Catalysis of a Protein Folding Reaction: Thermodynamic and Kinetic Analysis of Subtilisin BPN′ Interactions with Its Propeptide Fragment†

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ABSTRACT: The in vivo folding of subtilisin is dependent on a 77 amino acid propeptide, which is eventually cleaved from the N-terminus of subtilisin to create the 275 amino acid mature form of the enzyme (Ikemura et al., 1987). We have cloned, expressed, and purified large quantities of the 77 amino acid subtilisin propeptide. This has enabled us to characterize its participation in the subtilisin folding reaction by spectroscopic and microcalorimetric methods. Unfolded subtilisin, when returned to native conditions, is kinetically isolated from its native state. Folding of subtilisin with the native calcium site-A is extremely slow even in the presence of a high concentration of isolated propeptide. The folding of a calcium-free mutant subtilisin, however, is readily catalyzed by the isolated propeptide. The propeptide–subtilisin folding reaction can be described as the following equilibrium: $P_u + S_u \Leftrightarrow P\text{–}S \Leftrightarrow P_f\text{–}S_f \Leftrightarrow P_u + S_f$, where S_u and P_u are subtilisin and propeptide, respectively, which are largely unstructured at the start of the reaction; P–S is a collision complex of unfolded subtilisin and propeptide; P_f–S_f is the complex of folded subtilisin and propeptide; and S_f is folded subtilisin. The rate-limiting step in the folding reaction of calcium-free mutant subtilisin is formation of the initial collision complex, P–S. The rate at which P_u and S_u form a productive collision complex is $\sim$500 M^{-1} s^{-1}. The collision complex appears to be an early folding unit which, once formed, results in rapid isomerization to the fully folded complex. The rate constant for isomerization of the collision complex to the folded complex is $\geq$0.5 s^{-1}. Once folded, propeptide and subtilisin form a tight complex with a K_a of 2×10^8 M^{-1} at 25 °C. The energetics of binding the propeptide to the folded forms of either calcium-free subtilisin or subtilisin with the native calcium A-site are similar and are typical of the folding reaction of a small globular protein.

The *Bacillus* serine protease subtilisin is an unusual example of a monomeric protein with a high kinetic barrier to folding and unfolding (Bryan et al., 1992). Biosynthesis of subtilisin is dependent on a 77 amino acid propeptide, which is eventually cleaved from the N-terminus of subtilisin to create the 275 amino acid mature form of the enzyme (Ikemura et al., 1987). The probable role of the propeptide in subtilisin folding was recently illuminated by analogy with another extracellular microbial protease, α-lytic protease from *Lysobacter* (Baker et al., 1992b). Both subtilisin and α-lytic protease are extracellular, bacterial, serine proteases, though they are not evolutionarily related. α-Lytic protease has a 166 amino acid propeptide which has been shown to catalyze folding as a fusion protein with glutathione transferase (Baker et al., 1992a). As in subtilisin, the folded and unfolded forms of α-lytic protease are separated by a high kinetic barrier. The propeptides of α-lytic protease and subtilisin are strong competitive inhibitors of their respective enzymes (Baker et al., 1992a; Zhu et al., 1989). The propeptide of α-lytic protease has been proposed by Baker et al. to bind to, and therefore stabilize, a native-like transition state in the folding reaction. The 77 amino acid propeptide of subtilisin has been shown to promote subtilisin folding, and mutations have been identified which abolish its function in vivo (Kobayashi & Inouye, 1992). The role of these propeptides in faciliating protein folding is different from the Gro E-type chaperons in two major respects. First, propeptides appear to promote folding by accelerating the folding reaction rather than preventing competing off-pathway reactions (e.g., aggregation). Second, the propeptides are protein specific in their function.

We have cloned, expressed, and purified large quantities of the 77 amino acid subtilisin propeptide. This has enabled us to characterize its interactions with subtilisin in the folding reaction by spectroscopic and microcalorimetric methods. These studies focus on (1) the physical nature and energetics of propeptide interactions with native subtilisin and (2) how the propeptide acts to catalyze folding.

A model for the propeptide–subtilisin folding reaction can be described as the following equilibrium:

$$P_u + S_u \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} P\text{–}S \underset{k_{-2}}{\overset{k_2}{\Leftrightarrow}} P_f\text{–}S_f \underset{k_{on}}{\overset{k_{off}}{\Leftrightarrow}} P_u + S_f$$

where P_u is propeptide and S_u is subtilisin, which are largely unstructured at the start of the reaction; P–S is a collision complex of subtilisin and propeptide; P_f–S_f is the complex of folded subtilisin and propeptide; and S_f is folded subtilisin. In this reaction scheme, the P_u is acting as an enzyme in the folding of subtilisin. The goal of this series of experiments is to characterize all the steps in this reaction. The first step will be to characterize the binding of propeptide to folded subtilisin. The second step will be to determine the energetics of subtilisin folding in the presence of the propeptide.

Since unfolded forms of subtilisin and propeptide are very sensitive to proteolysis, it was necessary to decrease the native proteolytic activity of subtilisin. This was accomplished by

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[1] The specific activities of all S221C mutants discussed here are similar against the synthetic substrate, sAAPFna. (S.A. $\sim$ 0.0025 unit/mg at 25 °C, pH 8.0).

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Table I: Subtilisin Mutations[a]

	S221C	Δ75–83	N218S	M50F	Y217K
S12	+	–	+	+	+
S15	+	+	+	+	+

[a] The plus signs show that a subtilisin contains a particular mutation. The wild-type numbering of amino acids is used for simplicity in defining mutations even when the nine amino acids (75–83) have been deleted. X-ray crystal structures of S12 and S15 have been determined to be 1.8 Å (Bryan et al., 1992; Gallagher et al., 1993).

converting the active-site serine 221 to cysteine by site-directed mutagenesis.[1] This mutation reduces peptidase activity to a level that is measurable but no longer problematic for folding studies. The S221C mutant has become a vehicle for all of our characterizations of the folding and unfolding reactions (Bryan et al., 1992).[2]

Previous efforts to understand the physical and energetic nature of the kinetic barriers to subtilisin folding have established that the high-affinity calcium site-A imposes a large enthalpic barrier to folding and unfolding (Bryan et al., 1992). Removing the calcium site-A from subtilisin by deleting amino acids 75–83 enormously accelerates both the unfolding and refolding reactions. In this report we investigate the interactions of the propeptide with two S221C subtilisins: one with the native calcium A-site (S12) and one without (S15). The mutations in S12 and S15 subtilisins are described in Table I.

