Prodomain Mutations at the Subtilisin Interface: Correlation of Binding Energy and the Rate of Catalyzed Folding†

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ABSTRACT: The *in vivo* folding of subtilisin is dependent on a 77 amino acid prosequence, which is eventually cleaved from the N-terminus of subtilisin to create the 275 amino acid mature form of the enzyme. The recent determination of the structure of a complex of the prodomain and a calcium-free subtilisin mutant has suggested how the prodomain may catalyze subtilisin folding [Bryan, P., Wang, L., Hoskins, J., Ruvinov, S., Strausberg, S., Alexander, P., Almog, O., Gilliland, G., & Gallagher, T. (1995) *Biochemistry 34*, 10310−10318]. In the complex, the prodomain packs against the two parallel surface helices of subtilisin (residues 104−116 and residues 133−144) and supplies caps to the N-termini of the two helices. The binding site is contained almost entirely in the linear sequence 100−144 of subtilisin. The C-terminus of the prodomain (residues 72−77) extends out from its central part to bind like a substrate in subtilisin's active site cleft. The simplest model of catalyzed folding is one in which the observed binding interaction in the complex accelerates folding by stabilizing an intermediate which includes the 45 amino acid $\alpha\beta\alpha$ substructure in subtilisin. According to our hypothesis, amino acids 100−144 would have a native-like fold in the intermediate which the prodomain stabilizes. Guided by the structure of the bimolecular complex of subtilisin and its prodomain, we have constructed mutations in the C-terminal region of the prodomain. Analysis of five mutants reveals a general correlation between the ability of the prodomain to bind to native subtilisin and its ability to accelerate subtilisin folding. Substitutions of the prodomain side chain in the S1 subtrate binding pocket (Y77) have relatively small effects on catalysis of subtilisin folding and binding to folded subtilisin (3-fold or less). Deletion of two or five C-terminal amino acids, which removes hydrogen bonds in the complex, has much larger effects (25−500-fold). The correlation between binding to native subtilisin and foldase activity suggests that the subtilisin intermediate which the prodomain binds and stabilizes in the folding reaction has native-like structural features.

The 275 amino acid serine protease subtilisin and several other extracellular microbial proteases are unusual but not unique examples of proteins with a stable native state which is difficult to access from the unfolded state (Baker & Agard, 1994). The biosynthesis of subtilisin is dependent on a 77 amino acid prosequence, 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; Power et al., 1986; Vasantha et al., 1984; Wells et al., 1983). Refolding of processed subtilisin is problematic. Without the prodomain, subtilisin refolds very slowly (τ > weeks in 0.1 M KP_i,[1] pH 7.0). Even when catalyzed by the isolated prodomain in a bimolecular reaction, refolding of subtilisin occurs at a rate of only 0.2 M^{-1} s^{-1} at 15 °C (Eder et al., 1993). In order to simplify the study of subtilisin folding, we have employed subtilisin mutants from which the high-affinity calcium binding site is removed (Bryan et al., 1992).[2] Unlike wild-type subtilisin, refolding of "calcium-free" mutants is efficiently catalyzed by the isolated prodomain in a bimolecular reaction (Strausberg et al., 1993b).

The folding of the inactive, calcium-free subtilisin mutant Sbt-15 is catalyzed by the isolated prodomain at a rate of 480 M^{-1} s^{-1} to form a tight bimolecular complex. A model of this folding reaction is described by the equilibria:

$$P + S_u \leftrightarrow P_f{-}S_I \leftrightarrow P_f{-}S_f \leftrightarrow P + S_f$$

where S_u and P are Sbt and prodomain, respectively, which are largely unstructured at the start of the reaction, $P_f{-}S_I$ is a collision complex of a partially folded Sbt and folded prodomain, and $P_f{-}S_f$ is the complex of folded Sbt and prodomain. Formation of the collision complex is rate limiting in the reaction at concentrations of prodomain ≤100 μM. Subsequent isomerization of the intermediate to the native complex occurs at a rate in the order of >0.5 s^{-1} (Strausberg et al., 1993b).

We have recently determined the structure of a complex between prodomain and calcium-free subtilisin at 2.0 Å resolution (Bryan et al., 1995; Gallagher et al., 1995). The prodomain, which is largely unstructured by itself, ($\Delta G_{folding}$ = 2 kcal/mol at 25 °C), folds into a compact structure with

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[1] Abbreviations: CD, circular dichroism; Δ75−83 subtilisin, subtilisin BPN′ with a deletion of amino acids 75 to 83; cps, counts per second; K_a, association constant for prodomain binding; [P], prodomain concentration; P_i, phosphate; [S], subtilisin concentration; Sbt-12, subtilisin BPN′ with the mutations M50F, A73L, Y217K, N218S, and S221C; Sbt-15, subtilisin BPN′ with the mutations M50F, A73L, Δ75−83, Y217K, N218S, and S221C; Tris, tris(hydroxymethyl)aminomethane; τ, reciprocal of the rate constant for folding.

