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Ecotin is a homodimeric protein from *Escherichia coli* that inhibits many serine proteases of the chymotrypsin fold, often with little effect from the character or extent of enzyme substrate specificity. This pan-specificity of inhibition is believed to derive from formation of a heterotetrameric complex with target proteases involving three types of interface: the dimerization interface, a primary substrate-like interaction, and a smaller secondary interaction between the partner ecotin subunit and the protease. A monomeric ecotin variant (mEcotin) and a single-chain ecotin dimer (scEcotin) were constructed to study the effect of a network of protein interactions on binding affinity and the role of dimerization in broad inhibitor specificity. mEcotin was produced by inserting a β-turn into the C-terminal arm, which normally exchanges with the other subunit. While the dimerization constant (K_d) of wild-type (WT) ecotin was found to be picomolar by subunit exchange experiments using FRET and by association kinetics, mEcotin was monomeric up to 1 mM as judged by gel filtration and analytical centrifugation. A crystal structure of uncomplexed mEcotin to 2.0 Å resolution verifies the design, showing a monomeric protein in which the C-terminal arm folds back onto itself to form a β-barrel structure nearly identical to its dimeric counterpart. The kinetic rate constants and equilibrium dissociation constants for nonmonomeric and dimeric ecotin variants were determined with both trypsin and chymotrypsin. The effect of the secondary binding site on affinity was found to vary inversely with the strength of the interaction at the primary site. This compensatory effect yields a nonadditivity of up to 5 kcal/mol and can be explained in terms of the optimization of binding orientation. Such a mechanism of adaptability allows femtomolar affinities for two proteases with very different specificities.  

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Introduction  

Macromolecular recognition is critical for nearly all biological processes, yet the basis for specificity  

Abbreviations used: HPLC, high-pressure liquid chromatography; MUGC, 4-methylumbelliferyl p-guanidinobenzoate; MUTMAC, 4-methylumbelliferyl p-trimethylammonium cinnamate chloride; succ-AAPN-pNA, N-succinyl-Ala-Ala-Pro-Phe p-nitroanilide; N-CBZ-GPR-pNA, N-carbobenzoxy-Gly-Pro-Arg p-nitroanilide; FRET, fluorescence resonance energy transfer; WT, wild-type; MALDI-TOF, matrix-assisted laser desorption time-of-flight; CDR, complementarity-determining region; mEcotin, monomeric ecotin; scEcotin, single-chain ecotin.  

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in protein-protein interactions is not fully understood. Our knowledge of how proteins interact with each other has been advanced by detailed thermodynamics in several binary systems, including antibody/antigen (Dall’Acqua et al., 1996; Kelley & O’Connell, 1993), hormone/receptor (Cunningham & Wells, 1989), ribonuclease/inhibitor (Chen & Shapiro, 1999; Schreiber & Fersht, 1995), colicin DNase/immunity protein (Wallis et al., 1998), and protease/inhibitor complexes (Castro & Anderson, 1996; Empie & Laskowski, 1982). The main objective in these studies was generally to define the importance of individual residues to the binding epitope, and to relate the structural elements of the protein interfaces to thermodynamic stability in terms of hydrogen bonds, ion pairing, van der Waals interactions, and burial
of surface area. While this combination of structural, mutagenic, and kinetic experiments has illuminated the nature of individual protein interfaces, most biological processes are carried out by macromolecular assemblies of multiple proteins. However, the cooperative nature of protein-protein interactions in these protein networks has been difficult to study quantitatively.

The formation of protein homodimers to bind to a target ligand is clearly critical in a number of cellular processes, and two systems in which the binding energetics have been studied are hormone receptor and transcription factor dimerization. Human growth hormone can bind to the extracellular domain of its receptor (hGHbp) in a 1:1 complex through an interaction at site 1; dimerization of the receptor then adds a much weaker hormone interaction with the second receptor molecule at site 2 (Wells, 1996). The affinity at site 1 has been measured as 0.9 nM (Cunningham & Wells, 1993) and has been studied extensively through homolog- and alanine-scanning mutagenesis (Bass et al., 1991; Cunningham et al., 1989; Cunningham & Wells, 1989). While the binding of the second receptor is estimated to occur with a $K_d$ of between 0.5 and 5.0 nM (Pearce et al., 1999; Uchida et al., 1999), the interaction of hormone with pre-formed dimer is too tight to measure (Cunningham & Wells, 1993), and receptor dimerization is not measurable in the absence of hormone (Wells, 1994). These limitations prevent a study of the strength and cooperativity of protein interactions at each of the three interfaces.

Transcription factors are often observed to bind to DNA as dimers, but exist in solution as monomers (Sauer et al., 1986). For this reason, dimerization and DNA binding are energetically coupled, leading to cooperativity in the binding curve. In the case of bacteriophage $\lambda$ Cro repressor, use of monomeric and single-chain variants has shown that DNA binding is limited by Cro dimerization. By covalently tethering the two subunits, the full DNA binding energy could be realized (Jana et al., 1997, 1998). Engineered monomers, however, have not been observed to bind to DNA with measurable affinity (Daly & Matthews, 1986; Mossing & Sauer, 1990), presumably because they have to overcome roughly the same entropic penalty to binding as pre-formed dimers with only half of the favorable interactions and because monomers are often partially unfolded. This, again, prevents a determination of the energetic contribution of individual protein interactions to the complex.

The binding of ecotin, a 16 kDa protein from the *Escherichia coli* periplasm, to chymotrypsin-fold serine proteases represents an attractive system for investigating binding energetics in multi-protein complexes. Unlike traditional protease inhibitors, which bind only at the active site, ecotin forms a homodimer, allowing the partner subunit to make interactions with the protease at a discrete secondary site (Figure 1). The ecotin/protease complex offers two advantages as a model system. First, because ecotin inhibition of proteases can be easily monitored in solution by following substrate proteolysis, kinetic and equilibrium experiments permit measurement of binding affinities over ten orders of magnitude. Additionally, the secondary site interaction is weak enough that protease affinity can be measured both in its presence and absence.

Like other protease inhibitors that interact in the "standard mechanism" (Bode & Huber, 1991), ecotin inserts an exposed and stabilized loop into the protease active site. The P1 residue (Schechter & Berger, 1967) of inhibitors fits into the primary specificity pocket of the protease and normally provides much of the free energy for binding. Within a family of inhibitors the P1 residue often varies, changing the inhibitory specificity (Laskowski & Kato, 1980). Because the sequence of the substrate-like loop is usually complementary to the substrate specificity of the target protease, a given inhibitor will strongly inhibit only proteases with similar substrate specificities.

Eco1n is an exception to this rule in that it can potently inhibit serine proteases with unrelated specificity, such as trypsin, chymotrypsin, and elastase. This inhibitory profile led to the suggestion...
that ecotin may protect E. coli against external proteolytic attack in the mammalian gastrointestinal tract (Chung et al., 1983). The P1 residue of ecotin was found to be Met84 in all protease complexes studied (McGrath et al., 1991b; Seymour et al., 1994; Ulmer et al., 1995). Methionine may represent a good compromise residue that is able to fit into many disparate primary specificity pockets. In contrast to other serine protease inhibitors, mutating the P1 residue usually does not affect inhibition drastically (Pil et al., 1994; Seong et al., 1994; Yang et al., 1998).