MATERIALS AND METHODS

Cloning, Mutagenesis, and Expression of Subtilisin Mutants. The subtilisin gene from *Bacillus amyloliquefaciens* (subtilisin BPN′) has been cloned, sequenced, and expressed at high levels from its natural promoter sequences in *Bacillus subtilis* as previously described (Bryan, 1992; Vasantha et al., 1984; Wells et al., 1983).

Mutant genes for S12 and S15 (Table I) were recloned into a pUB110-based expression plasmid and used to transform *B. subtilis*. The *B. subtilis* strain used as the host contains a chromosomal deletion of its subtilisin gene and therefore produces no background wild-type activity. Oligonucleotide mutagenesis was carried out as previously described (Bryan et al., 1986). S12 and S15 subtilisins were expressed in a 1.5 l New Brunswick fermenter at a level of ~150 mg of the correctly processed mature form/L. The addition of wild-type subtilisin to promote production of the mature form of S221C subtilisin was not required in our bacillus host strain as was the case for Abrahmsen et al. (1991). Variant S221C subtilisins S15 and S12 were purified and verified for homogeneity essentially as described (Bryan et al., 1986; Pantoliano et al., 1987, 1988).

Cloning of the Propeptide of Subtilisin. The propeptide region of the subtilisin BPN′ gene was subcloned using the polymerase chain reaction in an Eppendorf MicroCycler according to conditions outlined in the GeneAmp PCR reagent kit. Oligonucleotides were synthesized which amplified the coding sequence for two versions of the propeptide: the 77 amino acid version, corresponding to the normal C-terminal cleavage site, and an 81 amino acid version which includes the first four amino acids of mature subtilisin (AQSV) at the C-terminus. Digestion of the amplified product with the appropriate restriction enzymes allowed a precise excision of the DNA that codes for either the 77 or 81 amino acid propeptide which was precisely fused to the ATG initiation codon of an *Escherichia coli* expression plasmid with an IPTG-inducible promoter.[3] The strategy is identical to that described in detail for high-level production of the 56 amino acid protein G, B-domain (Alexander et al., 1992a).

Fermentation and Expression of Propeptide. The *E. coli* production strain was grown at 37 °C in a 1.5-L BioFlo Model fermenter until an A600 1–1.5 was attained, at which time 1 mM IPTG was added to induce the production of T7 RNA polymerase that directs synthesis of target DNA message (Alexander et al., 1992). Two hours after induction the cells were harvested.

Protein Purification of Propeptide. *E. coli* paste from a 1.5-L fermentation (5 g) was suspended in 50 mL of cold phosphate-buffered saline (PBS), and PMSF was added to a final concentration of 1 mM. DNase I (1 mg) in 2 mL of 40 mM Tris-HCl and 1 M $MgCl_2$ was also added. This suspension was heated to 80 °C for 5 min. After the reaction was cooled on ice, another addition of PMSF and DNase I was made. This mixture was centrifuged at 25000*g* for 30 min (Alexander et al., 1992a).

The soluble, heat-released protein was dialyzed extensively against 20 mM HEPES, pH 7.0, and purified to homogeneity by anion-exchange chromatography with DE52 followed by cation-exchange chromatography using SE53. Five grams of *E. coli* paste yields 30 mg of purified propeptide.

N-Terminal Analysis. The first five amino acids of the purified propeptide were determined by sequential Edman degradation and HPLC analysis. This revealed that >95% of the purified material had the amino acid sequence expected from the DNA sequence of the gene (N-terminal sequence of AGKSN). The N-terminal methionine synthesized in the *E. coli* production system was absent in the final product. A 56 amino acid propeptide breakdown product was also purified. The N-terminus of this fragment begins at the sequence SAAKK.

Determination of Extinction Coefficient. The propeptide has four tyrosines and no tryptophans. Since the peptide is disordered at low ionic strength, the extinction of free tyrosine (ϵ = 1413 M^{-1}) was used to estimate the extinction of propeptide (5650 M^{-1}). The molecular weight of the 77 amino acid propeptide is 8475. This yields A_{275} of 0.67 = 1 mg/mL.

Titration Calorimetry Measurements. Calorimetric titrations were performed with a Microcal Omega titration calorimeter as described in detail by Wiseman et al. (1989). The titration calorimeter consists of a matched reference cell containing the buffer and a solution cell (1.374 mL) containing the subtilisin solution. Aliquots of the pro solution can be added to the cell through a rotating stirrer syringe operated with a plunger driven by a stepping motor. The accompanying heat change per injection is determined by a thermoelectric sensor between the cells. The area of each peak represents the amount of heat accompanying binding of the added ligand to the protein. The total heat, Q, is then fitted by a nonlinear least squares minimization method (Wiseman et al., 1989) to

[2] A shorthand for denoting amino acid substitutions employs the single-letter amino acid code as follows: Y217K denotes the change of Tyr 217 to Lys.

[3] Abbreviations: CD, circular dichroism; Δ75–83 subtilisin, subtilisin BPN′ with a deletion of amino acids 75–83; cps, counts per second; EDTA, disodium salt of ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; K_a, association constant for propeptide binding; [P], propeptide concentration; [S], subtilisin concentration; sAAPFna, succinyl-(L)-Ala-(L)-Ala-(L)-Pro-(L)-Phe-*p*-nitroanilide; Tris, tris(hydroxymethyl)aminomethane; $t_{1/2}$, half-life for a kinetic experiment.

the total ligand concentration, $[P]_{total}$, according to the equation:

$$dQ/d[P]_{total} = \Delta H[1/2 + (1 - (1 + r)/2 - X_r/2)/ (X_r - 2X_r(1 - r) + 1 + r^2)^{1/2}] \quad (1)$$

where $1/r = [S]_{total} \times K_a$ and $X_r = [P]_{total}/[S]_{total}$.

The titration calorimeter is sensitive to changes in K_a under conditions at which the product of $K_a \times$ [subtilisin] is between 1 and 1000 (Wiseman et al., 1989). The subtilisin concentrations ranged from 15 to 25 μM while the concentrations of the propeptide solutions were about 10–20 × the protein concentrations. Each binding constant and enthalpy were based on two titration runs at each temperature. Titration runs were performed until the titration peaks were close to the base line.