[2] A major part of the kinetic barrier to folding subtilisin involves formation of the calcium A-site ($K_a = 10^7$ M^{-1} at 25 °C) (Bryan et al., 1992). This has been demonstrated by deleting the calcium binding loop (amino acids 75 to 83) from subtilisin. This deletion eliminates calcium binding at the A-site and destabilizes subtilisin but greatly accelerates both uncatalyzed folding and bimolecular, prodomain-catalyzed folding (Strausberg et al., 1993b).

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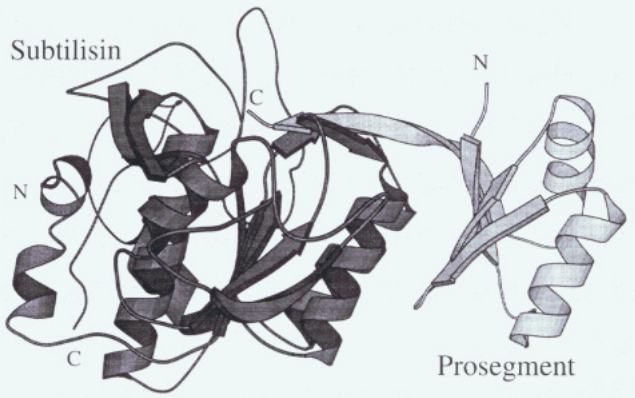

FIGURE 1: Prodomain−subtilisin complex. Ribbon drawing depicting the α-carbon backbone of the bimolecular complex of Δ75−83 subtilisin (darker shading) and the prodomain (lighter shading). The C-terminus of the prodomain is in the subtilisin active site.

a four-stranded antiparallel β-sheet and two three-turnα-helices when complexed with subtilisin (Figure 1). In the complex, the β-sheet packs against the two parallel surface helices of subtilisin (residues 104−116 and residues 133−144). Pro residues E69 and D71 form helix caps for the N-termini of the two subtilisin helices as shown in Figure 2. The C-terminus of the prodomain (residues 72−77) extends out from its central part to bind as a substrate in subtilisin's active site cleft. Residues Y77, A76, H75, and A74 of the prodomain occupy subsites S1−S4 of subtilisin, respectively. Subtilisin's binding interface comprises the linear sequence 100−144 (Figure 2).

The simplest model of catalyzed folding is one in which the observed binding interaction in the complex accelerates folding by stabilizing an intermediate which includes the 45 amino acid αβα substructure in subtilisin. The prodomain bound to this substructure is proposed to correspond to the collision complex, P_f-S_I, in our model. According to our hypothesis, amino acids 100−144 would have a native-like fold in the intermediate which the prodomain stabilizes. If this is true, mutations in the prodomain which slow the rate of bimolecular folding as a result of weaker binding to the αβα substructure would be expected to correlate with weaker binding to native subtilisin. Alternatively, a lack of correlation between binding to native subtilisin and catalysis would imply that the binding site of the prodomain on the intermediate is either non-native or different from its site on the native protein. Guided by the structure of the bimolecular complex of subtilisin and its prodomain, we have constructed mutations in the C-terminal region of the prodomain and measured their effects on the rate of catalyzed folding. The ability of mutants of the prodomain to catalyze the folding of subtilisin is compared to their ability to bind to folded subtilisin.

MATERIALS AND METHODS

Engineering Subtilisin for Facile Folding. The subtilisin mutant used in these studies is denoted Sbt-15 and contains the following mutations: Δ75−83 (to eliminate calcium binding and accelerate the folding rate); S221C[3] (to remove the active site nucleophile and greatly reduce proteolytic activity); and the stabilizing mutations M50F, Y217K, and N218S (to partially compensate for stability lost due to the calcium loop deletion) (Bryan et al., 1992).

