

Further Evidence for the Structure of the Subtilisin Propeptide and for Its Interactions with Mature Subtilisin*

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Evidence is presented for some secondary structure, very likely α -helical, of the propeptide of subtilisin E in aqueous salt solution, as well as for strong intermolecular interactions between the propeptide and the mature sequence both in the processed and unprocessed states (i.e. in prosubtilisin). Prosubtilisin is shown to exist as a dimer according to size exclusion high performance liquid chromatography under nondenaturing conditions; that dimer may be on the autoprocessing pathway. According to such a model, the prosequence of one prosubtilisin molecule is the template for the refolding of the mature sequence of the second, and, in turn, the hydrolytic process is intermolecular as well. Support for such an intermolecular folding model also includes potent slow binding inhibition of subtilisin by the propeptide, specific proteolysis of the propeptide by subtilisin, and evidence for intermolecular processing under a variety of conditions.

Subtilisin (EC 3.4.21.62), a bacterial alkaline serine protease cannot undergo refolding by itself, once it is denatured (1). The yield of spontaneous refolding was less than 0.1% when denatured subtilisin at a concentration of 0.1 mg/ml was diluted into high salt buffer, and most of the protein precipitated due to aggregation.¹ Renaturation of subtilisin BPN' in the absence of its propeptide (2) did result at very low protein concentration (<1.0 μ g/ml) (3). Subtilisin is synthesized in *Bacillus subtilis* as a preproprotein: the presequence of 29 amino acid residues is essential for secretion and is hydrolyzed by a signal peptidase (4), the prosequence of 77 amino acids is required for proper folding of the mature subtilisin (5, 6) and is autoprocessed hydrolytically by active, mature subtilisin.

In *Escherichia coli* (5, 7), prosubtilisin could be expressed as inclusion bodies, that when solubilized in 6.0 M guanidinium HCl undergo refolding and autoprocessing, once dialyzed against renaturing buffer (6, 8). The propeptide-assisted refolding to active enzyme was observed *in trans* by exogenously added prosubtilisin active-center mutant (Asp³² → Asn; the active center triad of subtilisin is comprised of Asp³², His⁶⁴, and Ser²²¹) or synthetic propeptides (9–15). In proproteins, the information required to attain the native tertiary structure of the mature sequence is encoded in the linear amino acid sequence encompassing the entire proprotein. Accordingly, prosubtilisin undergoes structural and functional refolding spontaneously in a matter of seconds, just as other single chain

globular proteins do (16, 43).

Whether autoprocessing takes place by an inter- or intramolecular pathway is still in dispute (6, 17, 18). Since the N terminus of subtilisin is 26 Å from the active site (19), *in trans* (18, 20), rather than *in cis* (6) processing would appear energetically less costly.

Subtilisin is an ideal and unique representative of a large group of proteases from different classes (e.g. α -lytic protease, papain, cathepsin B, carboxypeptidase Y, and pepsin) whose prosequence is essential for the production of enzymatically active protein but not for maintaining its enzymatic activity.

We present novel evidence pertinent to the prosequence-assisted folding of subtilisin. Several results favor the intermolecular autoprocessing/folding pathway, while we do not rule out a parallel intramolecular pathway.

EXPERIMENTAL PROCEDURES

Materials

Guanidine HCl was purchased from ICN; subtilisin Carlsberg and phenylmethanesulfonyl fluoride (PMSF)² were purchased from Sigma; urea was from Fisher; and succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide (s-AAPF-pNA) was from Bachem Bioscience.

Methods

Protein Assay—The propeptides were weighed and the $A^{0.1\%}$ (absorbance of a 1.0 mg/ml solution) of E-pro and BPN'-pro at 275 nm were determined to be the same value of 0.496 in water compared to 0.67 in 6.0 M guanidinium HCl. The measured extinction coefficients are 4280 M⁻¹ cm⁻¹ for E-pro (M_r = 8627) and 4250 M⁻¹ cm⁻¹ for the BPN'-pro (M_r = 8568). Concentrations of subtilisins were estimated either by weight or were determined spectroscopically. The $A^{0.1\%}$ values at 280 nm are 1.17, 1.20, and 0.96 for subtilisins BPN', E, and Carlsberg, respectively (21, 40).

Assay of Subtilisin Activity and Inhibition Studies—Subtilisin activity was assayed spectrophotometrically by monitoring the release of p-nitroaniline due to enzymatic hydrolysis of succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide (s-AAPF-pNA, M_r = 624.5) (22). The λ_{\max} of p-nitroanilide is at 380.4 nm. However, 400 nm was used (ϵ_{400} = 1.69×10^4 M⁻¹ cm⁻¹) to minimize absorption by the substrate. A COBAS-Bio centrifugal UV-vis analyzer (Roche Diagnostics) was used for kinetic data collection, and the slope of the linear region of the absorbance *versus* time curve was used as the initial velocity. The steady state kinetic constants K_m , V_{\max} and K_i were always determined simultaneously using the same enzyme solutions at the same time on the COBAS-Bio at 25 °C.

Cloning of Propeptide of Subtilisin E—By changing the GCG (coding

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¹ Z. Hu, K. Haghjoo, and F. Jordan, unpublished results.

² The abbreviations used are: PMSF, phenylmethanesulfonyl fluoride; prosubtilisin-S49C, the Ser⁴⁹ → Cys mutant of prosubtilisin E; (prosubtilisin-S49C)₂, the disulfide-linked dimer of the Ser⁴⁹ → Cys mutant of prosubtilisin E; prosubtilisin-S221A, the Ser²²¹ → Ala mutation at the active center of prosubtilisin E; prosubtilisin-S221C, the Ser²²¹ → Cys mutation at the active center of prosubtilisin E; APCS, the autoprocessing-competent state of prosubtilisin; PAGE, polyacrylamide gel electrophoresis; E-pro and BPN'-pro, -1 to -78 N-terminal extensions of the corresponding proteases; subtilisin-PMS, active mature subtilisin covalently inhibited at Ser²²¹ by phenylmethanesulfonyl fluoride; s-AAPF-pNA, succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide; HSQC, heteronuclear single quantum coherence.

Ala) at the start of the mature subtilisin coding region to a stop codon TGA, a prosequence gene was created from the prosubtilisin gene. At the same time, a *Bam*HI site after the stop codon was also created by site-directed mutagenesis. The gene was subsequently cloned into pET11a's ATG cloning site using *Nde*I and *Bam*HI restriction endonucleases to give pET11a:pro.

Protein Expression and Purification—Subtilisin BPN' and subtilisin E were purified from culture supernatants of a protease-deficient *B. subtilis* strain DB104 (23) harboring pPG588 (generously provided by Dr. Charles Saunders of Procter & Gamble) or pKWZ (24), respectively, according to the previously described protocols (7). BL21(DE3) carrying pET11a:E-Pro or pET11a-BPN'-pro (courtesy of Yuyun Li of the R. W. Johnson Medical School) were used to express E-pro and BPN'-pro, respectively. The propeptides, found in the soluble cellular extract, constitute 30% of the total soluble extract. A single clone was used to inoculate 25 ml of LB containing 100 μ g/ml ampicillin. After overnight incubation at 37 °C, the culture was spun down at 7,000 rpm in a sterile centrifuge tube and the cell pellet was resuspended in 1.0 liter of Penassay broth with 100 μ g/ml ampicillin, supplemented with 10 g of yeast extract. The culture was incubated at 37 °C with shaking at 300 rpm for 4 h until an $A_{550} = 1.8$ was reached, then induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside followed by a 3.0-h incubation. Five grams of cell paste could be obtained by centrifugation at 7,000 rpm for 5 min. The cells were resuspended in 25 ml of lysis buffer (50 mM Tris, pH 8.0, containing 1 mM EDTA, 100 mM KCl), and fresh PMSF was added to 1 mM. The cells were disrupted by ultrasonication for 20 min without cooling at the beginning so that the lysate temperature could rise 50–60 °C. On completion, a flocculent precipitate was seen and occupied almost one-half volume of the cell lysate. Twenty microliters of 0.1 M PMSF (in *n*-propyl alcohol) was added, the lysate was centrifuged at 40,000 rpm for 2 h, and the supernatant was retained. The supernatant was centrifuged again at 100,000 rpm for 2 h using a Beckman TLA-100.2 rotor before being injected into a pre-equilibrated CM-HR 10 \times 100 column. The propeptides were eluted at 0.36 M NaCl using a gradient starting with 100 mM NaCl at pH 7.0. About 6 mg of BPN'-pro could be obtained from a 2-ml injection. Purified propeptides showed a single band on 18% SDS-PAGE with an apparent molecular weight of 13,000 and were 99% pure according to reverse phase (218TP, 10 mm \times 25 cm C18 from Vydac) and/or size exclusion (TSK G3000SWx1, 0.75 \times 30 cm) HPLC analysis monitoring the absorbance at either 220 nm or 280 nm.

