of wild-type ecotin, Δ133–142 ecotin, and their trypsin complexes were calculated by linear regression analysis of a plot of the log molecular weight versus ratio of elution volume to void volume (Ve/Vo).

2.5. Fluorometric assay

Two-fold serial dilution of 12 mM wild-type ecotin and Δ133–142 ecotin was made in 0.15 M NaCl, 50 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.5. Prior to dilution the buffer was filtered through a 0.25 μm cellulose acetate filter (Millipore). The diluted samples were centrifuged at 60000 rpm for 10 min in Beckman TL-100 centrifuge. Corrected tryptophan fluorescence spectra were recorded by a SPEX FluoroMax spectrofluorometer at 25°C with excitation at 280 nm and an emission range of 290–450 nm. The ratio of fluorescence intensities for wild-type and Δ133–142 ecotin at 320 nm was plotted against concentration. Data points are the average of three independent measurements. The dissociation constant (K_d) of wild-type ecotin was calculated from a non-linear fit of data points to Eq. 1:

$$F = F_m + (F_d - F_m)\left\{-K_d + \sqrt{K_d^2 + 8K_d[E_1]}\right\}/4[E_1] \quad (1)$$

where F_d and F_m are the fluorescence intensity ratios of wild-type dimer/Δ133–142 ecotin and wild-type monomer ecotin/Δ133–142 ecotin, respectively.

2.6. Enzyme and inhibitor standardization

Trypsin stock solutions were made by dissolving protein in 1 mM HCl containing 20 mM $CaCl_2$. Concentration of trypsin was determined by active site titration [12]. The ecotin samples were standardized by titration with trypsin at 10^{-7} M final concentration using 10^{-4} M *N*-benzoyl-L-arginine-*p*-nitroanilide. The production of *p*-nitroaniline was measured at a wavelength of 410 nm as a function of time by a Shimadzu UV-2101PC spectrophotometer.

2.7. Measurement of the equilibrium inhibition constant (K_I)

K_I values were determined by the method of Green and Work [17] as modified by Empie and Laskowski [18]. A constant amount of trypsin was incubated with incremental amounts of inhibitor. After equilibrium was reached, the residual enzyme activity was determined as follows: fluorescence spectroscopy was used to follow the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Arg-AMC in 50 mM Tris-HCl, 20 mM $CaCl_2$ and 0.005% (w/v) Triton X-100, pH 8.0. The substrate concentration was 10^{-4} M. The production of free AMC was measured at excitation and emission wavelengths of 366 and 440 nm, respectively, by a SPEX FluoroMax spectrofluorometer.

Initial enzyme concentrations were chosen which – reaching the equilibrium – left 10–15% residual enzyme activity when stoichiometric quantities of trypsin and inhibitor were present.

3. Results

3.1. Mutagenesis, expression and purification of Δ133–142 ecotin

PCR mutagenesis successfully removed 10 codons from the 3′ end of the ecotin gene (Fig. 1a). The truncated gene was inserted into pT7-7 vector. The sequence was determined, demonstrating that no undesired mutations had occurred. Competent cells of the BL21(DE3) strain were transformed with this plasmid. The construct encodes a leader sequence which directs the recombinant protein into the periplasmic space, where it accumulates to high levels. The yield of truncated ecotin (Δ133–142 mutant) in the periplasm is about 50 mg/l culture. However, a large amount of the recombinant protein (carrying the signal peptide) remains in the cytoplasm of the *E. coli*. The gel pattern clearly shows that the recombinant protein expressed by the truncated gene is smaller than the original wild-type form (Fig. 1b). Comparable yields of the truncated form of the $Met^{84}Arg$ mutant were obtained following a parallel isolation procedure.

3.2. Δ133–142 mutant ecotin is monomer

The most straightforward method to determine the subunit composition of Δ133–142 ecotin is analytical gel filtration chromatography. As shown in Fig. 2, the elution volume of Δ133–142 ecotin is significantly larger than that of the wild type. The apparent molecular weights of wild-type and Δ133–142 ecotin were found to be 40600 and 22900, respectively (Fig. 3). In agreement with earlier observations [5], the retardation coefficient of ecotin yields an overestimate of its di-

meric molecular weight of 32000. The observed deviation from the calculated value can be explained by the highly asymmetric shape of the ecotin dimer. The value we obtained for Δ133–142 ecotin is about half of the apparent molecular weight of the dimer but still much higher than the calculated molecular weight of its monomer (15000). When analyzing these data we must remember that, on the time-scale of our experiments, ecotin exists in a state of dimerization equilibrium. The dissociation constant of wild-type ecotin has been determined by fluorescence titration [8] as approximately 390 nM in the absence of proteinase. In the same work the dissociation equilibrium was found to be a rapid process.