A high-resolution crystal structure of ecotin bound to rat anionic trypsin revealed that ecotin makes significant contacts outside the primary specificity pocket (McGrath et al., 1994). Ecotin dimerizes by a "domain swap" mechanism (Bennett et al., 1994) in which the C-terminal arm reaches across the partner subunit, creating a two-stranded antiparallel β-ribbon (Figure 1). The interactions with these arms account for most of the greater than 2800 Å² total buried surface area at the dimer interface (McGrath et al., 1994). The two tryptophan residues at positions 130 and 130' form a hydrophobic core at the center of the complex. This core appears to be important for the folding of ecotin, since deletions of the C terminus before residue 133 are not expressed to high levels (S.Q. Yang, S.A. Gillmor & C.S.C., unpublished results).

Each monomer of ecotin interacts with the protease active site through a primary binding site consisting of the substrate-like 80s loop and its supporting 50s loop. Additionally, the partner ecotin monomer interacts with the same protease through a secondary binding site, including the 60s and 110s loops on the opposite side of the molecule, which are functionally analogous to the complementarity-determining regions (CDR) of antibodies (McGrath et al., 1995). A combination of site-directed mutagenesis and in vitro selection of ecotin variants using bacteriophage display has shown that each of these four surface loops plays a role in protease binding (Yang & Craik, 1998; Yang et al., 1998; M.C.A. Laboissiere, C.T.E. & C.S.C., unpublished results). A dimer of ecotin can bind to two protease molecules, with each inhibitor molecule contacting both proteases. Ecotin therefore binds through a network of interactions at the primary binding site, secondary binding site, and dimer interface.

To understand how a network of protein interactions affects affinity, allowing tight inhibition of proteases of different specificities, we have constructed both a monomeric variant and a covalently tethered single-chain variant of ecotin. We have utilized the monomeric variant to quantify the strength of the primary inhibitor interaction in the absence of secondary site interactions. This represents the first instance in which the free energy change for an accessory protein-protein interaction has been rigorously measured. Our results show that the apparent strength of the secondary site can change dramatically, varying inversely with the strength of the primary site. This compensatory effect, which can be explained as the change in binding orientation required to optimize interactions at multiple sites, is a mechanism by which ecotin broadly inhibits a diverse class of proteins.

Results
Design and preparation of ecotin variants
An obligate monomer variant of ecotin was designed so as to maintain many of its important intermolecular contacts while sterically occluding the binding of the other subunit. A three-residue insertion, Ala-Asp-Gly, was introduced after Trp130 to create the four-residue sequence, Ala-Asp-Gly-Lys, known to form a type I β-turn in staph nuclease (Hydes & Fox, 1991; Loll & Latman, 1989). This variant was denoted mEcotin, for monomeric ecotin (Figure 1). Modeling studies indicated that the main-chain conformation of ecotin matched well with the conformation of staph nuclease surrounding the β-turn. This β-turn approach has been used successfully to construct a monomeric variant of λ Cro, which also features an antiparallel β-ribbon at the dimerization interface (Mossing & Sauer, 1990).

Two additional mutations were then introduced into mEcotin to reduce any secondary site interaction or to alter the primary binding site. The 60A₄ mutation replaces residues 67-70 with alanine residues. These residues have been shown to be important for the ecotin secondary site interaction (Yang et al., 1998). The M84R mutation replaces the P1 Met with Arg, which is optimal against trypsin due to a salt bridge with the aspartate residue at the base of the primary specificity pocket. All variants were stable in a purification protocol that includes boiling, acidifying to pH 3.0, and reverse-phase HPLC, indicating that the design did not compromise the exceptional stability of ecotin. In fact, the apparent melting temperature of mEcotin is approximately 74 °C, within error of that of wild-type (WT) ecotin at 73 °C (data not shown). While the fluorescence spectrum of native mEcotin is red-shifted compared to WT by about 8 nm due to the more exposed Trp130, the protein is not destabilized by the exposure of the normally buried hydrophobic surface, suggesting that the dominant energetic interaction at the dimer interface is the insertion of the C-terminal β-strand into the β-barrel structure, which is present in both WT and mEcotin.

To make a covalent ecotin dimer, two ecotin subunits were tethered together in a head-to-tail conformation with a Gly-Gly-Gly linker (Figure 1). This single-chain ecotin variant is denoted scEcotin. The N terminus of one subunit is approximately 10 Å from the C terminus of the second subunit in the WT structure. Furthermore, the first few residues are often disordered in crystal structures and are presumably flexible, so a three-residue linker was not expected to strain the structure.
of ecotin. This variant also expressed to high levels, but was less stable to reverse-phase HPLC, forming an insoluble product upon lyophilization. The stability of this variant may be compromised by a tendency to polymerize after partial denaturation. While both WT and scEcotin denatured around 73°C, scEcotin precipitated while WT remained soluble (data not shown). The DNA of all ecotin variants was sequenced and the protein was analyzed by MALDI-TOF mass spectrometry to confirm their identities. Measured masses and predicted masses (in parentheses) were as follows: WT, 16,090.8 (16,097.5); mEcotin, 16,337.6 (16,340.7); mEcotin 60A4, 16,099.4 (16,103.6); scEcotin, 32,331.7 (32,384.3); Ecotin M84R, 16,111.2 (16,122.5); mEcotin M84R, 16,366.3 (16,365.7); mEcotin M84R 60A4, 16,124.7 (16,128.6).

Analysis of ecotin oligomerization

The apparent molecular masses of WT and mEcotin were not affected by varying the loading concentrations on an analytical gel filtration column. At concentrations from 80 nM to 4 μM, WT ecotin eluted at an apparent molecular mass of 39,000 Da, somewhat higher than its predicted dimeric mass of 32,195 Da. The mEcotin variant appeared monomeric, with an apparent molecular mass of 20,600 Da, nearly half that of WT ecotin, even at concentrations from 25 μM up to 1 mM. The elution volume for each protein was unchanged within 0.01 ml over the entire concentration range. The scEcotin variant ran, as expected, as a dimer, with an apparent molecular mass of 37,600 Da (Figure 2(a)).

The oligomeric states of several ecotin variants complexed with rat trypsin were determined. Both WT rat trypsin (data not shown) and the significantly less active trypsin D102N (Craig et al., 1987), were used in complex formation studies with similar results, except that the D102N trials showed less proteolytic degradation product. When trypsin was in excess of WT ecotin, a single complex peak of apparent molecular mass 81 kDa was present, corresponding to the tetramer. As the molar ratio of ecotin to trypsin was raised, a second peak of 59 kDa emerged (Figure 2(b)), corresponding to the trimer (Chung et al., 1983). Ecotin M84R and scEcotin also formed tetrameric and trimeric complexes with rat trypsin (data not shown).

The mEcotin M84R 60A4 variant and rat trypsin formed a heterodimeric complex of about 38 kDa, with no higher mass species present. Surprisingly, mEcotin M84R, in contrast, formed a tetramer-sized complex with trypsin. However, upon adding excess mEcotin M84R, an additional complex of approximately 40 kDa appeared, which corresponds closely to the heterodimer. Apparently, at the high concentrations used for gel filtration, the binding energy from two secondary sites was enough to overcome the weakened dimerization interface to form a tetramer. Utilization of the ecotin variants, therefore, allowed the separation by gel filtration of the six species present in the model of the ecotin-protease equilibrium: protease, ecotin monomer, ecotin dimer, heterodimer, trimer, and tetramer (Figure 2(b)).