Incorporation of 15 N in the Propeptide—To incorporate the 15 N label, the propeptide was expressed on M9 minimum medium containing either NH_4 or $^{15}\text{NH}_4$ salts (25). It was purified on a SP-Sephadex C-25 column using a linear gradient of 10 mM phosphate, pH 6.0, to 10 mM phosphate, pH 8.0, containing 0.5 M KCl. The propeptide was eluted at 0.2 M KCl. The purified sample was dialyzed against 10 mM phosphate, ultrafiltered on an Amicon instrument using YM3 membrane, then concentrated, and lyophilized.

Proton Nuclear Magnetic Resonance— ^1H NMR spectra were recorded at 500 and 600 MHz on instruments specified in the figure legends. Typical spectra were recorded on 2 mM propeptide solutions, dissolved in 0.2 M phosphate, pH 7, in 90:10 $\text{H}_2\text{O}/\text{D}_2\text{O}$.

Circular Dichroism—These studies were performed on a Jobin Yvon (JY) Dichrographe Mark VI spectrophotometer at 20 °C. A 0.1-cm path length cell was used for the far-UV (200–250 nm) spectra, and a 1.0-cm path length cell for the near-UV (250–320 nm) region. The wavelength increment was set at 0.5 nm, and the integration time was 1.0 s. For each spectrum, eight scans were averaged in the near-UV and four in the far-UV region, and spectra were corrected by subtraction of the spectrum of an appropriate buffer. Results are expressed as mean residue ellipticity $[\Theta]_{\text{MRW}}$ (in degrees cm^2/dmol), calculated from the formula

$$[\Theta]_{\text{MRW}} = 3300 \Delta A/(cl)\text{MRW} \quad (\text{Eq. 1})$$

where ΔA is the difference in absorbance between left and right circularly polarized light, c is the concentration of protein in mg/ml, l is the path length in cm, and MRW is the mean residue molecular weight of the sample (MRW equals 103.0 for prosubtilisins, 100.8 for mature subtilisins, and 110 for the propeptides).

Size Exclusion Chromatography—Size exclusion chromatography was performed on a Waters HPLC system, using a TSK G3000SWx1 (0.75 \times 30 cm) column. Elution was done at a flow rate of 0.7 ml/min, with 50 mM Tris, pH 7.0, containing 0.2 M $(\text{NH}_4)_2\text{SO}_4$, using a protein concentration of 0.5–1.0 mg/ml and an injection volume of 10 μ l. Molecular mass standards were from Pharmacia Biotech Inc.: ribonuclease (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine

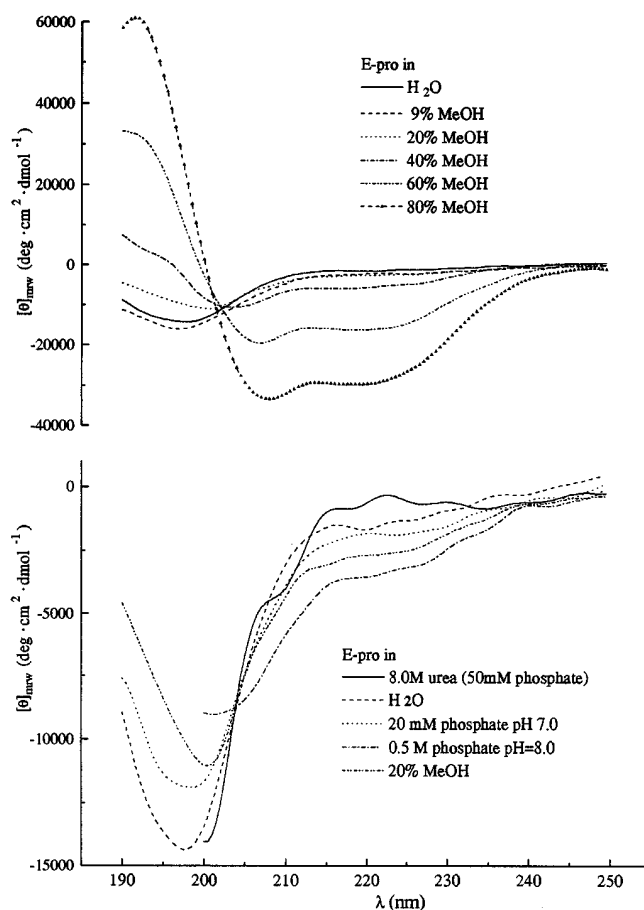


FIG. 1. Far UV-CD spectra of E-pro in different solutions. Top, α helix content increases with increasing MeOH concentration; bottom, α helix content induced by 0.5 M phosphate is higher than that induced by 20% methanol. Spectra are the average of 8 scans at 20 °C, in a 0.1-cm pathlength cell, E-pro concentration was 0.13 mg/ml.

serum albumin (67 kDa), and aldolase (158 kDa). The void volume emerged at 7.89 min as measured by blue dextran 2000 under the specified conditions and gave an equation $\log(M_r) = 5.83 - 0.039t$, where t is the retention time in minutes. As will be seen in the figures, the retention times are very highly reproducible under the specified conditions.

RESULTS AND DISCUSSION

Structure of Propeptide per se

While the sequences of propeptides E (E-pro) and BPN' (BPN'-pro) are predicted to have a high propensity to form α helix (61–66%), β sheet (approximately 13–16%), and "turn" (6.5–10%) according to various algorithms (26, 41, 42), they exhibit no α helical structure in pure water. According to CD spectra, E-pro acquires α helical content with added MeOH (Fig. 1). The high ionic strength required for the refolding of prosubtilisin enhances the α and β footprint region (more negative ellipticity around 220 nm). More α helix content is induced by 0.5 M phosphate than by 20% MeOH. The propeptides have no tertiary structure according to their near-UV CD spectra (identical in 8.0 M urea or in refolding buffer of 0.5 M phosphate, data not shown) and hydrodynamic volume (see below). The retention time of the propeptides on gel filtration chromatography is lower (higher MW_{app}) than that of active subtilisin.

A number of NMR studies were conducted on uniformly ^{15}N -enriched propeptide. That there is some structure in the propeptide was already evident from a one-dimensional spectrum in D_2O , that indicates dispersion of the 2 His C2H reso-

