

Chemically Mediated Site-Specific Proteolysis. Alteration of Protein–Protein Interaction[†]

Bixun Wang,[‡] Kathlynn C. Brown,[§] Michiel Lodder,[‡] Charles S. Craik,[§] and Sidney M. Hecht^{*,‡}

Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22901, and Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143

Received September 5, 2001; Revised Manuscript Received December 5, 2001

ABSTRACT: The design and synthesis of a novel iodine-labile serine protease inhibitor was realized by the use of an ecotin analogue containing allylglycine at position 84 in lieu of methionine. Allylglycine-containing ecotins were synthesized by in vitro translation of the ecotin gene containing an engineered nonsense codon (TAG) at the positions of interest. A misacylated suppressor tRNA activated with the unnatural amino acid allylglycine was employed for the suppression of the nonsense codons in a cell-free protein biosynthesizing system, permitting the elaboration of ecotin analogues containing allylglycine at the desired sites. The derived ecotin analogues were capable of inhibiting bovine trypsin with inhibitory constants (K_i s) comparable to that of wild-type ecotin. Iodine treatment of ecotin analogue Met84^AGly resulted in the deactivation of ecotin, caused by peptide backbone cleavage at its P1 reactive site. Upon iodine treatment, active trypsin could be released from the protein complex with ecotin analogue Met84^AGly. This constitutes a novel strategy for modulation of serine protease activity and more generally for alteration of protein–protein interaction by a simple chemical reagent.

We have recently described a novel strategy for site-specific cleavage of proteins (1). This technique allows cleavage of the protein amide backbone at a single, predetermined site with a simple chemical reagent. The strategy involves the use of protein analogues containing the non-native amino acid allylglycine (^AGly)¹ at a specific site. This amino acid can be attached to suppressor tRNA_{CUA} as an activated ester by chemical misacylation (2–9); readthrough of a nonsense (UAG) codon (10–15) at a predetermined site by the use of a suitable protein synthesizing system afforded full-length protein containing ^AGly at that site. Subsequent iodine treatment resulted in protein cleavage at the expected site through a presumed iodolactone intermediate. This strategy has been employed successfully to activate a trypsin from its zymogen form (trypsinogen) by chemical removal

of the activation peptide, suggesting the compatibility of this technique with the integrity of functional proteins (16).

The introduction of allylglycine expands the functionality of native proteins. One possible application of this new functionality includes the systematic removal of individual protein domains of putative higher order structures such that the functional contribution(s) of these structures can be dissected. Especially in complicated protein–protein complexes, there is presently no simple method either to inactivate one of the proteins or to remove a specific structural element from the protein complex in order to address structure–function questions.

To test the feasibility of this strategy at the level of a protein complex, the ecotin–trypsin complex was chosen as a model system. Ecotin, a homodimer protein present in *E. coli*, is a potent, competitive inhibitor of serine proteases (17). Ecotin is unique in its ability to inhibit a range of serine proteases having different substrate specificities, including trypsin, chymotrypsin, and elastase (17); moreover, it does so by a novel mechanism. Monomers of ecotin, having 142 amino acid residues, are held together by their long C-terminal strands (residues 125–142) that are arranged as a 2-stranded antiparallel β -sheet in the functional dimer (18). The ecotin dimer binds to two trypsin molecules at opposite ends of the dimer to form a heterotetramer with a 2-fold symmetry axis. The interaction of ecotin with trypsin relies on two distinct and interacting binding sites (19). The reactive site P1 residue has been determined to be Met84, which lies within a disulfide-bonded protein segment (20, 21).

Presently we describe the elaboration of an allylglycine-containing analogue of ecotin and illustrate its utility as an iodine-labile inhibitor of the serine protease trypsin, thus

[†] This work was supported at the University of Virginia by NIH Research Grant CA77359 from the National Cancer Institute, and at the University of California by National Science Foundation Grant MCB9604379. K.C.B. was a Daymon Runyon–Walter Winchell Foundation Fellow (DRG-1297).

* Address correspondence to this author at the Department of Chemistry, University of Virginia, Charlottesville, VA 22901. Email: smh9u@virginia.edu. Phone: (434) 924-3906. Fax: (434) 924-7856.

[‡] University of Virginia.

[§] University of California, San Francisco.

¹ Abbreviations: ^AGly, allylglycine; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NVOC, nitroveratryloxycarbonyl; THF, tetrahydrofuran; IPTG, isopropyl- β -D-thiogalactoside; LB, Luria–Bertani Broth; Et, wild-type ecotin; Et Met84^AGly, ecotin analogue with a substitution of allylglycine for methionine at position 84; Et Met84Val, ecotin analogue with a substitution of valine for methionine at position 84; Et Lys126^AGly, ecotin analogue with a substitution of allylglycine for lysine at position 126; Et Δ 84–142, ecotin analogue lacking residues 84–142; Et Δ 126–142, ecotin analogue lacking residues 126–142.

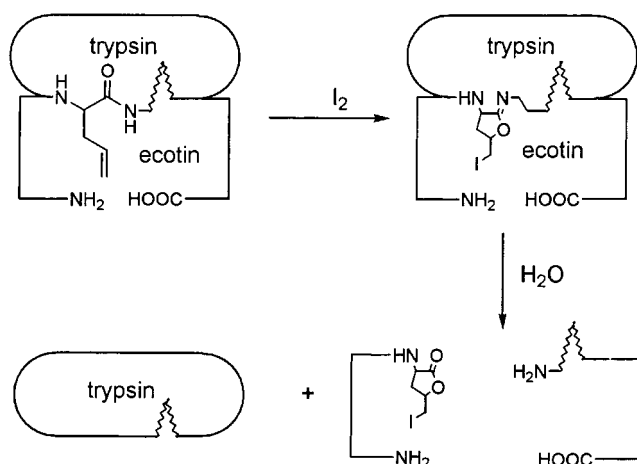


FIGURE 1: Scheme illustrating treatment of the ecotin analogue–trypsin complex with iodine, resulting in the release of trypsin. The protein complex was formed between trypsin and an ecotin analogue containing the unnatural amino acid allylglycine at position 84.

affording a novel method for the modulation of this serine protease activity. Toward this end, allylglycine was incorporated in lieu of Met84, the P1 reactive site of ecotin. As anticipated, iodine treatment resulted in the deactivation of the ecotin analogue and the release of active protease (Figure 1). Also described is the elaboration of an ecotin analogue having ^AGly in place of Lys126. Since Lys126 is located at the beginning of the dimer interface (18), elaboration of this analogue permitted a test of in situ removal of the dimer interface from the protein complex, such that its functional contribution could be studied.

EXPERIMENTAL PROCEDURES

General Methods and Materials. [³⁵S]Methionine (1000 Ci/mmol, 10 μ Ci/ μ L) was purchased from Amersham Corp. Ni-NTA resin was obtained from QIAGEN. Restriction endonucleases, purified acylated bovine serum albumin (BSA), T4 DNA ligase, and T4 RNA ligase were obtained from New England Biolabs (Beverly, MA). *E. coli* JM109 competent cells were from Stratagene Cloning Systems (La Jolla, CA). Kits for plasmid isolation were purchased from PGC Scientific (Gaithersburg, MD). AmpliScribe transcription kits and T7 RNA polymerase were purchased from Epicentre Technologies (Madison, WI). Plasmid pALTER-Ex1 was from Promega Corp. (Madison, WI). Acrylamide, *N,N*-methylenebisacrylamide, urea, Tris base, NTPs, DEAE Sepharose CL-6B, isopropyl β -D-thiogalactopyranoside, GMP, amino acids, bovine trypsin, bovine trypsin attached to cross-linked beaded agarose (trypsin–agarose resin) as well as *N* $^{\alpha}$ -benzoyl-*S*-arginine 7-amido-4-methylcoumarin, *N* $^{\alpha}$ -benzoyl-*S*-arginine-*p*-nitroanilide, and 4-methylumbelliferyl *p*-guanidine benzoate were from Sigma Chemicals. Nitroveratryloxycarbonyl (NVOC)-protected pCpA derivatives of valine and allylglycine were prepared as described previously (1).