Analytical centrifugation was used to measure the sedimentation equilibrium of WT and mEcotin and to monitor the oligomeric state of each. The molecular mass of WT ecotin was found to be 30,700(±1000) Da, which is in good agreement with the predicted mass of the dimer at 32,195 Da. There was no evidence of protein association at the concentration used. The data for mEcotin fit to a single species with a molecular mass of 17,300(±200), which compares favorably to the predicted mass of 16,341 Da (Figure 3).

Because WT ecotin is dimeric at concentrations below the absorbance detection limit, fluorescence resonance energy transfer (FRET) was used to determine the stability of the ecotin dimer by monitoring subunit exchange of two populations of labeled ecotin molecules. The change in the fluorescence spectra upon formation of an ecotin het-
Figure 3. Sedimentation equilibrium analysis of WT ecotin and mEcotin. (a) The absorbance of WT ecotin at a total protein concentration of 1 μM was measured at 215 nm at 15,000 (black), 18,500 (red), 22,000 (green), 26,000 (blue), and 30,000 rpm (cyan). The curves represent simulated data from the best-fit parameters of a single particle of 30,700 Da. (b) The absorbance of mEcotin at 300 nm with a total protein concentration of 343 μM was measured at 15,000 (black), 18,500 (red), 22,000 (green), and 26,000 rpm (blue). Curves represent simulated data from the best-fit molecular mass of 17,300 Da, which was obtained from the 343 μM data shown, as well as the data from 172 μM and 86 μM protein concentrations.

Erudin dimer consisting of one subunit labeled with a FRET donor and one subunit labeled with a FRET acceptor was measured over time. WT ecotin was labeled with the succinimimidyl ester of either 5-(and 6)-carboxy fluorescein as the donor or 5-(and 6)-carboxytetramethyl rhodamine as the acceptor.

The maximum emission wavelength for fluorescein-ecotin was 520 nm and for rhodamine-ecotin was 580 nm. A 20-fold excess of rhodamine-ecotin was added to 0.5 μM fluorescein-ecotin. A time-course of the fluorescence at 520 nm upon excitation at 488 nm showed that the fluorescence intensity of fluorescein-ecotin dropped 50% after formation of a heterodimer with rhodamine-ecotin. This subunit exchange occurred at a rate of 7.0(±0.2) × 10^{-5} sec^{-1} (Figure 4(a)). The exchange is completely reversible, since addition of 150 μM of unlabeled ecotin to the preformed donor/acceptor heterodimers gave a corresponding fluorescence increase of 100% with an identical rate constant of 7.0(±0.3) × 10^{-5} sec^{-1} (Figure 4(b)).

Since dissociation under these conditions should be much slower than association with rhodamine-ecotin monomers, this value should accurately reflect the dissociation rate constant for an ecotin dimer. The $k_{on}$ values for proteins of this size of ecotin are generally around $10^{6} M^{-1} sec^{-1}$ (Janin & Chothia, 1990). This is true for association of a variety of ecotin variants with proteases of a similar size (Table 1). Given the assumption of a $k_{on}$ within a few fold of $10^{6} M^{-1} sec^{-1}$ and the measured $k_{off}$ for the ecotin dimer, the ecotin dimerization
constant can be estimated to be approximately 70 pM.

A markedly different \( K_{\text{dim}} \) for ecotin dimerization, 300 nM, has been measured by monitoring the change in ecotin fluorescence upon serial dilution (Pál et al., 1996; Seymour et al., 1994; Yang et al., 1998). For this reason, the fluorescence titration curve of WT ecotin was compared to that of mEcotin and sEcotin, which should be monomeric and dimeric, respectively, over the entire observed concentration range. The three ecotin variants were dialyzed extensively, and the fluorescence of each was measured at 280 nm excitation and 340 nm emission upon dilution into dialysis buffer. To correct for inner filter effects and photomultiplier sensitivity, ecotin fluorescence was compared to a serial dilution of t-tryptophan at a concentration giving an equivalent absorbance at 280 nm. The intrinsic fluorescence of each variant decreased similarly through the upper nanomolar range (Figure 5). Since the \( K_{\text{dim}} \) of mEcotin was shown by analytical centrifugation and gel filtration to be greater than 1 mM and sEcotin is incapable of dissociation, the observed fluorescence change, whatever its cause, must not be due to dimer formation. Monitoring the change in intrinsic fluorescence during dilution, therefore, does not appear to be a valid method for determining ecotin dimerization constants.

Crystal structure verifies the design strategy

The goal of the design of a monomeric ecotin was to create a stable variant that would be incapable of dimerizing, even at high concentrations, and would bind to proteases only at its primary site. The crystal structure of a 100 nM solution of the dimeric ecotin variant showed that the molecular structure of the dimeric variant was similar to that of the monomeric variant, but the intermolecular contacts were different. The crystal structure also showed that the dimeric ecotin variant was more stable than the monomeric ecotin variant, which was consistent with the fluorescence data. The crystal structure data provided a molecular basis for understanding the design of a monomeric ecotin variant that was incapable of dimerization.

Figure 4. Subunit exchange of WT ecotin measured by FRET. (a) An excess of ecotin labeled with a FRET acceptor (A) was added to ecotin labeled with a FRET donor (D). The change with time of the fluorescence emission at 520 nm upon excitation at 488 nm was measured. The data was fit to a single exponential for a rate of \( 7.0 \times 10^{-5} \) sec\(^{-1} \). (b) An excess of unlabeled ecotin was added to pre-formed heterodimers of ecotin molecules labeled with either FRET donor or acceptor. The increase in fluorescence at 520 nm was fit to the single exponential rate constant \( 7.0 \times 10^{-5} \) sec\(^{-1} \).
mEco tin structure, with additional water-mediated hydrogen bonds compensating for the missing interactions. The C-terminal arm (residues 132-142) shows an r.m.s. deviation of 0.36 Å and 0.52 Å for complexed and uncomplexed e co tin, respectively.

Effect of the secondary site on protease binding

The binding constants of both monomeric and dimeric e co tin variants were measured to proteases with different specificities in order to determine the effect of the secondary binding site. The affinity of e co tin variants was measured toward rat trypsin and bovine chymotrypsin, two possible physiological targets of the wild-type inhibitor (Chung et al., 1983). These enzymes display different substrate specificities, but nevertheless both form high-affinity complexes with e co tin. Because many of these protease-inhibitor complexes are extremely stable, the dissociation constants were determined in most cases by taking the ratio of the dissociation and association rate constants. The kinetic and equilibrium constants are shown in Table 1.

Dissociation rate constants

To measure the unimolecular dissociation rate constant, $k_{off}$, of protease from a given e co tin variant, the protease-inhibitor complex was formed at micromolar concentrations and then diluted to low nanomolar concentrations into a buffer containing a large excess of an e co tin scavenger. Two different scavengers were used to prevent e co tin reassociation with free protease: rat trypsin S195A and human neutrophil elastase. Rat trypsin S195A, a nearly inactive mutant in which the active-site serine residue is replaced with alanine (Corey & Craik, 1992), was used to measure the $k_{off}$ values of all variants except mEco tin and mEco tin 60A, for which values were not determined. Human neutrophil elastase, which has been used previously as an e co tin scavenger (Seymour et al., 1994), was used as a second scavenger for WT e co tin, giving results similar to those of trypsin S195A.