Cloning and Expression of Subtilisin Mutant Sbt-15. The mutant gene for Sbt-15 was cloned into a pUB110-based expression plasmid and used to transform *Bacillus subtilis* (Bryan et al., 1992). The *B. subtilis* strain used as the host contains a chromosomal deletion of its subtilisin gene and therefore produces no background wild-type activity. Sbt-15 was expressed in a 1.5 L New Brunswick fermentor at a

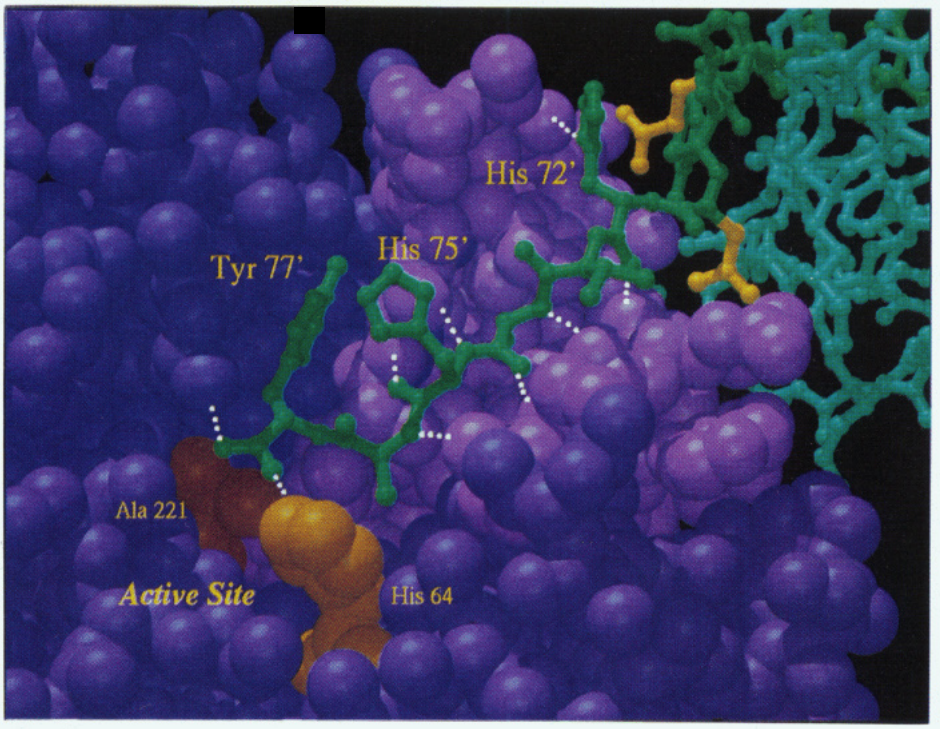

FIGURE 2: Subtilisin−prodomain interface. Amino acids 72−77 of the prodomain are bound in the substrate binding pocket of subtilisin and form the central strand in a three-stranded antiparallel β-sheet. The nine hydrogen bonds involving residues 72−77 are depicted by white dots. The remainder of the binding surface on subtilisin is its two surface α-helices. The binding site comprises the linear sequence 100−144 of subtilisin, which is colored the lighter shade of purple. Note the prodomain's α-helix capping residues in yellow. The crystal structure was determined for a complex in which the active site serine 221 was substituted by alanine, shown in red (Bryan et al., 1995; Gallagher et al., 1995).

level of ~150 mg of the correctly processed mature form per liter. Sbt-15 was purified and verified for homogeneity essentially as described (Bryan et al., 1986).

Cloning and Expression of the Prodomain of Subtilisin. The prodomain region of the subtilisin BPN′ gene was subcloned using the polymerase chain reaction as described (Strausberg et al., 1993b). Mutagenesis of the cloned prodomain gene was performed according to the oligonucleotide-directed *in vitro* mutagenesis system, version 2 (Amersham International plc). Single-strand plasmid DNA was sequenced according to Sequenase (United States Biochemical).

The *Escherichia coli* production strain was grown at 37 °C in a 1.5 L BioFlo Model fermenter and purified as described (Strausberg et al., 1993b).

Determination of Extinction Coefficient. The prodomain has four tyrosines and no tryptophans. Since the prodomain is disordered in aqueous solution, the extinction of free tyrosine ($\epsilon = 1413\ M^{-1}$ at 275 nm) was used to estimate the extinction of prodomain ($5650\ M^{-1}$). For mutants of the prodomain with substitutions or deletions of tyrosine 77, $\epsilon = 4240\ M^{-1}$ at 275 nm.

Kinetics of Subtilisin Folding. A stock solution of Sbt-15 at a concentration of 100 μM in 100 mM KP_i, pH 7.0, was prepared for refolding studies. Sbt-15 was denatured by diluting 3.3 μL of the Sbt-15 stock solution into 33 μL of HCl solution (final HCl concentration was 0.25 M). Sbt-15 is completely denatured in less than 1 s by these conditions (Bryan et al., 1992). After 5 s, the sample was neutralized by diluting the Sbt-15 and HCl solution into a total volume of 333 μL of Tris−base, KP_i, and prodomain (final buffer concentrations were 30 mM Tris-HCl and 5 mM KP_i, pH 7.5). The final concentration of Sbt-15 was 1 μM, while the prodomain concentration was varied from 5 to 30 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 prodomain. Data were obtained using a SPEX FluoroMax spectrofluorometer. Renaturation rates determined by fluorescence correlate exactly with rates measured by CD (i.e., the increase in negative ellipticity at λ = 222 nm).

