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Folding pathway mediated by an intramolecular chaperone

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ABSTRACT **The N-terminal propeptide of subtilisin, a serine protease, functions as an intramolecular chaperone which is crucial for proper folding of the active enzyme. This nascent N-terminal propeptide is removed after completion of the folding process. Here we present a possible pathway by which intramolecular chaperones mediate protein folding. Using circular dichroism to analyze acid-denatured subtilisin we have identified a folding-competent state which can refold to an active conformation in the absence of the propeptide. Earlier work had shown that guanidine hydrochloride-denatured subtilisin was in a state incapable of folding in absence of its propeptide. Comparison of the folding-incompetent and folding-competent states indicates that refolding is facilitated by the presence of residual structure present only in the folding-competent state. The analysis further indicates that the propeptide is essential for inducing this state. Therefore the folding-competent state may lie on—or be in rapid equilibrium with an intermediate on—the folding pathway of subtilisin. In the absence of the propeptide, formation of such a state—and hence refolding—is extremely slow.**

A large number of proteases, both in prokaryotes and in eukaryotes, are synthesized as precursors with N-terminal prosegments which play a vital role in the folding pathway (1). Subsequently, these propeptides are cleaved proteolytically to generate active enzymes. Hence, proteins such as subtilisins (2, 3), α-lytic protease (4), and carboxypeptidase (5), when unfolded in guanidine hydrochloride (Gdn·HCl) solution, refold only in the presence of their propeptides. Using subtilisin E as a model system, work in our laboratory has unambiguously shown that the 77-amino acid propeptide plays a vital role in folding the 275 amino acids of the mature enzyme (6). Because the propeptide is covalently attached to the N terminus of the mature enzyme prior to its maturation, and because each propeptide molecule mediates the folding of one enzyme molecule (generally the corresponding molecule), the term *intramolecular chaperones* has been coined for such propeptides to distinguish them from chaperones (6–8). Concurrent with this proposition, it was shown that the 13-amino acid propeptide of bovine pancreatic trypsin inhibitor facilitated the *in vitro* folding pathway by acting as a tethered, solvent-accessible, intramolecular thiol–disulfide reagent (9). Recently, it was shown that the propeptide of α-lytic protease helped to overcome a kinetic block in the folding pathway (10). The mature region refolded in the absence of the propeptide exists in a catalytically inactive "molten globule" state. On addition of the propeptide, this molten globule state changes to an enzymatically active form. Moreover, the propeptide functions at only one point in the folding pathway, an observation suggesting that the propeptide-mediated folding pathway is under kinetic and not thermodynamic control (10, 11). Attempts in our laboratory to trap a similar intermediate in the case of subtilisin were unsuccessful. This prompted us to believe that the propeptide of subtilisin facilitates folding in a manner different from that of α-lytic protease.

Earlier work in our laboratory showed that *in vitro* the propeptide from subtilisin E could refold not only subtilisin E but also subtilisin BPN′ and subtilisin Carlsberg. All three of these subtilisins are highly homologous and have very similar three-dimensional structures. In the present work, we have taken advantage of the ability of the propeptide of subtilisin E to facilitate refolding of subtilisin BPN′. Comparing the structures of subtilisin denatured by acid and by Gdn·HCl, we have identified and analyzed a state capable of refolding in absence of the propeptide. Refolding studies carried out in both the presence and the absence of the 77-amino acid propeptide emphasize that the propeptide enhances folding by a factor of about 1000. The rate of folding is increased, possibly by induction of some helical structure in unfolded protein molecules which in turn may propagate secondary and tertiary structure along other unfolded domains. In the absence of the propeptide, formation of such a structure and, hence, refolding are extremely slow. Thus, formation of this structure may be a rate-limiting step in propeptide-mediated folding. Moreover, the interaction between the propeptide and the mature region is dynamic. The 77-amino acid propeptide is required throughout the folding pathway. Structure in the propeptide is induced by its interactions with the mature region, and as it adopts its α-helical structure, it facilitates folding of mature subtilisin.

MATERIALS AND METHODS

Materials. Gdn·HCl, subtilisin BPN′, chymotrypsin inhibitor, EGTA, and succinyl-L-Ala-L-Ala-L-Pro-L-Phe *p*-nitroanilide were from Sigma. Membranes (type VM, 0.05 μm) for drop dialysis were from Millipore. All other chemicals were of reagent grade and were used without further purification.

