Inhibition of α-Lytic Protease by Pro Region C-Terminal Steric Occlusion of the Active Site†

Julie L. Sohl,‡ Andrew K. Shiau,§ Stephen D. Rader,‡ Barry J. Wilk,|| and David A. Agard*,‡,§

Howard Hughes Medical Institute and Department of Biochemistry and Biophysics, Graduate Group in Biophysics, University of California at San Francisco, San Francisco, California 94143-0448

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ABSTRACT: α-Lytic protease, a chymotrypsin-like serine protease, is synthesized with an N-terminal 166 amino acid pro region which is absolutely required for folding of the protease. The pro region is also the most potent inhibitor of the protease known with a K_i of $\sim 10^{-10}$ M. Compared to its role in the folding reaction, relatively little is known about the mechanism by which the pro region inhibits the mature protease. While proteinaceous protease inhibitors generally function by occluding the active sites of their respective targets [Bode, W., & Huber, R. (1992) *Eur. J. Biochem. 204*, 433−451], the pro region of α-lytic protease with its dual roles in folding and inhibition might be expected to show a novel mechanism of inhibition. However, experiments that probe both the structural and enzymatic consequences of pro region binding indicate that the pro region does not measurably distort the protease active site. Instead, the catalytic site is fully functional in the complex. Pro region inhibition of the protease is due to simple steric obstruction; the pro region C-terminus lies in the substrate binding sites of the protease. The implications of these results are discussed with regard to α-lytic protease maturation and folding. In addition, the proposed mechanism of α-lytic protease pro region inhibition is discussed with respect to data from other pro region families.

α-Lytic protease (αLP)[1] from *Lysobacter enzymogenes* is a 198 amino acid serine protease with specificity for peptides with small hydrophobic residues adjacent to the scissile bond (Whitaker, 1970). High-resolution X-ray crystallography has shown that αLP and chymotrypsin have nearly identical three-dimensional folds despite only moderate sequence homology (Brayer et al., 1979). In vivo, αLP is synthesized as a pre-pro-protease precursor containing an export signal sequence and a large amino-terminal pro region which is not part of the active protease (Silen et al., 1988; Whitaker, 1970). Heterologous expression experiments with αLP in *Escherichia coli* indicate that αLP activity is required for cleavage at the pro region−protease junction in the periplasm (Silen et al., 1989). The pro region, expressed either *in cis*, as a contiguous polypeptide with the protease, or *in trans*, as a separate polypeptide, is *essential* for secretion of active protease in vivo (Silen & Agard, 1989). Since the pro region is not part of the active protease but is required for obtaining protease activity, it follows that the pro region must function to promote folding and/or targeting of the protease domain.

In vitro experiments show that the αLP pro region has at least two distinct functions: catalyzing protease folding and inhibition of the native protease. A stable, inactive folding intermediate of the protease domain can be trapped by omitting the pro region from an in vitro refolding reaction. This intermediate is stable for months, yet folds rapidly to the native state upon addition of the pro region (Baker et al., 1992b). These studies demonstrate that the pro region does not function by blocking off pathway reactions such as aggregation (as do the molecular chaperones), but instead functions much like an enzyme to directly stabilize the rate-limiting folding transition state. However, the pro region is not able to perform multiple rounds of catalysis due to its tight binding to the folded protease product (Baker et al., 1992a,b).

While much effort has gone into understanding the mechanism by which pro region folds the protease, very little is known about its mechanism of inhibition. The αLP pro region inhibits the folded protease with a very high affinity, $K_i \leq 10^{-10}$ M (Baker et al., 1992a). Pro regions of aspartic, cysteine, metallo, and other serine proteases have been shown to be potent inhibitors of their cognate proteases, and where examined, the pro region inhibition mechanism is competitive (Fox et al., 1992; Fusek et al., 1991; Ohta et al., 1991; Segundo et al., 1982; Strausberg et al., 1993; Taylor et al., 1995; Winther & Sorensen, 1991). Many well-characterized protease inhibitors are small proteins which competitively inhibit proteases by blocking substrate access to the active site (Bode & Huber, 1992). However, all small protein protease inhibitors lack the dual functionality of folding and inhibition characteristic of pro regions (Baker et al., 1993).

This paper is a spectroscopic and biochemical exploration of the αLP pro region mechanism of inhibition. Given the αLP pro region's role in stabilization of the folding transition state, one plausible mechanism of inhibition would be for

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* To whom correspondence should be addressed. Telephone: (415)-476-2521. FAX: (415)476-1902. Email: agard@msg.ucsf.edu.

‡ Graduate Group in Biophysics.

§ Department of Biochemistry and Biophysics.

|| Howard Hughes Medical Institute.

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[1] Abbreviations: αLP, α-lytic protease; OAc, acetate; Pro-wt, wild-type pro region; Pro-1 to Pro-4, pro region mutants with one to four amino acids deleted from its C-terminus; Pro+5 and Pro+5G, pro region mutants with -APNSS and -GGGGG added to the Pro-wt C-terminus; CD, circular dichroism; SA195, αLP active site serine to alanine mutation; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; [1]S and P will be used to define the substrate and protease binding sites, respectively, according to the conventions outlined in Schechter and Berger (1967).

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the pro region, upon binding, to alter the protease structure toward that of the transition state. The αLP pro region stabilizes the folding transition state by a free energy of >17 kcal/mol which is at least 3 kcal/mol more tightly than it binds the folded native state (Sohl & Agard, 1995). Therefore, the increased affinity of the pro region for the folding transition state could drive conformational changes which could either destroy or distort the active site. Alternatively, the pro region could simply block the active site like many small protein protease inhibitors. Both of these mechanisms could manifest themselves as competitive inhibition. Data presented here prove that the pro region inhibits αLP largely, if not exclusively, by sterically occluding the substrate binding sites. The protease active site is fully formed and functional within the pro region−protease complex. Further, this study indicates that in the bound complex, the C-terminal residue of the pro region is located in the P1 site[1] of the enzyme. The implications of these results for the processing and folding of α-lytic protease are discussed. In addition, the proposed mechanism of αLP pro region inhibition is compared to data from other pro region families.