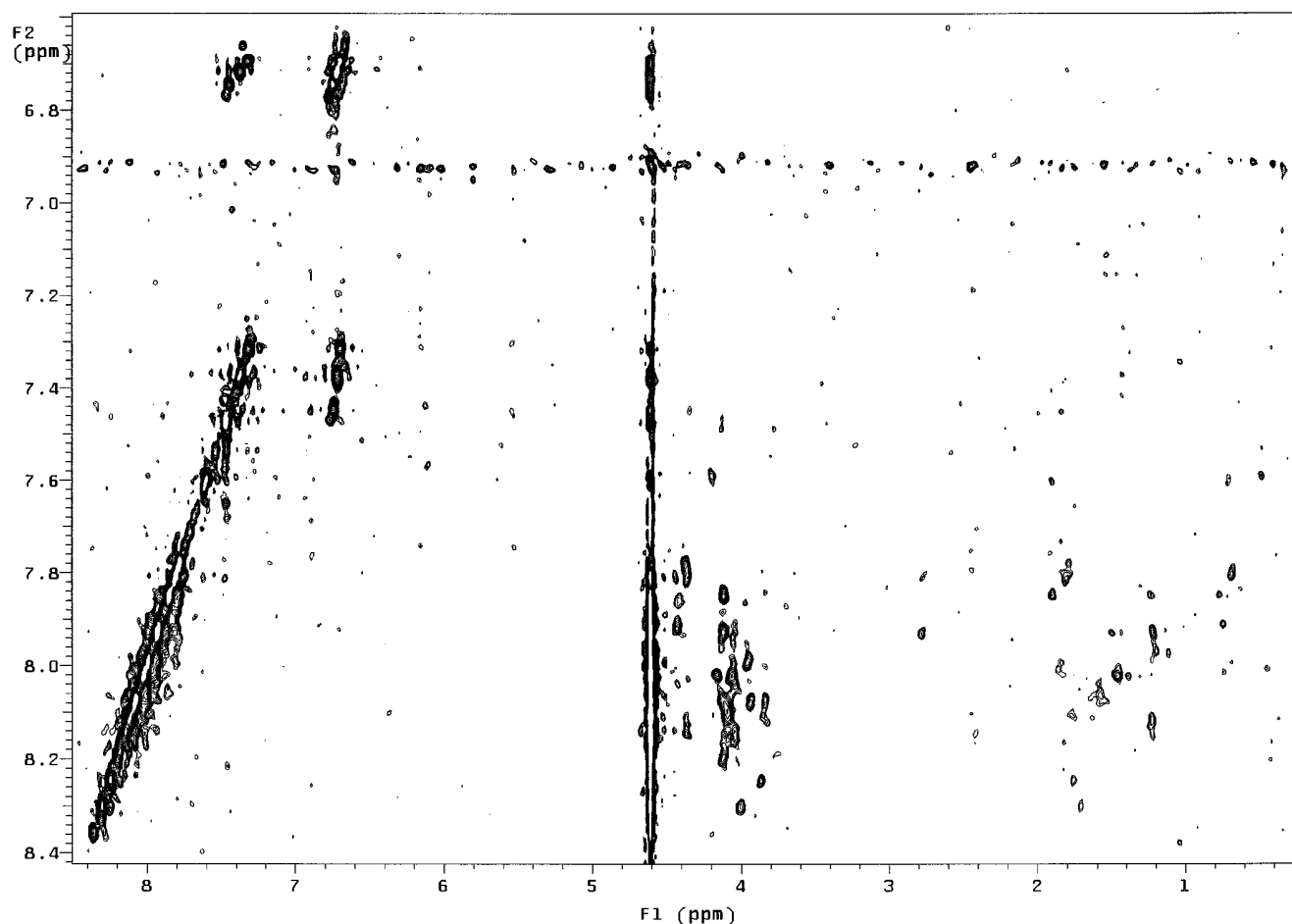


FIG. 2. Two-dimensional NOESY spectrum of uniformly ^{15}N -labeled BPN'-pro. Solution is 2 mM peptide in 0.2 M phosphate, pH 7.0, recorded on a Varian UNITY PLUS 600-MHz instrument.

nances (data not shown, there are only 2 His in the propeptide). A two-dimensional NOESY experiment in 0.2 M phosphate, pH 7.0, shows the presence of at least 15 cross-peaks in the amide NH-C α H region at 600 MHz (Fig. 2). This is strongly suggestive of helical secondary structure. Next, a three-dimensional NOESY-HSQC experiment was performed (Fig. 3) providing evidence for NH-NH, NH-C α H, NH-C β H interactions. The chemical shifts of the C α H interactions in the NOESY experiments are all upfield from water, again providing evidence for the presence of some helical secondary structure (typical chemical shifts of C α H in β sheets would be expected downfield from the water resonance).

Third, a series of experiments were performed to test how many NH resonances may be involved in stronger hydrogen bonds, that would be indicative of secondary structure. First, the spectrum of a 2 mM solution of the E-pro in 0.2 M phosphate, pH 7.0 at 10 $^{\circ}\text{C}$ was recorded, then it was rerecorded at 20, 30, 40, and 50 $^{\circ}\text{C}$ in the two-dimensional HSQC mode. Next, the temperature of the sample was returned to 10 $^{\circ}\text{C}$ to test the reversibility of the unfolding reaction (Fig. 4). Finally, the solution was made 6 M in guanidine hydrochloride, and the experiment was repeated at 10 $^{\circ}\text{C}$. While all of the NMR results presented are still qualitative (total assignment and structure determination will require ^{15}N , ^{13}C double labeling to achieve sufficient dispersion of signals under all conditions), they inform us of very significant changes both with increasing temperature and with addition of guanidine hydrochloride, changes that lead to similar conclusions.

The HSQC spectra readily enabled us to distinguish at least

63 of 76 backbone cross-peaks at 10 $^{\circ}\text{C}$. As the temperature is increased, one can detect differential exchange of the NHs. At 50 $^{\circ}\text{C}$, we observe at least 15–16 cross-peaks that we believe are pertinent to the hydrogens involved in secondary structure, while those resonances that represent NHs hydrogen-bonded to water have melted. We also note that the behavior is reversible, since the HSQC spectrum of the sample that was cooled back to 10 $^{\circ}\text{C}$ from 50 $^{\circ}\text{C}$ was essentially superimposable on the one prior to heating. When this sample at 10 $^{\circ}\text{C}$ is made 6 M in the guanidine hydrochloride denaturant, we retain more than 50 of the backbone and 3 of the 4 side chain resonances.

All of the resonances that are still resolved in guanidine hydrochloride appear to be shifted downfield, perhaps experiencing the positive charge of the denaturant that at such high concentrations surrounds the protein at close range. The 4 side chain NHs are at Asn⁻³³, Gln⁻³⁸, Gln⁻⁴⁰, and Gln⁻⁶², and give rise to two resonances each, separated by 0.6–0.9 ppm. The numbering of the propeptide extends from -1 to -78, with the tyrosine adjacent to the N-terminal residue of the mature sequence being numbered -1. On addition of guanidine hydrochloride, some of the side chains disappear, while there is a new NH resonance visible at 8.75 ppm, appropriate for the NH at an Asn (27) in the denatured state. Also, on heating the sample to 50 $^{\circ}\text{C}$, there are still two side chain NHs visible, suggesting that two of the four are involved in hydrogen bonds. Given the distribution of these side chains, we conclude that the helical segment is located somewhere in the N-terminal region of the propeptide, more specifically between residues -33 and -62. That there is a structural change on addition of

