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Folding Pathway Mediated by an Intramolecular Chaperone: Dissecting Conformational Changes Coincident with Autoprocessing and the Role of Ca^{2+} in Subtilisin Maturation

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Subtilisin is produced as a precursor that requires its N-terminal propeptide to chaperone the folding of its protease domain. Once folded, subtilisin adopts a remarkably stable conformation, which has been attributed to a high affinity Ca^{2+} binding site. We investigated the role of the metal ligand in the maturation of pro-subtilisin, a process that involves folding, autoprocessing and partial degradation. Our results establish that although Ca^{2+} ions can stabilize the protease domain, the folding and autoprocessing of pro-subtilisin take place independent of Ca^{2+} ion. We demonstrate that the stabilizing effect of calcium is observed only after the completion of autoprocessing and that the metal ion appears to be responsible for shifting the folding equilibrium towards the native conformation in both mature subtilisin and the autoprocessed propeptide:subtilisin complex. Furthermore, the addition of active subtilisin to unautoprocessed pro-subtilisin *in trans* does not facilitate precursor maturation, but rather promotes rapid autodegradation. The primary cleavage site that initiates this autodegradation is at Gln19 in the N-terminus of mature subtilisin. This corresponds to the loop that links α-helix-2 and β-strand-1 in mature subtilisin and has indirect effects on the formation of the Ca^{2+} binding site. Our results show that the N-terminus of mature subtilisin undergoes rearrangement subsequent to propeptide autoprocessing. Since this structural change enhances the proteolytic stability of the precursor, our results suggest that the autoprocessing reaction must be completed before the release of active subtilisin in order to maximize folding efficiency.

Key words: autoprocessing, intramolecular chaperone, propeptide, protein folding, subtilisin.

Propeptides are known to be required for catalyzing the folding of a large number of proteases (*1*). There are examples of serine (*2*), aspartyl (*3*), cysteine (*4*), and metalloproteases (*5*) that are synthesized as precursors. Upon the completion of folding, the propeptides are autoproteolytically removed because they are not necessary for the activity or stability of their cognate folded enzymes (*1*). Furthermore, propeptides function as potent competitive inhibitors of enzymatic activity; in subtilisin this affinity is in the nano-molar range (*6*). Due to their covalent attachment to the proteins they help to fold, propeptides are termed intramolecular chaperones (*7*). The requirements of propeptides for promoting folding have been demonstrated both *in vitro* and *in vivo* (*8*), and a covalent attachment between the propeptide and its associated protein is not necessary for the chaperone function (*8, 9*). Subtilisin represents one well-studied example of propeptide-mediated protein folding, and serves as an ideal model for the very large "subtilase" family that includes members in prokaryotes, eukaryotes, as well as archea (*10–12*). Subtilisin and its homologues adopt highly stable conformations and this stability is mediated in large part by the presence of a high affinity Ca^{2+} binding site (*13*).

Ligands, such as co-factors, substrates, or specific ions, that can stabilize the native structure of proteins can often affect their attainment of a functional state (*14*). Such factors may enhance the folding kinetics either by lowering the activation energy of the rate-limiting step or by shifting the conformational equilibrium towards the native state (*14, 15*). The *in vitro* folding of subtilisin is extremely slow due to a high kinetic energy barrier between the folded and unfolded states (*16*). Removal of the calcium-binding site through mutagenesis enables the protease domain of subtilisin to fold independent of its propeptide (*16, 17*), suggesting that Ca^{2+} ions play an important role in subtilisin folding (*18*). Furthermore, the pro-subtilisin precursor con-

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Abbreviations: AAPF, *N*-succincyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; CD, circular dichroism; EGTA, ethyleneglycol-bis-(beta-aminoethyl-ether)-*N,N,N′,N′*-tetraacetic acid; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; T_M, melting temperature.