Kinetics of Subtilisin Folding. For refolding studies subtilisin was maintained as a stock solution in 5 mM HEPES, pH 7.5, at a concentration of ~100 μM. To measure subtilisin renaturation rates as a function of propeptide concentration, subtilisin was denatured by mixing 25 μL of protein solution with 12.5 μL of 5 M HCl in a total volume of 100 μL (pH ~1.8). The final HCl concentration was 0.625 M. The Δ75–83 subtilisin mutant S15 is completely denatured in less than 1 s by these conditions (Bryan et al., 1992). Complete denaturation of S12 subtilisin requires 1 h. The rate of denaturation was determined by the 15% decrease in intrinsic tryptophan fluorescence (excitation λ = 300 nm, emission λ = 345 nm), which occurs upon unfolding of subtilisin. The completeness of denaturation was also verified by comparing the far-UV CD spectra of the proteins at pH 1.8 with their spectra in 6 M guanidine hydrochloride, pH 7.0.

Acid-denatured S15 protein was neutralized after 5 s by diluting 100 μL of denatured protein to 2.5 mL in 30 mM Tris base and 5 mM KPO_4, with rapid stirring (final pH = 7.5). Acid-denatured S12 protein was neutralized in the same manner but after 1 h. The desired concentration of propeptide was then added. The final concentration of subtilisin was 1 μM.

The rate of renaturation was determined by an increase in intrinsic tryptophan fluorescence of 1.7-fold (excitation λ = 300 nm, emission λ = 345 nm), which occurs upon folding of subtilisin into a complex with propeptide. Data were obtained using a SPEX FluoroMax spectrofluorimeter for manual mixing experiments. Rates determined by fluorescence increase correlate exactly with rates measured by CD (i.e., the increase in negative ellipticity at λ = 222 nm). The CD spectra of renatured S15 subtilisin complexes were compared to the spectra of native subtilisin plus propeptide and found to be identical. Thermal denaturation profiles of renatured and native S15 complexes, monitored by CD (λ = 222 nm) or tryptophan fluorescence, were also identical. In 30 mM Tris-HCl, 5 mM KPO_4, pH 7.5, and 15 μM propeptide, the melting of the complex is biphasic with a small transition at ~55 °C and the main transition at 64 °C.

Kinetics of Binding the Propeptide to Folded Subtilisin. The rate of folding of the propeptide in the presence of folded S15 or S12 subtilisin was monitored by fluorescence using a KinTek stopped-flow Model SF2001. The reaction was followed by the 1.2-fold increase in the tryptophan fluorescence of subtilisin upon its binding of the propeptide. Propeptide solutions of various concentrations in 0.1 M KPO_4, pH 7.0, were mixed with an equal volume of 0.67 μM subtilisin, and 0.1 M KPO_4, pH 7.0, in a single mixing step.

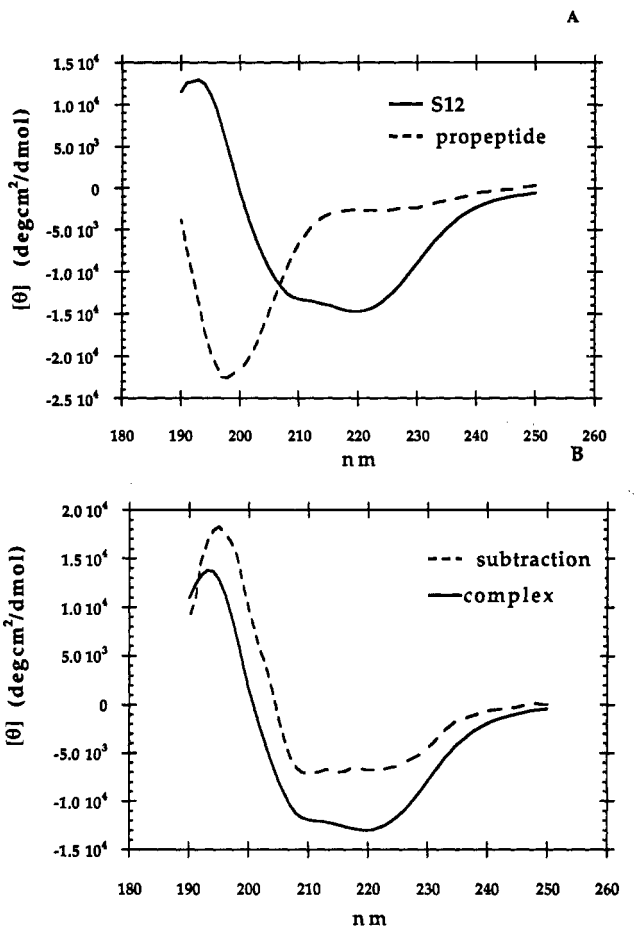

FIGURE 1: Circular dichroism spectra of subtilisin–propeptide complex. (A) Spectra of 100 μM S12 subtilisin and 100 μM isolated propeptide are shown. (B) Spectrum of 100 μM propeptide–S12 complex is shown with a subtracted spectrum of 100 μM propeptide–S12 complex minus 100 μM S12 subtilisin. All spectra were measured in 0.1 M KPO_4, pH 7.0, using a 0.05-mm cylindrical cuvette. Mean residue ellipticity is plotted versus wavelength.

RESULTS

Structure of the 77 Amino Acid Propeptide

The circular dichroism (CD) spectra of S12 subtilisin and the isolated propeptide in 0.1 M KPO_4, pH 7.0, are shown in Figure 1A. The spectrum of the isolated propeptide is typical of a largely random coil structure with a minimum ellipticity at 198 nm. To gain information about the native structure of the propeptide, S12 subtilisin and propeptide were mixed to a final concentration of 100 μM of each. The CD spectrum of the resulting complex is shown in Figure 1B. If no changes in the native subtilisin structure are induced by propeptide binding, then the difference spectrum of the complex minus S12 subtilisin will correspond to the structure of the bound propeptide. Comparison of the subtracted CD spectrum to reference spectra indicates that, in the complex, the propeptide acquires regular secondary structure. Further evidence that the propeptide goes from a disordered to highly ordered state is provided by calorimetric studies described below in detail.