Dissociation from inhibitor was measured as increasing proteolytic activity with time. Activity was related to the fraction of free enzyme by a standard curve with known protease concentrations. While protease dissociation from monomeric variants could be described by a single exponential, dimeric inhibitors showed a small burst phase, followed by slower dissociation. These bursts normally accounted for less than a few percent of the total enzyme. In these cases, the data were fit to a double exponential curve, and the $k_{off}$ was taken to be the rate of dissociation for the majority of the enzyme. Such burst phases have been observed before in the dissociation of tight complexes, where they were often attributed to minor contaminants or partial protein denaturation (Green, 1963; Kaplan & Bartlett, 1991; Schloss, 1988). In this case, the burst may represent the

Figure 5. Fluorescence titration of e co tin variants. The intrinsic fluorescence of e co tin variants was measured at 280 nm excitation and 340 nm emission upon dilution, relative to a standard of l-tryptophan.
heterogeneous nature of the inhibited complexes, since one would expect to find enzyme not only in tetrayers, but also in trimers or in tethrayers that include one molecule of scavenger. However, since the complex was formed at high concentrations with only a slight excess of inhibitor, tetramer is the predominant species for the dimeric ecotin variants. The calculated $K_d$ values should represent the affinity for protease within the tetramer. It is possible that the burst phase reflects a positive

Figure 6. Structure of mEcotin. (a) Ribbon plot of a 2,0 Å structure of mEcotin, showing β-sheets in yellow, helices in magenta, and flexible loops and turns in blue. The introduced β-turn is labeled red. The structure demonstrates the conservation of the flattened β-barrel fold and the folding back of the C-terminal arm onto the β-barrel. The figure was prepared using BOBSCRIPT (Bacon & Anderson, 1988; Merritt & Murphy, 1994). (b) Simulated annealing $3F_o - 2F_c$ difference map showing electron density around the inserted β-turn of mEcotin. The three inserted residues after Trp130, Ala-Asp-Gly, are labeled 130A, 130B, and 130C, respectively. The difference map was calculated using CNS program suites (Brünger et al., 1998), and the electron density map was visualized by Quanta 98 (Molecular Simulations Inc., San Diego).
cooperativity in the binding of trypsin and chymotrypsin, where protease is bound several orders of magnitude more tightly in the tetramer than in the trimer.

Ecotin can form strikingly stable complexes with proteases. The half-life of trypsin in WT ecotin or ecotin M84R complexes is 69 days and 4.5 years, respectively. The stability of this complex is reflected in a melting temperature of nearly 92°C for the WT ecotin-trypsin complex (M. Doyle, personal communication). Half the chymotrypsin dissociates from WT and M84R in 2.6 years and 77 days, respectively. The proteases bind equally tightly to WT and scEcotin, indicating that the covalent tether does not alter the conformation of the protease-binding sites. Likewise, the addition of the 60A_A mutation, which significantly decreases secondary site binding, has no effect on the affinity of mEcotin variants with trypsin or chymotrypsin. This reinforces the point that mEcotin forms heterodimeric complexes with proteases that involve only the primary binding site.

**Association rate constants**

The association rate constant, $k_{on}$, was determined by monitoring the decrease in enzymatic activity with time after mixing known concentrations of protease and ecotin variant. Dissociation of the enzyme-inhibitor complex was neglected, since it would not occur to a significant extent over the time of the assay. As expected, the $k_{on}$ values for all measured ecotin variants against trypsin and chymotrypsin were within fivefold of each other in a narrow range around 10^3 M^{-1} sec^{-1}. The scEcotin and 60A_A variants had essentially identical association rates to WT ecotin and the corresponding mEcotin variants, respectively. Ecotin was diluted into the reaction mix from a high concentration so that WT ecotin and ecotin M84R would be dimeric and the rate would reflect association with pre-formed dimers. There was not a noticeable change in association rate over the course of the assay, implying that the rates of association into a trimer and tetramer are comparable. The $k_{on}$ for each interaction is assumed here to be equal. Since WT ecotin dimers, but not monomers, form stable complexes with trypsin, the rate of WT ecotin association with trypsin is dependent on the dimer concentration. When WT ecotin monomers and dimers were allowed to equilibrate overnight before the addition of trypsin, the apparent $k_{on}$ decreased with concentration through the picomolar range to a value half as fast at around 20-50 pM, again suggesting a picomolar $K_{on}$. The covalent dimer of scEcotin, however, showed no decrease in association rate upon dilution (data not shown).

**Equilibrium dissociation constants**

While nearly all ecotin variants studied were tight inhibitors of both chymotrypsin and trypsin, there was a notable exception. Both mEcotin and 60A_A mEcotin were weak inhibitors of trypsin. The $K_i$ values for both were determined by traditional Michaelis-Menten kinetics at multiple substrate and inhibitor concentrations to be 1.4 μM. The $K_i$ values for other combinations of monomeric ecotin variants and protease were determined using equations for tight-binding inhibitors, since the inhibitor concentrations used were not in vast excess of enzyme concentrations. In each case, these equilibrium $K_i$ values were within twofold of the $K_d$ values determined from the ratio of $k_{cat}$ and $k_{on}$.

Because dimeric ecotin variants inhibited at sub-picomolar concentrations, measuring the apparent inhibition constants, or $K_i$ values, was difficult. The $K_i$ value does not necessarily represent a single binding affinity, but instead involves multiple equilibria with all six protease and inhibitor species. Using low concentrations of protease and incubating for long periods of time showed that the $K_i$ values for some inhibitors were higher than the $K_d$ values calculated for protease dissociation from the tetramer. In the case of WT ecotin and scEcotin binding to trypsin, the equilibrium $K_i$ is five- to sixfold higher than the $K_d$ value. The ecotin M84R $K_i$ for chymotrypsin was some ninefold higher than the $K_d$ value. Two causes could be expected for the rise in the apparent equilibrium inhibition constant. At low picomolar concentrations, some of the binding energy would be utilized to dimerize ecotin below its $K_{on}$ value, lowering the apparent affinity. Since both WT and the covalently tethered scEcotin variant have higher $K_i$ values against trypsin, however, it is likely in this case that the major cause is that the trimer complex has lower affinity than the tetramer for protease.

**Discussion**

**Ecotin interactions as a model for multi-protein complexes**

The ecotin-protease complex offers the opportunity to study how a network of interactions affects protein binding. The inhibitory activity at the primary site, as the substrate-like loop inserts into the protease active site, is modulated by interactions at two other interfaces. Ecotin dimerization adds a discrete secondary interaction with the protease, increasing the surface area of protease contact from 1900 Å^2 to around 2850 Å^2 (McGrath et al., 1994). The affinity of ecotin to chymotrypsin and trypsin is correspondingly tight, in the low to mid-picomolar range, making it one of the tightest reported protein interactions. By comparison, the 2 mM $K_i$ of ecotin M84R with rat trypsin is more than an order of magnitude lower than the 50 mM $K_i$ of bovine trypsin with the Kunitz domain inhibitor BPTI (bovine pancreatic trypsin inhibitor), which also inhibits in the “canonical” manner through an
exposed substrate-like loop (Castro & Anderson, 1996; Vincent & Lazdunski, 1972). While BPTI binds much more weakly to bovine chymotrypsin at 10 nM (Castro & Anderson, 1996), ecotin M84R continues to bind extremely tightly to this protease with a 34 fM affinity.