tains a tightly bound Ca^{2+} ion, suggesting that the regions of super-secondary structure are already present in the unautoprocessed conformation (*19, 20*). Early pro-subtilisin refolding studies have employed step-wise dialysis procedures (*2, 21–25*). As a result, the role of the Ca^{2+} ion in pro-subtilisin maturation has not been investigated due to the inability to monitor the kinetics of the individual stages of this pathway. A recently developed rapid dilution refolding method (*26*) allows real-time monitoring of the maturation kinetics of wild-type pro-subtilisin. This circumvents the introduction of mutations within the primary sequence and makes it possible to analyze the effect of calcium ions and the role of the folding environment on pro-subtilisin maturation. Since specific metal ions that can stabilize the native structure of proteins often affect their attainment of a functional state, we investigated the role of the metal ligand in the maturation of pro-subtilisin, a process that involves folding, autoprocessing and partial degradation. Our results show that the N-terminus of mature subtilisin undergoes rearrangement subsequent to propeptide autoprocessing, and that the scope of this structural change is substantially greater than earlier proposed (*23, 27*).

MATERIALS AND METHODS

Materials—Ethyleneglycol-bis-(beta-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and the synthetic substrate for subtilisin, *N*-succincyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF), were purchased from Sigma (St. Louis, USA).

Circular Dichroism Studies—Circular dichroism (CD) measurements were performed on an automated AVIV 60DS spectrophotometer (Precision Cells, Hicksville, USA) maintained at 4°C. Spectra were recorded in the range of 260 to 190 nm. The protein concentration was 0.3 mg/ml and a pathlength of 0.1 cm was used. In thermal denaturing measurements, the temperature was increased from 20 to 90°C at 1.0°C intervals, with equilibration for 15 s at each temperature. Data were collected at each temperature for 5 s.

Folding of Denatured Precursors—Wild-type pro-subtilisins E were expressed and purified as described earlier (*27*). Folding was initiated by rapidly mixing 30 μl of 65 μM denatured precursor in 6 M guanidine-HCl, pH 4.8, into 2,970 μl of folding buffer [50 mM Tris-HCl, pH 7.0, 0.5 M $(NH_4)_2SO_4$, 2 mM β-mercaptoethanol] containing either 1 mM $CaCl_2$ or 1 mM EGTA. CD measurements were recorded as described above. Folding kinetics were monitored using a quartz cuvette (1 cm pathlength) and simultaneously recording the changes in the CD spectra at 225 nm as described earlier (*26*). Prism Graph-pad software Version 2.01 was used for data fitting analysis and graph plotting. After completion of the folding experiments, aliquots of the sample were removed and assayed separately for proteolytic activity and the extent of precursor autoprocessing as described below.

Precursor Autoprocessing—Two hundred microliters aliquots of the folded precursor (from buffers containing 1 mM EGTA or 1 mM $CaCl_2$) were removed at fixed time intervals, and the reaction was stopped by adding 22 μl of 100% trichloroacetic acid. The precipitated samples were washed with 100% acetone and then air-dried. The pellet was dissolved in 10 μl of loading dye and subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The extent of precursor autoprocessing was quantitated by densitometry.

Protease Stability in the Presence and Absence of Ca^{2+} Ions—Mature subtilisin was diluted (final concentration 30 nM) into 5 ml folding buffer containing 1 mM EGTA and incubated at room temperature. Aliquots (100 μl) were removed at different time intervals and assayed for enzymatic activity as well as protein concentration by SDS-PAGE analysis.

Protease Activity—An aliquot of the sample was incubated at 25°C in 200 μl of protease assay buffer (folding buffer containing 0.5 mM AAPF). The enzymatic activity of subtilisin was estimated by monitoring (over a 5 min time interval) the release in *p*-nitroaniline by changes in the absorbance at 405 nm using a BioRad UV-microplate reader (Hercules, USA) as described earlier (*26*).