Plasmid DNAs were isolated using a Wizard Miniprep purification system (Promega) or a JetStar plasmid midi kit (PGC Scientific) according to the protocols provided. Sequence analysis was performed using a Sequenase version 2.0 DNA sequencing kit (US Biochemical). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out using the standard Laemmli procedure (22). Gels were

visualized and quantified utilizing a Molecular Dynamics 400E PhosphorImager equipped with ImageQuant version 5.0 software. Protein was dialyzed using Spectra/Por2 dialysis tubing, molecular mass cutoff 12–14 kDa (Spectrum Medical Industries), and concentrated using a micro-concentrator (Microcon YM-3, Millipore Corp.). Radioactivity measurements were made using a calibrated phosphorimager screen, permitting direct correlation of the pixel density of the image to the amount of radioactivity present in the sample. A defined number of methionines was incorporated into ecotin during in vitro translation (five for wild type and Lys126^AGly ecotin; four for Met84^AGly). Since the specific activity of [³⁵S]methionine was known, this permitted quantification of the amount of ecotin synthesized.

UV spectral measurements were made using a Perkin-Elmer Lambda 20 UV/VIS spectrometer. Radioactivity measurements were performed with a Beckman LS-100C liquid scintillation counter. Fluorescence spectral measurements were made using a Hitachi F2000 fluorescence spectrophotometer. All aqueous manipulations employed distilled, deionized water from a Milli-Q system.

Construction of Expression Plasmids for the in Vitro Elaboration of Ecotin Analogues. The plasmid pGem-Et(Wt) was constructed by subcloning the ecotin gene from the pBS his6-ecotin into the pGEM vector between the *Bam*HI and *Xba*I sites. The plasmid pGEM-Et(84) was constructed using site-directed mutagenesis by the method of Kunkel (23). Single-stranded pBS his6-ecotin was employed as the template, and the mutation was introduced using the primer CGC GTT TCA ACG TAG ATG GCA TGC CCG GAT GGC. The underlined region represents the newly introduced stop codon while the italicized base represents a silent mutation that introduces a unique *Sph*I site. Upon obtaining the correct plasmid, the gene was amplified by PCR and subcloned into the pGEM vector between the *Bam*HI and *Xba*I sites. The sequence was confirmed by DNA sequencing.

The same approach was used to construct the pGEM-Et(126) plasmid. The mutation was introduced using the primer GAC AAT GTA GAC GTC TAG TAC CGC GTC TGG AAG. The underlined region represents the newly introduced stop codon while the italicized base represents a silent mutation that introduced a unique *Aat*II site. The sequence was confirmed by DNA sequencing (24).

Plasmids pGem-Et(Wt), pGem-Et(84), and pGem-Et(126) were each digested with *Nde*I and *Nsi*I, while plasmid pALTER-Ex1 was digested with *Nde*I and *Pst*I. The small fragment isolated from pGem-Et(Wt), pGem-Et(84), or pGem-Et(126) digestion contained the ecotin gene and was subcloned into the digested pALTER-Ex1 vector to give plasmids pTac-Et(Wt), pTac-Et(84), and pTac-Et(126), respectively. The nucleotide sequences of pTac-Et(Wt), pTac-Et(84), and pTac-Et(126) were verified by restriction analysis and Sanger DNA sequencing.

In Vivo Synthesis and Purification of Ecotin. *Escherichia coli* strain JM109 was freshly transformed with expression plasmid pTac-Et(Wt). A single colony selected from tetracycline plates was used to inoculate 3 mL of Luria Broth (LB) containing 10 μ g/mL tetracycline. The cultures were grown at 37 $^{\circ}$ C for 9 h and diluted to 1 L of Luria Broth (LB)/tetracycline. Following growth at 37 $^{\circ}$ C for 1 h, IPTG was added to the cultures to a final concentration of 0.5 mM; the cultures were maintained at 37 $^{\circ}$ C for 8 h. Cells were

harvested by centrifugation (5000g, 10 min), washed, and resuspended in a buffer consisting of 300 mM NaCl, 10 mM imidazole, 10% glycerol, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 100 μ g/mL BSA in 50 mM sodium phosphate, pH 8.0. Lysozyme was added to a final concentration of 1 mg/mL, and the cells were incubated on ice for 30 min. All further steps were performed at 4 °C. The cells were disrupted by sonication on ice. The cell extract was prepared by two successive centrifugation steps (30000g for 30 min), followed by centrifugation at 32000g for 1 h to effect complete separation. The supernatant was applied to a Ni-NTA agarose column equilibrated with a solution containing 300 mM NaCl, 10 mM imidazole, and 100 μ g/mL BSA in 50 mM sodium phosphate, pH 8.0. After the column was washed with 10 volumes of this buffer, the protein was eluted with 8 volumes of 50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl and 250 mM imidazole. Fractions were analyzed by SDS–polyacrylamide gel electrophoresis (22), and the fractions containing pure ecotin were pooled and dialyzed against 10 mM sodium phosphate, pH 7.0, containing 50 mM NaCl and 10% glycerol at 4 °C for 4 h. The samples were then concentrated to 10 μ g/ μ L using a microconcentrator and stored at –20 °C. The purified ecotin showed a single band when analyzed by a Coomassie Blue-stained SDS–polyacrylamide gel.

Preparation of Misacylated Suppressor tRNA. Abbreviated suppressor tRNA (tRNA_{CUA}-C_{OH}) used in the chemical misacylation reactions was prepared as described previously (1, 16, 25). Chemical misacylation reactions were carried out in 100 μ L (total volume) of 50 mM Na Hepes, pH 7.5, containing 0.5 mM ATP, 15 mM MgCl₂, 50 μ g of suppressor tRNA-C_{OH}, 1.0 A₂₆₀ unit of protected aminoacyl-pdCpA (5–10-fold molar excess), 10% dimethyl sulfoxide, and 200 units of T4 RNA ligase. After incubation at 37 °C for 45 min, the reaction was quenched by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and the tRNA was precipitated with 2.5 volumes of cold ethanol, collected by centrifugation, washed with 70% ethanol, and dried. The product was redissolved in 1 mM KOAc, pH 4.5, to a final concentration of 5 μ g/ μ L. The efficiency of ligation was judged by gel electrophoresis at pH 5.0 (26). Deprotection of NVOC-containing aminoacyl-tRNAs was carried out at a tRNA concentration of 5 μ g/ μ L in 1 mM potassium acetate, pH 4.5. The aminoacyl-tRNAs were cooled to 2 °C and irradiated with a 500 W mercury–xenon lamp using both Pyrex and water filters. After 2 min of irradiation, the deprotected aminoacyl-tRNAs were used in in vitro suppression experiments immediately following deprotection.