Titration Calorimetry Measurements. Calorimetric titrations were performed with a Microcal Omega titration calorimeter as described (Strausberg et al., 1993b; Wiseman et al., 1989). Binding constants were determined by fitting the heat per injection, $dQ/d[P]_{total}$, 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 $1/r = [S]_{total} K_a$ and $X_r = [P]_{total}/[S]_{total}$ (Wiseman et al., 1989). Each binding constant and enthalpy determination were based on at least two titration runs at each temperature. Titration runs were performed until no heat was produced by further addition of prodomain.

RESULTS

Design of Mutations. Five mutants in the C-terminal tail of the prodomain were constructed for this study. Three of these are substitutions of the the C-terminal amino acid, Y77, by R, F, and E. The other two mutants are deletions of C-terminal amino acids: (1) the last two amino acids A76 and Y77 (Δ76−77) and (2) the last five amino acids V73, A74, H75, A76, and Y77 (Δ73−77).

The prodomain, which is largely unstructured by itself, folds into a compact structure with a four-stranded antiparallel β-sheet and two three-turn α-helices when complexed with subtilisin. Amino acids 72−77 are bound in the substrate binding pocket of subtilisin and form the central strand in a three-stranded antiparallel β-sheet. The two outer strands in this sheet are formed by subtilisin residues 100−104 and 125−131 (Figure 2). The remainder of the binding surface on subtilisin is its two surface α-helices 105−116 and 133−144. A total of 1100 Å^2 of subtilisin's surface area is buried at its interface with the prodomain. Roughly half of this area is in the substrate-binding region. Almost all of the binding interface is contained in the linear sequence 100−144 of subtilisin. The only interactions with the prodomain outside of this 45 amino acid sequence involve Y77 at the C-terminus of the prodomain. The S1 pocket for the tyrosine side chain is formed by amino acids 125−127, within the 45 amino acid $\alpha\beta\alpha$ substructure, and also by amino acids 152−156. The carboxylate of Y77 is hydrogen bonded to amino acids H64 and N155 (Figure 2).

Effects of Prodomain Mutations on the Kinetics of Catalyzed Folding: $P + S_u \leftrightarrows P_f{-}S_I \leftrightarrows P_f{-}S_f$. The folding reaction of subtilisin, in the presence of prodomain, 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 prodomain. The prodomain does not contain tryptophan residues and thus has no intrinsic fluorescence at 345 nm upon excitation at 300 nm. Therefore, fluorescence increases observed at 345 nm are due solely to the conversion of S_u to $P_f{-}S_f$.

If denatured and returned to native conditions at low ionic strength, Sbt-15 is kinetically isolated from the native state. For example, at 30 mM Tris and 5 mM KP_i, pH 7.5, the rate of uncatalyzed folding of Sbt-15 is $< 5 \times 10^{-5}\ s^{-1}$ at 25 °C. When the isolated prodomain is added, the rate of subtilisin folding increases rapidly with the concentration of prodomain, [P]. The folding reaction was followed using $[S_u]$ = 1 μM and varying [P] from 5 to 30 μM for each of the mutant prodomains. Under these conditions a single cycle of Sbt-15 folding is measured, and the reactions are a pseudo-first-order kinetic process. The folding curves were fit with a single exponential equation to determine a $k_{observed}$ for each [P] (Figure 3A). The $k_{observed}$ plotted as a function of [P] is linear up to [P] = 30 μM (Figure 3B). The absence of curvature in this plot implies that the isomerization of $P_f{-}S_I$ to $P_f{-}S_f$ is rapid relative to its formation (Strausberg et al., 1993b). Over the range of [P] examined here the formation of a productive collision complex, $P_f{-}S_I$, appears to be the limiting step in the reaction.

The slope of $k_{observed}$ vs [P] is equal to the second-order rate constant for catalyzed subtilisin folding. The second-order rate constants for the prodomain mutants are summarized in Table 1. None of the mutants studied here catalyze folding as well as the native prosequence. The three substitutions for Y77 have relatively small effects on catalysis. Y77E is the poorest catalyst among the three with a rate constant 3 times less than the wild-type Y77.

[3] 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.