Degradation of Unautoprocessed Precursor and Autoprocessed Complex—The mutants, pro-Ser_{221}Ala-subtilisin and pro-Ser_{221}Cys-subtilisin, were expressed and purified as described earlier (*27*). The folded pro-Ser_{221}Ala-subtilisin precursor and propeptide:Ser_{221}Cys-subtilisin complex were prepared by rapidly mixing 30 μl of 40 μM denatured precursor in 6 M guanidine-HCl, pH 4.8, into 2,970 μl of folding buffer containing 1 mM $CaCl_2$. The folded proteins (1,070 μl) were then mixed with 82 μl of 2.56 μM active subtilisin and 348 μl of refolding buffer. Aliquots (100 μl) were removed at different time intervals and the reaction was stopped by adding trichloroacetic acid. The samples were subjected to SDS-PAGE analysis and several bands were subjected to N-terminal sequencing and mass spectrometric analysis.

RESULTS AND DISCUSSION

Pro-subtilisin maturation involves several distinct stages. The first stage represents the folding of the protease domain mediated by the propeptide (*9, 23*). Upon the completion of folding, the peptide-bond between the propeptide and subtilisin is cleaved by auto-catalysis (*1, 2, 23*), coinciding to structural reorganization (*23, 27*). This change leads to the eventual degradation of the propeptide (*25*), which presumably locks the protease into a stable conformation (*1, 28*). Degradation is required since the propeptide can inhibit the active site of subtilisin with an affinity in the nanomolar range (*6*) to form a stable propeptide:subtilisin complex (*29, 30*). Since mature subtilisin uses Ca^{2+} ion as a stabilizing ligand, we have investigated its role in the maturation pathway of pro-subtilisin. Furthermore, the exact nature and location of the structural changes that coincide with propeptide autoprocessing (*23, 27*), and their relation to Ca^{2+} ions are presently unknown.

Ca^{2+} Does Not Influence the Folding and Autoprocessing of Wild Type Pro-Subtilisin—The refolding of denatured pro-subtilisin can be initiated by removal of the denaturant, either by step-wise dialysis (*2*) or rapid dilution (*23*). The rapid dilution method overcomes the inherent difficulties posed by the step-wise dialysis procedure, and enables quantitative measurements of the kinetics of the individual stages of precursor maturation. Precursor folding was performed using folding buffers with and without Ca^{2+} ions. Buffers without Ca^{2+} ions contain 1 mM EGTA to remove contaminating metal ions. The kinetics of pro-subtilisin folding was monitored by CD spectroscopy as discussed in

"MATERIALS AND METHODS." Rapidly diluted pro-subtilisin adopts a complete secondary structure within 5 min of folding initiation (Fig. 1a). The secondary structural contributions (Fig. 1a, inset) and the rate of pro-subtilisin folding (Table I) are both unaffected by the presence/absence of Ca^{2+} ligand in the folding reaction. Since, precursor maturation requires the cleavage and subsequent degradation of the propeptide, we measured the effects of calcium ions on the rate of pro-subtilisin autoprocessing. The conditions in these experiments were identical to those employed in the folding studies. Figure 1b (inset) demonstrates that pro-subtilisin autoprocessing is evident within 2 min of folding initiation and is complete within 30 min. The rates of folding and autoprocessing were fitted to exponential rate equations, and the rate constants obtained are shown in Table I. It is important to note that Ca^{2+} ion affects neither the folding nor the autoprocessing of pro-subtilisin, even though the unautoprocessed protein has been demonstrated to bind calcium ion with high affinity (*13, 16*). However, the yield of active subtilisin, which is 80% under normal refolding conditions, drops to approximately 55% when folding is carried out in the absence of Ca^{2+} ions (Fig. 2a). This suggests that either the stability of the precursor and/or mature subtilisin may be affected by the metal ligand.