Isolation of the 77-Amino Acid Propeptide. The 77-mer propeptide from subtilisin E was expressed in *Escherichia coli* using a high expression vector, pET11a, and subsequently purified to homogeneity (Y.L. and M.I., unpublished work).

Denaturation of Active Subtilisin. Subtilisin BPN′ (1 mg) was dissolved in 1 ml of ice-cold 25 mM H_3PO_4 (pH 1.8) and incubated for 30 min at 4°C. The time of incubation with the acid was critical. Decreasing the time of exposure of the protein to the acid resulted in incomplete denaturation, whereas prolonged incubation with acid resulted in a substantial decrease in the efficiency of salt-induced refolding. The solution was then drop-dialyzed for 60 min at 4°C against excess 50 mM Tris·HCl (pH 7.0) with constant stirring. The retentate was then spun at 222,000 × *g* for 20 min to remove any precipitated protein. The supernatant was then stored at −20°C. Gdn·HCl-denatured subtilisin was prepared as follows. Subtilisin BPN′ (1 mg) was dissolved in 1 ml of 50 mM

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Abbreviations: Gdn·HCl, guanidine hydrochloride; FC, folding-competent; FIC, folding-incompetent.

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Tris·HCl, pH 7.0/6 M Gdn·HCl. The solution was incubated for 3 hr at room temperature. This solution was then drop-dialyzed as described earlier and stored at −20°C. For double-denatured subtilisin, Gdn·HCl-denatured subtilisin (1 mg/ml) was first dialyzed against 50 mM Tris·HCl (pH 7.0). Then cold 25 mM H_3PO_4 was added until the pH dropped to ≈1.8; the material was then dialyzed against excess cold 25 mM H_3PO_4 for 30 min. This double-denatured subtilisin was once again dialyzed against excess 50 mM Tris·HCl (pH 7.0). To assess the role played by calcium ions in salt-induced folding, subtilisin was denatured in acid as described above. The only difference was in the dialysis buffers [50 mM Tris·HCl (pH 7.0)], which contained 1 or 3 mM EGTA. Final concentrations of all protein solutions were adjusted to 5.0 μg/ml.

Protein Determination. Protein concentration was estimated by measuring OD_{280} with bovine serum albumin as a standard. One OD_{280} unit corresponded to a protein concentration of 1.0 mg/ml.

Assay of Subtilisin Activity. Subtilisin activity was assayed spectrophotometrically by monitoring the release of *p*-nitroaniline at 410 nm due to the enzymatic hydrolysis of the substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe *p*-nitroanilide (12). The activity was measured using 0.15 mM substrate in 50 mM Tris·HCl, pH 8.5/1 mM $CaCl_2$ in a final volume of 1.0 ml. The reaction was terminated by addition of 0.25 ml of 2 M sodium citrate (pH 5.0). One unit of enzyme activity is defined as the amount of enzyme that produces 1 mmol per hour.

Refolding of Denatured Subtilisin BPN'. Salt-induced refolding of acid- and Gdn·HCl-denatured subtilisin was studied by addition of KCl to 0.6 M, followed by incubation at 4°C for 1 hr. Propeptide-mediated folding was achieved by addition of subtilisin E propeptide to denatured subtilisin in a 1:1 molar ratio and incubation at 4°C for 12 hr unless otherwise indicated. All renaturation experiments were repeated five times, and the data presented are the averaged values.

Preparation of Subtilisin BPN'–Propeptide Complex. Subtilisin–propeptide complex was prepared by adding 56 μl of propeptide (1 mg/ml) to 200 μl of subtilisin E (1 mg/ml) in a final volume of 1 ml of 10 mM phosphate buffer (pH 7.0), to give a 1:1 ratio of propeptide to subtilisin E. The complex thus prepared, containing 256 μg of protein, was used immediately for structural studies.

Circular Dichroism (CD) Studies. CD measurements were performed on an automated Aviv 60 DS spectrophotometer with a thermostatted cell holder controlled by an on-line temperature-control unit. Quartz rectangular cells (Precision Cells, Hicksville, NY) with a path length of 1 mm were used. For CD studies, protein solutions were dialyzed against excess 10 mM potassium phosphate (pH 7.5). Solutions were filtered through a 0.22-μm filter before measurements. Protein concentrations were 0.2 mg/ml. For the subtilisin BPN'–propeptide complex, the total protein was 256 μg. Each protein solution was freshly prepared and was scanned five times from 260 to 190 nm and at 4°C; the scans were averaged before baseline subtraction.