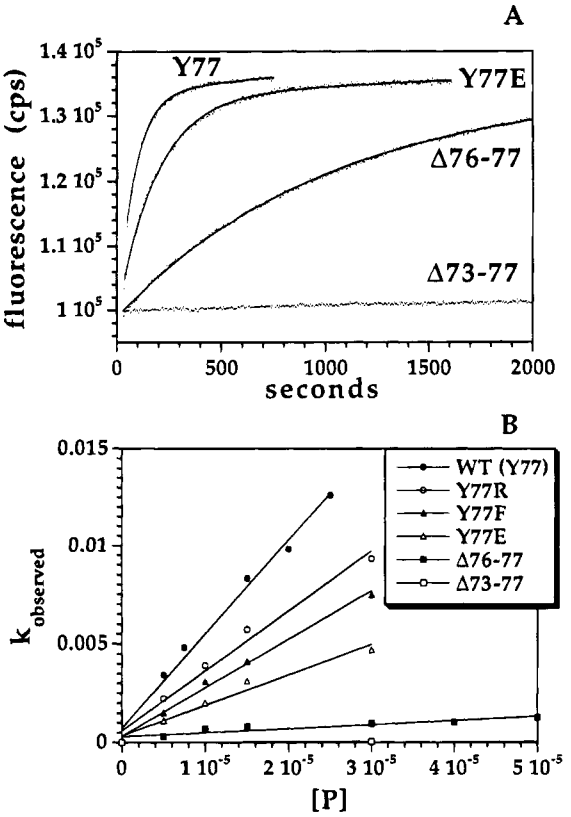

FIGURE 3: Kinetics of prodomain-catalyzed folding. (A) 1 μM denatured Sbt-15 and 30 μM prodomain were mixed in 5 mM KP_i and 30 mM Tris, pH 7.5 at 25 °C. The final prodomain concentration was 30 μM in each case. After manual mixing the reaction was followed by the increase in tryptophan fluorescence which occurs upon folding of subtilisin into the prodomain−subtilisin complex. The data are fit to a single exponential equation to determine a pseudo-first-order rate constant for folding (solid lines). (B) The pseudo-first-order rate constant for folding, $k_{observed}$, is plotted as a function of [P]. The slope of $k_{observed}$ vs [P] is equal to a second-order rate constant for catalyzed subtilisin folding.

Table 1: Effects of Mutations in the Prodomain on Binding and Catalysis[a]

	K_a (M^{-1})	$\Delta\Delta G_{binding}$ (kcal/M)	$k_{folding}$ (M^{-1}/s^{-1})	$\Delta\Delta E_a$ (kcal/M)
wild type (Y77)	1×10^8		480	
Y77F	6×10^7	0.3	250	0.4
Y77R	3×10^7	0.5	300	0.3
Y77E	1.5×10^7	1.1	150	0.7
Δ76−77	1.5×10^6	2.5	20	1.9
Δ73−77	$\sim 10^5$	4	1	3.7

[a] K_a of the prodomain to folded Sbt-12 at 25 °C was calculated from titration calorimetric data using eq 1 in the text. $k_{folding}$ for each prodomain was determined by measuring the rate of Sbt-15 folding vs [P]. The changes in binding energy and activation energy were calculated from the equations $\Delta\Delta G = RT \ln K_{binding(wt)}/K_{binding(mut)}$ and $\Delta\Delta E_a = RT \ln k_{folding(wt)}/k_{folding(mut)}$.

The two deletions from the C-terminus result in greater impairment of catalysis. Subtilisin folding in the presence of the Δ76−77 mutant is 25 times slower than WT and ~500 times slower for the Δ73−77 mutant (Table 1). We have previously shown that a version of the prodomain truncated by 21 amino acids on its N-terminus has no detectable foldase activity (Strausberg et al., 1993b).