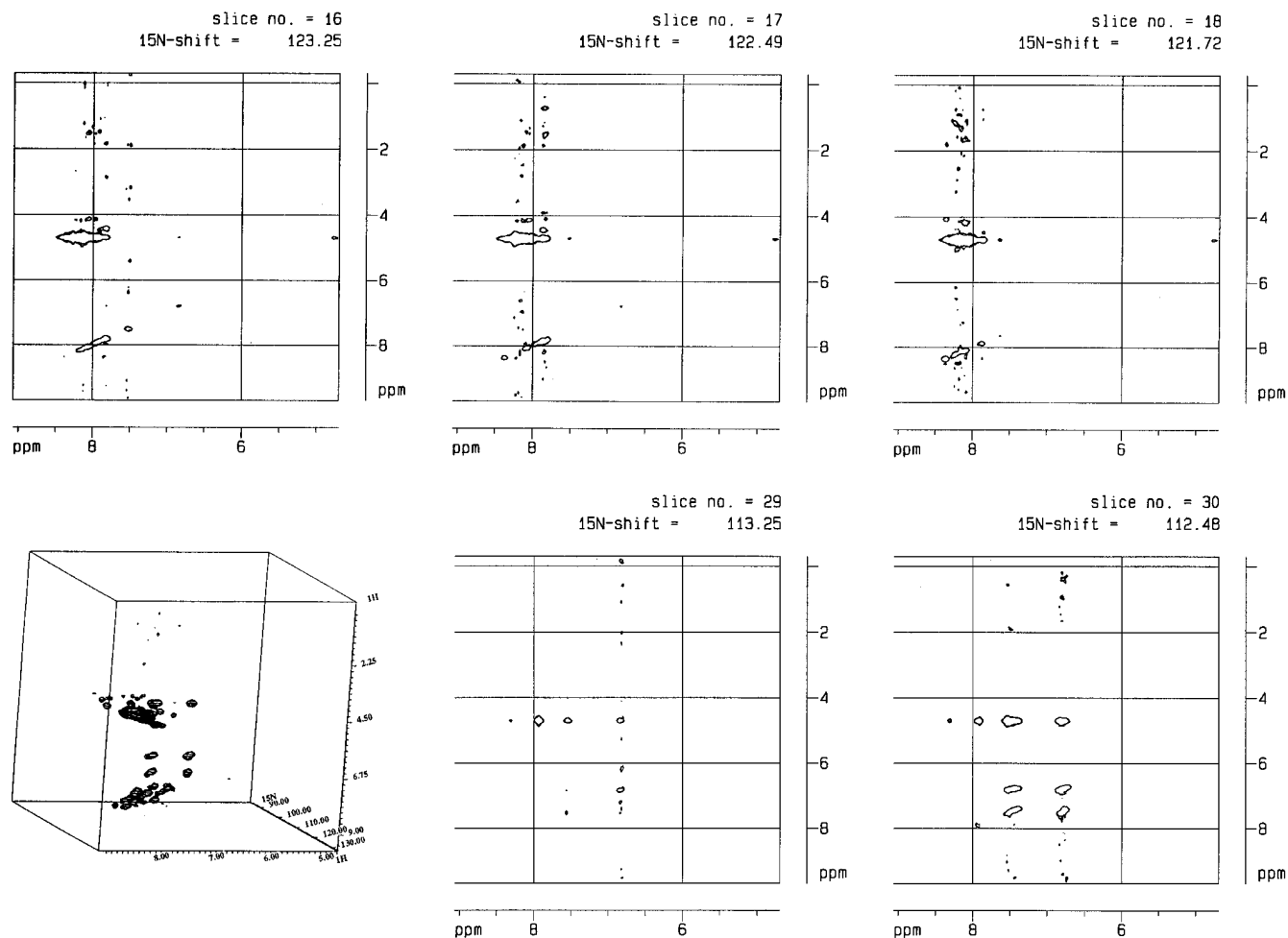


FIG. 3. Three-dimensional NOESY-HSQC spectrum of uniformly ^{15}N -labeled BPN'-pro in 0.2 M phosphate at pH 7.0 at 25 °C, recorded on the Bruker DXR 500-MHz instrument. Acquisition time = 0.0515 s, number of transients = 8, proton dimension spectrum width = 9.977 ppm, nitrogen dimension spectrum width = 49.227 ppm; number of increments = 512 (^1H), 64 (^{15}N), and 128 (^1H); mixing time = 200 ms.

guanidine hydrochloride is also evident from the wider range of chemical shifts of the backbone NHs in the absence than in the presence of the denaturant, as well as from the clearly different appearance of the two HSQC spectra (Table I).

We conclude that in refolding buffer the propeptide likely exists in an extended form, with essentially no tertiary, but with a significant amount of helical secondary structure. Because of the coincidence of the number of NH-C α H NOE cross-peaks and the number of resonances that survive to 50 °C, we tentatively suggest that as many as 14–16 backbone NHs may be involved in the helical structure, along with two of the four amidated side chains (Asn and Gln). The fact that less than 20% of the amino acids participate in the helix probably accounts for the weak CD signals. The presence of any secondary structure on the propeptide had gone undetected in other studies. For example, Bryan and co-workers reported (28) that there is secondary structure induced in the propeptide only on binding to the mature protein. In the subtilisin-bound form, there is found α -helical structure in the N-terminal region of the propeptide, as deduced here for the free propeptide.

Evidence for Intermolecular Interactions of Pro- and Mature Sequences

Size Exclusion HPLC—Formation of a strong subtilisin-propeptide complex could be demonstrated directly by HPLC on a TSK3000 size exclusion column. The MW_{app} of prosubtilisin, subtilisin, propeptides, and the propeptide-subtilisin complex were estimated as 130,000, 12,000, 34,000, and 22,000,

respectively (Fig. 5). The MW_{app} of the propeptide is 10,000 (molecular weight of the complex – molecular weight of subtilisin or 22,000 – 12,000) upon binding to the mature enzyme, compared with 34,000 before binding. Since active subtilisin hydrolyzes the propeptide (see below), it was inhibited with phenylmethanesulfonyl fluoride (PMSF) prior to being mixed with propeptides. The PMS group covalently attached to Ser²²¹ did not affect the shape of the enzyme (not shown), nor did it affect complex formation, as the complexes formed between subtilisin-PMS and E-pro and subtilisin-S221C and E-pro have similar retention times (14.55 min, Fig. 5). Even though the propeptide ($\text{pI} = 10.1$) is positively charged while the mature enzyme ($\text{pI} = 6.8$) is weakly negatively charged, the complex is not stabilized purely by electrostatic interactions, since it is stable when 0.5 M phosphate is used as eluent. Mutations in the hydrophobic region of the prosequence decrease, while the Lys³⁶ → Glu mutation does not affect the stability of the complex (29). As the S221C mutation abolishes subtilisin's protease activity, the propeptide remains intact after autoprocessing.

The results suggest that prosubtilisin itself may exist as a dimer or a multimer. Had the prosubtilisin formed a compact, highly ordered structure, it should have similar hydrodynamic volume to that found for the subtilisin-propeptide intermolecular complex, clearly not the case. The refolded prosubtilisin-S221A has an apparent molecular weight of 130,000, much higher than that of E-pro (34,000) or of the subtilisin-propeptide complex (22,000) (Fig. 5). Refolding of prosubtilisin-S221A

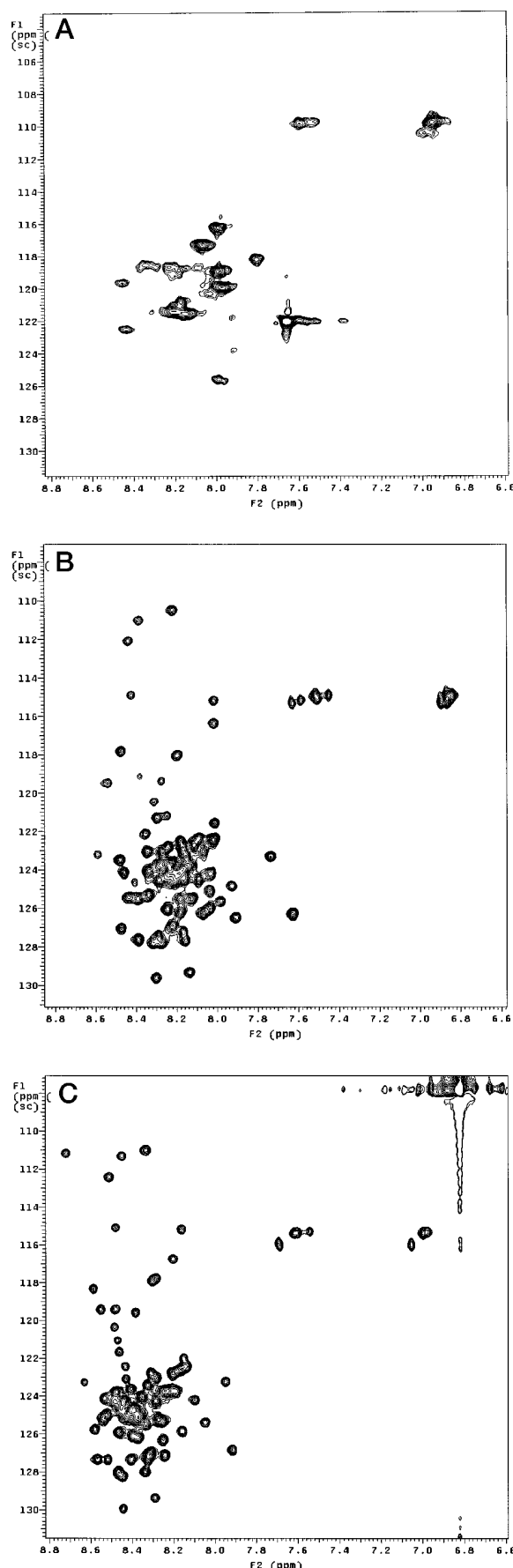


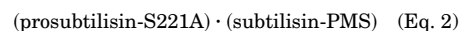
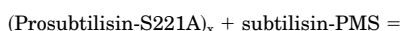
FIG. 4. Two-dimensional HSQC spectra of uniformly ^{15}N -labeled BPN'-pro. Spectrum B, at 10 °C, 0.2 M phosphate, pH 7.0; spectrum A, the same sample at 50 °C; spectrum C, the same sample