RESULTS AND DISCUSSION

Work conducted in our laboratory on the role of the propeptide in subtilisin E has shown that its complete removal results in production of misfolded, inactive subtilisin (3). Partial removal of the propeptide (i.e., residues −77 to −68 or −77 to −64) results in the production of active subtilisin. The efficiency of these shorter peptides is 30–40% of that of the 77-mer. However, removal of amino acids −77 to −63 results in a complete loss in folding efficiency (13). Furthermore, covalent attachment of the propeptide to the N terminus of subtilisin is not a necessary criterion for refolding, and hence exogenously added propeptide can also effect folding (1). *In vivo*, the covalently attached propeptide is cleaved by an intramolecular mechanism. Under certain conditions, however, it is possible for an intermolecular cleavage to occur. In both cases the propeptide acts as a substrate for subtilisin (7). Moreover, the cleavage is mediated by the same catalytic triad—namely, Asp^{32}, His^{64}, and Ser^{221}. The propeptide binds to the active-site region of subtilisin and acts as a competitive inhibitor with a K_i comparable with that of *Streptomyces* subtilisin inhibitor, the strongest known inhibitor of subtilisin (14).

Earlier work had shown the inability of Gdn·HCl-denatured subtilisin to refold by itself to a biologically active state (6–8, 15). We call this state a folding-incompetent (FIC) state. Refolding of the FIC state was achieved only in the presence of its propeptide (3). An acid-denatured (25 mM H_3PO_4, pH 1.85) subtilisin mutant in which one of the calcium binding sites was removed was shown to refold completely under high salt concentrations (0.6 M KCl) in the absence of the propeptide (16). However, recently it has been suggested that this state may correspond to a molten globule state (17). The fact that subtilisin could refold in the absence of its propeptide was rather surprising and appeared to be contradictory to earlier work in our lab. When Gdn·HCl-denatured subtilisin was allowed to refold over 20 days in the absence of the propeptide in ionic concentrations close to those *in vivo*, only up to 1% of the activity was recovered. In the case of acid-induced denaturation, recovery under similar conditions was 2–3% (Fig. 1). Extensive dialysis of Gdn·HCl (6 M)-denatured subtilisin against a refolding buffer produced active subtilisin only in the presence of the propeptide. Efforts to trap an intermediate in the folding pathway as shown by Baker *et al.* (10) were unsuccessful when subtilisin was denatured by 6 M Gdn·HCl. Fig. 2*A* depicts the CD profiles of subtilisin denatured by various methods. Both rapid dilution and slow dialysis show that subtilisin is still in an unfolded, random coil structure, suggesting that in the case of subtilisin this intermediate may be a very labile and transient one.

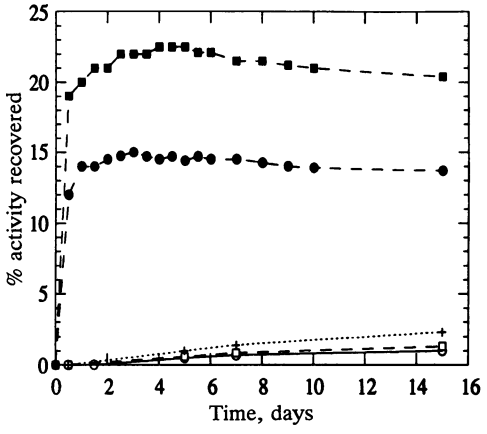

FIG. 1. Refolding kinetics of subtilisin. Refolding of denatured subtilisin was carried in 50 mM Tris·HCl at pH 7.0 and activity was estimated as described. ○ and ●, subtilisin unfolded by Gdn·HCl, refolded without and with the 77-amino acid propeptide, respectively; □, refolding in the presence of chymotrypsin inhibitor; ■, acid-denatured subtilisin refolded with the propeptide; +, acid-denatured subtilisin refolded in the absence of the propeptide. Percent recovery was estimated by calculating the ratio of activity of refolded subtilisin to the activity of native subtilisin under identical conditions. The folding rates were estimated during the first 3 hr because the active subtilisin produced degrades unfolded subtilisin. Refolding rate of subtilisin alone was 5.10×10^{-7} units/sec while that of Gdn·HCl-denatured subtilisin in presence of the propeptide was 4.85×10^{-4} units/sec, giving a calculated rate enhancement of 950.