In Vitro Synthesis of Ecotin Analogues. Syntheses of ecotin and ecotin analogues were performed in a bacterial S-30 extract containing diminished levels of release factor I (25). In vitro translation reactions carried out in the presence of S-30 extract were performed in 10–8000 μ L reaction mixtures that containing the following per 100 μ L: 10 μ g of plasmid DNA dissolved in diethyl pyrocarbonate-treated water, 40 μ L of premix [35 mM Tris–acetate, pH 7.0, 190 mM potassium glutamate, 2 mM dithiothreitol, 30 mM ammonium acetate, 11 mM magnesium acetate, 20 mM phospho(enol)pyruvate, 0.8 mg/mL *E. coli* tRNA, 0.8 mM isopropyl β -D-thiogalactopyranoside, 20 mM each of GTP and ATP, 5 mM each of UTP and CTP, and 10 mM cAMP], 100 μ M of amino acids minus methionine, 50 μ M methio-

nine, 40 μ Ci of [³⁵S]-S-methionine, and 30 μ L of an S-30 extract that had been heat-shocked at 42 °C for 6 min (25). Suppression reaction mixtures (100 μ L) contained 25 μ g of deprotected misacylated tRNA_{CUA}, and were incubated at 37 °C for 90 min. As a control, in vitro translations were also carried out without the addition of misacylated tRNA_{CUA}. Aliquots were taken for analysis by 20% SDS–PAGE. Autoradiography of the gels was carried out to determine the location of ³⁵S-labeled protein; quantification of the bands was carried out using a phosphorimager.

Purification of in Vitro-Synthesized Ecotin Analogues. In vitro translation mixtures (200 μ L) containing ³⁵S-labeled protein were centrifuged at 14000g for 30 min. The supernatant was applied to a 200- μ L DEAE Sepharose CL-6B column that had been equilibrated with 5 mM potassium phosphate buffer, pH 7.0. The column was washed with three 300- μ L portions of 5 mM potassium phosphate buffer, pH 7.0, containing 10 mM β -mercaptoethanol, followed by three 300 μ L portions of 5 mM potassium phosphate buffer, pH 7.0, and then with three 300 μ L portions of 5 mM potassium phosphate buffer, pH 7.0, containing 30 mM KCl. The remaining proteins (including the desired product) were then eluted from the column by washing with six 100 μ L portions of 5 mM potassium phosphate buffer, pH 7.0, containing 1 M KCl.

The fractions containing radioactivity were applied to a 100 μ L Ni-NTA agarose column equilibrated with 300 mM NaCl, 10 mM imidazole, and 100 μ g/mL BSA in 50 mM sodium phosphate, pH 8.0. After the column was washed with 5 volumes of equilibration buffer, the protein was eluted with 8 volumes of 50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl, 250 mM imidazole, and 10 μ M BSA. The radioactive fractions were combined and dialyzed against 50 mM NaCl in 20 mM sodium phosphate, pH 7.0. The purified proteins were then concentrated to the desired concentration using a microconcentrator. The amount of ³⁵S-labeled protein in each fraction was determined by liquid scintillation counting of a portion of each.

Binding of Ecotin Analogues to a Trypsin–Agarose Resin. One hundred microliters of trypsin–agarose resin was equilibrated with 20 mM Tris-HCl, pH 4.5. Aliquots (50–100 ng) of ecotins purified on Ni-NTA agarose were taken such that they contained the same amount of protein. The buffer for the proteins was changed to 20 mM Tris, pH 4.5, by using a 3000 MW cutoff microconcentrator (Microcon YM-3, Millipore Corp.). The protein samples were then combined with 100 μ L of equilibrated trypsin–agarose resin; the mixture was maintained at 4 °C for 10 min. The resin was then washed successively with five 100 μ L aliquots of equilibration buffer. Fifty microliters of 25 mM HCl was added to the resin; the mixture was maintained at 4 °C for 10 min. The resin was then eluted with six 100 μ L portions of 25 mM HCl. An aliquot (10 μ L) of each of the six fractions was used for determination of radioactivity and analyzed by SDS–PAGE.

Determination of Trypsin and Ecotin Concentration. A stock solution of bovine trypsin was quantified by active site titration (27) with the fluorogenic substrate 4-methylumbelliferyl *p*-guanidine benzoate (λ_{ex} 360 nm; λ_{em} 450 nm) to determine the concentration of the enzyme active sites accurately. Active site-titrated trypsin was used to quantitate ecotin using *N* $^{\alpha}$ -benzoyl-S-arginine-*p*-nitroanilide as the

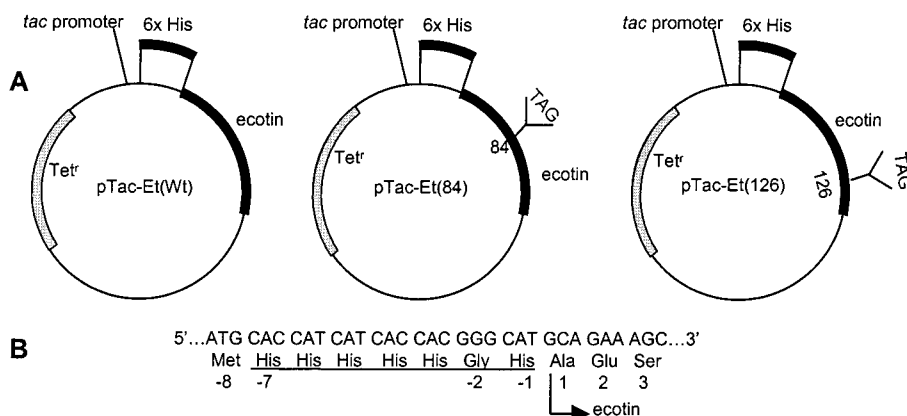


FIGURE 2: (A) Plasmid constructs used for the *in vitro* elaboration of wild-type ecotin [pTac-Et(Wt)] and ecotin analogues modified at either Met84 [pTac-Et(84)] or Lys126 [pTac-Et(126)]. (B) Nucleotide and amino acid sequence at the 5'-end of the ecotin gene. A hexahistidine sequence added to the N-terminus to facilitate affinity purification is underlined. Amino acids are numbered starting from the first Ala of wild-type ecotin. The nonsense codon TAG was introduced into the ecotin gene at codon position 84 or 126.

substrate; an ecotin/trypsin stoichiometry of 1:1 was assumed (17). For *in vivo* synthesized ecotin, there was good agreement with protein concentrations determined by Bradford analysis (28), titration with trypsin, and the calculated molar extinction coefficient at 280 nm ($21\,920\text{ cm}^{-1}\text{ M}^{-1}$). The concentrations of *in vitro* synthesized ecotin and ecotin analogues were calculated on the basis of [^{35}S]methionine content; these were normalized to the specific activity of an *in vivo* synthesized sample of wild-type ecotin by making the assumption that *in vivo* and *in vitro* generated wild-type ecotin have the same specific activity for bovine trypsin inhibition.

Determination of Ecotin Dissociation Constant by Fluorescence Titration. Measurement of the change in fluorescence of Trp130 with ecotin concentration was carried out on a Hitachi F2000 fluorescence spectrophotometer (excitation at 280 nm, emission at 340 nm). Ecotin (10 μM) and *S*-tryptophan (40 μM) were each consecutively diluted 2-fold in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 2 mM CaCl_2 . The absorbance of the initial tryptophan solution was equivalent to that of the initial ecotin solution. Fluorescence emission was recorded within 15 s of dilution. The concentration dependence of the ratio of the ecotin fluorescence emission to the *S*-tryptophan fluorescence emission was used to determine the dissociation constant for ecotin dimerization (29).