MATERIALS AND METHODS

Production and Purification of α-Lytic Protease. Lysobacter enzymogenes 495 (ATCC 29487) is used to produce wild-type α-lytic protease. Liquid cultures of *Lysobacter* were grown in modified Whitaker's medium according to the protocol of Hunkapiller et al. (1973). *E. coli* strain D1210 (Sadler et al., 1980) is used for expression of SA195 α-lytic protease. *E. coli* transformed with the bicistronic pro region SA195 construct (see below) were grown at 12 °C in pH 6.8 60 mM ACES (Sigma, St. Louis, MO) buffered LB media to an absorbance of 1.5 at 600 nm and then induced with 0.1 mM IPTG. SA195 was harvested 6 days postinduction. Both wild-type and SA195 proteases were purified from culture supernatants using ion exchange chromatography and HPLC (Mace & Agard, 1995).

Production and Purification of Pro Region. E. coli strain BL21(DE3) pLysS is used to produce wild-type and mutant pro regions as inclusion bodies. *E. coli* transformed with the appropriate pro region plasmid (see below) were grown in LB media at 37 °C. One liter of culture, shaken at 250−275 rpm, was induced with 0.1 M IPTG when the cells reached an absorbance of ∼0.8 at 600 nm. The cultures were allowed to continue shaking at 37 °C for 3−12 h after induction and then harvested by centrifugation at 4000*g* for 20 min at 4 °C. The cell pellet was subsequently frozen with a dry ice/ethanol bath, thawed, and resuspended in 15 mL of sonication buffer [50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 200 mM NaCl, 2.5 mM benzamidine hydrochloride, and 1 mM AEBSF (CalBiochem, La Jolla, CA)] per gram of wet cell pellet. The Branson Sonifier 250 was used to lyse the cells and to promote chromosomal shearing. Using a 50% duty cycle, sonication was done until the absorbance at 600 nm dropped to 10% of its expected value. After sonication, the cell extract was treated with 10 mM $MgCl_2$ and ∼10 mg of DNase and incubated at room temperature with stirring for at least 2 h. The DNase-treated cell extract was then spun down at 12000*g* for 10 min at 4 °C. The resulting pellet was resuspended by sonication in 2 mL of ddH_2O, then layered over 20 mL of 53% sucrose/1 mM EDTA/1 mM Tris, pH 8, in centrifuge tubes, and spun at 27000*g* for 30 min at 4 °C. The resulting pellet was washed with 10 mL of ddH_2O and then spun at 12000*g* for 10 min at 4 °C. After the supernatant was discarded, the pellet was gently resuspended in 2 mL of fresh 6 M urea/10 mM sodium citrate, pH 4.5. One milliliter of distilled water was added to the resuspension to reduce the urea concentration to 4 M, and any precipitate was removed by spinning at 27000*g* for 10 min at 4 °C. The resulting supernatant was loaded onto a fast flow S-Sepharose (Sigma, St. Louis, MO) column preequilibrated in 4 M urea/10 mM sodium citrate, pH 4.5, column buffer (binding capacity >10 mg of pro region/mL of resin). The column was extensively washed with column buffer prior to pro region elution via steps of 50, 100, 200, and 400 mM NaCl in the 4 M urea/10 mM sodium citrate, pH 4.5, column buffer. Pro region normally elutes in 200 mM NaCl column buffer. Urea was removed from the purified pro region fractions by dialysis against 20 mM $NaPO_4$, pH 7.2, at 4 °C using Spectra/Por dialysis tubing with a 10 000 molecular weight cutoff (Spectrum, Houston, TX).

SA195 Bicistronic Construct. In order to construct the SA195 protease mutant, a *Bam*HI/*Dra*III fragment containing part of the pro region and the protease domain open reading frames from pALPE (gift from Jack Richards of CalTech) was ligated into our high-level expression vector, pP_{A1}phoALP12 (Mace et al., 1995), also digested with *Bam*HI and *Dra*III. The resulting construct is named pP_{A1}phoALP14. pALPE provides a synthetic protease gene which results in lower G+C content and more unique restriction sites near the protease catalytic residues. Then a double-stranded oligonucleotide cassette which contains the substitution of the nucleotides GCT (Ala) for TCT (Ser) at position 195 (chymotrypsin numbering) was ligated into pP_{A1}phoALP14 digested with *Sph*I and *Dra*III. This created the *cis* construct, pP_{A1}phoALP14(S195A), which encodes for the pro region and SA195 mutant αLP as a contiguous polypeptide.

A construct in which the pro region and the wild-type protease are expressed *in trans* (as separate polypeptides) under the control of the same promoter was then created. The bicistronic plasmid, pP_{A1}phoPro-2+3phoALP12, was made by ligating two complementary oligonucleotides into pP_{A1}phoALP12 digested with *Xma*I and *Hin*dIII. The inserted oligonucleotides create a pro region with an altered C-terminus (Thr Thr → Lys Leu Lys) followed by a stop codon. The pro region open reading frame is then followed by a short linker, a second ribosome binding site, and an αLP open reading frame containing its own initiator methionine as well as a *pho*A signal sequence.

pP_{A1}phoPro-2+3phoALP14(S195A), a bicistronic construct encoding the SA195 mutant, was then created by ligating a *Bam*HI and *Age*I fragment from pP_{A1}phoPro-2+3phoALP12 into pP_{A1}phoALP14(S195A) digested with the same enzymes. The SA195 modification was confirmed by automated gene sequencing (Biomedical Resource Center DNA Sequencing Facility, UCSF, San Francisco, CA).

Pro Region C-Terminal Deletion and Extension Mutants. In order to facilitate the rapid construction of pro region C-terminal deletion and extension mutants by oligonucleotide cassette mutagenesis, a T7 polymerase-based expression vector containing a pro region open reading frame with a restriction site upstream of the 3′ end of the gene was created. pT7XmaCysPro (A.K.S., S.D.R., and D.A.A., unpublished results) contains the pro region open reading frame with a

silent mutation creating an *Xma*I site 23 base pairs from the 3′ end of the gene. Using PCR, a fragment encoding the pro region (166 residue pro region with 2 residues from the signal sequence, not including the start methionine) was created using pALP10 (John Reidhaar-Olsen and D.A.A., unpublished results) as the template. This fragment was digested with *Nde*I and *Xho*I and ligated into pHB40P (Studier & Moffatt, 1986) digested with the same enzymes creating pT7Pro. A *Bam*HI fragment from pT7XmaCysPro (containing the *Xma*I site) was ligated into pT7Pro cut with *Bam*HI, creating pT7XmaPro. All of the pro region C-terminal deletion and extension mutant constructs (encoding Pro −1, −2, −3, −4, +5, and +5G) were created by ligating the appropriate double-stranded oligonucleotide cassettes into pT7XmaPro digested with *Xma*I and *Xho*I. The local sequence surrounding the mutations was verified by double-stranded sequencing (Sanger et al., 1977).