Effects of Prodomain Mutations on Binding to Folded Subtilisin: $P + S_f \overset{K_a}{\rightleftharpoons} P_f{-}S_f$. Titration calorimetry was used to determine the enthalpies of binding ($\Delta H_{binding}$) and the association constants (K_a's) of mutant prodomains for folded subtilisin. The C221 subtilisin Sbt-12 was used in the titrations. Sbt-12 is identical to Sbt-15 but has the native calcium loop. Sbt-12 is more stable than Sbt-15 and therefore more suitable for titrations at higher temperatures. The prodomain has no contacts with the calcium loop when complexed to subtilisin. Accordingly, the wild-type prodomain has the same binding affinity and enthalpy of binding to Sbt-12 as it has to Sbt-15 at 25−35 °C (Strausberg et al., 1993b). Titrations were performed at Sbt-12 concentrations, [S], = 25 μM, over the temperature range 25−45 °C (Table 2). K_a's for prodomain binding to Sbt-12 subtilisin were determined by curve fitting the data at temperatures from 35−45 °C using eq 1 (Figure 4). At the lower temperatures the smaller amount of heat produced per titration, coupled with tighter binding, makes direct determination of K_a less accurate. At 20 °C $\Delta H_{binding}$ is ~0 kcal/mol for all prodomain variations. Because of the decrease in heat capacity (ΔC_p) of −1 kcal M^{-1} deg^{-1} upon binding prodomain to subtilisin, the negative heat of binding increases with increasing temperature. Therefore, at 40 °C, $\Delta H_{binding}$ is ~−20 kcal/mol, making the determination of K_a more accurate. Titration curves of Sbt-12 with Y77 (wild type), Y77R, Y77F, Y77E, and Δ76−77 prodomains at 40 °C are shown in Figure 4. The data points correspond to the negative heat of binding associated with each addition of prodomain to Sbt-12. 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). The K_a of the various prodomains for Sbt-12 subtilisin ranges from 5×10^5 to 3×10^7 M^{-1} at 40 °C; therefore, $K_a \times$ [S] ranges from 12.5 to 750.

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:

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

where K_{eq}° is the equilibrium constant at T_0 (Brandts & Lin, 1990; Connelly et al., 1990). The K_a's at the reference temperature of 25 °C are recorded in Table 2.

The binding affinity of the Δ73−77 prodomain was too weak to measure calorimetrically. Its association constant was estimated be $\sim 10^5$ M^{-1} at 25 °C from measurements of the the change in tryptophan fluorescence which occurs upon binding.

The strength of binding for mutations at postition 77 is as follows: Y > F ~ R ≫ E. Since amino acid 77 of the prodomain occupies the S1 subsite of subtilisin, one might expect a correlation between binding of Y77 mutants of the prodomain to Sbt-12 and substrate binding to native subtilisin BPN′. The catalytic constants were determined for subtilisin BPN′ against the synthetic tetrapeptides s-A-A-P-X-*p*-nitroanilide, where X, the P1 amino acid, is varied (Estell et al., 1986). The value of K_m for each of the tetrapeptide substrates, as determined by Estell et al. follows the same order as the $1/K_a$ for mutant prodomains with the same P1 amino acid.

DISCUSSION

Mature subtilisin lacks the proenzyme's facility for folding and appears to be an example for which an efficient folding pathway is not a necessary consequence of a stable native