It is quite likely that the dimeric nature of ecotin originated from a “domain swap” mechanism, similar to that seen in diphteria toxin and many other proteins (Bennett et al., 1995). Protein dimerization is frequently achieved by extending a terminal arm to embrace the other subunit (Richardson, 1981). This arm-exchange mechanism is observed in such proteins as methyamine dehydrogenase, tumor necrosis factor, and bleomycin-resistance protein, where a conserved profline residue at the hinge of the arm has been proposed to help keep the arm in a conformation suitable for oligomerization (Bergdoll et al., 1997). The hinge residue of ecotin appears to be Trp130, but it is unclear what prevents the C terminus of the wild-type protein from folding back on itself before dimerization can occur. Both the concentration dependence of association rates and subunit-exchange experiments using FRET suggest that the dimerization constant is in the mid-picomolar range, which is consistent with the large dimerization interface. This value is significantly lower than the approximately 300 nM value previously reported using fluorescence titration, a method that does not appear to measure dimerization, since both mEcotin and scEcotin show fluorescence changes over the same concentration range.

Monomeric ecotin reveals contribution of second interface

To create a monomeric variant of ecotin, mEcotin, that would retain its primary substrate-like interaction with the target protease while preventing the formation of trimers or tetramers, we took advantage of the fact that most of the dimer interface in the WT protein consists of the exchanged C-terminal arms. We reasoned that an inserted β-turn could force the arm to loop back on itself, fulfilling those stabilizing interactions intramolecularly while occluding binding of a second subunit. This mEcotin variant was shown by both analytical centrifugation and gel filtration to be monomeric at concentrations as high as 1 mM, indicating that reversing the domain swap found in WT ecotin raised the $K_{d_{\text{dim}}}$ at least seven orders of magnitude. Our crystal structure of mEcotin verifies this design strategy, revealing a monomeric protein with a β-turn of the same type as modeled. Furthermore, it shows that the basic structure of ecotin, including the primary site loops, remains essentially unchanged, making it likely that mEcotin binds to the active sites of proteases with the same affinity as a WT ecotin monomer. The simplicity of the mutation needed to change ecotin from a monomer to a dimer or vice versa highlights the efficiency of domain swapping as a mechanism for evolution of oligomeric proteins.

In addition to behaving as monomers in solution, mEcotin variants are capable of forming stable heterodimers with proteases. Gel filtration analysis shows that the mEcotin M84R 60Δ4 variant, which has both a disrupted secondary site and dimer interface, forms heterodimers with trypsin with no further oligomerization. The mEcotin M84R variant, on the other hand, does appear to form tetramers, but not trimers, with trypsin at high concentrations. The energy from two secondary sites, but not one, is apparently capable of driving dimerization of the disabled dimer interface. It is worth noting, however, that disruption of the secondary site had no effect on the measured affinities of monomeric ecotin variants under our assay conditions, indicating that the heterodimer was the only relevant complex present while measuring binding affinities.

The mEcotin variants, then, can be used to measure the energetics of protease binding to the primary site, separate from the secondary site. The $ΔG$ value calculated from the mEcotin $K_d$ in Table 1 can be considered to be the primary site binding energy, and the $ΔG$ value for dimeric ecotin can be considered the binding energy of the primary plus the secondary site. The difference between these two values, $ΔG_{\text{secondary}}$ represents the additional binding energy conferred by the secondary site. This value signifies a lower limit for the “intrinsic binding energy,” which is the theoretical Gibbs free energy change representing all the chemical forces at a protein interface in the absence of destabilization or losses in translational and rotational entropy (Jencks, 1975, 1981). Since the $K_d$ value for dimeric ecotin variants was calculated from kinetic constants, it is a measure specifically of protease dissociation from the tetrameric complex.

The affinity of trypsin for both dimeric and monomeric ecotin was measured with either the wild-type Met or the optimal Arg at the P1 position. In the context of monomeric ecotin, providing this important electrostatic interaction with the M84R mutation adds 7.57±(0.03) kcal/mol of binding energy. With dimeric ecotin, however, this decrease in the $ΔG$ is only 2.6±(0.3) kcal/mol. Correspondingly, the secondary site adds 9.6±(0.3) kcal/mol of binding energy in WT ecotin, but only 4.6±(0.09) kcal/mol in ecotin M84R (Figure 7(a)). These results indicate that the secondary site compensates for changes in primary site affinity. The stronger the primary site interaction, the less binding energy is gained from the addition of the secondary site.

Against chymotrypsin, Met is only slightly more optimal than Arg in the P1 position. The $K_d$ is lower with P1 Met than with P1 Arg by a factor of 23(±7) in monomeric ecotin and 8(±5) in dimeric ecotin. Perhaps because of the similar primary affinities, the secondary site binding energy only increases from 3.9(±0.3) to 4.5(±0.08) kcal/mol with the less optimal P1 residue (Figure 7(a)).
and those with an arginine residue prefer trypsin, the selectivity of inhibitors is much greater in monomeric form. The ratio of the chymotrypsin $K_d$ over the trypsin $K_d$ increases nearly seven orders of magnitude upon changing P1 Met to Arg in monomeric ecotin, but fewer than three orders of magnitude in dimeric ecotin. The secondary site, therefore, has a definite role in buffering changes in affinity at the primary site.

**Non-additivity from optimization of binding orientation**

The change in the strength of the secondary site can also be described as non-additivity in a double mutant cycle (Carter et al., 1984; Horowitz, 1996), where removing the secondary site can be considered one mutation and changing to the less-optimal P1 residue can be considered the second. The P1 residue and the secondary site are separated by more than 20 Å, and simple additivity has generally been observed for distant residues that do not interact directly or indirectly (Wells, 1990). However, careful measurements in some systems have discovered small-magnitude non-additivities between distant sites in protein interfaces (LiCata & Ackers, 1995). In the case of human hemoglobin, virtually every significant nonadditivity has been found to be subadditive, meaning that the double mutant has less of an effect on the free energy than would be expected from the sum of the single mutants (LiCata & Ackers, 1995; LiCata et al., 1990).

A strikingly analogous case to ecotin inhibition of proteases is found in the binding of RNase inhibitor (RI) to members of the RNase superfamily (Shapiro et al., 1995). The horseshoe-shaped RI virtually engulfs its target, burying 2,551 Å² of total surface and making many different interactions with RNase targets (Kobe & Deisenhofer, 1995). Double mutants in the complex of RI with angiogenin, many spaced more than 13 Å apart, were often found to be superadditive (Chen & Shapiro, 1999; Shapiro et al., 2000), signifying that the effect of the double mutation was greater than the sum of the single mutations. Superadditivity of mutations can also be described as negative cooperativity between two binding sites, as broken interactions are compensated for by forming new bonds or strengthening others. Such adaptability of the protein interface may be important for the broad recognition by RI of many different RNases (Chen & Shapiro, 1999).