Energetics of Propeptide Binding to Folded Subtilisin

$$P_u + S_f \overset{K_a}{\Leftrightarrow} P_f{-}S_f$$

Calorimetric Studies. Titration calorimetry was used to determine thermodynamic state functions ΔG, ΔH, ΔS, and ΔC_p for the reaction of the disordered propeptide with folded

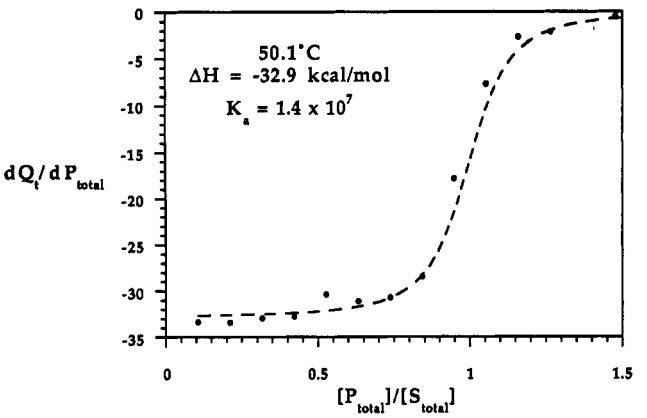

FIGURE 2: Titration calorimetry of subtilisin S12 with isolated propeptide. The heats of binding for successive additions of propeptide are plotted vs the ratio of [pro]/[S12]. The data are best fit by a calculated binding curve assuming a binding constant of 1.4×10^7 and ΔH equal to −32.9 kcal/mol using eq 1 from the text. In this titration [S12] = 15 μM in 0.1 M KPO_4. Temperature was 50.1 °C.

subtilisin. This experiment is analogous to the calorimetric studies performed on the S-peptide of ribonuclease A in complex with the S-protein (Connelly et al., 1990; Varadarajan et al., 1992).

Titrations of S12 and S15 were performed at protein concentrations [S] = 15 and 25 μM and over the temperature range of 25–50 °C. Titration of the S12 subtilisin with propeptide at 50 °C is shown in Figure 2. The data points correspond to the negative heat of binding associated with each addition of propeptide. The titration calorimeter is sensitive to changes in K_a under conditions at which the product of $K_a \times$ [S] is between 1 and 1000 (Wiseman et al., 1989). Since the K_a of S12 subtilisin for propeptide is about 1×10^7 M^{-1} at 50 °C, these protein concentrations result in values of $K_a \times$ [S] = 150 and 250. S12 subtilisin is fully native throughout the temperature range of 25–50 °C. Titrations of S15 subtilisin were not carried out above 37 °C because of its lower stability (Bryan et al., 1992). From the temperature dependence of ΔH, the heat capacity change (ΔC_p) upon binding was determined to be −1.03 kcal/(deg·mol) from the equation:

$$\Delta H = \Delta H_0 + \Delta C_p(T - T_0) \quad (2)$$

The plot of ΔH vs temperature is shown in Figure 3A.

The binding parameters obtained for S12 and S15 are similar: $\Delta H = \sim -7.5$ kcal/mol, $\Delta C_p \sim 1$ kcal/(deg·mol), and $K_a \sim 2 \times 10^8$ M^{-1} at 25 °C, and a stoichiometry of binding of 1 propeptide per subtilisin. The results of titrations of S12 and S15 are summarized in Table II.

Association constants for propeptide binding to S12 subtilisin were determined by curve fitting the data at temperatures of 40, 45, and 50 °C using eq 1. At the lower temperatures the smaller amount of heat produced per titration, coupled with tighter binding, makes direct determination of association constants less accurate. Nevertheless, since ΔH at a reference temperature and ΔC_p are known, the equilibrium constant can be calculated as a function of temperature from the equation:

$$K_{eq} = K_{eq}^0\{\exp[(-\Delta H_0/R)(1/T - 1/T_0) + (\Delta C_p T_0/R)(1/T - 1/T_0) + (\Delta C_p/R)\ln(T/T_0)]\} \quad (3)$$

where K_{eq}^0 is the equilibrium constant at T_0 (Brandts & Lin, 1990; Connelly et al., 1990). A profile of K_a vs T for propeptide binding was calculated from eq 3, using the experimentally determined K_a's at 40, 45, and 50 °C and $\Delta C_p = -1.03$ kcal/(deg·mol) (Figure 3B). The calculated curve is analogous to a stability curve for protein denaturation (Becktel & Schellman, 1987). The maximum K_a of the complex (2.3×10^8 M^{-1}) occurs at ~20 °C. At this temperature $\Delta H = 0$ and binding energy is entirely due to a positive entropy change of 38 cal/(deg·mol). The net positive entropy change upon binding presumably results from the large gain of entropy from hydrophobic burial exceeding the loss of conformational entropy. The overall thermodynamics of propeptide binding to folded subtilisin are typical of the folding reaction of other small globular proteins (Becktel et al., 1987; Privalov, 1979).

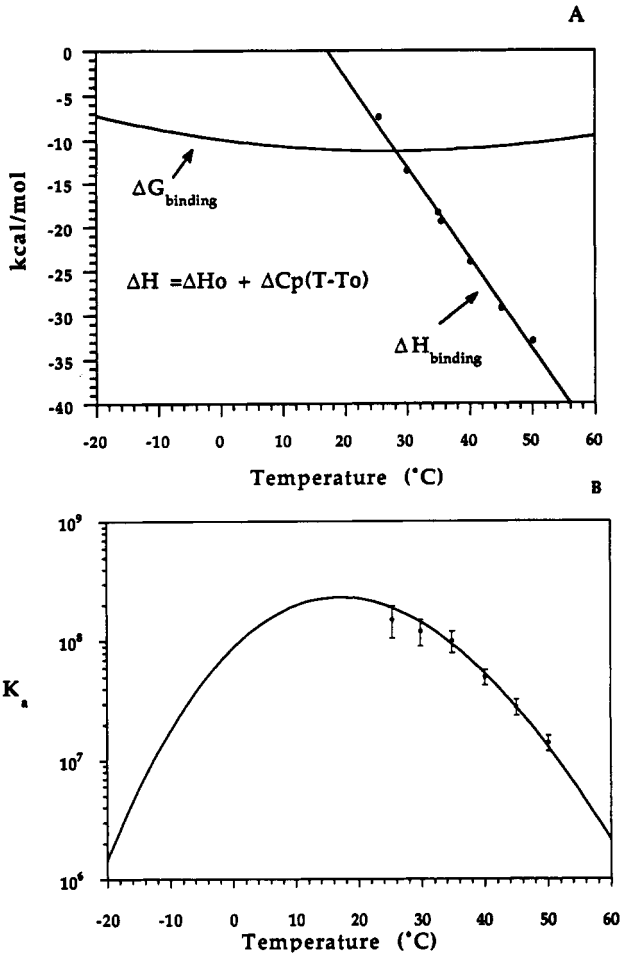

FIGURE 3: Energy of binding propeptide to S12 subtilisin vs temperature. (A) A plot of ΔH vs temperature is linear over the range of 25–50 °C and allows determination of the heat capacity change upon binding from eq 2 in the text ($\Delta C_p = -1.03$ kcal/mol). The ΔG curve is calculated from eq 3 in the text. (B) The temperature dependence of K_a of the propeptide–S12 complex is shown. The K_a's were determined by curve fitting titration data using eq 1 as described in the text. The curve shown above is calculated from eq 3, using $\Delta C_p = -1.03$ kcal/(deg·mol).