All oligonucleotides were synthesized at The Howard Hughes Medical Institute DNA Facility, UCSF, San Francisco, CA, and designed from the published sequence (Silen et al., 1988). All restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and calf intestinal phosphatase, and Vent DNA polymerase were purchased from New England Biolabs Inc. (Beverly, MA) and/or Boehringer Mannheim Biochemicals (Piscataway, NJ). All enzymes were used in accordance with the manufacturers' instructions and buffers.

Circular Dichroism Studies. CD measurements were performed with a Jasco 710 spectropolarimeter at 5 °C. Far-UV CD spectra were collected using a 1 mm path length cuvette, and near-UV CD were measured in a 10 mm cuvette. For all spectra, sample concentrations were 12.5 μM αLP and 12.5 μM Pro-wt in 20 mM KPO_4 buffer, pH 7.2. The spectra of the αLP/pro region complex contain both at 12.5 μM concentration.

SDS−PAGE Gels. Discontinuous 5−15% SDS−PAGE gels were prepared and run as described by Laemlli (1970). Samples of αLP and pro were either acidified with 0.1 M HCl or not prior to mixing with 4× Laemlli sample buffer and boiling for 2 min. Special sample buffer was prepared with water in place of buffer in order to assess the pH dependence of complex formation. The pH was adjusted in the αLP + pro samples using a 25 mM KPO_4 and 25 mM KOAc mixed buffer. All gels were stained with 0.1% Coomassie brilliant blue R-250 (Sigma) in a mixture of 40% methanol and 10% glacial acetic acid.

Sample Preparation for Mass Spectroscopy. 1:1 molar quantities of αLP and wild-type or mutant pro region were mixed in 25 mM KPO_4/25 mM KOAc, pH 3, buffer and equilibrated for 9 min at room temperature prior to injection onto a C18 (Vydac, Hesperia, CA) column. Protein elution was followed by the absorbance at 280 nm using a 0−100% acetonitrile gradient. Pro region and protease elute separately. No additional peaks were observed as the result of protease treatment for any of the pro region samples. Purified pro region samples were lyophilized and sent for mass spectroscopic analysis.

Inhibition Measurements. α-Lytic protease activity was assayed at room temperature in pH 8 0.1 M Tris buffer with 0.1 mg/mL bovine serum albumin (fraction V, Boehringer Mannheim) and 1% DMSO. Reaction volumes of 1 mL were used. Hydrolysis of the substrate *t*-boc-Ala-*p*-nitrophenyl ester (Sigma) was monitored in a 1 cm path length cuvette by the *p*-nitrophenyl absorbance at 410 nm using a Hewlett Packard 8452A UV-VIS spectrophotometer. Pro region inhibition was assayed by substrate addition. All reactions were preincubated for 5 min at room temperature to allow for complete pro region binding (Baker et al., 1992a) prior to initiation of the assay by addition of substrate. Inhibition studies typically used a fixed protease concentration of 8 nM, pro region concentrations from 1 to 250 nM, and substrate concentrations from 30 to 484 μM, corresponding to approximately 5−70% of the observed K_m. Background ester hydrolysis in the absence of protease was subtracted from all measured rates. Lineweaver−Burk plots (Segel, 1993) were constructed for Pro-wt, Pro-3, and Pro-4, and the plot for each pro region was found to intersect on the 1/v axis. Given the high affinity of Pro-wt for αLP, the velocity data for Pro-wt were analyzed according to the Henderson equations for tight binding inhibition (Segel, 1993). The competitive form of the Henderson slope replot was found to be linear and was used for the calculation of the Pro-wt K_i (Segel, 1993). The K_i of Pro-3 was determined using the equations for partial competitive inhibition (Segel, 1993). All data fitting was done with Kaleidagraph (Synergy Software, Reading, PA).

RESULTS

Secondary and Tertiary Structural Consequences of Pro Region Binding. The interaction of the pro region with αLP was first explored utilizing peptide and aromatic circular dichroism (CD). As indicated by the crystal structure, αLP is a predominantly β-sheet protein with one short α-helix at its C-terminus (Brayer et al., 1979). Lack of significant helical content is reflected in the protease peptide CD spectrum by the absence of strong signals at 208 and 222 nms (Figure 1A), the characteristic wavelengths of helical structure. In contrast, the pro region appears to be largely helical (Figure 1A). The peptide CD spectrum of the complex is nearly identical to the sum of the individual pro region and protease spectra (Figure 1A,B), indicating that no large changes in secondary structure occur upon complex formation. The small positive peak in the peptide region difference spectrum (complex less sum, Figure 1B) may indicate the loss of some β-turn structure and/or an alteration in the environment of aromatic side chains upon complex formation. Aromatic side chains can contribute to the peptide region CD spectrum (Freskgard et al., 1994; Vuilleumier et al., 1993).

Tertiary structure changes in complex formation were probed by aromatic CD (Figure 1C). Although qualitatively similar, the complex spectrum is significantly less intense than the spectrum calculated for the sum of the αLP and pro region spectra (Figure 1D). This could be the result of tertiary structural reorganization in the complex and/or burial of surface aromatics upon complex formation. Tryptophan and tyrosine side chains are the major contributors to aromatic CD (Freskgard et al., 1994; Vuilleumier et al., 1993). While the locations of the two tryptophans and two tyrosines in the pro region are unknown, one of the two tryptophans and three of the four tyrosines in αLP are located on the protease surface. The second tryptophan of αLP is buried beneath the active site. The decreased far-UV signal of the complex may be due to rearrangements in tertiary structure which could account for pro region inhibition and/or it could be a consequence of the burial of surface aromatics and/or quenching upon complex formation.