The ecotin double mutant cycle data show a superadditivity of 5 kcal/mol for trypsin and 0.6 kcal/mol for chymotrypsin. The apparent change in the strength of the secondary site upon mutation of the primary site implies some sort of change in binding conformation between protease–inhibitor complexes in the double mutant cycle. This may be a difference in the mode of binding between monomers and dimers or between WT and M84R or both. It seems likely that the

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**Figure 7.** Energetic compensation by the secondary site. (a) Stacked bar graph of the measured binding free energies of ecotin WT and M84R with trypsin and chymotrypsin in terms of primary and secondary site energies. The primary site binding energy, blue, is measured as the $\Delta G_{\text{binding}}$ of the corresponding mEcotin variant. The secondary site binding energy, yellow, is the $\Delta G_{2\text{primary}}$ calculated as the difference between the free energies of the dimer and the monomer. The combined energy is the $\Delta G_{\text{binding}}$ of the dimeric ecotin variant. (b) Model of proposed change in orientation upon mutation of the primary site. When the primary site interaction is sub-optimal, ecotin changes its orientation to take advantage of secondary site contacts.
interaction at each of the two protease binding sites is sub-optimal in the context of the dimer. The complex that forms should represent the compromise orientation that maximizes the combined binding energies of the two sites. When the primary site interaction is weak, the dimer may bind in an orientation that takes full advantage of the secondary site. Likewise, if the primary interaction is strong, the secondary site may essentially disengage, allowing the primary site to realize its full potential binding energy (Figure 7(b)). This model, which involves changes in the quaternary structure of the complex, but not in the tertiary structure of the proteins, is supported by two recent crystal structures of ecotin variants bound to rat trypsin (Gillmor et al., 2000). One variant has Met at P1 and an optimized secondary site, while the second has an optimal P1 Arg, but an alanine-shaved secondary site. The second of these structures shows a dramatic rotation and translation of ecotin with respect to trypsin compared to the first structure, as ecotin essentially moves away from the secondary site and toward the more optimal primary site.

Mutational non-additivity would be predicted in a model of protein interactions in which many different binding orientations are possible, the stability of each binding determined by the sum of the intrinsic binding energies of multiple sub-optimal interactions. If the interface can be described as consisting of a small number of sites, especially if those sites are distributed over a large portion of the target protein, one might expect superadditivity of deleterious mutations. Disruption of one site would be expected to cause those orientations that favor the remaining sites to become more stable. The observed effect of the single mutant would be low, because the binding energy would be partially recouped by strengthening other interactions. By debilitating those interactions that could potentially be strengthened in another orientation, combinations of mutations would be observed to have a much greater effect than the sum of the individual mutations. As the number of interacting sites increases, the non-additivity may be expected to decrease and then become subadditive as a more favorable orientation is more likely to exist after disruption of two sites than one site.

Because individual protein interfaces in a multi-protein complex may rarely be geometrically oriented in an optimum fashion, an inherent negative cooperativity would be expected between them that would make additional protein interactions weaker than would be expected given their intrinsic strength. It is important to note the distinction between the positive cooperativity derived from adding extra bonds without having to pay the entropic penalty twice and the negative cooperativity resulting from the inability of two interfaces to bind optimally at the same time. Both of these effects can be seen in the early observation that bivalent antibodies displayed two to three orders of magnitude better target affinity than monovalent fragments (Greenbury et al., 1965; Hornick & Karuch, 1972). Positive cooperativity causes the increase in affinity because the antibody is already greatly immobilized before the second Fab arm binds to the target. However, an enhancement of as much as eleven orders of magnitude might be expected if no distortion or further immobilization were necessary for the second Fab arm to bind (Erickson, 1989). Likewise, while the positive cooperativity from ecotin dimerization can be given by the \( \Delta G_{\text{2ndary}} \), this gain in affinity is smaller than the maximum possible increase because of negative cooperativity between the sites, part of which is observed in the mutational superadditivity.

The secondary site of ecotin appears to add 4-5 kcal/mol of binding energy to protease binding in many cases, but it is capable of adding nearly 10 kcal/mol when the primary site is very weak, perhaps acting as an inhibitory “safety net” to chelate a protease using two low-affinity interactions rather than one high-affinity interaction. These results represent one of the first instances that the affinity of a protein-protein interaction has been measured in both the presence and absence of a second protein interface. Given the structural data available, the relative rigidity of ecotin, and the opportunity to analyze multiple protein interfaces, ecotin inhibition of proteases represents an excellent system for computational and experimental work on protein-protein interactions.

The data presented here help explain how addition of a general, low-affinity protein interaction can result in the extremely tight and relatively non-specific binding observed with ecotin. Such inhibitory properties would be ideal for a protein whose proposed role is to protect the bacterium against the digestive proteases of multiple species of mammalian hosts. The negative cooperativity observed between distant binding sites in the ecotin-protease complex, as well as the RNase-RNase inhibitor complex, may represent a general mechanism for generating high affinity to a diverse family of proteins. Evolutionary considerations argue that ecotin evolved from a monomeric precursor to a dimeric inhibitor through the domain swap of a flexible arm, which permitted a second interaction with a target protease. Instead of utilizing a separate inhibitor for each target protease, E. coli might counter the digestive environment of the mammalian gut more efficiently by utilizing a single dimeric inhibitor, whose chelation of proteases with two distinct binding sites allows potent inhibition of many digestive proteases.

Materials and Methods

Materials

Ecotin variants were expressed in the E. coli ecotin gene deletion strain IM101 (Yang et al., 1998). All ecotin variants except sEcotin were expressed and purified essentially as described (Yang et al., 1998). WT and mEcotin were
further purified for analytical centrifugation on a HiLoad 26/60 Superdex 75 preparative gel filtration column (Pharmacia), as were mEcoTIN and 60A<sub>a</sub> mEcoTIN for kinetics against trypsin. The EcoTIN variant was purified by preparative isoelectric focusing, followed by acidification to pH 3.0, centrifugation, and dialysis into water. A 65% (w/v) ammonium sulfate precipitation was performed, and the pellet was resuspended and dialyzed into 10 mM Tris (pH 8.0). After concentration, the protein was loaded onto a Mono Q HR 10/10 strong anion exchange column (Pharmacia). At a constant 10 mM Tris (pH 8.0), a slow gradient of NaCl was run up to 13 mM, where it was held until the EcoTIN eluted from the column. The molecular masses of EcoTIN variants were determined by matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry on a BioSpectrometry Workstation (PerSeptive Biosystems, Inc., Framingham, MA). Approximately 1 µg of protein was analyzed on a matrix of sinapinic acid with a laser intensity of 2350. Apparent melting temperatures were determined by measuring the peak fluorescence wavelength and intrinsic fluorescence intensity upon excitation at 280 nm over a temperature range of 25-90°C.

The gene for rat trypsinogen was subcloned into the expression vector pPICZA (Invitrogen, San Diego, CA) utilizing an EcoRI site at the first two residues and after the stop codon to insert the gene at the EcoRI site directly after the Saccharomyces cerevisiae α-factor signal peptide. Trypsinogen S195A was constructed through site-directed mutagenesis by overlap extension PCR, utilizing the 5' and 3' AOX1 primers specified by Invitrogen along with the mutagenic primers 5' GAG GAC AGC TGC CAG GGT GCC CCT GTG TGT GTC TGT AAT CGG 3' and 5' CCA GCG TAC CCC TGC CAG CTT TTC CC 3'.