Determination of Equilibrium Dissociation Constants. Ecotin or ecotin analogues were incubated at various concentrations with a constant amount of trypsin in a total volume of 190 μL of 50 mM Tris-HCl, pH 8.0, containing 10 mM CaCl_2 and 100 mM NaCl. Following equilibration at room temperature for 20 min, 10 μL of 4 mM *N*-benzoyl-L-arginine 7-amido-4-methylcoumarin was added, and the rate of substrate hydrolysis was measured by monitoring the change in emission at 460 nm over a 2 min period at 25 $^{\circ}\text{C}$ using a Hitachi F2000 fluorescence spectrophotometer. The assays were repeated 3 times at different ecotin concentrations. The data were fit to the equation derived for kinetics of reversible tight-binding inhibitors (30) by nonlinear regression analysis using the program Kaleidagraph (19).

Iodine Treatment of Ecotins. Ecotin or ecotin analogues were treated with iodine in an aqueous solution (400 μL total volume) containing 10 pmol of ecotin or an ecotin analogue,

1 mM I_2 , and 15% THF. The solution was incubated at 25 $^{\circ}\text{C}$ for 60 min with constant mixing. Excess iodine was removed quickly by a buffer exchange process using a 3000 MW cutoff microconcentrator. The buffer for the ecotin analogues was changed to 50 mM Tris-HCl, pH 8.0, containing 10 mM CaCl_2 and 100 mM NaCl. Each sample was then used immediately in trypsin inhibition assays.

Iodine Treatment of Trypsin–Ecotin Complexes. Fifty picomoles of ecotin or ecotin analogues was incubated with 40 pmol of bovine trypsin in a total volume of 200 μL of 50 mM Tris-HCl, pH 8.0, containing 10 mM CaCl_2 and 100 mM NaCl. Following equilibration at 25 $^{\circ}\text{C}$ for 1 h, a 20 μL aliquot was used to verify that trypsin activity (for catalyzing the hydrolysis of *N* $^{\alpha}$ -benzoyl-*S*-arginine 7-amido-4-methylcoumarin) had been completely inhibited. Iodine was dissolved in 1:1 THF/ H_2O and added to the protein complex to a final concentration of 1 mM (15% THF). After a 1 h incubation at 25 $^{\circ}\text{C}$, the protein complex was dialyzed for 6 h at 4 $^{\circ}\text{C}$ against 50 mM Tris-HCl, pH 8.0, containing 10 mM CaCl_2 and 100 mM NaCl. Trypsin activity for the resulting protein complex was analyzed as described above.

RESULTS

Construction of Expression Plasmids for *In Vitro* Elaboration of Ecotin Analogues. Plasmids pTac-Et(84) and pTac-Et(126) were constructed (Figure 2) in order to permit the incorporation of the unnatural amino acid allylglycine ($^{\Delta}\text{Gly}$) into ecotin. Modified ecotin genes were placed under the control of both tac and SP6 promoters, as well as efficient translation initiation signals. A DNA sequence coding for the peptide sequence MHHHHHGH was introduced immediately prior to the first codon of the ecotin gene. The inclusion of the hexahistidine moiety was intended to facilitate purification of the synthesized ecotin by affinity chromatography on Ni-NTA agarose (31). The nonsense codon TAG was introduced to replace ATG (Met84) or AAG (Lys126). Additionally, the authentic stop codon for the gene was changed from TAG to TGA to preclude readthrough by the misacylated suppressor tRNA. As a control, a plasmid containing the wild-type ecotin gene was also constructed. Subcloning of the modified ecotin genes from the intermediate vectors pGem-Et(Wt), pGem-Et(84), and pGem-Et(126) into the expression vector pALTER-Ex1 afforded final

expression plasmids pTac-Et(Wt), pTac-Et(84), and pTac-Et(126), respectively.

In Vivo Expression and Characterization of Ecotin. To verify that the inclusion of the hexahistidine moiety could simplify the purification procedure of ecotin without affecting its ability to inhibit trypsin, ecotin containing the hexahistidine peptide (HisEt) was synthesized *in vivo*. The expression plasmid pTac-Et(Wt) was transformed into *E. coli* JM109. Expression of HisEt was induced by addition of 1 mM isopropyl- β -D-thiogalactoside. A cell extract was prepared and applied directly to a Ni-NTA column for purification by affinity chromatography (31). As judged by SDS-PAGE, greater than 98% purity could be achieved by this simple chromatographic step (Supporting Information, Figure 1). The yield of purified protein was about 12 mg per liter of culture.

To investigate whether the histidine fusion peptide influenced the dimerization of ecotin, the monomer-dimer dissociation constant, K_d , was measured for HisEt. Each ecotin monomer contains two tryptophan residues (Trp67 and Trp130) with Trp130 located near the dimer interface. The fluorescence of Trp130 is sensitive to the oligomerization state of the molecule; changes in its emission spectrum are dependent upon the monomer-dimer equilibrium (29). By monitoring the fluorescence of Trp130, a K_d value of 260 ± 110 nM was determined for HisEt, in good agreement with the value (220 ± 130 nM) reported previously (19, 29) (Supporting Information, Figure 2). The inhibition constant (K_i) of HisEt for blocking trypsin function was determined to be 0.33 ± 0.01 nM, also comparable to the value (0.31 ± 0.06 nM) reported previously (19). These results demonstrated that the presence of the histidine fusion peptide did not significantly affect the self-association of ecotin or its ability to inhibit bovine trypsin.

Synthesis of Allylglycyl-tRNA_{CUA}. The suppressor tRNA used in this study was a modified yeast tRNA^{Phe} transcript (6, 32) that contains a CUA anticodon. A truncated form of the tRNA lacking the 3'-terminal pCpA was prepared by *in vitro* T7 transcription of linearized pYRNA8 plasmid DNA (14) bearing a gene for the yeast tRNA^{Phe}_{CUA}. The misacylated tRNA_{CUA} used for readthrough of the TAG codon was prepared by T4 RNA ligase-mediated coupling of *N*-(6-nitroveratryloxycarbonyl)-(R,S)-allylglycyl pCpA with the truncated tRNA transcript (Figure 3). Photolytic deblocking of the ligation product gave allylglycyl-tRNA_{CUA}. Valyl-tRNA_{CUA} was prepared similarly.

In Vitro Synthesis and Characterization of Ecotin Analogues. The synthesis of ecotin analogues containing ^AGly in lieu of either Met84 or Lys126 was effected using the general strategy for the synthesis of proteins containing unnatural amino acids at specific sites (2–15). The ecotin gene constructs were transcribed and translated in a coupled XAC-RF S30 bacterial extract containing diminished levels of release factor 1 (25). Translation of pTac-Et(Wt)-derived mRNA afforded a new protein, relative to those produced from pALTER-Ex1. This product migrated as a protein having molecular mass of 17 kDa and was attributed to full-length ecotin produced from the wild-type ecotin gene (Figure 4, lane 1). As anticipated, expression of the other gene constructs [pTac-Et(84) or pTac-Et(126)] to afford a comparable full-length ecotin band was dependent on the presence of a suppressor tRNA that had been activated with

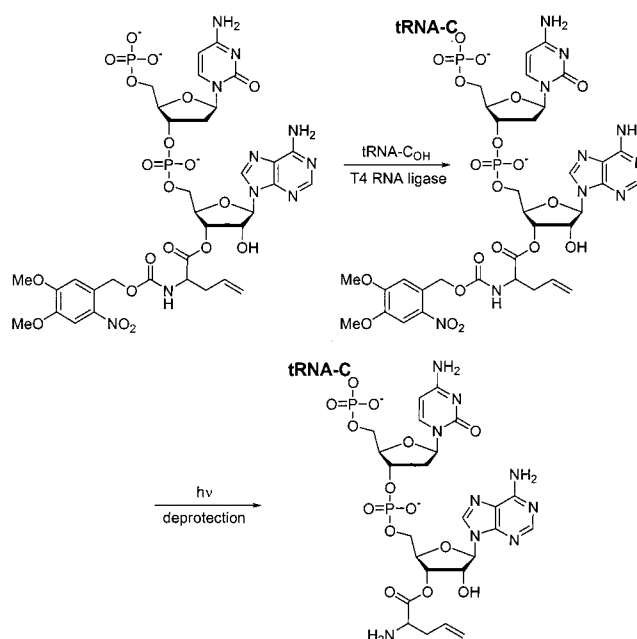


FIGURE 3: “Chemical aminoacylation” strategy employed for the preparation of misacylated suppressor tRNA_{CUA}s used for the elaboration of ecotin analogues.