When a single molecular species has two forms with different hydrodynamic radii (let it be a dimerization or an unfolding equilibrium), in rapid equilibrium it is impossible to separate these species by analytical gel filtration. Instead, a single peak is observed, the position of which depends on the conditions [19]. In the case of a dimerization equilibrium, protein concentration is the major factor determining the extent of association. At concentrations 20–50 times above the K_d the peak corresponds to the dimer. If the concentration is well below the K_d the protein behaves as a monomer. In the concentration range around the K_d the position of the peak is between these two extremes.

We found that the elution volume of wild-type and Δ133–142 ecotin loaded onto a Superdex 75 column at 12 μM initial concentration is 10.68 ± 0.02 ml (n = 8) and 11.97 ± 0.03 ml (n = 5), respectively. Then we ran identical chromatograms with varied initial protein concentrations. The results of these experiments are summarized in Table 1; some typical chromatograms are shown in Fig. 4. We conclude that Δ133–142 ecotin exists as a monomer under the above conditions, since the position of its peak does not shift towards higher elution volume values following dilution. On the other hand, there was a significant decrease in the elution volume when a highly concentrated sample (500 μM) of Δ133–142 ecotin was applied to the column. Therefore this truncated mutant ecotin is still capable of dimerization (Fig. 4a), although the K_d of this equilibrium is in the 100 μM range, i.e. higher than 1 mg/ml protein concentration.

Similar experiments with wild-type ecotin show that the dimer is more stable than previously reported [8]. When serially diluted samples were loaded onto the column, the first indication of partial dissociation was found at 80 nM. This protein concentration is so low that it can only be measured at 215 nm. Chromatogram B shown in Fig. 4b is a smoothed average of three separate runs. Further dilution of wild-type ecotin resulted in a signal below the detection limit. This experiment suggests that under our experimental conditions the dissociation constant of wild-type ecotin is below 80 nM.

Another, more direct measure of the dissociation constant of ecotin is based on the concentration dependence of its tryptophan fluorescence [8]. We recorded fluorescence spectra of wild-type and Δ133–142 ecotin at identical protein concen-

Table 1. Elution volume of Δ133–142 ecotin and wild-type ecotin on Superdex 75 column at different initial concentrations

Initial concentration	Elution volume (ml)
Δ133–142 ecotin	
500 μM	11.41
26 μM	11.96
12 μM	11.94
1.2 μM	11.98
Wild type ecotin	
2600 nM	10.68[a]
650 nM	10.70[a]
325 nM	10.66[b]
162 nM	10.75[b]
81 nM	10.90[b]

[a]Detection at 280 nm, elution with buffer 1.
[b]Detection at 215 nm, elution with buffer 2.