The vector containing WT or trypsinogen S195A was linearized with Pmel and transformed into the X33 strain of Pichia pastoris, selecting for gene integration by resistance to Zeocin<sup>TM</sup>. Colonies were picked into 10 ml of BMGY/Zeocon and grown overnight at 30°C with shaking. This was used to inoculate one liter of BMGY, which was shaken for one day at 30°C and then spun down and resuspended in 200 ml of BMMY. The culture was shaken at 30°C for four days, supplementing each day with methanol up to 0.5%.

Trypsinogen was purified from the media on a Phenyl Sepharose column (Pharmacia Biotech). The column was equilibrated with 50 mM Mes (pH 6.0), 4.5 M NaCl and the supernatant was brought up to a concentration of 4.5 M NaCl. The supernatant was loaded, the column was washed with the equilibration buffer, and a gradient was run from 4.5 M NaCl to 50 mM NaCl. During purification, the enzyme autoactivated and was affinity purified on a p-aminoazobenzamide column (Higaki et al., 1987). Purified trypsin was dialyzed and concentrated in storage buffer containing 10 mM citrate, 100 mM NaCl, 2 mM CaCl<sub>2</sub> (pH 4.0). Trypsin S195A was purified in the same way, except that following the Phenyl Sepharose column, the protein was brought to 50 mM Tris (pH 8.0), 10 mM CaCl<sub>2</sub> and 1/20 (w/w) enterokinase (Biozyme) was added and incubated overnight at 37°C to convert trypsinogen to trypsin. Trypsin D102N was prepared previously (Gillmor et al., 2000). The concentrations of trypsin S195A and D102N were determined by absorbance at 280 nm using a predicted molar extinction coefficient of 34,440 M<sup>-1</sup> cm<sup>-1</sup> calculated from the amino acid sequence (Gill & von Hippel, 1989).

The concentration of WT trypsin active sites was determined by titration with MUGB. Bovine α-chymotrypsin was obtained from Sigma and was active-site titrated with MUFMAC. The chromogenic substrate Z-GPR-pNA was purchased from Sigma. The chromogenic substrate suc-AAPF-pNA was purchased from Bachem. The succinimidyl esters of 5-(and 6-)-carboxyfluorescein and 5-(and 6-)carboxyfluorescamine were purchased from Molecular Probes (Eugene, OR).

**EcoTIN mutagenesis**

EcoTIN variants were constructed in the pTatTac vector (McGrath et al., 1991a), which is based on the bacterial expression vector pFISe5 (Muchmore et al., 1989). Site-directed mutagenesis was performed by overlap extension PCR. The first half of the mEcoTIN gene was constructed off the template pTacTac: ecotin K131A-[DKG] [Δ138-142 (mutation of Lys131 to Ala, insertion after residue 131 with DKG, and deletion of residues 138-142)] using the reverse mutagenic primer 5' CTC CTC GGC CTT TCC ATC AGC CCA GAC GCC GTA CTT CAC 3' and the second half was constructed off the template pTatTac: ecotin A132-[DKG] [Δ135-142 using the forward mutagenic primer 5' GAT GGA AAG GCC GAC GAG AAA ATT GAC AAC GCC GCC GTA GTT CCG 3']. A second PCR using these two products and the outside primers was performed and the product was digested and ligated into the pTatTac vector to produce the mEcoTIN construct, which inserted ADG after Trp130. The ecotin constructs M84R, 60A<sub>a</sub>, and M84R 60A<sub>a</sub> were previously constructed (Yang et al., 1998), mEcoTIN M84R, mEcoTIN M84R 60A<sub>a</sub>, and mEcoTIN 60A<sub>a</sub> were generated by digesting constructs with BamHI and ligating the inserts into the pTatTac: mEcoTIN vector.

The scEcoTIN variant was constructed in the pBlue-script-ecotin vector (Wang et al., 1995). A new HindIII site was introduced at the first codon of ecotin using the mutagenic primer 5' CT TCC GCC TGG GAA GCT TAA AGC GTC CAG 3' (Kunkel, 1985). The HindIII fragment containing ecotin was removed and ligated into the HindIII site of a non-modified pBS-ecotin vector to form a construct with two tandem ecotin genes. A Gly-Gly-Gly linker was then inserted between the two genes using the primer 5' CG GTA GTT CCC GCC GCC GGA GCT GAA AGC GTT CAG 3', which also removed the new HindIII site. A BamHI/HindIII fragment containing the tethered ecotin genes was ligated into the pTatTac vector.

**Gel filtration**

Ecolin-protease complexes were analyzed on a Pharmacia Superdex 200 10/30 column run at 0.5 ml/minute in 50 mM Tris (pH 8.0), 150 mM NaCl, 20 mM CaCl<sub>2</sub>. Wild-type ecotin and mEcoTIN were analyzed on a Pharmacia Superdex 75 10/30 column run at 1.0 ml/minute in 50 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> (pH 7.5), 150 mM NaCl. Apparent molecular masses were calculated from a standard curve of the following proteins: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and myoglobin (17.6 kDa). Peaks were assigned by absorbance at 280 nm, except for WT ecotin on the Superdex 75, which was measured at 215 nm.
Analytical centrifugation

Sedimentation equilibrium experiments were run on a Beckman XL-A ultracentrifuge, using 6-position cells at 15,000, 18,500, 22,000, 26,000, and 30,000 rpm. The buffer used was 50 mM NaH2PO4/Na2HPO4 (pH 7.5), 150 mM NaCl. Sednterp 1.01 software (Hayes, 1997) was used to estimate the following values based on amino acid and buffer composition: WT partial specific volume = 0.7435, mEctoin partial specific volume = 0.7422, buffer density = 1.00956. Global data analysis was performed by non-linear least-squares fitting using Origin 4.1 software (Microcal Software, Inc.). Both proteins were fit to single-particle models. WT ectoin data analyzed was at 1 µM total protein concentration at 215 nm at all five speeds. The mEctoin data analyzed was collected at 343 µM at 300 nm, and 172 and 86 µM at 295 nm. These three concentrations were monitored at 15,000, 18,500, 22,000, and 26,000 rpm.

Fluorescence resonance energy transfer

A 0.29 mg sample of the succinimidyl ester of 5-(and 6)-carboxyfluorescein or 1.0 mg of the succinimidyl ester of 5-(and 6)-carboxytetramethylrhodamine in 0.1 M NaHCO3 (pH 8.3). The protein and dye were incubated at room temperature for one hour, and then hydroxylamine (pH 8.5) was added to 0.15 M and incubated one hour. The reaction protein was extensively dialyzed against 10 mM Tris (pH 8.0). The degree of labeling was determined by measuring the absorbance at 280 nm and at the absorbance maxima of the two dyes as approximately half for fluorescein-ectoin and one-third for rhodamine-ectoin.