Kinetics of Binding the Propeptide to Folded Subtilisin

$$P_u + S_f \underset{k_{off}}{\overset{k_{on}}{\Leftrightarrow}} P_f\text{–}S_f$$

The kinetics of folding the propeptide in the presence of native subtilisin were measured by stopped-flow mixing methods. The reaction was followed by the increase in tryptophan fluorescence upon binding. The purpose of the experiment was to measure the second-order rate constant, k_{on}, for binding propeptide to folded subtilisin. From this measurement, the off-rate can be determined using the equilibrium constant for binding from the titration calorimetry

Table II: Titration Calorimetry of Subtilisin Mutants S12 and S15 with Propeptide

		parameters calculated from fit			
[S]	T (°C)	n	K_a (M^{-1})	ΔH (kcal/mol)	ΔC_p [kcal/(deg·mol)]
S12, 15 μM	25.4	0.95 ± 0.03	$\sim 10^8$	−7.4 ± 0.5	
	29.9	0.96 ± 0.02	$\sim 10^8$	−13.6 ± 0.2	
	34.9	0.92 ± 0.05	$\sim 10^8$	−18.4 ± 0.3	
	40.1	0.97 ± 0.02	$(5.0 \pm 1.5) \times 10^7$	−24.0 ± 0.3	
	45.1	0.97 ± 0.04	$(2.8 \pm 1.0) \times 10^7$	−29.2 ± 0.3	
	50.1	0.95 ± 0.03	$(1.4 \pm 0.5) \times 10^7$	−32.9 ± 0.4	
					−1.03
S15, 25 μM	25.4	0.85 ± 0.05		−7.5 ± 1.2	
	36.2	0.82 ± 0.03	$(5.0 \pm 2.5) \times 10^7$	−22.7 ± 0.2	
					−1.0

[a] Binding parameters for stoichiometric ratio (n), binding constant (K_a), and binding enthalpy (ΔH) were determined using nonlinear least-squares minimization of the titration data to eq 1 in the text (Wiseman et al., 1989). Measurements for each experimental condition were performed in duplicate at each temperature. K_a's at the lower temperatures cannot be determined precisely by curve fitting due to the decreasing enthalpy of binding. At 20 °C the ΔH is ~0.

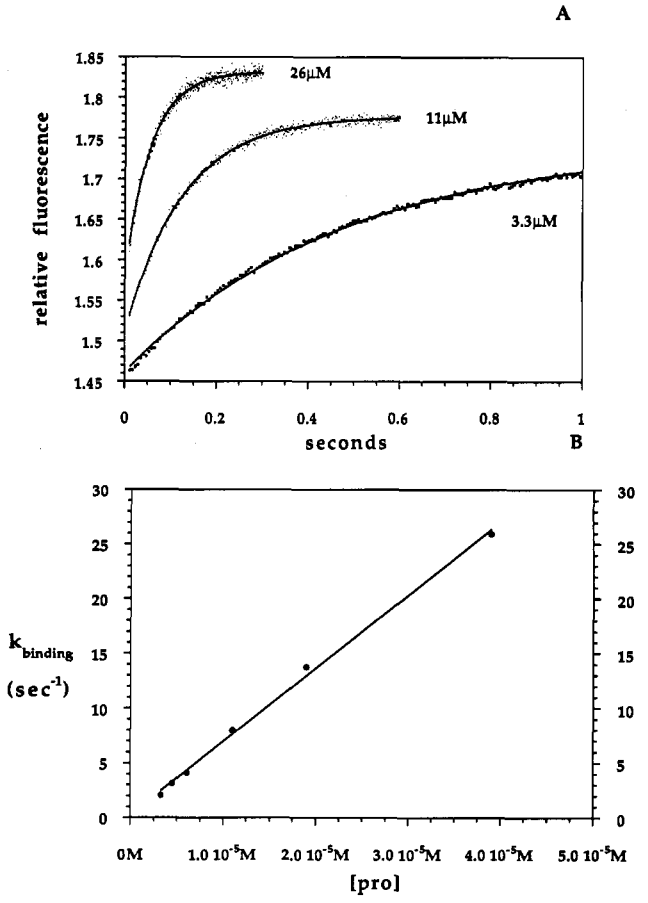

FIGURE 4: Folding rate of the propeptide in the presence of folded S12 subtilisin. (A) Folding was monitored by fluorecence using a KinTek stopped-flow Model SF2001. Propeptide solutions of various concentrations in 0.1 M KPO_4, pH 7.0, were mixed with an equal volume of 0.67 μM S12 subtilisin and 0.1 M KPO_4, pH 7.0, in a single mixing step. The final propeptide concentrations are indicated on the graph. The reaction was followed by the 1.2-fold increase in tryptophan fluorescence, which occurs upon formation of the propeptide–subtilisin complex. The data are fit to a single exponential equation to determine a pseudo-first-order rate constant for folding (solid lines). Temperature was 25 °C. (B) The pseudo-first-order rate constant for folding and binding, $k_{observed}$, is plotted as a function of [P_u]. Over the 10-fold range of [P_u], $k_{observed}$ was a linear function of [P_u] and could be fit to the equation: $k_{observed} = 0.27 + (6.74 \times 10^5)[P_u]$.

experiments. The propeptide does not contain tryptophan and has no intrinsic fluorescence at 345 nm if an excitation wavelength of 300 nm is used, as in these experiments. Subtilisin contains three tryptophans. Fluorescence at 345 nm increases by 1.2-fold due to the change in environment of tryptophans in S_f upon the binding of propeptide. If the reaction is carried out with a 10-fold or greater excess of P_u, then one observes a pseudo-first-order kinetic process with a rate of $k_1[P_u]$. The folding reaction was followed using [S_f] = 0.33 μM and varying [P_u] from 5 to 50 μM (Figure 4A). The $k_{observed}$ was plotted as a function of [P_u]. Over the 10-fold range of [P_u], $k_{observed}$ was a linear function of [P_u] and could be fit to the equation: $k_{observed} = 0.27 + (6.74 \times 10^5)[P_u]$ (Figure 4B). The absence of noticeable curvature in the plot implies that the isomerization of the collision complex of P_u and S_f is rapid relative to its formation. The second-order rate constant $k_{on} = 6.74 \times 10^5$ M^{-1} s^{-1}. The y-intercept in this plot is equal to the off-rate, k_{off}. The value determined from this plot is imprecise, however, because the off-rate is small. The off-rate can be better estimated using the relationship $K_a = k_{on}/k_{off}$. The calorimetrically determined association contant for the complex at 25 °C, 1.9×10^8 M^{-1}, yields k_{off} equal to 0.0035 s^{-1}.