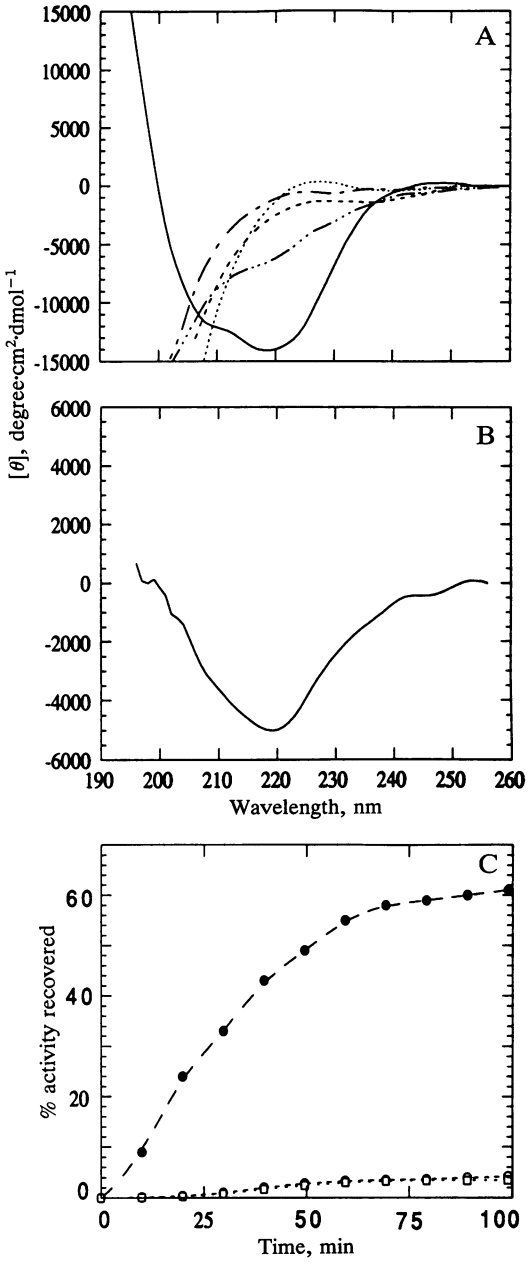

FIG. 2. Characterization of unfolded subtilisin. (*A*) CD profiles of unfolded subtilisin. —, profile for native subtilisin; —·—, subtilisin unfolded by 6 M Gdn·HCl and dialyzed with 10 mM phosphate buffer at pH 7.0; -----, 6 M Gdn·HCl-denatured subtilisin after rapid dilution; ···—···, [25 mM H_3PO_4 (pH 1.85)]-unfolded subtilisin; ·····, Gdn·HCl-unfolded subtilisin dialyzed against phosphate buffer and subsequently incubated with acid for 1 hr and then neutralized to pH 7.0. (*B*) CD difference spectrum between acid-denatured and Gdn·HCl-denatured subtilisin. (*C*) Kinetics of 0.6 M KCl-induced refolding of unfolded subtilisin. Unfolding was effected by using 6 M Gdn·HCl (□), Gdn·HCl followed by 25 mM H_3PO_4 (pH 1.85) (○); ●, refolding of acid-denatured subtilisin.

Fig. 2*C* describes refolding profiles (measured in terms of activity) of denatured subtilisin. Acid-denatured subtilisin refolds within 60 min of addition of 0.6 M KCl. However, Gdn·HCl-denatured subtilisin barely refolds. One of the possible reasons may be that acid denaturation does not completely unfold the protein, which therefore retains some small element of structure that may facilitate folding of other domains in the protein. Alternatively, at pH 1.85, certain amino acid side chains may be protonated and crucial for folding. To verify this, subtilisin was denatured first by Gdn·HCl and dialyzed with a buffer at pH 7.0. This dialysate was then subjected to a second denaturation, this time by acid as described in *Materials and Methods*. The subtilisin which was subjected to double denaturation was dialyzed against excess 50 mM Tris·HCl (pH 7.0) for 60 min. Folding of this Gdn·HCl/acid-denatured subtilisin with 0.6 M KCl did not occur with the same efficiency as acid-denatured subtilisin (Fig. 2*A*). This ruled out the second possibility. Moreover, acid denaturation resulted in aggregation which, for the same protein concentration, was not observed with Gdn·HCl. However, when subtilisin first denatured with Gdn·HCl was dialyzed against 50 mM Tris·HCl (pH 7.0) and then 25 mM H_3PO_4 (pH 1.85), no aggregation was observed. Thus, acid-denatured subtilisin is likely to retain a small amount of structure (18, 19). This small element of structure was indeed detected by the difference between the CD profiles of Gdn·HCl and acid-denatured subtilisin (Fig. 2*B*). However, note that subtilisin has two calcium binding sites, and these may be responsible for the residual structure observed in acid-denatured subtilisin.