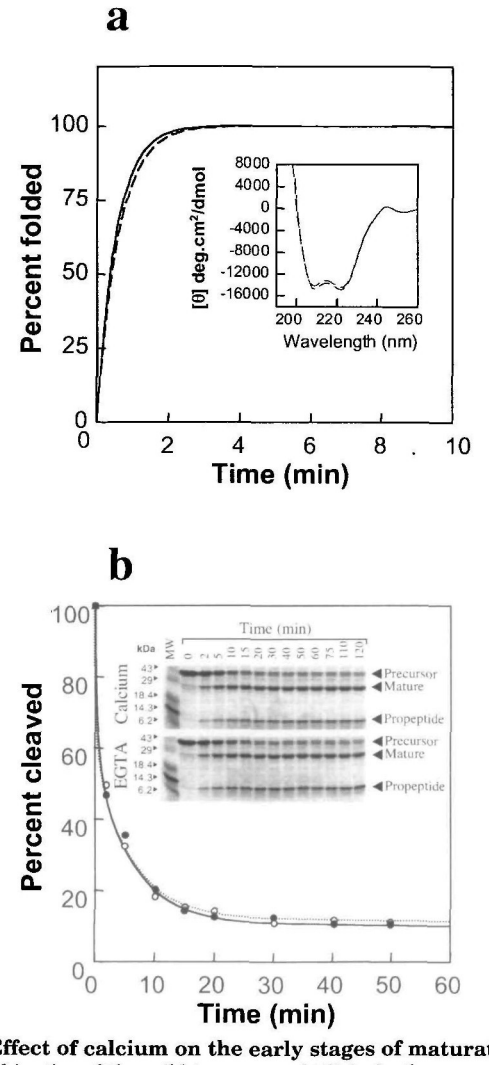

Fig. 1. **Effect of calcium on the early stages of maturation.** (a) Folding kinetics of the wild-type pro-subtilisin in the presence and absence of calcium. Folding was carried out as described in "MATERIALS AND METHODS." Rapidly diluted wild-type pro-subtilisin acquires a complete native structure within 4 to 5 min in the presence of Ca^{2+} ions (depicted by the solid line). The profile is almost unchanged in the absence of Ca^{2+} ions (depicted by the dashed line). The kinetic constants are listed in Table I. Percent folding was calculated as $\%F \simeq [(A-A_D)/(A_F-A_D)] \times 10^2$, where A represents ellipticity at 225 nm at a give time, while A_F and A_D represent the signals of the fully folded and completely denatured precursors. Inset describes the CD spectra of the precursors with (solid line) and without (dashed line) Ca^{2+} ions. (b) Autoprocessing of pro-subtilisin (precursor). The reaction was monitored as described in "MATERIALS AND METHODS." Autoprocessing of pro-subtilisin in the presence (filled circles) and absence of calcium ions (open circles) fits an exponential rate equation. The propeptide produced by autoprocessing is maximum after 30 min. Approximately 80% of the theoretically expected amount of mature subtilisin is produced through this reaction. SDS-PAGE of the maturation reaction is shown in the inset.

The Stability and Activity of Mature Subtilisin and the Autoprocessed Complex Are Ca^{2+} Dependent—We next monitored the stability of wild-type subtilisin E in the presence and absence of Ca^{2+} ions. Pro-subtilisin was renatured under normal refolding conditions to obtain active subtilisin, and subsequently diluted (10-fold) into folding buffer lacking calcium, but containing 1 mM EGTA instead. A 10-fold dilution of active subtilisin into folding buffer containing Ca^{2+} ions was used as the control. The samples were incubated at room temperature and aliquots were removed at various time intervals, and the proteolytic activity was plotted as a function of time. Figure 2b demonstrates that in the absence of Ca^{2+}, subtilisin loses more than 75% of its activity in 500 min. This loss of activity occurs due to autodegradation and not due to denaturation (data not shown). The half-life of the inactivation reaction with and without Ca^{2+} ions was 8,139 and 329 min, respectively. This suggests that Ca^{2+} ions stabilize subtilisin by approximately 25-fold, a results consistent with earlier work that demonstrated that Ca^{2+} affects the stability of subtilisin (*13, 16, 17*).