Energetics of Propeptide-Catalyzed Subtilisin Folding

$$P_u + S_u \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P\text{–}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P_f\text{–}S_f$$

Kinetic Measurements. The folding reaction of subtilisin, in the presence of propeptide, can be followed by an increase in tryptophan fluorescence of 1.7-fold due to changes in the environments of the three tryptophans in subtilisin upon its folding and binding of the propeptide. As described above, the propeptide does not contain tryptophan and has no intrinsic fluorescence at 345 nm if an excitation wavelength of 300 nm is used. Therefore fluorescence increases observed at 345 nm are due to the conversion of S_u to P_f–S_f.

We attempted to follow the propeptide-catalyzed folding reactions of subtilisin S12 and S15 (the Δ75–83 version). No significant folding of 1 μM S12 subtilisin could be measured over a time scale of hours, even in the presence of 100 μM propeptide. We believe that covalent attachment of the propeptide to subtilisin may be required to avoid a kinetic trap involving premature folding of the high-affinity A-site region, as discussed below. In contrast, the folding of Δ75–83 subtilisin is efficiently catalyzed by the detached propeptide.

Folding experiments for S15 subtilisin were carried out at lower ionic strength than the binding studies to decrease the rate of uncatalyzed folding. In 0.1 M KPO_4, the uncatalyzed rate of S15 folding is 0.002 s^{-1} at 25 °C. At 30 mM Tris and 5 mM KPO_4, pH 7.5, S15 folds at a rate of $\leq 6 \times 10^{-5}$ s^{-1} at 25 °C (Figure 5A). The rate of folding in either case is accelerated as a function of [P_u]. The folding reaction at low

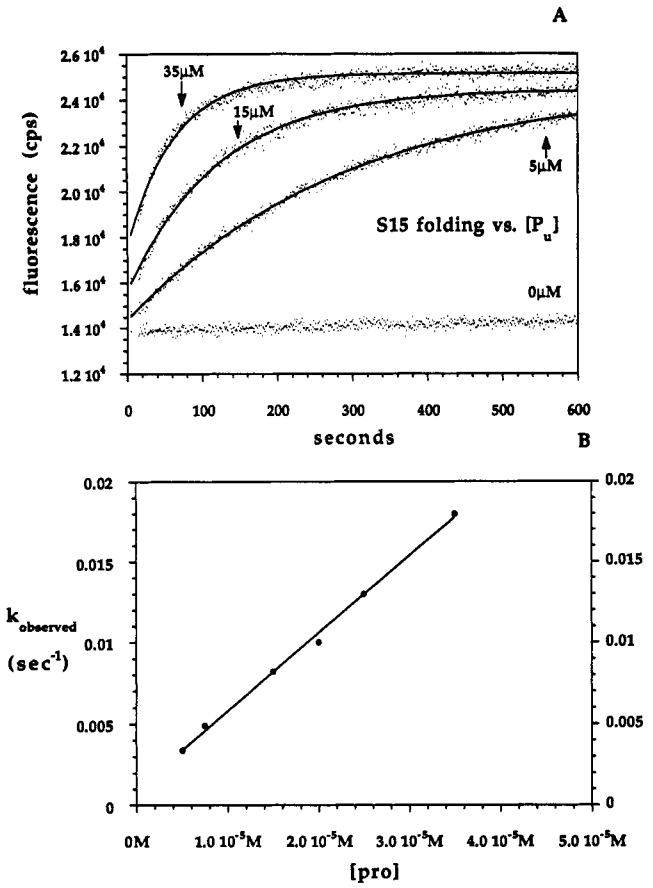

FIGURE 5: Folding rate of S15 subtilisin in the presence of the propeptide. (A) Folding was monitored by fluorescence using a SPEX Fluoromax. Propeptide solutions of various concentrations and 1 μM denatured S15 subtilisin were mixed in 5 mM KPO_4 and 30 mM Tris, pH 7.5. The final propeptide concentrations are indicated on the graph. The reaction was followed by the 1.7-fold increase in tryptophan fluorescence, which occurs upon folding of subtilisin into the propeptide–subtilisin complex. The data are fit to a single exponential equation to determine a pseudo-first-order rate constant for folding (solid lines). Temperature was 25 °C. (B) The pseudo-first-order rate constant for folding, $k_{observed}$, is plotted as a function of $[P_u]$. Over the 10-fold range of $[P_u]$, $k_{observed}$ was a linear function of $[P_u]$ and could be fit to the equation: $k_{observed} = 0.0011 + 478[P_u]$.

ionic strength was followed using $[S_u] = 1$ μM and varying $[P_u]$ from 5 to 35 μM. As described above, the reaction is a pseudo-first-order kinetic process when P_u is in sufficient molar excess of S_u (Figure 5A). The $k_{observed}$ was plotted as a function of $[P_u]$ (Figure 5B). We have modeled the reaction assuming that the formation of the collision complex can be approximated as a rapid equilibrium reaction, with the equilibrium constant, K_1, equal to $[P–S]/[S_u][P_u]$ (Anderson et al., 1991). The rate of formation of S_f–P_f will depend on the concentration of P–S. Since $[S–P]/[S_{total}] = K_1[P_u]/(1 + K_1[P_u])$, the observed rate of formation of S_f–P_f would be equal to

$$\{k_2K_1[P_u]/(1 + K_1[P_u])\} + k_{-2} \quad (4)$$

The concentration dependence of the rate should follow a hyperbola, which is a function of the saturation of [S–P] (Anderson et al., 1991). Since the folding of subtilisin in the absence of propeptide is a slow process, we expected that isomerization of the collision complex, P–S, to the fully folded complex, P_f–S_f, might become the rate-limiting step in the reaction at high $[P_u]$. The absence of curvature in the plot implies, however, that the isomerization of P–S is rapid relative to its formation. Fitting the plot of $k_{observed}$ vs $[P_u]$ in Figure 5B to eq 4 indicates that $K_1 \leq 10^3$ and that $k_2 \geq 0.5$ s^{-1}. If