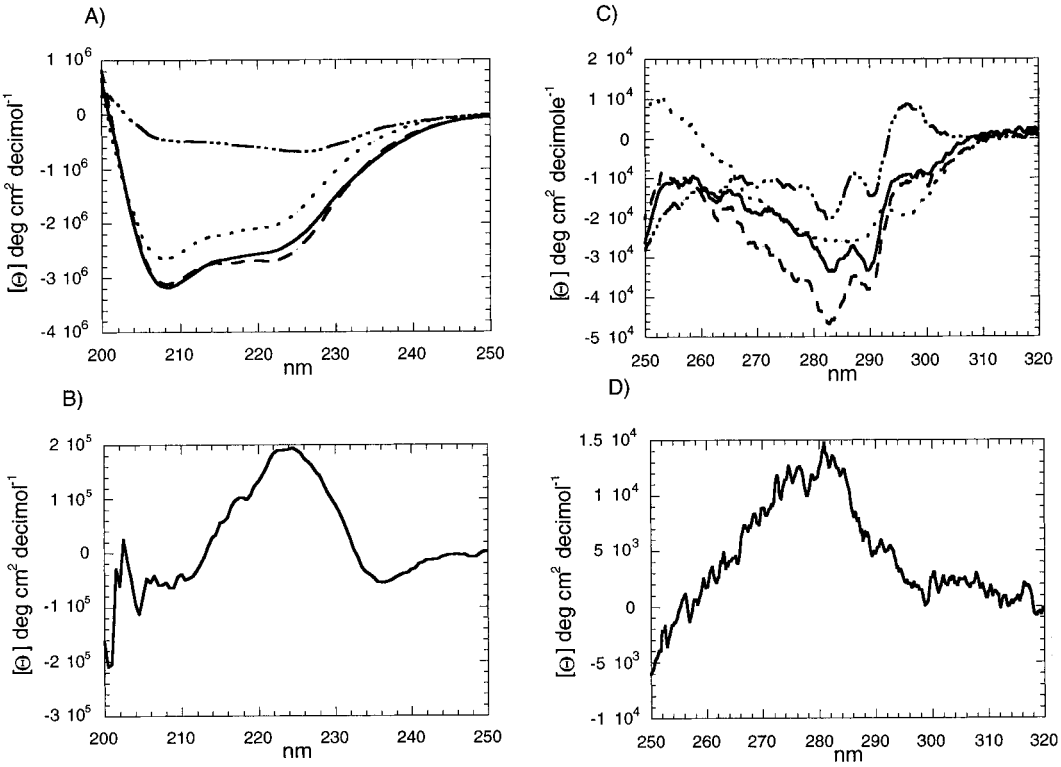

FIGURE 1: Circular dichroism of αLP and its pro region in the far-UV (A and B) and in the near-UV (C and D). In panels A and C, the following line types designate the sample: (—···—) αLP; (···) Pro-wt; (—) complex of αLP and Pro-wt. (— —) indicates the sum of the individual αLP and Pro-wt spectra. Panels B and D are difference spectra of the αLP/pro complex less the spectra of αLP and pro region individually.

Pro Region Competitively Inhibits the Protease. In order to determine if pro region binding distorts the protease active site, the mechanism of pro region inhibition was investigated. The initial characterization of pro region protease inhibition was limited by the extremely tight binding of the pro region and the sensitivity limits of the assay using the amide substrate, succinyl-Ala-Pro-Ala-*p*-nitroaniline (sucAPApNA) (Baker et al., 1992a). The data were fit to a simple equation for tight binding inhibition which yields the inhibition constant but no information on the mechanism of inhibition. However, the pro region was found to be a competitive inhibitor of the highly homologous protease, SGPB (Baker et al., 1992a), which suggests a similar mechanism for pro region inhibition of αLP. In order to assess the mechanism by which the pro region inhibits αLP, a more sensitive ester substrate, *t*-boc-Ala-*p*-nitrophenyl ester (t-bocApNPE), is used in this study. Using this substrate, Lineweaver−Burk plots are consistent with a mechanism of competitive inhibition for Pro-wt, Pro-3, and Pro-4 (data not shown); V_{max} is not changed by the presence of any of these pro regions. However, a closer examination of the data reveals that there are significant differences between the wild-type pro region and its mutants (Figure 2). As the concentration of Pro-wt increases, the initial velocity approaches zero, which is expected for competitive inhibition in the case where substrate and inhibitor compete for free enzyme (squares, Figure 2A). In contrast, high concentrations of Pro-3 do not fully inhibit substrate turnover. Instead, the velocity is eventually reduced to a nonzero minimum which is unaffected by further increases in the concentration of Pro-3 (circles, Figure 2A). The inability of Pro-3 to fully compete with substrate indicates that Pro-3 is a partial competitive inhibitor (Segel, 1993). In contrast to pure competitive inhibition, the substrate can bind to a partial competitive

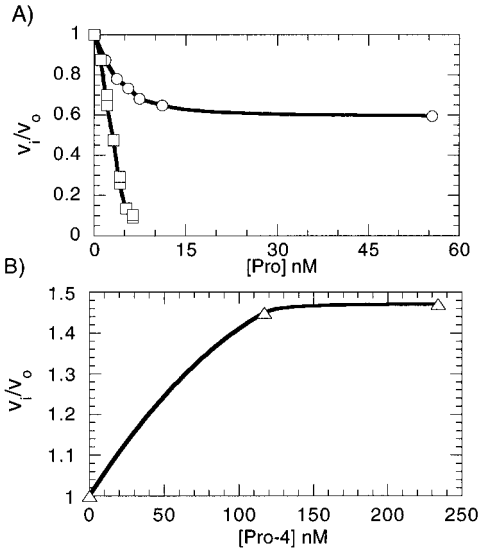

FIGURE 2: Initial velocity as a function of pro region concentration. At constant t-bocApNPE (120 μM), varying amounts of pro region were added, and the resulting initial velocities (v_i) are plotted for Pro-wt (squares) and Pro-3 (circles) in panel A and for Pro-4 (triangles) in panel B. Velocities are normalized by the activity in the absence of pro region (v_0). Interpolated linefits are shown.

inhibitor−enzyme complex, EI, forming ESI. In addition, the inhibitor can bind the enzyme−substrate complex, forming ESI. V_{max} is unchanged in partial competitive inhibition because both ESI and ES turn over at the same rate. Therefore, as the inhibitor concentration is increased, the velocity cannot be driven to zero because ESI can turn over substrate. However, the observed velocity is reduced in the presence of the Pro-3 inhibitor due to the ∼1.8 times higher K_m of the substrate for the EI complex (data not shown). Pro-4 addition does not inhibit substrate turnover

Table 1: Inhibition as a Function of Substrate Length

	sucAPApNA[a]		t-bocApNPE	
	K_i (nM)	mechanism	K_i (nM)	mechanism
Pro-wt	0.1	tight binding[b]	0.1	competitive
Pro-3	1.7	tight binding	2.4	partial competitive
Pro-4	~100	tight binding	−	none[c]

[a] Pro-wt K_i value is taken from Baker et al. (1992a). Pro-3 and Pro-4 K_i values are taken from R. J. Peters, A.K.S., J.L.S., and D.A.A., unpublished data. [b] K_i values with sucAPApNA were determined using equations for tight binding inhibition as described in Baker et al. (1992a). [c] Pro-4 binding leaves k_{cat} unchanged. However, instead of increasing the apparent substrate K_m, which would be the case for a competitive inhibitor, Pro-4 actually decreases the K_m.