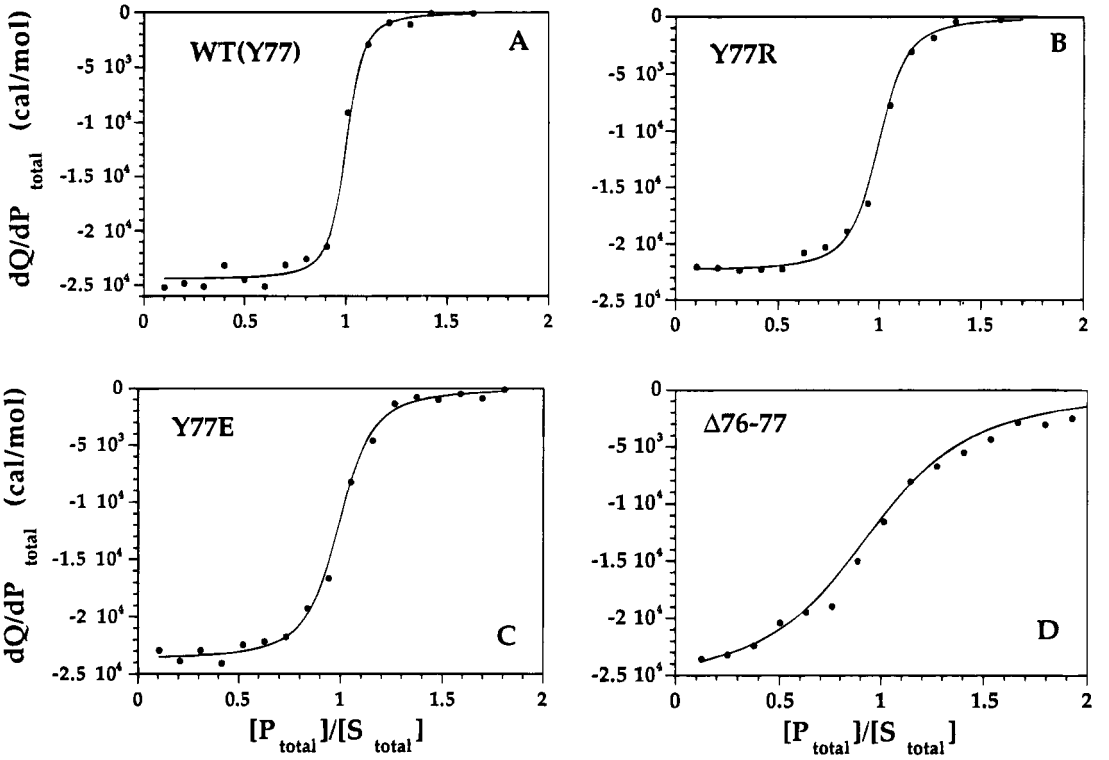

FIGURE 4: Titration calorimetry of subtilisin Sbt-12 with mutant prodomains. The heats of binding for successive additions of prodomain are plotted vs the ratio of [P]/[Sbt-12]. Binding curves were determined by the nonlinear least squares minimization method using eq 1 in the text (Wiseman et al., 1989). The temperature was 40 °C.

Table 2: Titration Calorimetry of Sbt-12 with Mutants of the Prodomain[a]

	T (°C)	K_a (M^{-1})	$\Delta H_{binding}$ (kcal/M)	ΔC_p (kcal M^{-1} deg^{-1})
wild type (Y77)	25.4		−7.4	−1.0
	30.0		−17.1	
	35.0		−20.4	
	39.8	3×10^7	−24.4	
	45.1	1.5×10^7	−27.2	
Y77F	35.0		−17.7	
	40.6	2×10^7	−21.6	
	45.1	2×10^7	−23.0	
Y77R	35.0		−16.8	
	39.8	1×10^7	−22.5	
	45.1	6×10^6	−28.8	
Y77E	35.0	1×10^7	−13.8	
	39.8	5×10^6	−23.7	
	45.1	2×10^6	−28.0	
Δ76−77	34.9	7×10^5	−21.7	
	39.8	5×10^5	−27.6	
Δ73−77	40.0	$<10^5$		

[a] Binding constant (K_a) and binding enthalpy ($\Delta H_{binding}$) were determined using nonlinear least squares minimization of the titration data to eq 1 in the text (Wiseman et al., 1989). The stoichiometry of binding is 1. 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.

state. Denatured Sbt-15, when returned to native conditions at low ionic strength, remains largely disordered and kinetically isolated from the native state, even though the native form is the global thermodynamic minumum. Circular dichroic (CD) spectroscopy suggests that this isolated, denatured state is a heterogeneous collection of relatively unfolded conformations (Bryan et al., 1995). An efficient folding pathway implies that productive intermediates are significantly more stable than the surrounding landscape of unfolded and misfolded conformations. The difficulty in folding the mature protease appears to be a lack of partially folded structures of sufficient stability to initiate folding (Strausberg et al., 1993a).

The structure of the prodomain−subtilisin complex suggests that the prodomain promotes folding by stabilizing the 100−144 αβα motif in subtilisin. The folded prodomain is a compact structure with shape complementarity and high affinity to native subtilisin. The prodomain binds on subtilisin's two parallel surface α-helices and supplies caps to the N-termini of the two helices and may selectively stabilize the αβα motif relative to other partially folded states. We propose that the prodomain bound to the αβα substructure corresponds to the collision complex, P_f-S_I, whose formation is rate limiting in the bimolecular reaction. Both the prodomain and and the 45 amino acid αβα motif are proposed to have a more or less native fold in the bimolecular intermediate. We would define the intermediate S_I as minimally comprising only amino acids 100−144. It is possible that it includes more structure, however.

Analysis of the five mutants described here shows a general correlation between binding of the prodomain to native subtilisin and its ability to accelerate folding (Figure 5). This correlation is consistent with our hypothesis and suggests that the intermediate (P_f-S_I) has native-like structural features. Substitution of the prodomain side chain in the S1 substrate binding pocket (Y77) does not have a large effect on either binding to folded subtilisin or catalysis of subtilisin folding (3-fold or less). Deletion of C-terminal amino acids, which removes hydrogen bonds in the complex, has a much larger effect on catalysis (Table 1). On the basis of the structure of the complex one would predict that four prodomain−subtilisin hydrogen bonds are lost upon deletion of amino acids 76−77 and eight hydrogen bonds are lost upon deletion of amino acids 73−77. Even without these interactions the Δ73−77 prodomain catalyzes folding by a factor of $\geq 10^4$ M^{-1} relative to uncatalyzed folding, illustrat-