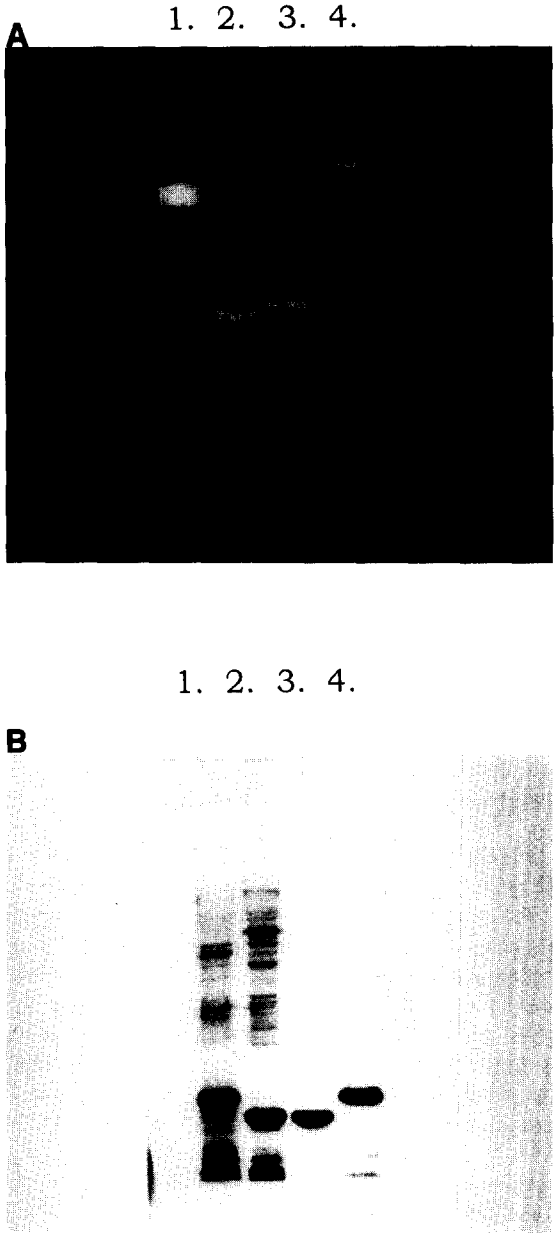

Fig. 1. (a) PCR amplified products were run on 1.5% agarose gel. Lane 1: 100 bp ladder, lane 2: PCR amplification product of the Δ133–142 ecotin gene, lane 3: PCR amplification product of the wild-type ecotin gene, lane 4: 1 kb ladder. (b) Purification of ecotin samples was followed by 17.5% SDS PAGE. Lane 1: Cytoplasmic fraction of Δ133–142 ecotin producing *E. coli* BL21 cells, lane 2: periplasmic fraction of Δ133–142 ecotin producing *E. coli* BL21 cells, lane 3: 5 μg of Δ133–142 mutant ecotin, purified by affinity chromatography, lane 4: 3 μg of wild-type ecotin, purified by affinity chromatography.

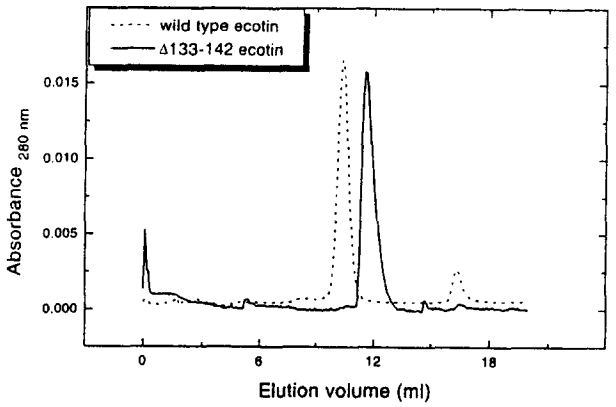

Fig. 2. Analytical gel filtration profile of wild-type and Δ133–142 ecotin on a Superdex 75 column. 25 μl of 12.5 μM wild-type (dotted line) or Δ133–142 ecotin (solid line) was loaded onto a column equilibrated with buffer 1 (see section 2.4) at room temperature. Rate of elution was 0.5 ml/min.

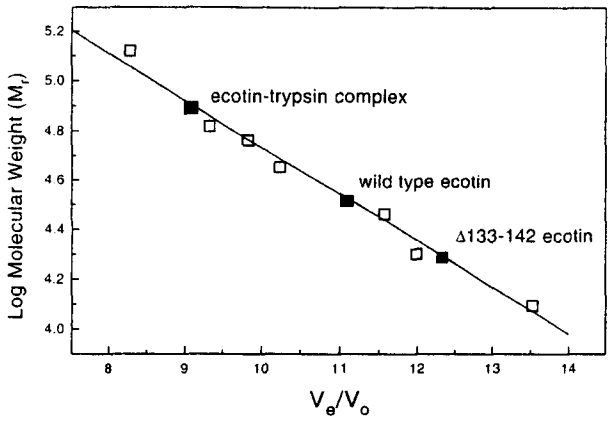

Fig. 3. Determination of the molecular weight of wild-type ecotin, Δ133–142 ecotin and their trypsin complexes by gel filtration.

trations in the range from 12 μM to 24 nM. Fig. 5 shows the ratio of their fluorescence intensities, *Fwt*/*F*Δ133–142 at each concentration. The experimental points were fitted to Eq. 1, assuming that the change in the fluorescence ratio comes entirely from the dissociation of wild-type ecotin. The best fit was obtained with K_d = 90 nM. The experimental error at the last two points is 32% and 55%, respectively.