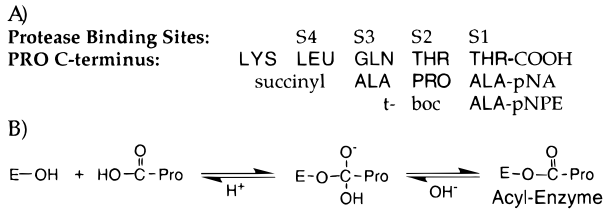

FIGURE 3: Inhibition data suggest pro region active site interactions. (A) The observation that Pro-4 is a strong inhibitor using the long substrate but shows no inhibition with the short substrate suggests that the pro region C-terminus may bind in the S-sites on the protease as shown. The longer substrate would not be able to efficiently bind in the presence of Pro-4 due to steric clashes with the succinyl group. However, no steric conflict would be present with the short substrate and Pro-4. (B) If the pro region binds in a product-like manner, it should be in equilibrium with the covalent acyl-enzyme intermediate formed between the pro region C-terminus and the catalytic serine of the protease as diagrammed here.

whatsoever. Instead, addition of Pro-4 increases the observed velocity (Figure 2B). This is achieved by Pro-4 increasing the affinity of the substrate for the protease, thereby reducing the apparent K_m. The Lineweaver−Burk plots show that Pro-4 binding does not change V_{max}. Therefore, by the extremely sensitive parameter of V_{max}, both Pro-3 and Pro-4 clearly do not perturb the catalytic machinery upon binding to the protease. We are unable to determine if the competitive inhibition of Pro-wt leaves the active site intact or not. Competitive inhibition can be achieved either by blocking substrate binding (as Pro-3 is partially able to do) or by distortion of the active site such that its catalytic activity is not detected in the assay.

The calculated K_i values for the ester substrate, t-bocApNPE, are shown in Table 1 (data not shown). The K_i values from the tight binding analysis with longer amide substrate, sucAPApNA, are listed for comparison. K_i values for both Pro-wt and Pro-3 show little change between the amide and the smaller ester substrate. Pro-4, however, is an inhibitor with the amide substrate but shows no inhibition with the ester substrate. Based on the ester substrate analysis above, Pro-4 binding of the protease does not alter the protease V_{max} value. Assuming this is also true for the longer amide substrate, the Pro-4 inhibition of the amide substrate must be due to steric interactions that do not occur with the shorter ester substrate. A model which is consistent with this would place the pro region C-terminus in a product-like conformation in the P sites of the protease (Figure 3A). The ester substrate length, which is less than four amino acids, would be able to access the P-sites on the protease needed for effective binding with Pro-4 bound to αLP. In contrast, the amide substrate binding to αLP would be altered with Pro-4 due to the increased size of the substrate (effective length is approximately five amino acids due to the succinyl group). Modeling studies suggest that the t-boc substrate group could extend as far as the S3 site of the protease which would be partially blocked by the carboxylate of Pro-3 (data not shown). This may account for the partial competitive inhibition seen in Pro-3 with the ester substrate.

The Pro Region C-Terminus Lies in a Product-like Conformation on αLP. The kinetic data suggest that the pro region C-terminus lies in the S-sites of the protease with the C-terminal pro region residue in S1 (Figure 3A). The positioning of the pro region is predicted to be similar to that of a proteolysis product in the binding pocket. However, unlike a normal proteolysis product, the pro region does not rapidly dissociate from the protease but remains tightly bound. If this model of pro region/active site interactions is correct, we should be able to trap an acyl-enzyme between the pro region and protease. The acyl-enzyme is a covalent intermediate formed between the catalytic serine and the carbonyl of the scissile peptide bond in the course of a proteolysis reaction. In the absence of product release after proteolysis, the acyl-enzyme should remain in equilibrium with the reaction products (Figure 3B). The acylation and deacylation rates which form the basis of this equilibrium are reduced at acidic pH. Therefore, it should be possible to trap an acyl-enzyme product in a preexisting equilibrium by dropping the pH.

Pro region and αLP form a high molecular weight complex at acidic pH not separable by SDS−PAGE (Figure 4A, WT_A). The molecular weight of this complex is similar to that of the covalent proαLP precursor (PC). Formation of the αLP and pro region complex at neutral pH does not yield the high molecular weight band (WT_N). Instead, degradation products are observed due to αLP proteolytic activity during denaturation. Prolonged sample boiling or neutralization of the low-pH sample after boiling (to promote disulfide reduction) does not disrupt the covalent complex (data not shown). Therefore, the high molecular weight complex does not originate from incomplete denaturation and/or reduction of the three disulfides in the protease domain (there are no disulfides in the pro region). Analysis of the high molecular weight species by Western blotting with both anti-pro region and anti-αLP antibodies and N-terminal sequencing revealed that both molecules are present and that both N-termini are free (data not shown). Under conditions in which wild-type active protease can form the high molecular weight complex, no such complex was seen with a mutant in which the active site serine had been mutated to alanine, SA195 (Figure 4B, SA_A, SA_N). The same result was observed with protease inhibited by incubation with AEBSF which covalently modifies the active site serine (data not shown). Further investigation of the pH dependence of complex formation (Figure 4C) reveals that the amount of complex trapped by SDS denaturation increases with decreasing pH.