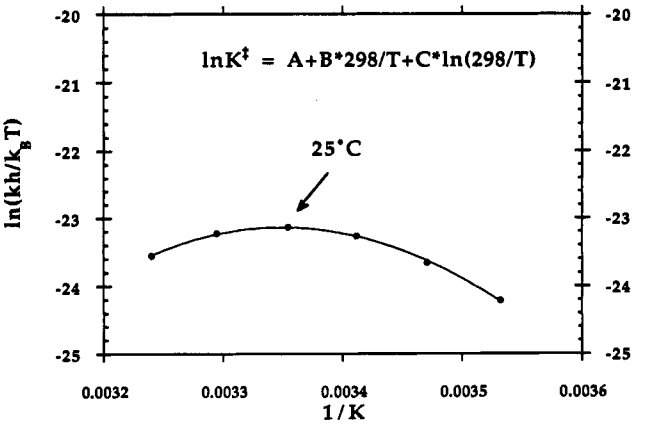

FIGURE 6: Temperature dependence of the refolding rate of S15 subtilisin plus propeptide. The natural log of the equilibrium constant for the transition state (calculated from the Eyring equation) is plotted vs the reciprocal of the absolute temperature. The line is fit according to eq 6 in the text, with $T_0 = 298$ K.

K_1 were as large as 10^4, then the plot would deviate noticeably from linearity at the highest $[P_u]$. The lower limit for K_1 is not known since we were not able to raise $[P_u]$ high enough to saturate the collision complex and cause k_2 to become limiting in the reaction. Over the $[P_u]$ examined here the formation of a productive collision complex is the limiting step in the reaction, and the data can be approximated to the simpler equilibrium:

$$P_u + S_u \underset{k_{unf}}{\overset{k_1}{\rightleftharpoons}} P_f\text{–}S_f$$

The second-order rate constant $k_1 = 480$ M^{-1} s^{-1}. The reverse rate for unfolding of the complex k_{unf} is equal to the y-intercept in this plot and is obviously very small, though an accurate value cannot be determined from these experiments.

Temperature Dependence of the Catalyzed Folding Reaction

Second-order rate constants (k_1) for folding were measured over the temperature range of 10–35 °C. A plot of ln k_1 vs $1/T$ is not linear, indicating that the folding reaction cannot be described in terms of simple Arrhenius theory. This behavior has been observed previously for the folding reaction of other proteins and is due to the decrease in heat capacity which occurs in going from the unfolded state to the transition state (Chen et al., 1989; Pohl, 1968).

We have fit our data to the Eyring equation:

$$\ln K^{\ddagger} = -RT \ln(kh/k_BT) \quad (5)$$

where k_B is the Boltzman constant, h is Planck's constant, and k is the second-order rate constant for folding of the subtilisin propeptide complex. The graph of $\ln(kh/k_BT)$ vs $1/T$ (K) is shown in Figure 6. The data are fitted to the equation:

$$\ln K^{\ddagger} = A + B(T_0/T) + C \ln(T_0/T)$$

where $A = [-\Delta C_p^{\ddagger} + \Delta S^{\ddagger}(T_0)]/R$; $B = -A - \Delta G^{\ddagger}(T_0)/RT_0$; and $C = -\Delta C_p^{\ddagger}/R$ (Chen et al., 1989). This treatment of the kinetic data is based on vibrational theory in which the frequency of breakdown of the transition state to product is equal to $\kappa k_BT/h$, where κ is a transmission coefficient assumed to be equal to 1. The curvature in the plot is due to the decrease in heat capacity [$\Delta C_p^{\ddagger} = -1.5$ kcal/(deg·mol)] in going from the unfolded state to the transition state. The overall $\Delta C_{p,folding}$ for subtilisin plus propeptide is ~−5 kcal/(deg·mol). The change in heat capacity associated with formation of the

transition state is therefore about 30% of the total heat capacity change for folding. The change in heat capacity upon protein folding is correlated with the change in environment of apolar groups (Livingstone et al., 1991). The maximum rate of catalyzed S15 folding occurs at ~25 °C. The slope of the curve at 25°C is almost zero, indicating that $\Delta H^{\ddagger}$ is small and that the activation barrier to catalyzed folding is almost completely entropic.

Influence of Altered Forms of the Propeptide and SSI on Subtilisin Folding

We have tested two altered versions of the propeptide in the subtilisin folding reaction. A 56 amino acid breakdown product of the propeptide was recovered from the *E. coli* expression strain and purified. This truncated version is missing the N-terminal 21 amino acids and was shown by peptide sequencing to begin with the amino acids SAAKK. A second 81 amino acid version of the propeptide was synthesized intentionally. In this version, the first four residues of mature subtilisin were included at the C-terminal end of the propeptide. Our reasoning was that an independently stable propeptide structure might be achieved if part of the mature subtilisin sequence were included. Neither of these peptides had any secondary structure detectable by CD, however, and neither has any effect on the rate of folding of S12 or S15 subtilisin. We also found that the streptomyces subtilisin inhibitor protein (SSI) has no detectable influence on the rate of S15 folding.

In these experiments, 5 μM of an altered propeptide or SSI was added to 1 μM of unfolded S15 subtilisin in 30 mM Tris and 5 mM KPO_4, pH 7.5, and 25 °C. The reactions were followed by fluorescence for 30 min. The change in fluorescence over this time period was similar to the uncatalyzed reaction rate. After 30 min, 5 μM of the 77 amino acid propeptide was added. Upon the addition of unaltered propeptide, folding of subtilisin occurred at a rate of 0.003 s^{-1}, which is the same rate observed if altered propeptide or SSI is not included in the reaction.