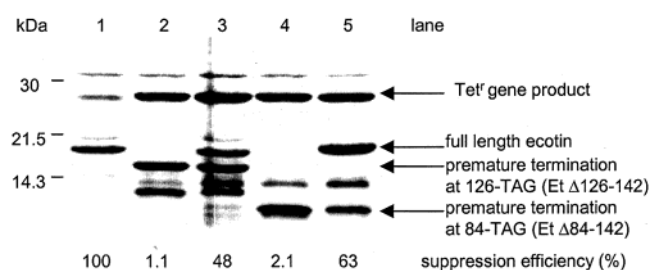


FIGURE 4: Phosphorimager analysis of a 15% SDS-polyacrylamide gel illustrating the *in vitro* synthesis of ecotin. Protein synthesis was carried out using an *E. coli* S30 system in the presence of [³⁵S]methionine and the following ecotin gene constructs: lane 1, pTac-Et(Wt); lane 2, pTac-Et(126) without suppressor tRNA_{CUA}; lane 3, pTac-Et(126) + allylglycyl-tRNA_{CUA}; lane 4, pTac-Et(84) without suppressor tRNA_{CUA}; lane 5, pTac-Et(84) + allylglycyl-tRNA_{CUA}. The suppression efficiency is indicated below each lane. Other [³⁵S]methionine-labeled protein bands were also observed following transcription-translation of plasmid pALTER-Ex1.

allylglycine (Figure 4, lanes 3 and 5). However, in the absence of this species, the translation product derived from the modified ecotin gene in pTac-Et(126) was a 15 kDa protein (Figure 4, lane 2), presumably formed by premature termination of protein synthesis at TAG codon at position 126 (denoted as Et Δ126–142). Likewise, premature termination product resulting from the translation of pTac-Et(84) (denoted as Et Δ84–142) also appeared as a protein having the anticipated molecular mass (Figure 4, lane 4). Other observed protein bands were also produced from vector pALTER-Ex1, and are thus unrelated to expression of the ecotin gene.

The ability of allylglycyl-tRNA_{CUA} to suppress stop codon TAG at position 84 or 126 was quantified by phosphorimager analysis, and the results are shown in Figure 4. The yield of wild-type ecotin produced by *in vitro* translation was determined to be 21 μg/mL by both active site titration and Bradford analysis (28). Thus, the yields for Et Met84^AGly and Lys126^AGly were 13 and 10 μg/mL, respectively, based on the suppression efficiencies shown in Figure 4.

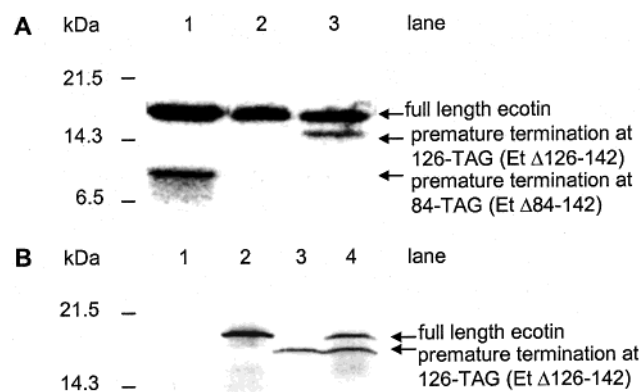


FIGURE 5: Autoradiogram of a 15% SDS-polyarylamide gel demonstrating the binding of in vitro synthesized ecotin variants to Ni-NTA agarose resin (A) and trypsin-agarose resin (B). Panel A: In vitro synthesized ecotins were applied to Ni-NTA columns. The proteins retained by the columns were analyzed: lane 1, translation mixture as described in Figure 4, lane 5; lane 2, wild-type ecotin; lane 3, translation mixture as described in Figure 4, lane 3. Panel B: Ecotin analogues produced by in vitro synthesis and purified on Ni-NTA agarose were applied to trypsin-agarose resin. The proteins retained by the resin were analyzed: lane 1, transcription/translation product from pTac-Et(84) in the absence of allylglycyl-tRNA_{CUA}; lane 2, transcription/translation product from pTac-Et(84) in the presence of allylglycyl-tRNA_{CUA}; lane 3, transcription/translation product from pTac-Et(126) in the absence of allylglycyl-tRNA_{CUA}; lane 4, transcription/translation product from pTac-Et(126) in the presence of allylglycyl-tRNA_{CUA}.

The protein mixtures derived from the in vitro transcription/translation of the ecotin expression plasmids were applied to Ni-NTA agarose columns. The proteins retained by the resin were analyzed by SDS-PAGE (Figure 5, panel A). Since Ni-NTA agarose specifically retains those proteins that have a spatially accessible hexahistidine moiety (31), one would expect that only the translation product derived from the ecotin gene would be retained on the Ni-NTA resin. The results shown in Figure 5A were consistent with this expectation. After Ni-NTA chromatography purification, only full-length ecotins as well as the premature termination products Et Δ 84–142 and Et Δ 126–142 were retained on resin; other proteins initially present in the translation mixture were eliminated.

This procedure provided access to several purified ecotin analogues. These are listed in Table 1. In the presence of allylglycyl-tRNA_{CUA}, translation of pTac-Et(84) and pTac-Et(126) afforded Et Met84^AGly and Et Lys126^AGly, respectively, while in the absence of misacylated suppressor tRNA_{CUA}, transcription/translation from the same expression plasmids afforded Et Δ 84–142 and Et Δ 126–142. Et M84V was used as a control and was obtained by transcription/translation of pTac-Et(84) in the presence of valyl-tRNA_{CUA}.

To test the binding capability of ecotin, the in vitro synthesized, Ni-NTA-purified ecotin analogues were applied to a trypsin-agarose resin. The proteins retained on the column were analyzed by SDS-PAGE. As shown in Figure 5B, all full-length ecotins (wild type, Met84^AGly, and Lys126^AGly), as well as truncated mutant Et Δ 126–142, were capable of binding to trypsin while mutant Et Δ 84–142 was not retained by trypsin. A comparison of Figure 5A and Figure 5B indicated that the amount of truncated mutant Et Δ 126–142 bound to the trypsin agarose column

Table 1: Definition and Characterization of Ecotin Analogues

analogue symbol	binding to trypsin	relative inhibitory efficiency (%) ^a	K _i (nM)	mutations
Et	+	100	0.34 ± 0.02	wild type
Et M84 ^A G	+	93	0.41 ± 0.05	single residue substitution at position 84
Et M84V	+	96	0.47 ± 0.03	single residue substitution at position 84
Et K126 ^A G	+	100	0.37 ± 0.06	single residue substitution at position 126
Et Δ 84–142	–	0	nd ^b	deletion of residues 84–142
Et Δ 126–142	+	52	0.91 ± 0.04	deletion of residues 126–142

^a Relative inhibitory efficiency was established by comparing the residual trypsin activity after adding the same amount of ecotin analogues to defined amounts of bovine trypsin. ^b Not detectable.