TABLE I
Cross-peaks observed in the HSQC experiments on the propeptide

Condition	Number of backbone cross-peaks	Number of side chain cross-peaks ^a
10 °C	63	4
50 °C	15–16	2
Cooled to 10 °C, then 6 M guanidinium hydrochloride added	>50	3

^a It is to be noted that there are two peaks for each side chain, since the chemical shifts of the two protons on the side chain amide differ by more than 0.6 ppm.

is confirmed by far-UV and near-UV CD (7). Therefore, refolded prosubtilisin-S221A must exist as a multimer. Since each prosubtilisin-S221A has a ligand (propeptide) and a binding site, it could form a linear polymer, while the interaction of prosubtilisin-S221A with mature subtilisin (PMSF-inhibited) would only lead to a dimer:



When subtilisin-PMS was added to refolded prosubtilisin-S221A, the retention time of the complex increased (MW_{app} decreased; Fig. 6, *top*), as expected. When subtilisin-PMS is in excess, Equation 2 is shifted to the right, irrespective of whether $(\text{prosubtilisin-S221A})_x$ is a dimer or a multimer. Addition of excess subtilisin-PMS shifted the MW_{app} from 130,000 to 120,000, only 10,000 lower than that of $(\text{prosubtilisin-S221A})_x$. The magnitude of the shift (10 kDa) is equal to the MW_{app} of the mature enzyme-propeptide complex minus the MW_{app} of mature enzyme (Fig. 5). We conclude that the refolded prosubtilisin-S221A is a dimer bridged by the prosequences. When active enzyme was added to refolded prosubtilisin-S221A, no dimer could be seen due to intermolecular processing of prosubtilisin-S221A (Fig. 6, *bottom*). However, the transient presence of the subtilisin-S221A-propeptide complex as an intermediate was evident at 14.5-min elution time. Thus the active enzyme cleaved the propeptide from prosubtilisin-S221A on initial complexation. Subsequently, the propeptide could bind to the mature subtilisin-S221A produced from prosubtilisin-S221A.

Interaction of Propeptide and Mature Subtilisin Preliminary NMR Results—Using the ^{15}N -enriched propeptide, one-dimensional HSQC experiments were performed on the propeptide in the absence (Fig. 7, *right-hand panel*) and in the presence of subtilisin BPN'-PMS (Fig. 7, *left-hand panel*). Only the amide region is shown with resonances that represent protons attached to ^{15}N of the propeptide. First, the fact that the propeptide is now bound is evident from the change in linewidths of the corresponding resonances (approximately 3 times larger in the complex). Second, there is more dispersion in the left-hand spectrum, in spite of the very much larger molecular weight in the complex (36,000 *versus* 8,500) and broader lines. Since in the propeptide there was evidence found for some helical character, we conclude that there is an additional structure being induced in the propeptide upon binding, although the nature of the induced structure is not evident at this level of analysis. In accord with these observations, the x-ray structure of the subtilisin-propeptide complex on a subtilisin BPN' variant from which the Ca(II) binding loop had been excised (28) showed a

cooled to 10 °C, then made 6 M in guanidine hydrochloride. These spectra were recorded at 500 MHz on a Varian Unity Plus spectrometer. Acquisition time = 0.386 s, number of points = 2048, $J_{\text{NH}} = 92$, $d_1 = 1.0$ s, and number of transients = 4.

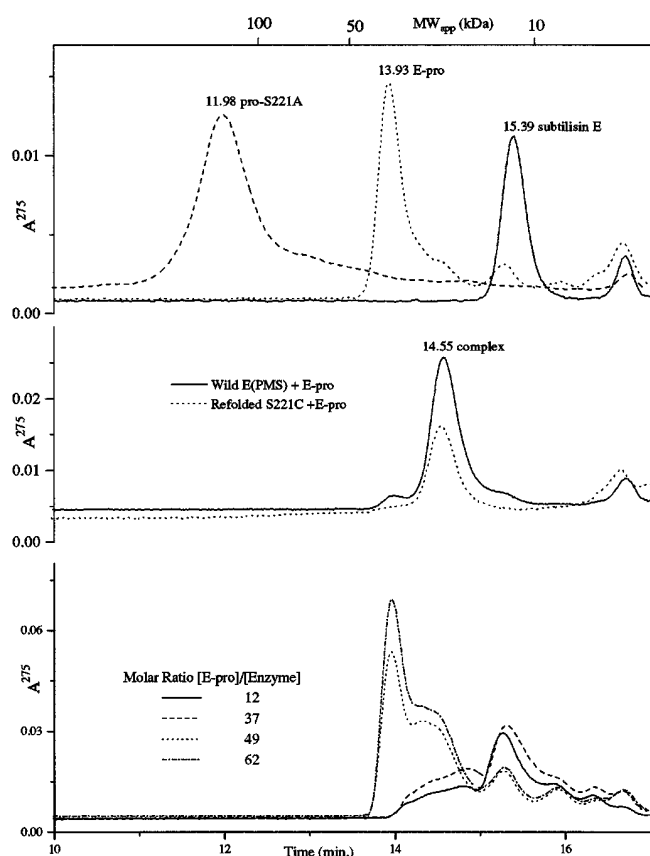


FIG. 5. Size exclusion HPLC of prosubtilisin, subtilisin, propeptide, and their complexes. *Top*, mature subtilisin, refolded prosubtilisin-S221A, and propeptide individually. *Middle*, the complexes of the propeptide with PMSF inhibited subtilisin E (solid lines) and with subtilisin-S221C mutant (dotted lines). *Bottom*, digestion of E-pro by subtilisin E. See "Methods" for details.

considerable amount of secondary structure induced in the propeptide on complexation.

Propeptide as a Specific Substrate of Subtilisin—Subtilisin is secreted when cells are being starved, no propeptide is found in the culture media or in the membrane of *B. subtilis* when subtilisin is secreted. The propeptide is found to be a substrate for subtilisin according to SDS-PAGE of propeptide-subtilisin mixtures. With a molar ratio of [BPN'-pro]/subtilisin E of 6:1, the BPN'-pro is completely digested in 10 min (Fig. 8A), and, even with a molar ratio as high as 310:1 (Fig. 8B), the digestion is clear. Subtilisin E digests its own E-pro much faster than it digests the BPN'-pro: after 2 h, the E-pro band became very faint (Fig. 9, lane 7), while the BPN'-pro band was still prominent (Fig. 9, lane 15). In the presence of excess bovine serum albumin, the propeptide was selectively digested while bovine serum albumin remained intact (data not shown).

Propeptide as a Potent Slow Binding Inhibitor of Active Subtilisin—Several propeptides have been shown to potently inhibit their mature enzymes (11, 12, 16, 29–32). Here we report experimental results with recombinant propeptides and subtilisin BPN' and Carlsberg. Elsewhere, we recently reported inhibition data with propeptide mutants (29). Above, it was demonstrated that the propeptides are excellent substrates of the active subtilisins, especially of subtilisin E. Therefore, studies with subtilisin E were difficult to interpret. Fig. 10 shows a typical progress curve for release of *p*-nitroaniline from s-AAPF-*p*NA by subtilisins BPN' and E in the presence of BPN'-pro. Similar results were seen for E-pro and with subtilisin Carlsberg.