3.3. Δ133–142 ecotin forms a tetrameric complex with trypsin

Gel filtration experiments on complexes of wild-type and Δ133–142 ecotin formed in the presence of excess trypsin are illustrated in Fig. 6. The elution volumes of the two complexes were identical within the limits of experimental error. The apparent molecular weight of the trypsin complex of both wild-type and Δ133–142 ecotin is 92 000. There was no change in the elution volume of the complexes upon dilution. Similar results were obtained using Superdex 75 and Superose 12 columns to those illustrate above with Superdex 200.

3.4. Determination of equilibrium inhibitory constants (K_I) on trypsin

Equilibrium inhibitory constants of the wild-type ecotin, its $Met^{84}Arg$ P1 site mutant and their Δ133–142 C-terminus truncated forms were measured on trypsin (Table 2). Our data show that the C-terminal truncation of the wild-type form significantly decreases the efficiency of the inhibitor, increasing the K_I value more than two orders of magnitude. On the contrary, the same truncation of the $Met^{84}Arg$ P1 site mutant caused just a slight increase of the K_I value.

4. Discussion

As has been shown earlier, in the ecotin dimer the two protomers are in a head-to-tail arrangement interacting via their extended C-terminal arms [10,11]. The size of this binding site is greater than 2800 Å^2. Removal of the last 10 amino acids of ecotin certainly reduces significantly the overlapping surface area.

Our results show that Δ133–142 ecotin is a monomer up to near the mM range, where it starts to dimerize. Using gel filtration chromatography we found that the elution volume of Δ133–142 ecotin injected at 500 μM is midway between that of the dimer and that of the monomer. It is known that this experimental technique does not allow for accurate estimation of the K_d, since the elution volume does not vary as a linear function of the extent of dimerization. A further complication is introduced by the progressive dilution of the sample in the course of the chromatographic run. However, we can estimate the K_d for the dissociation of two truncated ecotin molecules to be around 100 μM.

Parallel experiments were performed to determine the dissociation constant of wild-type ecotin. There was a clear indication of dissociation when wild-type ecotin was subjected to gel filtration at 80 nM initial concentration (see chromatogram B in Fig. 4b). Thus, the dissociation constant of wild-type ecotin must be less than 80 nM.

Tryptophan fluorescence of wild-type ecotin behaves characteristically upon serial dilution: around the K_d for dimer dissociation tryptophan fluorescence decreases rapidly, presumably because of solvent quenching upon exposure of Trp^{130} on the surface of the monomer. We compared fluorescence spectra of wild-type and Δ133–142 ecotin between 12 μM and 24 nM. We used Δ133–142 ecotin as the reference since it is monomeric throughout this concentration range, thus its tryptophan fluorescence is a simple linear function of dilution. By contrast, wild-type ecotin behaves as an equilibrium mixture of monomers and dimers with a $K_d \approx 90$ nM. This value is probably also an overestimate, because the last measured value in this series is at the detection limit. Together with the results of gel filtration experiments, the fluorescence measurements indicate a K_d value significantly lower than 80 nM. This is about one fifth of the value previously estimated for the protomers in an ecotin dimer [8]. However, deletion of the 10 C-terminal amino acid residues destabilizes the ecotin

Table 2. K_I values (M) of the wild-type, $Met^{84}Arg$ P1 site mutant and their truncated forms on trypsin

Met^{84} wild-type ecotin	Δ133–142 Met^{84} ecotin	$Met^{84}Arg$ ecotin	Δ133–142 $Met^{84}Arg$ ecotin
$\approx 10^{-13}$	2.0×10^{-11}	$\approx 10^{-13}$	6.0×10^{-13}

dimer increasing the K_d value by at least three orders of magnitude. Our result is a striking confirmation of this C-terminal peptide segment's role in subunit association, the function postulated on the basis of the crystal structure [10].