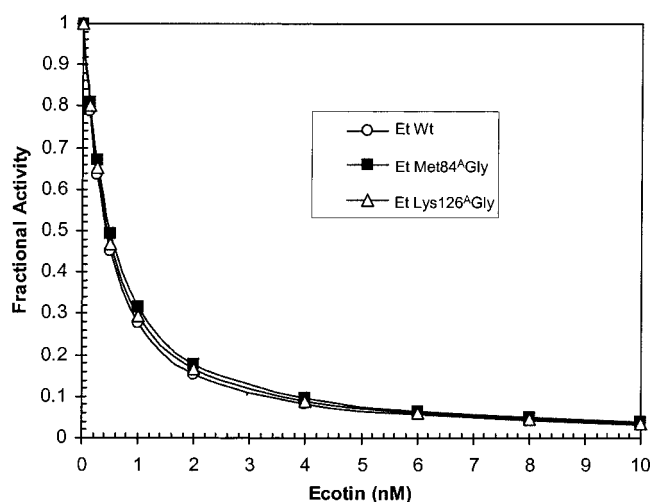


FIGURE 6: Determination of the ability of in vitro synthesized ecotin and ecotin analogues to inhibit bovine trypsin function. Inhibition of function is expressed as the ratio of the inhibited rate to the uninhibited rate (fractional activity) at varying ecotin (analogue) concentrations following formation of the ecotin–trypsin complex. The fluorogenic substrate used for the assay was *N*^α-benzoyl-S-arginine 7-amido-4-methylcoumarin.

was comparable to that of full-length ecotin, although more full-length ecotin had been retained on the Ni-NTA agarose column. Despite the apparently tighter binding of Et Δ 126–142 to trypsin, the truncated mutant had a diminished capacity to inhibit trypsin function (Table 1).

To investigate whether the introduction of ^AGly at Met84 (P1 reactive site) or Lys126 affected the potency of ecotin in trypsin inhibition, the inhibitory activities of ecotin analogues Met84^AGly and Lys126^AGly were compared to that of wild-type inhibitor against bovine trypsin. Figure 6 shows that both Et Met84^AGly and Et Lys126^AGly were capable of inhibiting bovine trypsin to nearly the same extent as wild-type ecotin. The inhibitory constants (*K*_is) of ecotin analogues toward bovine trypsin were measured and are listed in Table 1. The *K*_is for both Et Met84^AGly and Et Lys126^AGly are comparable to that of wild type, indicating that the allylglycine-containing ecotins are still potent trypsin inhibitors of trypsin function.

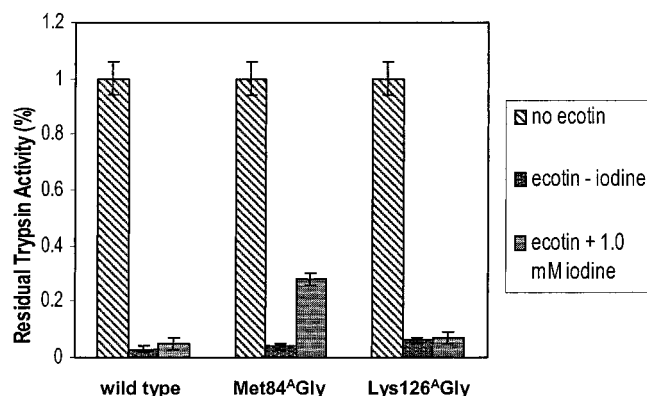


FIGURE 7: Effect of iodine treatment on the ability of ecotin and ecotin analogues to inhibit bovine trypsin function. Five picomoles each of wild-type ecotin, ecotin analogue Met84^AGly, or ecotin analogue Lys126^AGly was treated with 1 mM I₂ at 25 °C for 20 min, then used to inhibit bovine trypsin after removing residual iodine. The hydrolysis of 0.2 mM trypsin substrate *N*^α-benzoyl-*S*-arginine 7-amido-4-methylcoumarin was determined for bovine trypsin (3 pmol).

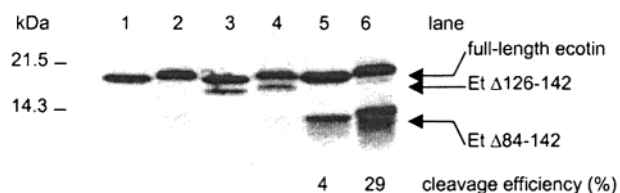


FIGURE 8: Iodine-induced cleavage of ecotin analogues. The Ni-NTA-purified proteins were treated with 1 mM iodine in 100:15 water/tetrahydrofuran at 25 °C for 30 min. Lane 1, wt Et without iodine; lane 2, wt Et with iodine; lane 3, Lys126^AGly without iodine; lane 4, Lys126^AGly with iodine; lane 5, Met84^AGly without iodine; lane 6, Met84^AGly with iodine.

The abilities of the other modified forms of ecotin to inhibit trypsin function were also determined (Table 1). That Et Δ84–142 did not inhibit trypsin function was consistent with the observation that this truncated ecotin analogue did not bind to trypsin–agarose resin (Figure 5B, lane 1). Analogue Et Δ126–142 exhibited a ~48% decrease in ability to inhibit trypsin function, while Et Met84Val had a *K_i* value comparable to that of wild-type ecotin.

To demonstrate that Et Met84^AGly was an iodine-labile inhibitor that could be deactivated by iodine treatment, purified Et Met84^AGly was treated with iodine, and then tested for its ability to inhibit bovine trypsin. The experiments were carried out under conditions shown previously not to affect trypsin activity (1, 16). The inhibitory activity was determined by measuring the fractional trypsin activity after admixture of inhibitor and was compared with that of Et Met84^AGly without iodine treatment. The same amount of wild-type ecotin as well as mutant Met84Val was also subjected to the same treatment and measurement. As shown in Figure 7, only Et Met84^AGly could be inactivated by iodine, thus demonstrating that this iodine-labile property is due explicitly to the introduction of ^AGly at position 84. Based on a 1:1 stoichiometry of trypsin and ecotin (16), it is estimated that 28% of Et Met84^AGly was inactivated by iodine treatment. This is consistent with the phosphorimager analysis of an SDS–PAGE showing the cleavage of Et Met84^AGly (Figure 8).

Contrary to expectation, iodine treatment of Et Lys126^AGly had little effect on the ability of this ecotin analogue to inhibit

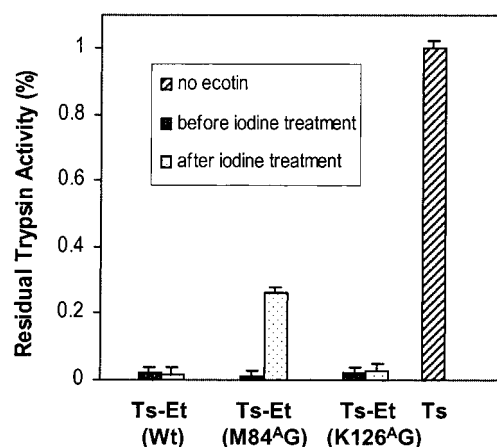


FIGURE 9: Activation of trypsin from trypsin–ecotin (analogue) complexes by iodine treatment. Wild-type ecotin and ecotin analogues Met84^AGly or Lys126^AGly were each used to form complexes with trypsin that lacked trypsin function. After equilibrium was reached, the protein complexes were treated with 1 mM I₂ at 25 °C for 20 min. The hydrolysis of 0.2 mM trypsin substrate *N*^α-benzoyl-*S*-arginine 7-amido-4-methylcoumarin was used to determine trypsin activity.

trypsin function (Figure 7). The reason for this became apparent when the cleavage of Et Lys126^AGly was analyzed by PAGE. Less than 5% cleavage was noted.