To examine the effects of Ca^{2+} ions on the unautoprocessed precursor and autoprocessed complex, we used pro-subtilisin mutants whose active site Ser221 residue was replaced by Ala and Cys, respectively. These precursors can adopt secondary structures similar to those of the wild-type precursors. However, as a consequence of Ser_{221}Ala and Ser_{221}Cys substitutions, the maturation reaction is blocked at the stages of autoprocessing and degradation, respectively (*23, 27, 29*). The unautoprocessed pro-Ser_{221}Ala-subtilisin adopts a classical α-β-conformation that is unaffected by the presence or absence of calcium (Fig. 3a). However, the thermal stability of this unautoprocessed pro-Ser_{221}Ala-subtilisin is significantly lower than that of mature

TABLE I. **Effect of calcium ions on the kinetics of maturation.**

Property	With calcium	Without calcium
Rate constant of folding (s^{-1})	0.061	0.058
Rate constant of autoprocessing (s^{-1})	0.1594	0.1523
Melting temperature of proteins (°C)		
Pro-Ser_{221}Ala-subtilisin	46.6	44.1
Propeptide:Ser_{221}Cys-subtilisin	49.7	42.5
Mature subtilisin	59.1	49.2

Ser_{221}Cys-subtilisin (Fig. 3b). Calcium ions appear to stabilize the unautoprocessed precursor marginally and increase the melting temperature (T_M) from 44°C (without Ca^{2+}) to 47°C (with Ca^{2+}), consistent with the finding that Ca^{2+} is associated with the unautoprocessed precursor (*19, 20*). It is also important to note that the thermostability of the unautoprocessed precursor is lower than that of the autoprocessed propeptide:Ser_{221}Cys-subtilisin complex, which is 50°C in the presence of Ca^{2+} ions. This suggests that autoprocessing of the peptide bond increases the cooperativity within the individual subdomains. Interestingly, the removal of the Ca^{2+} ion from the subtilisin domain dramatically destabilizes the autoprocessed complex and reduces the T_M to approximately 42°C. Degradation of the propeptide increases the thermostability of the protease domain to 59.5°C in the presence and 50°C in the absence of Ca^{2+} ions.

The N-Terminal Domain of Subtilisin Undergoes Structural Changes Subsequent to Autoprocessing—A comparison between the secondary structures of the unautoprocessed precursor and autoprocessed complexes suggests that minor secondary structural changes occur coincident with autoprocessing. In order to localize the area of these structural changes, we examined the autoproteolytic stability of the autoprocessed complex and unautoprocessed precursors. The unautoprocessed pro-Ser_{221}Ala-subtilisin precursor and the autoprocessed propeptide:Ser_{221}Cys-subtilisin complex were incubated with a half-molar amount of active subtilisin in buffer supplemented with Ca^{2+}. Aliquots of the protein were removed at different time intervals and the extent of degradation was examined. As seen in Fig. 4a, the addition of mature subtilisin results in the cleavage of the unautoprocessed precursor (lane 1) into at least 5 dif-