The hyperbolic progress curves for release of the *p*-nitroani-

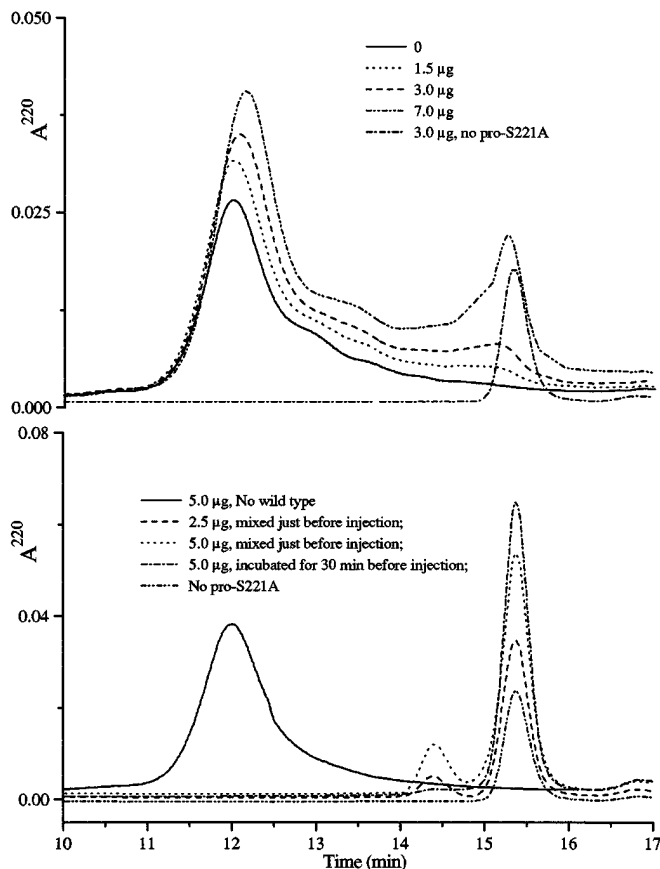
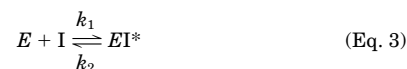


FIG. 6. Size exclusion HPLC of refolded prosubtilisin-S221A in the presence of inhibited and active mature subtilisin. TSK G3000SWxl (0.75 × 30 cm) was used under the conditions explained under "Methods" except that sample volume varied between 10 and 20 μ l. Baseline subtraction was performed. *Top*, the retention time of refolded prosubtilisin-S221A (11.98 min) increases with the amount of subtilisin-PMS added due to an exchange reaction explained in the text in Equation 2. Prosubtilisin-S221A (3.0 μ g) was mixed with subtilisin E-PMS in the amounts indicated above the chromatograms. *Bottom*, processing of the refolded prosubtilisin-S221A (11.98 min; present in the amounts indicated above the chromatograms) by exogenously added active subtilisin E. The peak corresponding to prosubtilisin-S221A disappeared when active subtilisin E was added, while the height of the peak corresponding to the mature enzyme (15.4 min) increased with increasing concentrations of prosubtilisin-S221A added, as well as with increasing incubation time, since this peak has contributions from both the wild type and the S221A mutants. Also, the transient appearance of a peak with a retention time appropriate for the propeptide-mature enzyme complex (14.5 min) is in evidence. There was 4.0 μ g of active subtilisin unless indicated otherwise.

line from s-AAPF-*p*NA by active subtilisin in the presence of E-pro indicated that E-pro is a slow and tight-binding inhibitor (33, 34) of both subtilisins BPN' and Carlsberg. A single-step inhibition mechanism was found to account for the observed progress curves (16, 29):



where EI^* is an altered conformation of EI , and

$$\nu = \nu_s + [\nu_o - \nu_s] \exp(-kt) \quad (\text{Eq. 4})$$

where ν , ν_o , and ν_s are the enzymatic hydrolysis rates (at time t), the initial rate, and the steady-state rate, respectively. The apparent first order rate constant, k , is given by Equation 5.

$$k = k_2 + \frac{k_1[I]}{1 + [S]/K_m} \quad (\text{Eq. 5})$$

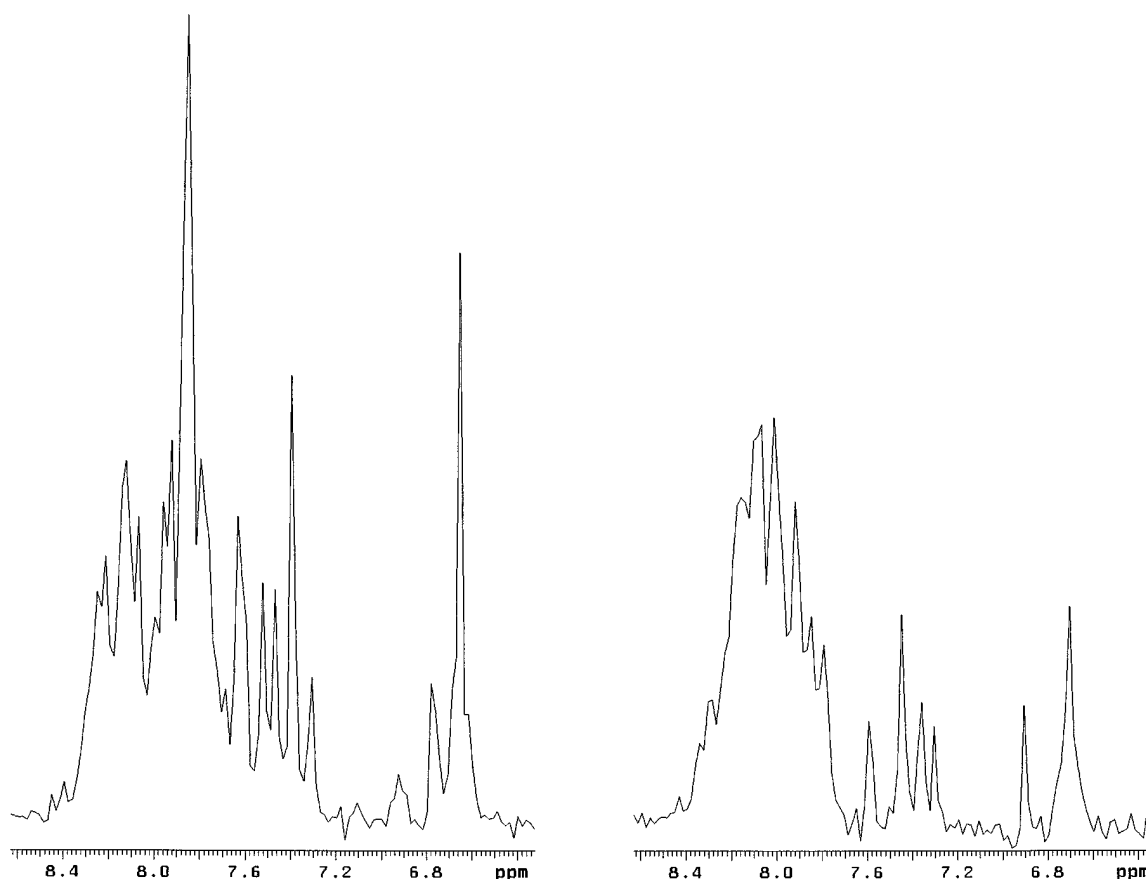


FIG. 7. HSQC filter experiment of uniformly ^{15}N -labeled BPN'-pro in the absence (right) and in the presence (left) of an equivalent of subtilisin BPN'-PMS. Spectra were recorded at 600 MHz on the Varian UNITY PLUS instrument. Acquisition time = 0.256 s, number of points = 5120, spectrum width = 16.666 ppm, number of transients = 4, line broadening = 1 Hz, 25 °C with presaturation of the water signal for suppression.

The single-step mechanism is supported by the progress curves, such as in Fig. 10, that show ν_o to be independent of $[\text{I}]$ for fixed concentrations of substrate for both subtilisins BPN' and Carlsberg (data not shown). Single-step inhibition was also reported for the inhibition of cathepsin B by its propeptide (32). Under these conditions,

$$\nu_o = V_{\max}[\text{S}]/(K_m + [\text{S}]) \quad (\text{Eq. 6})$$

At a constant substrate concentration $[\text{S}]$, integration of Equation 4 leads to Equation 7 for the increase of absorbance due to the appearance of the product chromophore,

$$A = \nu_s t + (\nu_o - \nu_s)(1 - \exp(-kt))/k + A_0 \quad (\text{Eq. 7})$$

where A and A_0 are the absorbance of the hydrolytic product at time t and at time zero. Hence, ν_s and k could be obtained from a nonlinear least squares fit of the progress curves (A versus t) resulting from reactions in which the propeptide and substrate were preincubated and the reaction was initiated by the addition of enzyme for the condition $[\text{I}]/[\text{E}] > 10$ (Ref 33.; typical values were: $[\text{S}]_0 = 0.3 \text{ mM}$, $[\text{Enz}] \sim 5 \text{ nM}$, $[\text{pro-BPN}'] \sim 250 \text{ nM}$, $K_m \sim 0.5 \text{ mM}$, $K_i \sim 10^{-9} \text{ M}$). The progress curves for the reactions in which the propeptide and the enzyme were preincubated and then the reaction was initiated by addition of substrate also confirmed the slow binding mechanism, but these gave less reliable data due to digestion of propeptide by the enzyme during the preincubation.