Ecotin has two binding sites for the proteinases it inhibits. The primary interaction site is composed of two loops. Loop 83–88, which includes the Met^{84} P1 site residue, associates with residues in the proteinase forming an extended anti-parallel β sheet. Additional contacts to trypsin involve the inner supporting loop (residues 54–59) of ecotin. The surface area buried in the primary interaction is about 1900 $Å^2$, which is a usual size for a proteinase-inhibitor interaction.

Ecotin's secondary interaction site is located at the opposite end of the molecule. The surface of this site is small, only 900 $Å^2$, i.e. about half the size of a typical protein-protein contact site. These two interactions between the inhibitor and the proteinase and the interaction between the two subunits of the wild-type homodimer ecotin constitute a special network-like arrangement of the complex that results in a unique quaternary structure. As discussed below, the presence of two modes by which ecotin binds its target proteinase may explain the promiscuity of this inhibitor.

Our results concerning the Δ133–142 mutant of ecotin show

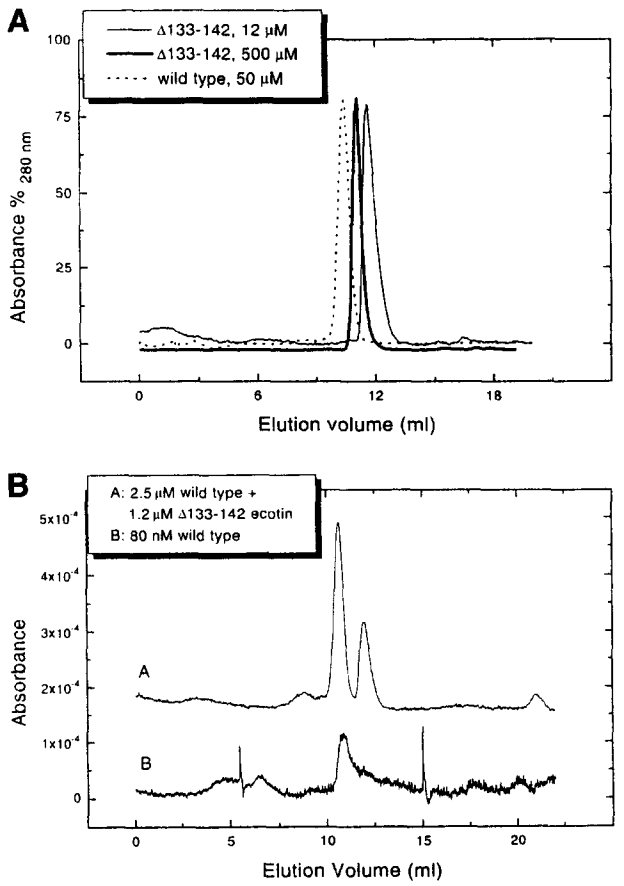

Fig. 4. (a) Effect of the increase in protein concentration on the elution volume of Δ133–142 ecotin on Superdex 75 column. (b) Effect of the dilution on the elution volume of wild-type ecotin on Superdex 75 column. A: Chromatogram of a mixture of 2.5 μM wild-type and 1.2 μM Δ133–142 ecotin recorded at 280 nm. The column was equilibrated with buffer 1 at room temperature. B: Chromatogram of 80 nM wild-type ecotin recorded at 215 nm. The column was equilibrated with buffer 2 at room temperature. (Composition of buffer 1 and buffer 2 is given in section 2.4.)

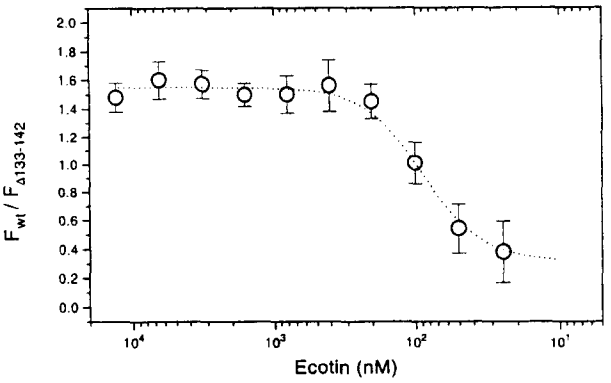

Fig. 5. Fluorescence titration of ecotin. The ratio of tryptophan fluorescence of wild-type ecotin to that of Δ133–142 ecotin is plotted against ecotin concentration. The dotted curve represents a non-linear regression analysis of the data fit to Eq. 1.