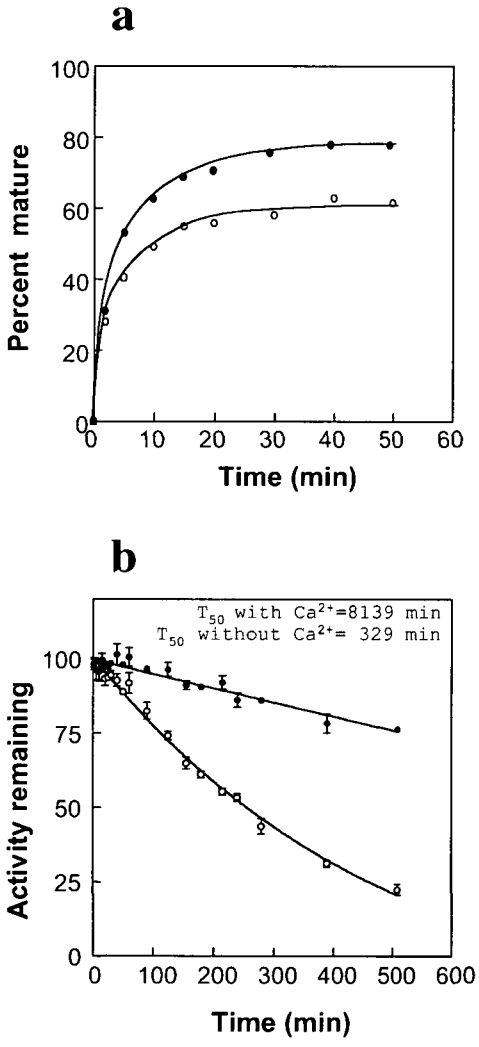

Fig. 2. **Effect of calcium on the yield and stability of mature subtilisin.** (a) The yield of subtilisin was estimated from the amount of mature subtilisin in Fig. 1b, inset, and plotted as a function of time after the initiation of folding. Filled circles represent folding in the presence of calcium while the open circles depict folding in the presence of 1 mM EGTA. (b) Stability of the protease domain. Subtilisin in the presence of calcium (open circles) displays a half-life of 8,139 min, 25-fold longer than in the absence of calcium (filled circles).

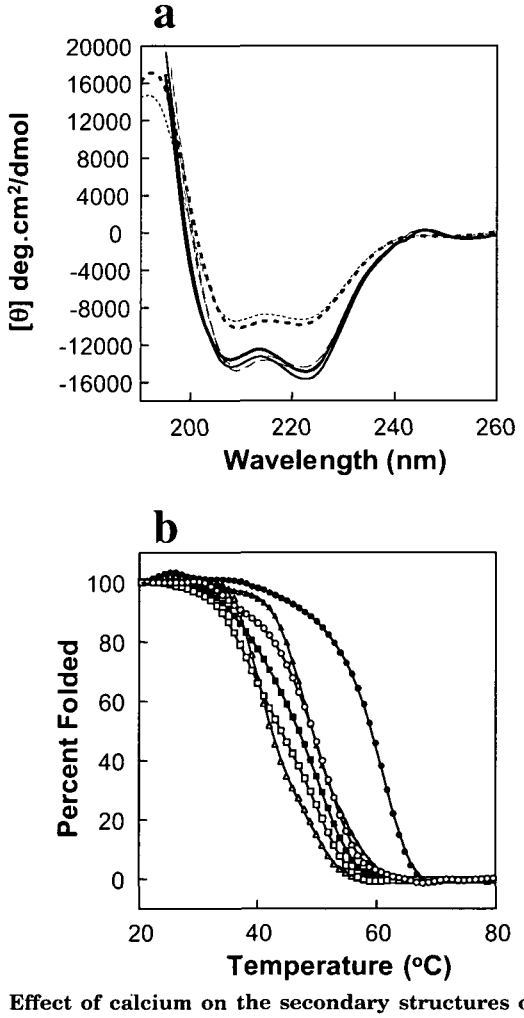

Fig. 3. **Effect of calcium on the secondary structures of the precursor and mature enzymes.** (a) CD spectra of pro-subtilisin. Thick lines represent proteins in the presence of calcium ions while thin lines depict proteins in the presence of 1 mM EGTA. The solid line represents pro-Ser_{221}Ala-subtilisin, the dashed line indicates mature subtilisin, and the dotted line shows the autoprocessed complex. (b) Thermal stability of pro-subtilisin in the presence and absence of calcium. Filled symbols represent proteins in the presence of calcium while open symbols indicate the presence of 1 mM EGTA. Mature subtilisin (circles) has a lower ellipticity than pro-Ser_{221}Ala-subtilisin (squares) or pro-Ser_{221}Cys-subtilisin (triangles).