For the single-step mechanism, the K_i can be extracted by a nonlinear least squares fit of a set of ν_o/ν_s versus $[\text{I}]$ to the competitive inhibition equation:

$$\nu_o/\nu_s = \frac{K_m(1 + [\text{I}]/K_i) + [\text{S}]}{K_m + [\text{S}]} \quad (\text{Eq. 8})$$

The constants k_1 and k_2 can be calculated from: $k_2 = k \nu_s/\nu_o$ and $K_i = k_2/k_1$.

The concentration dependence of the K_i values in Table II is due to the depletion of the propeptide during the kinetic experiments. The competitive inhibition constants displayed by E-pro and BPN'-pro toward subtilisins BPN' and Carlsberg are comparable to that afforded by *Streptomyces* subtilisin inhibitor, which is one of the strongest subtilisin inhibitors reported to date. *Streptomyces* subtilisin inhibitor is also shown to be slow and tight-binding (data not shown). The K_i values determined kinetically are more than an order of magnitude smaller than those determined spectroscopically (28). However, E-pro is a very weak inhibitor of subtilisin E with $K_i = 2 \times 10^{-5} \text{ M}$ which is comparable to the inhibition provided by bovine serum albumin ($5 \times 10^{-5} \text{ M}$). Since no steady state could be obtained due to the digestion of propeptides by subtilisin E, no attempt was made to calculate the exact K_i .

Additional Evidence for Intermolecular Processing of Prosubtilisin—Subtilisin could not be completely denatured even in 10 M urea (35). In our hands, the activity remaining in 8.0 M urea would autolyze the partially denatured enzyme according to SDS-PAGE. Autoprocessing and subsequent autolysis of prosubtilisin in aqueous urea is expected, providing that prosubtilisin undergoes spontaneous refolding. The high ionic strength, required for the refolding and autoprocessing of prosubtilisin, can be substituted for by 1.0 M urea (data not shown). Autoprocessing also proceeds in SDS. A prosubtilisin

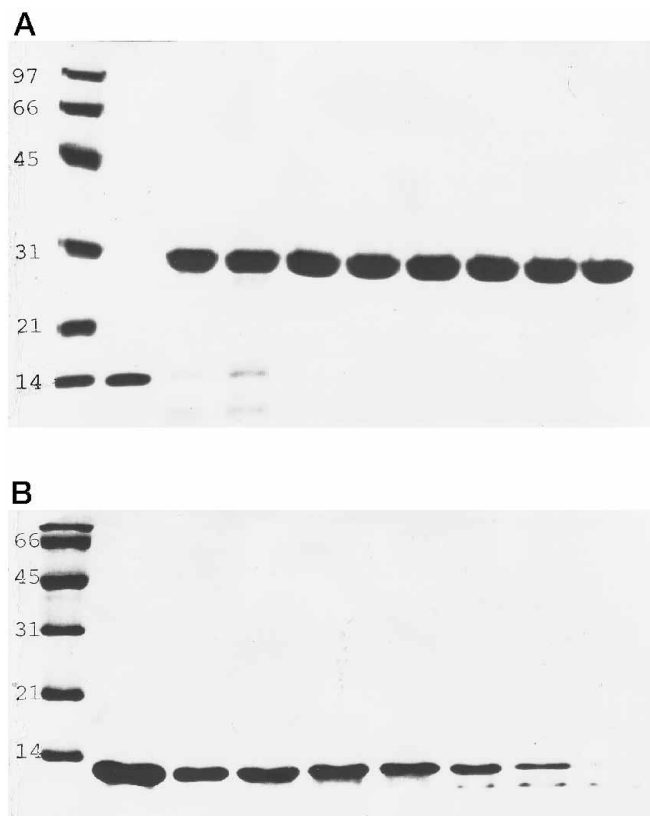


FIG. 8. BPN'-pro is a substrate of both subtilisins E and BPN' (18% SDS-PAGE). A, BPN'-pro was mixed with subtilisin E at a final concentration of 1.0 mg/ml propeptide and 0.5 mg/ml enzyme at room temperature. Aliquots were taken at 1, 10, and 30 min and mixed with PMSF and are shown from lanes 3–5, respectively. Lane 2 shows the position of BPN' propeptide. B, BPN'-pro was mixed with subtilisin BPN' at a final concentration of 1.0 mg/ml propeptide and 0.01 mg/ml enzyme at room temperature. Aliquots taken at 1, 10, 30, 60, 120, 240, and 420 min are shown from lanes 3–9. The subtilisin bands are at $M_r = 29,000$.

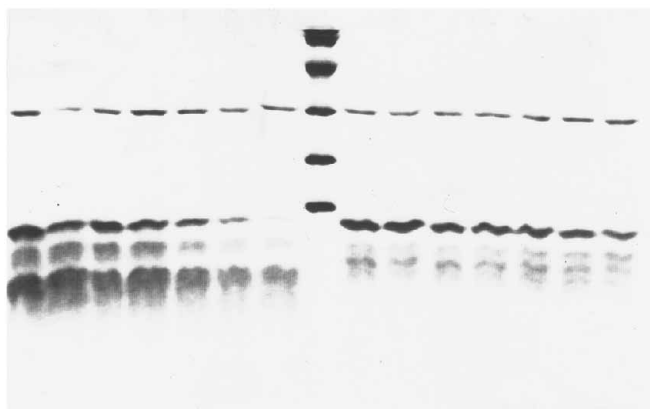


FIG. 9. E-pro is a better substrate than BPN'-pro of subtilisin E. Subtilisin E (20 μ l of 0.83 mg/ml) was mixed with 80 μ l of E-pro (A_{275} 1.15) or BPN'-pro (A_{275} 0.947), respectively. Samples taken at 0 (immediately after mixing), 1, 10, 20, 30, 60, and 120 min are shown from lanes 1–7 for E-pro or from lanes 9–15 for BPN'-pro. The top bands are subtilisin.

sample prepared for SDS-PAGE (in 2% SDS) showed the presence of the mature subtilisin band when stored overnight at 4 °C (results not shown). Apparently, under these conditions, prosubtilisin is in equilibrium with the folded form in the various denaturants, and the presence of the folded form leads to autoprocessing.

Autoprocessing could not be inhibited by PMSF, a serine

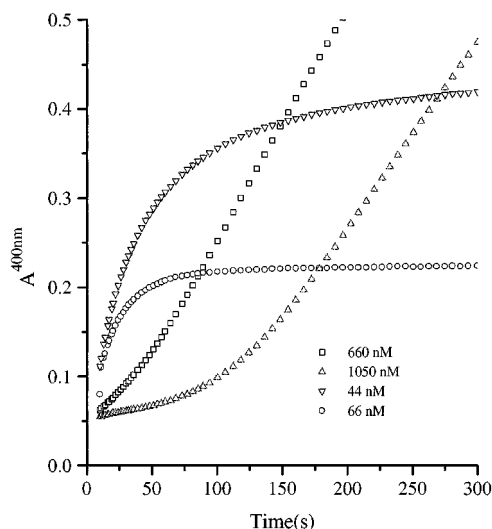


FIG. 10. Progress curves for inhibition of subtilisins BPN' and E by BPN'-pro. BPN'-pro at various concentrations was dissolved in the assay buffer described under "Experimental Procedures," then active subtilisins were added to initiate the reaction at 25 °C. The solutions contained: 50 mM Tris, pH 8.0, 0.10 M KCl, 1.0 mM CaCl₂, 0.20 mM s-AAPP-pNA, and BPN'-pro at the concentrations indicated in the figure. Inverted triangles and circles refer to subtilisin BPN' at 18 nM; triangles and squares to subtilisin E at 180 nM.