To initiate the subunit exchange experiment, 10 µM rhodamine-ectoin was added to 0.5 mM of fluorescein-ectoin. The following day, 200 µM of unlabeled ectoin was added to the sample. The fluorescence at 520 nm, F(t), at time t, was measured upon excitation at 488 nm. The rate of subunit exchange, kex, was obtained by fitting the data to the following equation, where C1 is the fluorescence at infinite time and C1 + C2 represents the fluorescence at time zero:

\[ F(t) = C_1 + C_2 e^{-kt} \]

The fluorescence was normalized to the initial value, C1 + C2, when rhodamine-ectoin was added and to the final value, C1, when unlabeled-ectoin was added. A similar technique has been used to measure the subunit exchange of α-cristallin (Bova et al., 1997).

Fluorescence Titration

Samples for fluorescence titration experiments were extensively dialyzed against 10 mM Tris (pH 8.0). The fluorescence intensity at 340 nm upon excitation at 280 nm was measured for WT, mEctoin, and scEctoin and for a sample of t-tryptophan of the same absorbance at 280 nm. Serial twofold dilutions of each sample were made with dialysis buffer, and the ratio of ectoin fluorescence to tryptophan fluorescence was fit to the following equation:

\[ F = F_M + (F_D - F_M) \left( \frac{-K_D + (K^*_D + 8K_D[H]^1/2)}{4[H]} \right) \]

where the relative fluorescence, F, is related to the monomer fluorescence, F_M, the dimer fluorescence, F_D, and the total inhibitor concentration, [H].

Crystallography

mEctoin was crystallized at concentrations between 12 and 15 mg/mL by the hanging drop method in the presence of 28-30% polyethylene glycol 4000, 0.2 M sodium acetate, 15% glycerol (v/v), and 0.1 M Tris (pH 8.0). A 1 ml aliquot of a 1:1 ratio of silicon oil and paraffin oil was placed over the 1 ml of well buffer to control the vapor diffusion rate. A full data set was collected at Stanford Synchrotron Radiation Laboratory beamline 7-1 using a Mar345 imaging plate system and was evaluated and integrated using SCALEPACK/DENOZO. The data set, which was 97% complete to 1.8 Å, consisted of 200,193 measurements of 15,534 reflections. The mean I/σ was 23.85 and the Rsym was 9.6%. Processing statistics indicated that the crystal had a space group of P4_2_2 with unit cell parameters of a = b = 97.2 Å, c = 37.2 Å, α = β = γ = 90°. Each asymmetric unit contained only one molecule of mEctoin, and the solvent content in the crystal was 55%.

A model of mEctoin was constructed based on a single molecule from the uncomplexed ectoin dimer structure (Shin et al., 1996). The N-terminal five residues and the C-terminal 12 residues were omitted from the model. The mEctoin structure was determined by molecular replacement using programs from the CNS1.0 program suites (Brünger et al., 1998). Data between 15.0 Å and 4.0 Å was used in the rotational search, and the top 20 solutions were selected for translational search using data to 2.0 Å. After the first round of rigid body refinement, the top solution from translational search resulted in an R value of 45.2%. The mEctoin structure was refined reiteratively using the CNS1.0 program suites and was visualized and rebuilt using Quanta98 (Molecular Simulations Inc., San Diego CA). The structure has been refined to 2.0 Å, with a final Rfree of 26.64% and an Rwork of 24.14% for data in the 2.6-Å range with a 2 σ cut-off. The r.m.s.d. values for bond lengths and angles are 0.007 Å and 1.4°, respectively. Structure comparison studies were performed using Insight II (Biosym, Inc.).

Kinetics

All reactions were performed in a buffer of 50 mM Tris (pH 8.0), 100 mM NaCl, and 20 mM CaCl2. Ectoin variants were quantified by titration with trypsin, except in the case of mEctoin and mEctoin 60ΔX, which were titrated against chymotrypsin. Equilibrium Kd values were determined at enzyme concentrations lower than 250 times the Ki, to assure measurable curvature in the inhibition curve. Protease and inhibitor were allowed to incubate for a time much longer than that calculated to be necessary given the measured kcat and km values to reach within 1% of equilibrium, as long as 16 days. Protease activity was found to be stable over this period of time as long as two weeks by leaving one of the assay tubes without inhibitor at 4°C for the duration of the incubation and by following the activity of a sample of protease at 25°C over time. For ectoin variants with subnanomolar Ki values, no substrate-induced enzyme dissociation was observed on the time-scale of the assay, so the fractional activity was equal to fractional free enzyme. Data was fit to the following equation for tight-
binding inhibitors:

\[ \frac{v}{v_0} = 1 - \frac{[E_0] + [I_0] + K_i - \sqrt{([E_0] + [I_0] + K_i)^2 - 4[E_0][I_0]}}{2[E_0]} \]

Variants with K_i values above 1 μM were analyzed at multiple substrate and inhibitor concentrations by traditional Michaelis-Menten kinetics:

\[ v = \frac{V_{\text{max}}[S]}{[S] + K_m (1 + [I]/K_i)} \]

Dissociation rate constants were determined by forming protease-inhibitor complexes at low micromolar concentrations with a slight excess of inhibitor. Protease and inhibitor were incubated at room temperature for over an hour and then diluted to low nanomolar concentrations in 25°C reaction buffer with either human neutrophil elastase or trypsin S195A as an ecotin scavenger. Elastase was used at either 125 or 250 nM and trypsin S195A was used at concentrations of 0.5 to 2 μM. At selected time points, 500 μl aliquots were taken, and substrate was added to 180 μM. The initial enzyme rate was determined by monitoring absorbance at 405 nm. A standard curve of enzyme activity was constructed so that the fractional free enzyme concentration could be determined. The enzyme rate, v, at time T was fit to the following equation:

\[ v = v_0 + v_{\text{burst}}(1 - e^{-k_{\text{burst}}T}) \]

where v_0 is the rate of substrate turnover at time zero, including scavenger activity, and v_{\text{burst}} is the expected rate if all of the enzyme were free, based on the standard curve. In the case of dimeric ecotin variants, a burst phase was observed, so the data was fit to the following biphasic equation:

\[ v = v_0 + v_{\text{burst}}(1 - e^{-k_{\text{burst}}T}) + v_{\text{burst}}(1 - e^{-k_{\text{burst}}T}) \]

where k_{\text{burst}} is the fraction of enzyme undergoing the burst phase and k_{\text{burst}} is the rate at which enzyme dissociates during the burst.

Association rate constants were calculated by mixing known concentrations of enzyme and inhibitor at time zero and monitoring the loss of enzymatic rate as enzyme formed stable inhibitor complexes. The time was recorded when substrate was added to a concentration many times the K_m, effectively stopping inhibitor association. Because of the slow dissociation rates of proteases from ecotin variants, dissociation could be neglected on the time scale of association assays. Rates were fit to the following equation:

\[ v = C \left( \frac{[E_0] - [I_0][E_0][e^{([I_0] + [E_0] + K_m)K_i} - 1]}{[I_0][E_0][e^{([I_0] + [E_0] + K_m)K_i} - [E_0]} \right) \]

where [E_0] and [I_0] are the initial concentrations of protease and inhibitor, respectively, and C is the conversion factor from enzyme concentration to activity. Uncertainty values given for all kinetics values in Table 1 are the sample standard deviations of independent determinations.

PDB accession codes

Coordinates have been deposited in the RCSB protein databank, with accession code 1HFG.

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