ferent bands (lane 2). The most prominent band occurs below the exogenously added mature subtilisin and was removed and analyzed by mass spectrometry and N-terminal sequencing. The molecular mass of this band corresponds to 25.737 kDa, demonstrating that the cleavage site [HSQ-GYTG] corresponds to Gln19 in mature subtilisin. This Gln19 is located in the loop that links the α-helix-2 and β-strand-1 in the N-terminus of subtilisin, as shown on the model of the pro-S_{221}A-subtilisin precursor (Fig. 4b). However, this cleavage does not occur in the autoprocessed propeptide:Ser_{221}Cys-subtilisin complex, and the addition of wild-type subtilisin causes the rapid degradation of the propeptide domain. This suggests that the N-terminus of the protease domain undergoes structural changes coincident with autoprocessing. Since it has been demonstrated that the unautoprocessed precursor contains the high affinity site (*19, 20*), and the deletion of this site allows propeptide-independent folding of subtilisin (*16, 17*), we have analyzed the structure of the complex. The X-ray structure of mature subtilisin reveals the structural details of two calcium ion binding sites (*31*), both of which are also present in the autoprocessed propeptide:subtilisin complex (*30*) (Fig. 4b). The high-affinity A-site in the autoprocessed complex binds Ca^{2+} through residues Gln2 $O^{\varepsilon 1}$, Asp41 $O^{\delta 1}$, Asp41 $O^{\delta 2}$, Leu75 O, Asn77 $O^{\delta 1}$, Ile79 O, Val81 O, while the low-affinity B-site makes contacts with the Ca^{2+} ion through the main chain carbonyl atoms of Ala169, Try171, and Thr174 (*30*). However, Gln2 $O^{\varepsilon 1}$ would be unable to contribute to the high affinity site in the unautoprocessed complex because Ala1 is covalently linked to the C-terminus of the propeptide (*23, 27*). This indicates that the N-terminal part of subtilisin may accommodate structural changes to enable intramolecular autoprocessing (*23, 27, 32, 33*).

Role of Ca^{2+} Ligand and the Propeptide-Domain in Conformation Locking—While the unautoprocessed precursor contains a high affinity Ca^{2+} binding site, we demonstrate that this site does not contribute to the folding and autoprocessing stages. This ligand is, however, crucial for stabilizing the mature subtilisin against autoproteolysis. This is unlike the case of proteinase K (a homologue of subtilisin) whose activity, but not stability, is modulated by Ca^{2+} ions (*36, 37*). Since the unautoprocessed precursor is proteolytically unstable, our results demonstrate that the inhibitory function of the propeptide is required to allow the folded precursor to complete its autoprocessing before active subtilisin is released. Therefore, the inhibitory function of the propeptide maximizes maturation efficiency. After propeptide cleavage, the N-terminus moves away from the active

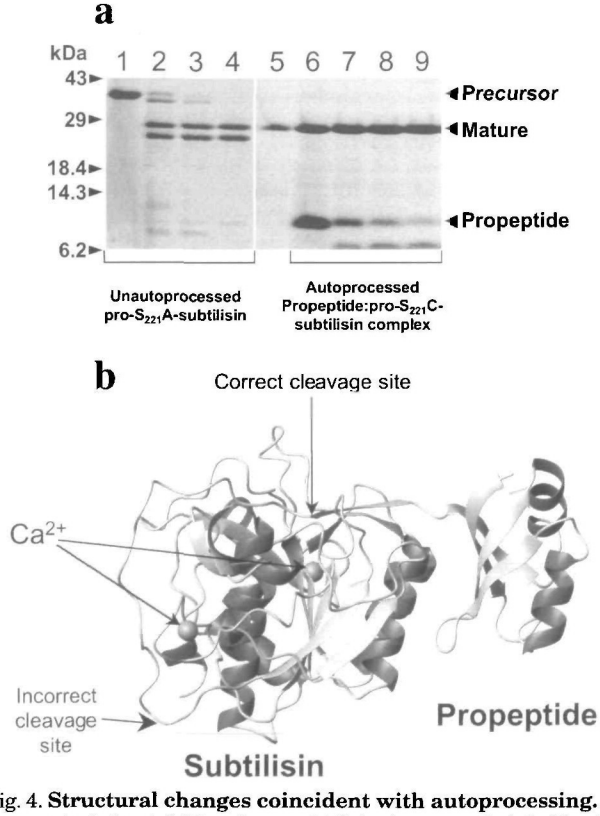

Fig. 4. **Structural changes coincident with autoprocessing.** (a) Autoproteolytic stability of pro-subtilisin (precursor). A half-molar amount of active subtilisin (lane 5) was added to folding buffer containing unautoprocessed pro-Ser_{221}Ala-subtilisin (lane 1), and degradation was monitored in aliquots taken at 0.5 min (lane 2), 1 min (lane 3), and 2 min (lane 4). Simultaneously, the same amount of active subtilisin (lane 5) was added to folding buffer containing propeptide:Ser_{221}Cys-subtilisin complex (lane 6), and aliquots were taken at 0.5 min (lane 7), 1 min (lane 8), and 2 min (lane 9). (b) A model of the unautoprocessed precursor indicating the correct and incorrect cleavage sites. Intramolecular autoproteolysis occurs at the correct site while transproteolysis can occur at the incorrect cleavage site.