DISCUSSION

Subtilisin has a high activation barrier between folded and unfolded state, so that the in vitro folding reaction is extremely slow. We estimate that the $t_{1/2}$ for in vitro folding of wild-type subtilisin, under optimal conditions, is weeks or longer. The Δ75–83 deletion, which removes the calcium A-site, accelerates uncatalyzed folding by an estimated 10^4- to 10^5-fold (Bryan et al., 1992; Gallagher et al., 1993). Denatured S15 subtilisin, when returned to native conditions at low ionic strength, is kinetically isolated from the native state and remains largely devoid of regular structure for hours. When the isolated and unstructured propeptide is added, folding of both occurs spontaneously. This foldase activity of the propeptide is a specific effect, however, since two altered versions of the propeptide are unable to catalyze the folding of S12 or S15 subtilisin. If the propeptide were a stable structure independent of subtilisin, then it would be easier to understand how it could catalyze folding by binding to and stabilizing a native-like transition state, as is proposed to be the case for α-lytic protease and its propeptide (Baker et al., 1992). The reaction of unfolded propeptide with unfolded S15 subtilisin to form a stable, folded complex is an intriguing case of complementation. On the basis of the results reported here, the energetics of the folding reaction of S15 subtilisin and propeptide can be summarized as follows:

$$P_u + S_u \underset{k_{-1}}{\overset{k_1}{\underset{\geq 0.5\ s^{-1}}{\overset{480\ M^{-1}\ s^{-1}}{\Leftrightarrow}}}} P\text{–}S \underset{k_{-2}}{\overset{k_2}{\underset{<0.001\ s^{-1}}{\overset{\geq 0.5\ s^{-1}}{\Leftrightarrow}}}} P_f\text{–}S_f \underset{k_{on}}{\overset{k_{off}}{\underset{7 \times 10^5\ M^{-1}\ s^{-1}}{\overset{0.0035\ s^{-1}}{\Leftrightarrow}}}} P_u + S_f$$

The rate-limiting step in the folding of S15 subtilisin is formation of the initial collision complex, P–S. A diffusion-controlled reaction involving macromolecules can occur at a rate of 10^9 M^{-1} s^{-1}. The rate at which P_u and S_u form a productive collision complex is very infrequent, however, with $k_1 \sim 500$ M^{-1} s^{-1}. The kinetic results indicate that once a productive collision complex is formed, subsequent isomerization is rapid. The rate constant for isomerization of the collision complex to the folded complex is not known but must be ≥0.5 s^{-1} in order for isomerization not to become the rate limiting step in folding at $[P_u] = 35$ μM.

Formation of a productive collision complex may require that a partially structured subtilisin molecule collide with structured propeptide, thus providing a nucleation point for subsequent folding. The extremely slow rate of uncatalyzed S15 folding at low ionic strength indicates that the probability of forming productive folding intermediates in subtilisin alone is low. CD experiments indicate that the equilibrium constant for the $P_u \Leftrightarrow P_f$ transition for isolated propeptide is also very small. We suspect that the collision complex is a stable early folding unit resulting from the collision of short-lived species of subtilisin and propeptide. This mechanism would explain why the second-order rate constant for folding S15 subtilisin with the detached propeptide is small. Reaction rate analysis shows that the activation barrier for forming the productive collision complex is almost entirely entropic at 25 °C (the temperature at which the catalyzed folding rate is fastest).

The change in heat capacity associated with formation of the transition state for catalyzed folding is consistent with the idea that the collision complex is an early folding unit. $\Delta C_p^{\ddagger}$ is about 30% of the total heat capacity change for folding. Since $\Delta C_{p,\text{folding}}$ is correlated with the change in environment of apolar groups, the transition state for the catalyzed folding reaction of subtilisin appears to be more similar in compactness to the unfolded state than to the native state. This result contrasts with observations made for the folding reactions of several smaller globular proteins. For example, $\Delta C_p^{\ddagger}$ of T4 lysozyme (Chen et al., 1989), chymotrypsin inhibitor 2 (Jackson & Fersht, 1991), and protein G, B domain (Alexander et al., 1992b), are all ~70% of the total ΔC_p for folding.

Structural complementarity of folded propeptide and folded subtilisin is evidenced by a K_a of the complex equal to 2.3×10^8 M^{-1} at 20 °C. The thermodynamic state functions for binding the propeptide to folded subtilisin are typical of the folding reaction of a small globular protein. The energetics of binding the propeptide to the folded forms of either S15 or S12 subtilisin are similar. Folding of S12 subtilisin, however, is extremely slow even in the presence of a high concentration of isolated propeptide. The high K_a of the complex means that recycling of the propeptide to fold additional subtilisin molecules is very slow in our in vitro reaction. Given the tight binding of propeptide to subtilisin, one wonders how mature subtilisin is released after processing and secretion in vivo. In vivo, additional proteolytic steps on the propeptide might be required after the initial processing step to disrupt the propeptide–subtilisin complex.

Why Doesn't the Detached Propeptide Fold Subtilisin with the Native Calcium A-Site? We have not been able to conclusively demonstrate folding of subtilisin S12, which has

the wild-type calcium A-site, even in the presence of 100 μM propeptide. It has been recently reported that a small percentage of native subtilisin is recovered in the presence of isolated propeptide after 8 days (Eder et al., 1993). Folding on this time scale would be unlikely to be of biological relevance, however. We believe that covalent attachment of the propeptide to subtilisin may be required to avoid a kinetic trap involving premature folding of the high-affinity A-site region. Eder et al. have reported that subtilisin without propeptide folds to a highly structured intermediate state which binds calcium. This partially folded state may serve as the kinetic trap, which blocks subsequent folding to the native state. The covalently attached propeptide may prevent the formation of this intermediate by dictating the sequence of folding events, such that the A-site region is folded late in the pathway.

The mechanism of processing the propeptide is consistent with this idea. In vivo processing is known to be autocatalyic, although it is uncertain whether it occurs intramolecularly or intermolecularly (Ikemura et al., 1987; Power et al., 1986). In either case, the N-terminus of mature subtilisin must occupy the substrate binding pocket of a subtilisin molecule for processing to occur. Since the N-terminal four amino acids of the mature subtilisin are involved in β-pair hydrogen bonds with the calcium binding loop and the side-chain carbonyl oxygen of Q2 is a ligand to the calcium in the A-site, it is hard to imagine how the N-terminus of completely native subtilisin could bind in the P1′ pocket of the subtilisin active site. Thus the covalent attachment of the propeptide to mature subtilisin may prevent the final folding of the calcium A-site region, until after its cleavage to produce the mature enzyme. This argument is supported by two experimental observations. First, the folding of Δ75–83 subtilisin, which lacks the A-site, is efficiently catalyzed by the detached propeptide. Second, the binding affinities of the propeptide for S12 and S15 subtilisins are similar, indicating that a native A-site is not part of the recognition surface of the propeptide. Purification of large quantities of the intact 352 amino acid prosubtilisin should allow the energetics of folding subtilisin with the native A-site to be better understood.

In summary, we have defined the overall energetics of interactions of the isolated 77 amino acid propeptide with subtilisin. The results suggest a general mechanism for a catalyzed protein folding reaction. Unfolded S15 subtilisin when returned to native conditions at low ionic strength is kinetically isolated from its native state. Upon the addition of isolated and unfolded propeptide, the two cooperate in folding, forming a simple self-assembly system.

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