The model of pro region S-site interactions predicts that the pro region C-terminus provides the necessary carboxylate for the acylation reaction. This hypothesis was tested using a series of C-terminal deletions in the pro region. The last five amino acids of the wild-type pro region are --KLQTT. Deletions of one to four amino acids from the pro region C-terminus were assayed for their ability to form the high molecular weight complex (Figure 5). Deletion of even one

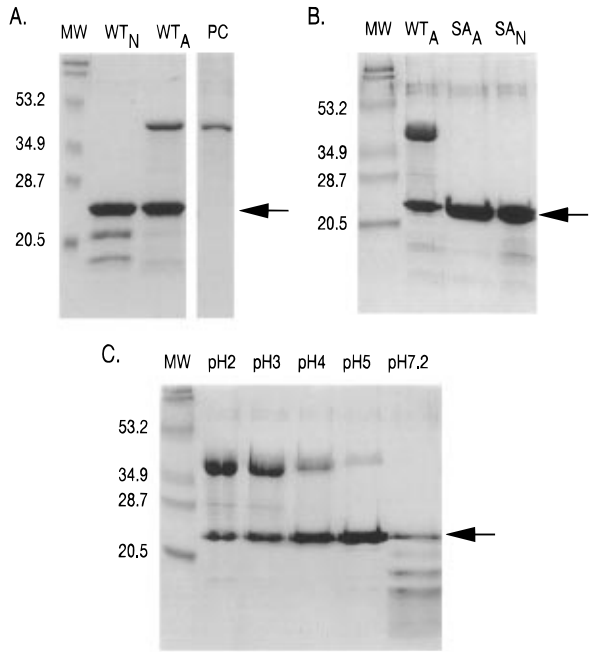

FIGURE 4: αLP and pro region form a putative acyl-enzyme complex trapped at low pH. (A) Addition of HCl prior to denaturation of αLP and pro region in trans complex results in a high molecular weight band (WT_A) of a similar size to the covalent proαLP precursor (PC). If no HCl is added, the precursor sized band is not observed (WT_N). Subscripts "A" and "N" denote acidic and neutral denaturation, respectively. (B) The active site mutant SA195/pro region complex cannot form the high molecular weight band (SA_A) under conditions in which wild-type protease/pro region can (WT_A). (C) The pH of the SDS−PAGE sample denaturation was varied as indicated. αLP and pro region migrate separately at approximately the same rate shown by the arrow. Denaturation at neutral pH results in additional low molecular weight bands produced by proteolysis.

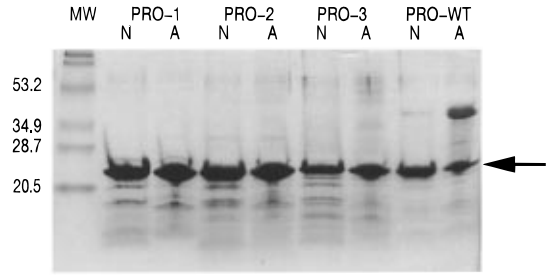

FIGURE 5: Pro region C-terminal deletion mutants. Pro region C-terminal deletions of one to four amino acids cannot form the putative acyl complex with αLP under conditions in which Pro-wt can (data not shown for Pro-4). "A" denotes addition of HCl which results in acidic sample denaturation. "N" denotes neutral pH sample denaturation. The arrow indicates the size at which αLP and pro region migrate when uncomplexed.

amino acid from the pro region C-terminus abolishes the ability of the pro region to form the covalent complex. All of these pro region mutants have been shown to tightly bind the protease (R. J. Peters, A.K.S., and D.A.A., unpublished results), yet none of them are able to form the covalent complex.

These data support the model of pro region C-terminal interactions in the protease active site. The pro region and protease are trapped in a putative acyl-enzyme at low pH which requires both the catalytic serine and the full-length wild-type pro region.

Protease Active Site Is Functional with the Pro Region Bound. The product-like positioning of the pro region

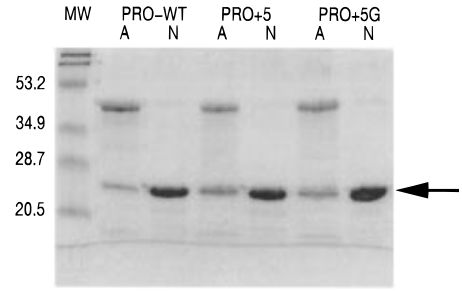

FIGURE 6: Pro region C-terminal extension mutants. Pro+5 (5) and pro+5G (5G) can form the acyl complex with αLP at low pH. "A" denotes addition of HCl which results in acidic sample denaturation. "N" denotes neutral pH sample denaturation. The arrow indicates the size at which αLP and pro region migrate when uncomplexed.

Table 2: Pro Extension Size[a] Before and After αLP Binding at pH 3

	calculated mass	mass before αLP	mass after αLP
Pro-wt	17914	17914	17914
Pro+5	18371	18371	17914
Pro+5G	18199	18199	17914

[a] All mass measurements were done by David King.

accounts for the inhibition patterns seen for Pro-4 and Pro-3 with the long and short substrates. The inhibition (or lack thereof) seen in both pro region mutants is the result of pro region C-terminal interactions with the S-sites on the protease which block these sites, thus preventing substrate binding. While the pro region mutant data support a similar mechanism of Pro-wt inhibition, it is possible that Pro-wt is able to elicit distortions in the active site not seen in the pro region C-terminal deletions. Pro-wt makes additional interactions immediately adjacent to the catalytic machinery which are not present in the pro region deletion mutants. The ability of the Pro-wt/αLP complex to form an acyl-enzyme suggests that the protease active site must be at least partially functional with Pro-wt bound. However, the most stringent test of active site functionality in the complex is to ascertain its ability to carry out a complete amide cleavage reaction. The experiments with the pro region C-terminal deletions indicate a strict length requirement for acyl-enzyme formation. This suggests that if additional amino acids are added to the C-terminus of the pro region, they will lie across the active site catalytic machinery in a substrate-like manner.

Two pro region variants having five additional amino acids on the C-terminus, -APNSS in Pro+5 and -GGGGG in Pro+5G, were assayed and found to form an acyl-enzyme complex (Figure 6). The pH dependence of acyl-enzyme formation for these mutants is similar to that of the wild-type pro region (data not shown). In order to determine which residue of each of the extended pro regions actually forms the complex, samples of these mutant pro regions were mixed with the protease at acidic pH and incubated at this pH prior to preparation for mass spectroscopy. Mass spectroscopic analysis of the pro C-terminal extensions before and after exposure to αLP revealed that the mutant masses shift entirely to the wild-type pro region mass when exposed to the protease (Table 2). Therefore, the acyl-enzyme complex is formed not at the extended pro region C-terminus but instead precisely at the wild-type C-terminus. Cleavage of the pro region extensions demonstrates that the protease is catalytically active when in complex with the pro region.