To assess the role of calcium ions in protein folding, acid-denatured subtilisin was dialyzed against 50 mM Tris·HCl (pH 7.0) containing 1 or 3 mM EGTA, a calcium-specific chelating agent. Fig. 3 compares the refolding rates of acid-denatured subtilisin with and without EGTA. No significant difference was seen, indicating that calcium ions are not responsible for the residual structure and are not required in salt-induced refolding. This may also suggest that calcium ions may be stabilizing the folded conformation of subtilisin.

One of the reasons why high-salt conditions facilitate folding is that the propeptide itself is highly charged (3). The amino acid sequence of the propeptide is unusual and extremely biased when compared with the sequence of mature subtilisin. The propeptide contains 17 basic amino acid residues (15 lysine and 2 histidine) along with 11 acidic residues (6). This makes more than a third (36%) of the propeptide highly charged. Moreover, the distribution of these residues is highly uneven, suggesting that these residues are on the surface of the three-dimensional structure of the propeptide. This highly ionic environment provided by the propeptide can to a certain extent be mimicked by high salt.

Subtilisins BPN′ (20), Carlsberg (21), and Novo (22) have been shown to be very similar, containing seven α-helical structures ranging from 7 to 18 residues. Two of these

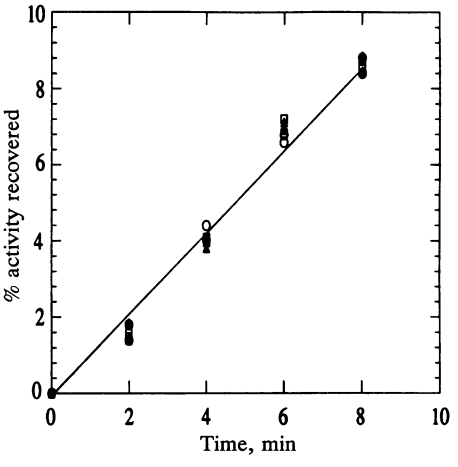

FIG. 3. Refolding of acid-denatured subtilisin dialyzed against 50 mM Tris·HCl (pH 7.0) without EGTA (○) or with 1 mM (□) or 3 mM (▲) EGTA. As no significant differences in the refolding rates with and without EGTA were observed, only one line depicting the best fit is shown.

α-helices, nos. 5 and 6, corresponding to amino acids 133–147 and 220–237, are largely hydrophobic and contain 6 and 5 alanine residues, respectively. Therefore, helices 5 and 6 are probably more stable than the other helices at pH 1.85. Moreover, stretches of alanine residues (as those found in helices 5 and 6) adopt an α-helical structure at low pH (23), suggesting that these helices could be retaining their structure and facilitating the refolding of subtilisin.