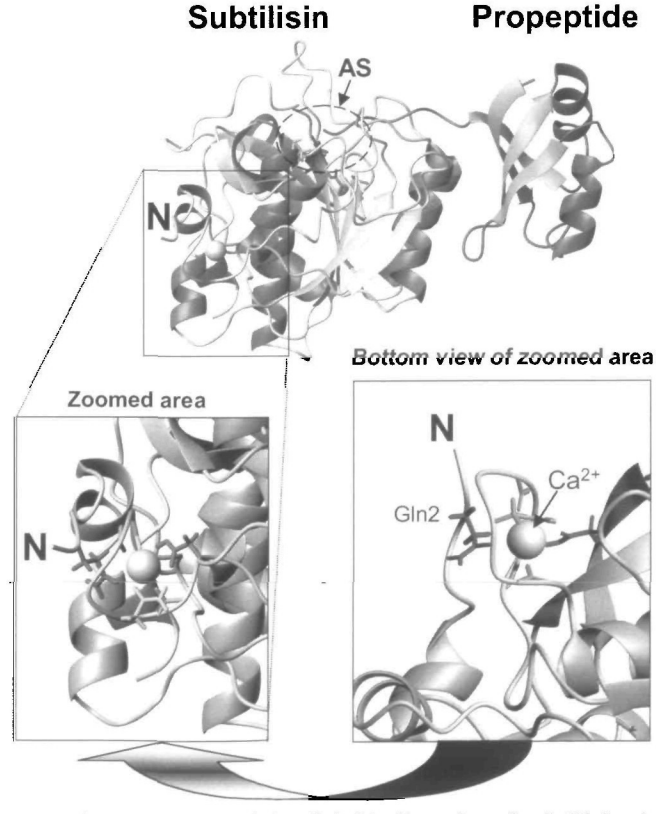

Fig. 5. **Close up view of the Ca^{2+} binding site of subtilisin.** A ribbon model of the crystal structure of the propeptide:Ser_{221}Cys-subtilisin complex is shown, and the area around the calcium binding site and N-terminus is enlarged in the two lower panels. The residues connected to the calcium ion are shown in neon. N indicates the N-terminus of subtilisin and AS shows the active center.

site (Fig. 5) and forms a hydrogen bond with the Ca^{2+} binding loop. This presumably stabilizes the autoprocessed complex.

Although many proteases require their propeptides to facilitate folding, these findings do not constitute a universal rule. For example, although α-lytic protease shares the same folding as trypsin and chymotrypsin (*34*), the latter proteases fold correctly when expressed independently of their propeptide-domains (*35*). Hence it is tempting to speculate that proteins that employ dedicated intramolecular chaperones do so because of their need to acquire a very stable protein conformation. Most proteases that function in harsh environments require propeptides to facilitate folding. Such proteases need to be stable towards autolysis or inactivation by other proteases in their environment. Proteolytic stability can be achieved through tight packing and reduced configurational entropy. The consequence of this rigidity often results in a high-energy unfolding transition state that requires the total disruption of an extremely tight conformation. Difficulty in unfolding tight conformations can also occur during folding, and, therefore, such proteases require the assistance of dedicated propeptides or intramolecular chaperones. Here we show that after the cleavage of the propeptide domain, the N-terminus of mature subtilisin undergoes conformational changes that contribute to the formation of the Ca^{2+} binding site, which, in turn, traps the folded subtilisin domain into a stable conformation. However, since the continued presence of the propeptide decreases the stability of the complex, this domain is destroyed through autoproteolysis. Hence propeptides can function as single-turnover folding catalysts in order to lock the protease domain into a stable conformation.

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