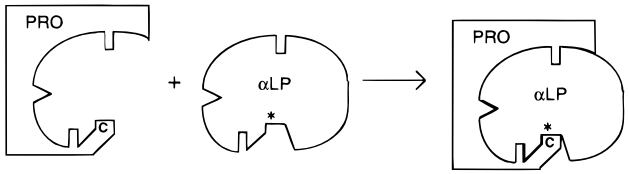

FIGURE 7: Model of αLP and pro region interactions. The asterisk denotes the active site serine; "C" symbolizes the pro region C-terminus.

DISCUSSION

Model for αLP Pro Region Mechanism of Inhibition. Spectroscopic, biochemical, and kinetic data suggest a model for pro region interactions in the active site of αLP (Figure 7). The pro region C-terminal threonine lies in the S1 site of the protease in an acylation-competent conformation. This model is supported by the requirement of both wild-type length pro region and the active site serine hydroxyl along with the patterns of pH dependence observed for covalent complex formation. Pro region C-terminal extensions are able to form the acyl-enzyme complex only after they are efficiently cleaved to the wild-type length. Unlike small substrates, the tight binding of the pro region prevents normal product release. Instead, the C-terminus of the pro region presumably remains in a constant state of cycling between being free and covalently coupled as an acyl-enzyme with the rate of cycling determined by the solvent pH.

Specific interactions between the pro region and αLP are suggested by the pro region mutant data. The C-terminus of the pro region must extend across the S-sites, making specific interactions in and around the S-sites on the protease. The nearly identical Pro-wt K_i values for the long and short substrates suggest that the C-terminus makes specific interactions with the S-sites proximal to the catalytic serine. If the C-terminus was not making persistent contacts with the active site, the inhibition constant measured with the shorter substrate should be greater than that measured with the longer substrate. In addition, Pro-3 is still a potent inhibitor of the protease, which suggests that the pro region is making specific interactions in P4. Interactions near the active site are supported by the observation that protease inhibition by Pro-4 is detected with the longer peptide substrate. This suggests that the final residue of Pro-4 interacts in a "P5"-like position which can interfere with longer substrate access to the active site. Specific pro region/active site interactions are further supported by the precise cleavage of the pro region extension mutants, Pro+5 and Pro+5G. If specific pro region/active site interactions were not present, the pro region C-terminal extensions, in particular the polyglycine mutant, would not be expected to be cleaved at the length of the wild-type pro region. Interactions distal to the active site are likely to be present as well which tether the C-terminus, thus preventing the Pro-1 mutant from forming the acyl-enzyme even though it presents the same C-terminal amino acid as Pro-wt.

The circular dichroism spectrum of the complex suggests that little secondary structure change occurs upon complex formation. Spectra which monitor tertiary structure do suggest that some alterations in the environments of aromatic side chains occur upon the binding of the pro region to αLP. It is unlikely that any large-scale tertiary structural changes have occurred in the protease given that the protease is fully active within the complex. Instead, the observed changes in the environments of the aromatic residues are likely to result from burial and/or quenching of surface aromatics in the complex due to interactions between the pro region and protease outside the active site. While changes in pro region structure cannot be ruled out, we favor a model in which complexation leads to little or no change in the secondary structure or tertiary structure of either the pro region or the protease (Figure 7).

It is clear that Pro-wt inhibition has a steric component. However, competitive inhibition could result from Pro-wt sterically blocking substrate binding and/or distortion of the active site. The observation of cleavage of the pro region C-terminal extensions when complexed to αLP argues that pro region binding across the active site does not destroy the catalytic activity. In fact, a comparison of the cleavage rates expected at pH 3 for free αLP to the estimated rate of pro region extension cleavage suggests that the active site is not significantly perturbed with pro region bound. The observed rate for cleavage of Pro+5 and Pro+5G at pH 3 is estimated to be $0.0008-0.002\ s^{-1}$ (taken from the incubation time to elution time off the reverse phase column). This is similar to the rate that would be expected for a good substrate at pH 3, 5 pH units from that optimal for protease activity (Mace et al., 1995; Whitaker, 1970). The similarity of the rates of pH 3 pro region extension cleavage to that of free αLP suggests that the protease catalytic machinery is not compromised when the pro region binds across the active site and that the mechanism of Pro-wt competitive inhibition is entirely steric as seen in Pro-3 and Pro-4.

Data Suggest an Intramolecular Processing Mechanism for the αLP Precursor. α-Lytic protease is made as a pre-pro-protease. Maturation of the protease domain involves removal of the pre sequence or export signal sequence by signal peptidase after targeting to the periplasm followed by cleavage at the junction between the pro region and protease domain. Previous experiments have shown that αLP activity is necessary for the processing reaction (Silen et al., 1989). However, it is not known whether processing of the αLP precursor is an inter- or intramolecular reaction. The αLP crystal structure reveals that its N-terminus is far from the active site and is partially buried (Brayer et al., 1979). The experiments presented in this paper suggest that the pro region C-terminus lies across the S-sites of the protease in a product-like manner. Therefore, if the processing event is intramolecular, it is most likely coupled to a significant conformational change of the αLP N-terminus, leaving the pro region C-terminus in the active site. Conversely, if processing is intermolecular, the pro region C-terminus must rearrange into the protease active site after processing. The crystal structure of subtilisin and its pro region in a *trans* complex shows that the subtilisin N-terminus is far from the active site and that its pro region C-terminus lies in the S-sites (Gallagher et al., 1995). For both αLP and subtilisin, rearrangement of the C-termini of their pro regions back into a substrate-like conformation following cleavage seems unlikely. As proposed for subtilisin based on the structure in complex with its pro region (Gallagher et al., 1995), the location of the αLP pro region C-terminus suggests an intramolecular mechanism of αLP processing.

In αLP, the folded protease is able to process pro region variants having additional C-terminal amino acids precisely at the wild-type pro region length. This was observed for pro region mutants whose extension sequences do not match

the αLP N-terminal sequence. This suggests that the interactions which specify cleavage at the correct pro region/αLP junction reside in the C-terminus of the pro region and possibly the main chain of the αLP N-terminus.