CD studies on the 77-mer propeptide reveal that it has largely a random coil structure (Fig. 4). However, in the presence of subtilisin, the propeptide adopts an α-helical structure. Fig. 4*B* shows the difference spectrum between native subtilisin and its complex with the 77-mer propeptide. If the formation of the complex does not alter the secondary structure of the mature region, it may be possible to make a rough estimate of the structure of the 77-mer propeptide. In a 1:1 complex the propeptide is only about 20% of the total mass (77/352) of the complex. Therefore, a mixture of 80% native CD and 20% propeptide-alone CD would give the same effect. To verify this, 80% of the amplitude of the spectrum of native subtilisin and 20% of the amplitude of the spectrum of the propeptide alone were added and compared with the CD spectrum of the 77-mer propeptide complexed with native subtilisin (Fig. 4*C*); the difference can clearly be seen. The ellipticity at 208 and 222 nm for the complex is lower than that of the native subtilisin and that of the 1:1 ratio mixture. Therefore, a difference in the spectra, assuming no secondary structural change occurs upon the formation of the complex, would give the structure of the propeptide in a complex. The approximate secondary structure in the propeptide complexed with native subtilisin as estimated by the method of Greenfield and Fasman (24) is 31% α-helix, 27% β-sheet, 33% random coil, and 9% β-turn. However, adding trifluoroethanol to the 77-mer propeptide, thereby increasing the hydrophobicity in the environment, increases the helical content to 38%. This indicates that somewhere in the folding pathway, the propeptide interacts with hydrophobic elements (possibly helices 5 and 6) along subtilisin and begins to adopt an α-helical structure to induce the mature region to start folding. Thus, the propeptide and the mature sequence work in a dynamic, symbiotic relationship. Comparing the rate of folding (Fig. 1) in the presence and absence of a competitive inhibitor (chymotrypsin inhibitor) and the propeptide gives an idea of the rate enhancements brought about by the propeptide. Percent recovery was estimated by calculating the ratio of the activity of refolded subtilisin to the activity of native subtilisin under identical conditions. The folding rates were estimated during the first 3 hr, as the active subtilisin produced degrades unfolded subtilisin. The refolding rate of subtilisin alone was 5.10×10^{-7} subtilisin activity units/sec while that of Gdn·HCl-denatured subtilisin in presence of the propeptide was 4.85×10^{-4} units/sec, giving a calculated rate enhancement of ≈1000-fold over that of the inhibitor and without the propeptide. This result also implies that the primary role of the propeptide is not a competitive inhibitor (11) of subtilisin to protect unfolded subtilisin from degradation.

We thus propose a folding pathway for subtilisin (Fig. 5). By appropriately manipulating denaturation, we can show that there are at least two different intermediates—namely the FIC state and the folding-competent (FC) state. Under any salt conditions there is a high barrier between the FIC and FC states, and under conditions of low salt there is a high barrier between the FC state and the native state. The propeptide, which is highly ionic, can interact with the FC state at low ionic strength and facilitate the conversion to the active form, perhaps in a manner analogous to the way high salt brings about folding. Moreover, the propeptide can also interact with the FIC state and facilitate its conversion to the active form. Thus the propeptide acts as an intramolecular chaperone and possibly works by reducing the energy of rate-limiting barriers. However, Gdn·HCl-treated subtilisin adopts a completely denatured state and, when allowed to fold in the absence of the propeptide, can adopt various conformations, probably 1% or less of which correspond to the FC state.