that an analogue tetramer complex of ecotin and trypsin forms even in the absence of effective binding between the two ecotin protomers. The formation of this tetrameric complex is remarkable, as it indicates that interactions exist between the inhibitor and the proteinase at both the primary and the secondary sites and these are sufficient to hold the two ecotin molecules together. As we mentioned, the surface of the secondary site is quite small. Thus, on the basis of the X-ray structure one might have suspected that in case of wild-type ecotin this site is unimportant as a determinant of tetramer stability, being a coincidental contact between the proteinase and the inhibitor due to the special geometry of the complex. The existence of the heterotetramer of Δ133–142 mutant ecotin and trypsin shows that the secondary binding site is a real recognition site. We propose the following scenario for the formation of the tetramer. When a monomer Δ133–142 mutant ecotin encounters a trypsin molecule, they first interact via the large primary binding site. In a subsequent collision of two such heterodimers, the proteinase subunits associate with the inhibitor subunits via the two secondary sites simultaneously. In this step the total buried surface area corresponding to two secondary binding sites is 1800 $Å^2$, which reaches the size of a usual protein-protein interface. Consequently, the trypsin molecules form a scaffold which holds the two ecotin protomers together. The stability of this complex confirms the role of the secondary site in the binding of target proteinases even in case of wild-type ecotin.

Our kinetic measurements showed that Δ133–142 monomer mutant form of ecotin is a fully active trypsin inhibitor. However, its K_I value on trypsin is about two orders of magnitude higher than that of its wild-type counterpart indicating a weakened interaction. We propose that this relative inefficiency of truncated ecotin results from the decreased stability of its tetrameric complex formed with trypsin, which lacks the contact between the C-terminal peptides of the ecotin protomers. The difference we found between the K_I values of intact and truncated ecotin can serve as circumstantial evidence that in those very diluted solutions where the K_I measurements were carried out, the tetrameric form can still exist. In these experiments the enzyme and inhibitor concentration was about 10^{-10}–10^{-12} M, much lower than the dissociation constant of the wild-type ecotin, found to be about 80 nM. This apparent contradiction can be easily resolved if we consider

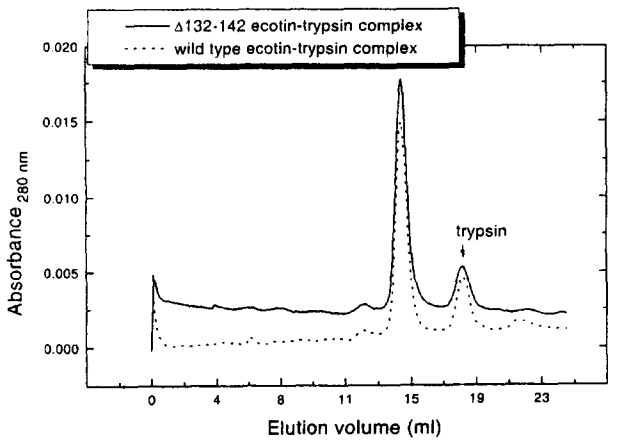

Fig. 6. Analytical gel filtration profile of the trypsin complexes of wild-type and Δ133–142 ecotin on Superdex 200 column.

that due to mutual stabilization 'a network is stronger than its strongest link' [11]. Interestingly enough, in the case of the $Met^{84}Arg$ mutant the C-terminal truncation caused a relatively small effect on the K_I value. Probably, the Arg^{84} P1 residue, which is more favorable for trypsin than Met^{84}, can partly compensate the loss of the dimerization contact.

The most remarkable feature of ecotin is its ability to inhibit a wide range of proteinases. The greater promiscuity of ecotin as compared to conventional proteinase inhibitors probably stems from its dual mode of contacting the target enzyme. We have demonstrated, at least in the case of trypsin, that both modes of binding are effective. With chymotrypsin, which is a more suitable target of the Met^{84} P1 site, ecotin's secondary site seems less important (manuscript in preparation). The relative importance of the two modes of ecotin binding is under current investigation using a variety of target proteinases.

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