Implications for the Pro Region Folding Mechanism. The observation that the αLP catalytic machinery is fully functional with Pro-3 and Pro-4 bound suggests that the pro region is highly complementary to the free folded protease. Given this observation, the simplest mechanism of pro region-assisted protease folding would be the pro region providing a template of the native state. The high affinity the pro region has for the native state suggests that the folding transition state must have native-like character. However, energetic analysis of the protease folding reaction with and without the pro region suggests that the pro region binds the folding transition state at least 3 kcal/mol more tightly than it binds the folded protein (Sohl & Agard, 1995). Though native state binding measurements must be reevaluated as K_i measurements only provide upper limits (see below), the magnitude of the transition state stabilization implies that the pro region makes additional favorable contacts with the protease which enthalpically stabilize the transition state and/or the presence of the pro region reduces the entropy of the transition state ensemble. Mutations have been identified which preferentially alter the free energy of the pro region mediated αLP folding transition state (R. J. Peters, A.K.S., and D.A.A., unpublished results). However, the data presented here indicate that structural distortions of the protease in the folding transition state are relieved once the final state of folded protease and pro region has been achieved.

Pro region C-terminal deletions have been assayed for their ability to refold the protease. Significantly, deletion of three amino acids from the pro region C-terminus reduces the rate of *trans* refolding by over 300-fold, and no refolding is detected using the Pro-4 mutant (R. J. Peters, A.K.S., and D.A.A., unpublished results). For subtilisin, the effects of C-terminal pro region deletions on folding and inhibition have also been assessed. Interestingly, as two and four C-terminal residues were removed from the subtilisin pro region, there was a concurrent loss in both inhibition, as measured by titration calorimetry, and folding capabilities (Wang et al., 1995). Deletions of ≥18 amino acids from the C-terminus of this pro region result in no detectable refolding or inhibitory activity (Li et al., 1995; Ohta et al., 1991). In contrast, αLP pro region C-terminal deletions do retain significant binding affinities (as measured with the large substrate) (Table 1). However, these measurements provide only a lower limit of the true binding affinity of the pro region for the protease due to their apparent dependence on substrate length as a result of steric effects. It is striking that in both systems, large decreases in refolding rates are seen when pro regions with small deletions in the C-termini are used. Given the location of these amino acids in the respective αLP and subtilisin pro region complexes, determined in this study and from the crystal structure of the subtilisin/pro region complex, the refolding data indicate that active site interactions with the pro region C-terminus are crucial for stabilizing both folding transition states. Data suggest that other pro regions interact with their protease's active sites (see below). Further experiments on these systems will reveal whether pro region/active site interactions are a general feature of pro region-mediated folding catalysis.

Steric Occlusion as a General Pro Region Inhibition Mechanism. The inhibition mechanism(s) of pro regions appear(s) to be conserved within protease families. Many serine, cysteine, aspartic, and metallo protease pro regions can inhibit homologous proteases with high affinity relative to their native protease (Baker et al., 1992a; Fox et al., 1992; Fusek et al., 1991; Segundo et al., 1982; Taylor et al., 1995; Li et al., 1995). For several proteases, structural and biochemical data are available which allow a comparison of inhibition mechanisms across these protease families.

Crystal structures of both the free protease and the covalent or *cis* proprotease complex have been determined for the metallo proteases carboxypeptidases A and B and the aspartyl protease pepsin (Coll et al., 1991; Guasch et al., 1992; Hartsuck et al., 1992; James & Sielecki, 1986). A comparison of the free protease to the pro region bound-protease structures, including the positioning of the catalytic residues, shows that the protease structures remain virtually unchanged. These proprotease structures reveal that pro region inhibition is most likely due to pro region-mediated occlusion of the active site catalytic residues and substrate binding cleft and not large distortions of the active site. Spectra of proCPA and CPA which are sensitive to the catalytic metal ion position are virtually identical, further supporting the existence of a fully functional active site in proCPA (Behnke & Vallee, 1972).

The structure of procathepsin B, a cysteine protease in the papain superfamily, reveals that its pro region blocks access to the active site by interactions with the substrate binding cleft in a reverse orientation relative to those of a typical substrate (Cygler et al., 1996). The structures of the protease in procathepsin B and cathepsin B are virtually identical with the exception of one surface loop. In fact, the catalytic cysteine of papain can be labeled by a specific inhibitor when in the *cis* propapain complex (Vernet et al., 1991). Taken together, these results suggest a functional active site in cysteine proprotease complexes.

The crystal structures of free subtilisin and subtilisin with its pro region *in trans* show no significant differences in the catalytic triad or elsewhere (Bryan et al., 1995; Gallagher et al., 1995). The structure of the complex reveals that the pro C-terminus lies as substrate would in the S-sites with the final pro region residue in the S1 site. No biochemical data are available to assess the contribution of catalytic strain to the observed pro region competitive inhibition of subtilisin (Ohta et al., 1991). However, the similarity of the positioning of their pro region C-termini strongly suggests that subtilisin and αLP, two nonhomologous serine proteases, share a highly conserved mechanism of pro region inhibition.

Biochemical studies of the pro region interactions with the active site have been published for the serine protease carboxypeptidase Y (CPY). The active site of procarboxypeptidase Y was probed for accessibility to bulky modification reagents. Interestingly, both S1 and S1′ were found to be modified but not the catalytic serine in the complex (Sorensen & Winther, 1994). These results led the authors to propose a catalytic strain mechanism of pro region inhibition for CPY. The CPY pro region may then in fact act differently than other pro regions. However, additional data will be necessary to fully address the contributions of small active site distortions in more pro region inhibition mechanisms before making such a distinction.

It is quite striking that not only within but also across protease families, pro regions are found which inhibit protease activity not by active site conformational rearrangement but by simply blocking access to the protease active site. In contrast to most small protein protease inhibitors, it is clear that interactions outside the active site make significant contributions to the pro region binding affinity. In the case of αLP, despite removal of four active site residues, the pro region still binds with at least 100 nM affinity as shown by the large substrate inhibition (R. J. Peters, A.K.S., J.L.S., and D.A.A., unpublished results). In addition, the structures of pro region−protease complexes all show extensive pro region contacts with the protease outside the active site. However, despite these additional contacts, the protease structures appear virtually unchanged with pro region bound. These data suggest that as seen for αLP, steric occlusion of the active site accounts for at least one general mechanism of pro region inhibition. Pro regions, even with their uniquely evolved roles in protease folding catalysis, do not appear to deviate from canonical protease inhibitory mechanisms.

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