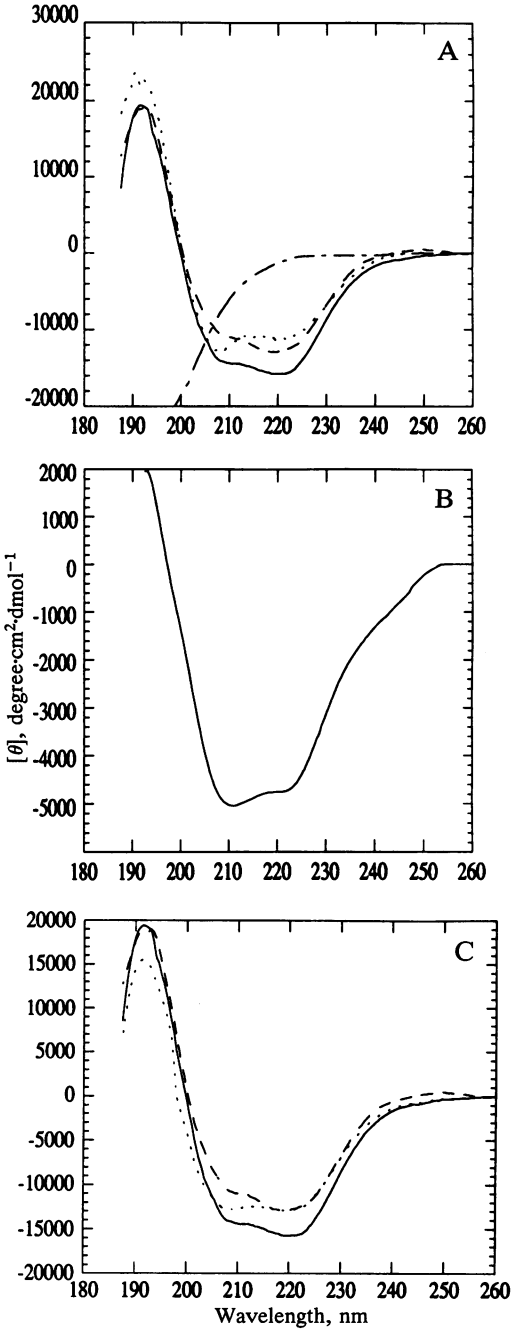

FIG. 4. Structural characterization of the propeptide. (*A*) CD profiles of the 77-amino acid propeptide in 10 mM phosphate buffer at pH 7.0 (—·—), in 50% trifluoroethanol (····), or in a 1:1 complex with mature subtilisin in buffer (– – –). Native subtilisin in 10 mM phosphate buffer is also represented (—). (*B*) Difference spectrum between native subtilisin and its complex with the 77-amino acid propeptide. (*C*) CD Profile of a theoretically computed 1:1 ratio of the propeptide to mature subtilisin (····) compared with spectra native subtilisin (– – –) and subtilisin complexed to the 77-amino acid propeptide (—). The profile was computed by summing the individual contributions of the propeptide alone and native subtilisin. The propeptide contributes about 20% whereas native subtilisin accounts for 80% of the total mass of the complex. The difference between the theoretically computed and experimentally obtained CD profiles suggests that the mature enzyme induces a conformation in the propeptide.

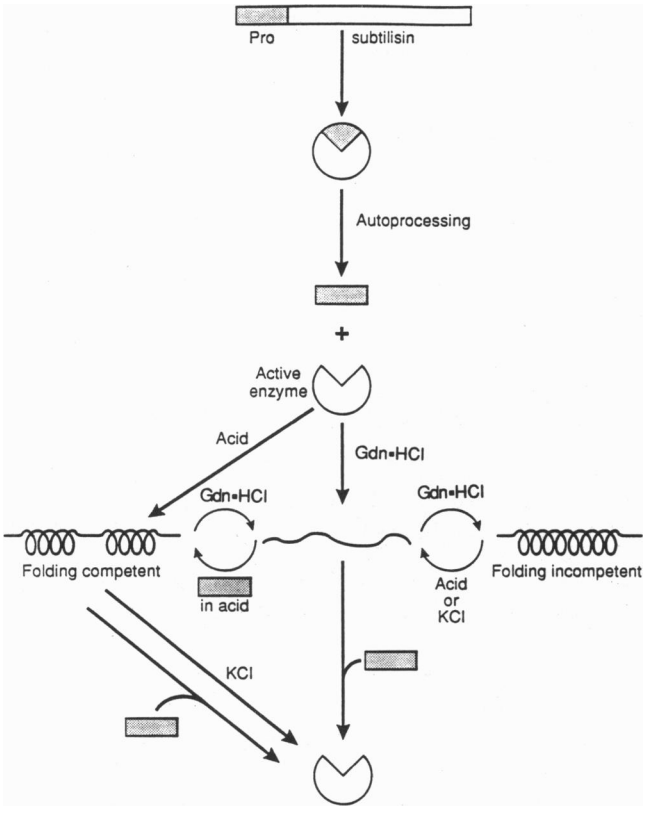

FIG. 5. Proposed pathway for propeptide (Pro)-mediated folding of subtilisin.

Preliminary studies indicate that the FC and FIC states may be interconvertible at a very low rate. This may be the rate-limiting step in the folding reaction, and the propeptide may act by (*i*) lowering the energy barrier between the FIC and the FC state or (*ii*) avoiding the formation of the FIC state. We believe the latter to be important because *in vivo*, the propeptide is synthesized first. Therefore when the mature region of subtilisin emerges from the cytoplasmic membrane, the propeptide may prevent the mature region folding to the FIC state, thus acting as an intramolecular chaperone in the folding pathway.

We propose that the propeptide acts as an intramolecular chaperone, enhancing the conversion of the FIC state to the FC state, as well as conversion of the FC state to the native state, by a factor of 1000. It is required probably throughout folding. The inhibitory function of the propeptide is important in delaying the proteolytic activity of folded subtilisin.

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