To demonstrate that active trypsin can be released from a complex with an iodine-labile ecotin analogue, Et Met84^AGly was used to form a 1:1 complex with bovine trypsin; the complex displayed no observable amide hydrolysis activity. The formed protein complex was then treated with iodine. The trypsin activity of this iodine-treated protein complex was then tested in the presence of a fluorescent substrate. As shown in Figure 9, destruction of Et Met84^AGly afforded trypsin activity equivalent to 26% of the activity observed in the absence of ecotin. Since the cleavage product was analogous to Et Δ84–142, the latter of which does not inhibit trypsin function (Table 1), it is logical to conclude that 26% of the trypsin molecules were available for enzyme turnover in their complexes with Et Met84^AGly. In fact, this figure matches closely the 29% cleavage of Et Met84^AGly noted (Figure 8). However, analogous iodine treatment of the protein complex formed by admixture of trypsin with Et Lys126^AGly had no effect on trypsin activity.

DISCUSSION

Introduction of the unnatural amino acid allylglycine into a protein expands the repertoire of chemical transformations accessible to that protein. Of particular interest in the present study is the observation that the amide backbone adjacent to the ^AGly residue was labile upon iodine treatment. This new property permitted cleavage of proteins at a single, predetermined site with a simple chemical reagent (1). Previously, this strategy has been employed successfully to activate trypsin from its inactive trypsinogen precursor using mild chemical treatment rather than proteolytic processing (1, 16). A more sophisticated application of this strategy may be envisioned in the manipulation and study of protein–protein interaction. In the present case, the elaboration of an allylglycine-containing ecotin afforded an iodine-labile serine protease inhibitor. The active trypsin could be released from a complex with this inhibitor by iodine treatment. To

our knowledge this constitutes the first report of chemical alteration of protein–protein interaction by cleavage of a protein chain containing a unique amino acid with a simple chemical reagent. The use of a chemical reagent to regulate protein function, and potentially to modulate cascade-activation events, provides new tools for the study of protein–protein interaction.

To incorporate ^AGly at predetermined sites in ecotin, the stop codon TAG (10–15) was placed in the ecotin gene at the position intended for ^AGly incorporation, and was employed in combination with allylglycyl-tRNA_{CUA}. Allylglycyl-tRNA_{CUA} was prepared by chemical misacylation (2–9) of a suppressor tRNA with synthetic allylglycine (Figure 3). Although the allylglycyl-tRNA_{CUA} synthesized in this fashion included both *R*- and *S*-isomers of allylglycine, this is of little consequence since ribosome-mediated protein biosynthesis utilizes virtually exclusively those tRNAs activated with *S*-amino acids (2).

The incorporation of allylglycine into ecotin was carried out in vitro in a coupled transcription–translation system, which allows direct and facile introduction of the misacylated suppressor tRNA during mRNA translation. While not a normal protein constituent, allylglycine is not dramatically different in physicochemical properties than leucine, isoleucine, and valine; it has been shown previously (1) that its incorporation into specific sites in a protein can be realized by readthrough of a UAG codon with a misacylated suppressor tRNA activated with allylglycine. The fact that inclusion of allylglycyl-tRNA_{CUA} was essential for the synthesis of full-length ecotin supports the assertion that allylglycine must be incorporated specifically into ecotin at the site of each UAG codon (Figure 4). The modified ecotins (Et Met84^AGly and Et Lys126^AGly) had the same electrophoretic mobility in SDS–polyacrylamide gels as wild-type ecotin; the mobility of the latter relative to a set molecular mass markers was consistent with the calculated molecular mass of 17.1 kDa expected for ecotin containing an eight amino acid fusion peptide at the N-terminus (Figures 2 and 4). Critically, in vitro derived wild-type ecotin containing the histidine fusion peptide was fully competent as a trypsin inhibitor relative to native ecotin isolated from *E. coli*.

Interestingly, in the absence of misacylated suppressor tRNA, in vitro transcription–translation of the modified ecotin gene containing stop codon TAG at either position 84 or position 126 afforded truncated ecotins Et Δ84–142 or Et Δ126–142, respectively. These two variants were formed because the in vitro translations were terminated prematurely at the respective TAG codons. Significantly, these deletion products lacked the same residues as Et Met84^AGly and Et Lys126^AGly, following treatment of the latter two with I₂, and could thus be used as standards to help characterize the cleavage products.

In addition to its utility in separating elaborated ecotin (analogues) from other proteins, it may be noted that the hexahistidine fusion peptide present in the constructs studied here also served a second purpose. Ecotin is an *E. coli* protein, and the protein synthesis mixtures undoubtedly contained some chromosomally derived ecotin in addition to that produced from the expression plasmids. Because wild-type ecotin does not bind to Ni-NTA agarose, the purification scheme utilized here resulted in the removal of such wild-type protein from the hexahistidine-containing ecotin.

The fusion peptide at the N-terminus influenced neither the dimerization of ecotin monomer nor the efficiency of bovine trypsin binding. The dimerization and binding functions of ecotin are mediated by three distinct interfaces: an ecotin–ecotin dimer interface (residues 125–142), a large primary ecotin–protease interface (residues 52–54; 81–86), and a smaller secondary ecotin–protease interface (residues 67–70; 108–113) (19). The N-terminus of ecotin is not involved in any of these interactions.

Substitution of ^AGly or Val for Met84, the P1 reactive site of ecotin, did not affect the binding of the proteins to bovine trypsin (Figure 5B and Table 1). Although the P1 amino acid side chain is often a major determinant of the inhibitory specificity and efficiency of many substrate-like serine protease inhibitors, Met84 has been shown not to be crucial for its specificity toward target proteases (19). The replacement of Met84 with Ile, Arg, Glu, or Tyr showed little or no effect on the ability of ecotin to bind to trypsin (33). The fact that significant changes in the electrostatic and hydrophobic properties of the P1 residue of ecotin had relatively little effect on trypsin binding demonstrates that the side chain of the P1 residue does not play an important role in determining the binding specificity and efficiency of ecotin.

By deleting residues 84–142, the inhibitory activity of ecotin was lost completely. This result was not unexpected since four out of seven strands were thereby deleted; these were necessary to form the overall seven-stranded β-barrel structure (18).

Iodine treatment of wild-type ecotin did not result in the loss of ecotin activity (Figure 7). Molecular iodine is an oxidant; at higher concentration, it may have detrimental effects on proteins (16). However, under the conditions of the experiments described here, its use was compatible with the functional integrity of both ecotin (Figures 7 and 9) and trypsin (1).