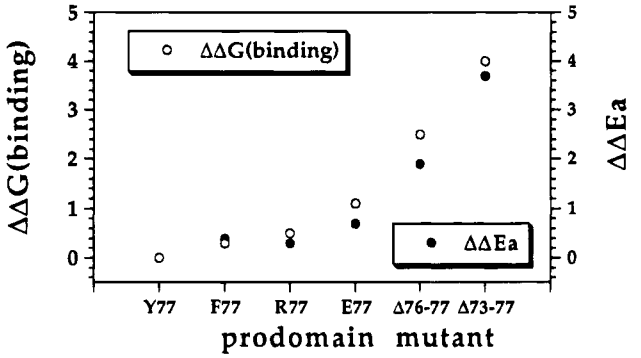

FIGURE 5: Comparison of $\Delta\Delta G_{binding}$ and $\Delta\Delta E_a$ for folding. The change in binding energy to folded subtilisin and the change in the energy of the transition state for catalyzed subtilisin folding are plotted for wild type and each of the five mutant prodomains.

ing the importance of interactions between the prodomain with subtilisin's surface helices.

Although there is a general correlation between the binding affinity of mutant prodomains to folded subtilisin and their ability to accelerate folding, high-affinity binding to the native fold is not sufficient to promote folding. The tight binding inhibitors eglin and SSI have $K_i < 10^{-9}$ M (Laskowski & Kato, 1980) but do not accelerate the rate of Sbt-15 folding (Strausberg et al., 1993b). These inhibitors bind exclusively in the subtilisin substrate binding cleft, occupying subsites S1′−S3′ and S1−S5 (McPhalen & James, 1988; Takeuchi et al., 1991). Apparently, this mode of binding does not stabilize the intermediate S_I or any other productive folding intermediates. This observation indicates that much of the substrate binding site of subtilisin, and therefore much of its tertiary structure, is not yet formed in S_I.

On the basis of the magnitude of the folding rate acceleration, it appears that the prodomain binds tightly to the folded form of S_I. Under the folding conditions employed here, the rate of uncatalyzed folding is $\leq 5 \times 10^{-5}$ s^{-1} at 25 °C. The bimolecular folding reaction of wild-type prodomain and Sbt-15 occurs at a rate of 500 M^{-1} s^{-1}, an acceleration of $\geq 10^7$ per mole of prodomain. The binding constant of the prodomain for folded Sbt-15 is 10^8 M^{-1} at 25 °C. In order to increase the concentration of S_I by 10^7-fold, the prodomain must bind to the folded form of S_I almost as tightly (within ~10-fold) as it binds to the completely folded protein. This does not imply that the intermediate P_f-S_I is independently stable but only that it is much more stable than S_I alone. In fact, P_f-S_I is only marginally stable, and its formation remains the rate-determining step in folding up to prodomain concentrations of 100 μM (Strausberg et al., 1993b).

For the purpose of analyzing the independent stability of S_I, the equilibrium for the formation of the bimolecular intermediate, P_f-S_I, can be divided into two steps:

$$P + S_u \overset{K_{SI}}{\rightleftharpoons} P + S_I \overset{K_{Pf-SI}}{\rightleftharpoons} P_f-S_I$$

where $K_{SI} = [S_I]/[S_u]$ and $K_{Pf-SI} = [P_f-S_I]/[S_I][P]$. The overall equilibrium constant for the formation of P_f-S_I ($[P_f-S_I]/[S_u][P]$) has been estimated from transient state kinetic analysis to be $<10^4$ (Strausberg et al., 1993b). Because of the linked equilibrium, the product of $K_{SI} \times K_{Pf-SI}$ is equal to overall equilibrium constant, that is, $<10^4$. At a hypothetical reference state of [P] = 1 M and [P] ≫ [S], K_{Pf-SI} is equal to 10^7, the rate acceleration of subtilisin folding per mole of prodomain. It then follows that the independent stability of the intermediate S_I is very low with an equilibrium constant, K_{SI}, $<10^{-3}$. Thus S_I is insufficiently stable to propagate folding very frequently in the absence of the prodomain.

The prodomain mutations described here are representative of a class which directly affects binding to subtilisin by altering the interface between the prodomain and subtilisin. Another class of prodomain mutations, which affects its binding to subtilisin less directly, are those which alter the independent stability of the prodomain. Although structured in complex with subtilisin, the isolated prodomain is largely unfolded ($\Delta G_{folding}$ = 2 kcal/mol at 25 °C). The low independent stability of the prodomain decreases its binding energy to subtilisin because the cost of folding the prodomain must be paid for with binding energy. A subsequent report will describe mutations of the prodomain which increase its independent stability and will discuss the correlation of prodomain stability with folding activity.

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