The availability of a serine protease inhibitor that can be deactivated by iodine treatment is attractive because it provides a novel strategy for modulating serine protease activity and altering protein–protein interaction by a simple chemical reagent. Deactivation of allylglycine-containing ecotin was mediated by the iodolactonization reaction which is believed to proceed via iminolactone formation, followed by hydrolysis of the formed intermediate (1). It has been speculated previously that the efficiency of iodine-induced cleavage might be based both on the solvent accessibility of the allyl group and on the protein secondary structure at the ^AGly site (1). Our previous studies suggested that the latter seems to be a more important determinant (1). In the present case, when ^AGly was located at position 84 or 126, the iodine-induced cleavage efficiencies were 29% or <5%, respectively. Met84 resides on a protruding loop. Based on a structural analysis of the trypsin–ecotin complex, the conformation of the central inhibitory residues (residues 83–85) which interact directly with the catalytic machinery of the protease is unchanged in all characterized ecotin structures with substitutions at Met84 (34). Since Et Met84^AGly had a *K_i* comparable to wild type for trypsin binding, the secondary structure at the ^AGly site should also be fundamentally analogous to wild type and the analogues studied previously. In contrast, Lys126 is located in the long C-terminal strand (residues 125–142) used to form the dimer

interface. In the dimer, these strands are arranged as a 2-stranded antiparallel β -sheet, with 10 main hydrogen bonds between residues 125 and 142 of each monomer. The surface area which is buried upon dimerization is $>1400 \text{ \AA}^2$ per monomer (18). Extensive hydrogen binding and hydrophobic interactions of this β -sheet dimer interface may well place constraints on $^A\text{Gly}126$ which make the formation of the requisite cyclic intermediate (1, 35) leading to cleavage difficult. The hydrophobic nature of this domain may also make subsequent hydrolysis of the formed iminolactone inefficient.

At present neither the currently available examples of iodine-induced protein cleavage nor the depth of analysis of the factors controlling cyclization and hydrolysis of specific peptide bonds permits a detailed understanding of the relationship between protein structure and the facility of iodine-induced cleavage. Clearly, better definition of the essential structural parameters would permit the realization of more efficient cleavage by a judicious choice of sites for ^AGly substitution, and potentially provide a high-resolution tool for verification of local protein structure elements in solution.

Despite the foregoing limitations, the present results demonstrate unequivocally that the introduction of allylglycine into key positions in a protein can permit important information to be gathered concerning the nature of protein–protein interaction. This includes factors essential for the activation of a zymogen (1, 16), as well as the release of a catalytically active protein from a complex with a second protein. Simple extension of the principles established experimentally thus far should provide access to folded fragments of proteins, and the removal of unwanted proteins from complexes.

SUPPORTING INFORMATION AVAILABLE

SDS–polyacrylamide gel analysis of expressed ecotin before and after purification on a Ni-NTA column, and fluorescence titration of ecotin. This material is available free of charge via the Internet at <http://pubs.acs.org>.

REFERENCES

- Wang, B., Lodder, M., Zhou, J., Baird, J. T. T., Brown, K. C., Craik, C. S., and Hecht, S. M. (2000) *J. Am. Chem. Soc.* 122, 7402–7403.
- Heckler, T. G., Zama, Y., Naka, T., and Hecht, S. M. (1983) *J. Biol. Chem.* 258, 4492–4495.
- Heckler, T. G., Chang, L.-H., Zama, Y., Naka, T., Chorghade, M. S., and Hecht, S. M. (1984) *Biochemistry* 23, 1468–1473.
- Baldini, G., Martoglio, B., Schachenmann, A., Zugliani, C., and Brunner, J. (1988) *Biochemistry* 27, 7951–7959.
- Bain, J. D., Glabe, C. G., Dix, T. A., Chamberlin, A. R., and Diala, E. S. (1989) *J. Am. Chem. Soc.* 111, 8013–8014.
- Robertson, S. A., Noren, C. J., Anthony-Cahill, S. J., Griffith, M. C., and Schultz, P. G. (1989) *Nucleic Acids Res.* 17, 9649–9660.
- Noren, C. J., Anthony-Cahill, S. J., Suich, D. J., Noren, K. A., Griffith, M. C., and Schultz, P. G. (1990) *Nucleic Acids Res.* 18, 83–88.
- Roeser, J. R., Xu, C., Payne, R. C., Surratt, C. K., and Hecht, S. M. (1989) *Biochemistry* 28, 5185–5195.
- Hecht, S. M. (1992) *Acc. Chem. Res.* 25, 545–552.
- Noren, C. J., Anthony-Cahill, S. J., Griffith, M. C., and Schultz, P. G. (1989) *Science* 244, 182–188.
- Bain, J. D., Diala, E. S., Glabe, C. G., Wacker, D. A., Lyttle, M. H., Dix, T. A., and Chamberlin, A. R. (1991) *Biochemistry* 30, 5411–5421.
- Bain, J. D., Switzer, C., Chamberlin, A. R., and Benner, S. A. (1992) *Nature* 356, 537–539.
- Cornish, V. W., Mendel, D., and Schultz, P. G. (1995) *Angew. Chem., Int. Ed. Engl.* 34, 621–633.
- Karginov, V. A., Mamaev, S. V., An, H., Van Cleve, M. D., Hecht, S. M., Komatsoulis, G. A., and Abelson, J. N. (1997) *J. Am. Chem. Soc.* 119, 8166–8176.
- Mamaev, S. V., Laikhter, A. L., Arslan, T., and Hecht, S. M. (1996) *J. Am. Chem. Soc.* 118, 7243–7244.
- Baird, T., Jr., Wang, B., Lodder, M., Hecht, S. M., and Craik, C. S. (2000) *Tetrahedron* 56, 9477–9485.
- Chung, C. H., Ives, H. E., Almeda, S., and Goldberg, A. L. (1983) *J. Biol. Chem.* 258, 11032–11038.
- McGrath, M. E., Erpel, T., Bystroff, C., and Fletterick, R. J. (1994) *EMBO J.* 13, 1502–1507.
- Yang, S. Q., Wang, C., Gillmor, S. A., Fletterick, R. J., and Craik, C. S. (1998) *J. Mol. Biol.* 279, 945–957.
- McGrath, M. E., Erpel, Y., Browner, M. F., and Fletterick, R. J. (1991) *J. Mol. Biol.* 222, 139–142.
- McGrath, M. E., Hines, W. M., Sakanari, J. A., Fletterick, R. J., and Craik, C. S. (1991) *J. Biol. Chem.* 266, 6620–6625.
- Laemmli, U. K. (1970) *Nature* 227, 680–685.
- Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82, 488–492.
- Sanger, F., Niklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463–5467.
- Short, G. F., III, Golovine, S. Y., and Hecht, S. M. (1999) *Biochemistry* 38, 8808–8819.
- Varshney, U., Lee, C. P., and RajBhandary, U. L. (1991) *J. Biol. Chem.* 266, 24712–24718.
- Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A., and Elmore, D. T. (1973) *Biochem. J.* 131, 107–117.
- Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254.
- Seymour, J. L., Lindquist, R. N., Dennis, M. S., Moffat, B., Yansura, D., Reilly, D., Wessinger, M. E., and Lazarus, R. A. (1994) *Biochemistry* 33, 3949–3958.
- Morrison, J. F. (1969) *Biochim. Biophys. Acta* 185, 269–286.
- Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R. A., Nordheim, A., and Stunnenberg, H. G. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 8972–8976.
- Sampson, J. R., and Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85, 1033–1037.
- Seong, I. S., Lee, H. R., Seol, J. H., Park, S. K., Lee, C. S., Suh, S. W., Hong, Y. M., Kang, M. S., Ha, D. B., and Chung, C. H. (1994) *J. Biol. Chem.* 269, 21915–21918.
- Gillmor, S. A., Takeuchi, T., Yang, S. Q., Craik, C. S., and Fletterick, R. J. (2000) *J. Mol. Biol.* 299, 993–1003.
- Lodder, M., Golovine, S., Laikhter, A. L., Karginov, V. A., and Hecht, S. M. (1998) *J. Org. Chem.* 63, 794–803.

BI011762P