TABLE II
 K_i (nM) of propeptides toward subtilisins
 K_i values are the average of two experiments.

Pro-peptides	Subtilisin BPN'				Subtilisin Carlsberg			
[I], μ M	0.292	0.438	0.584	0.219	0.292	0.438	0.584	
SSI	0.101	(@0.146 μ M) ^a			0.078	(@0.146 μ M) ^a		
BPN'-pro	0.563	0.423	0.410	1.67	0.462	0.160	0.142	
E-pro	4.85	2.09	1.40	— ^b	18.4	1.87	1.02	

^a Velocity was too low to be measurable at higher inhibitor concentrations.

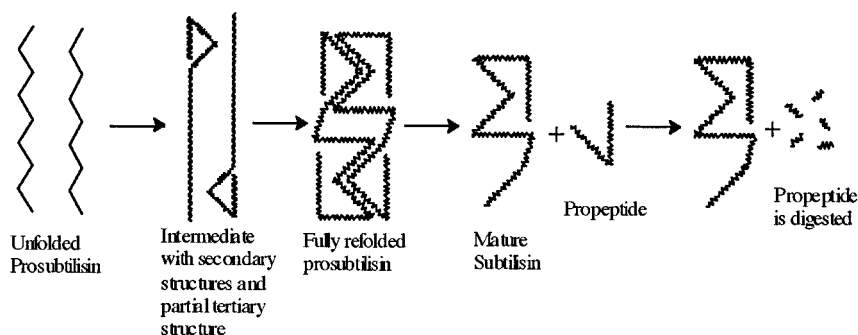
^b No velocity could be measured due to the depletion of inhibitor.

protease inhibitor. Apparently, the propeptide can successfully compete with PMSF for the active center of the refolded protein. Alternatively, the active center is assembled by using the propeptide as a template, then autoprocessing of the propeptide takes place by an active center with its full catalytic power recruited, to the exclusion of PMSF.

Autoprocessing of the prosubtilisin-S49C mutant (7) was found to take place even in saturated urea at 4 °C (approximately 6.0 M at this temperature). However, the intermolecular disulfide bond engineered into the (prosubtilisin-S49C)₂ dimer inhibited autoprocessing so that the refolded dimer is trapped as an intermediate in an autoprocessing-competent state (APCS). APCS is ready to be processed upon reduction (7). After mixing the prosubtilisin-S49C monomer with the (prosubtilisin-S49C)₂ dimer (1:10 molar ratio) in saturating urea at 4 °C, the propeptide band became intense with time (not shown) indicating the ongoing, slow intermolecular processing. No mature monomer or dimer could be detected by SDS-PAGE as they were autolyzed at such high concentrations of urea.

The APCS itself has no protease activity. Near-UV CD spectra did show that the APCS has tertiary structure, but less than that of refolded prosubtilisin-S221A or of mature subtilisin (7). The APCS could not undergo intramolecular autoprocessing, since this would have led to the formation of mature (subtilisin-S49C)₂ dimer. The disulfide bond should have had little effect on the autoprocessing, since Cys⁴⁹ is on the surface, far from the active site and the N terminus of subtilisin. If the prosubtilisin dimer is the intermediate prior to autoprocessing

SCHEME 1. Intermolecular template model for the processing of prosubtilisin.



as we believe to be the case for prosubtilisin-S221A, the APCS represents an unproductive dimer. That is, the secondary structures are completely formed, the active sites could not be properly aligned with the propeptides due to the restriction imposed by the disulfide hinge, and the propeptides of other dimers could not get near enough to be used as a template for refolding. When the disulfide bond is reduced, the mature portion of prosubtilisin-S49C could undergo reorganization and a cooperative interaction with the propeptide of another prosubtilisin-S49C, then refolding and autoprocessing would ensue.

The APCS may resemble that prosubtilisin-S221A folding intermediate detected in 1.0 M guanidinium HCl (14), given that both possess well-defined secondary and tertiary structures. The MW_{app} of 32,000 reported for prosubtilisin-S221A (14) is not consistent with MW_{app} expected for a dimer. The discrepancy may be due to the high ionic strength (0.5 M $(NH_4)_2SO_4$ plus 0.05 M phosphate).

The prosequence can guide the refolding of denatured enzyme without it being covalently bonded to the N terminus of the mature subtilisin. In the early report, we noted that exogenously added active site mutant prosubtilisin-D32N could refold the denatured mature subtilisin *in trans* (9), but we hadn't yet recognized the inhibitory and substrate-like behavior of the propeptide *vis à vis* the mature enzyme. No enzymatic activity should have been recovered in such *in trans* refolding experiments, had intermolecular processing of the propeptide been precluded (5, 15). The refolded enzyme should have been completely inhibited, had the propeptide been cleaved from prosubtilisin-D32N, but not digested subsequently. The long incubation times required for regain of even partial activity hinted at these complexities (36).

CONCLUSIONS

Based on the combined evidence we conclude the following. 1) The refolded form of prosubtilisin is in equilibrium with the unfolded state leading to autoprocessing even in saturated urea at 4 °C; (prosubtilisin-S49C)₂ dimer could be processed intermolecularly by active monomer in such a solution. 2) Propeptides are both substrates and strong, slow-binding inhibitors of subtilisins BPN' and Carlsberg. Their K_i values are in the range 1.0×10^{-9} M without correcting for the depletion of propeptides due to proteolysis. 3) Propeptides form strong complexes with PMS-modified or active center mutant subtilisins. Such complexes are stable at high ionic strength (0.5 M $(NH_4)_2SO_4$ plus 0.1 M phosphate at pH 7.0). The propeptides gain additional secondary structure (over and above the helical secondary structure reported here) when they bind to the mature enzymes according to x-ray and far-UV CD spectra (28, 37, 38), as well as the preliminary NMR results presented here. Their well defined tertiary structure is evident as the hydrodynamic volume is reduced dramatically concomitant with binding (Fig. 5). 4) Prosubtilisin-S221A exists as a dimer according to size exclusion HPLC. Formation of a tight prosub-

tilisin dimer is also supported by the observation that chymotrypsin inhibitor 2 (CI2) with a K_i of 2.9×10^{-12} M to subtilisin BPN' could not bind to the refolded prosubtilisin-S221A (14). However, CI2 could compete with propeptide ($K_i \sim 1.0 \times 10^{-9}$ M) favorably for the mature subtilisin. Consequently, there is evidence for an intermolecular pathway for both the refolding and autoprocessing of prosubtilisin. The intermolecular processing, while in agreement with experiments *in vivo* (18) and *in trans* refolding *in vitro* (9), is in conflict with some *in vitro* findings (6, 15, 28). We propose that the proregion acts as an intermolecular refolding template (Scheme 1). Initially, the unfolded prosubtilisin folds into a "molten globule"-like structure. Next, the catalytic machinery and substrate binding subsites are formed using the proregion of another prosubtilisin molecule as template and the dimer is generated as an intermediate. Finally, the propeptide is cleaved from the mature sequence and is subsequently digested. This digestion is advantageous for two reasons, it removes the potent inhibitor and it inhibits the unfolding reaction that may also be assisted by the propeptide, if it acts as catalyst. Hence, the propeptide can be considered as a "single-turnover" catalyst. Recently, several other examples of the intervention of protein dimers in cellular processes were emphasized (39).

Finally, we wish to speculate on the issue of inter- versus intramolecular processing. Given that one is typically dealing with Avogadro's numbers of molecules in a reaction flask, it is difficult to unequivocally differentiate intra- from intermolecular processing. We believe that perhaps both are feasible, the case for intermolecular processing via the various examples presented here, and presented earlier by other groups, makes the case for this option quite convincing. Because of the very tight binding between the various species, the usual tests, such as concentration dependence of the phenomenon, cannot unequivocally differentiate between the two pathways. On the other hand, perhaps very early on, when there is only a very low concentration of prosubtilisin molecules and there are essentially no enzymatically active mature molecules around, intramolecular processing, or adventitious processing by a different protease, may be a necessity. As more active mature molecules are produced, the intermolecular pathway should take precedent. This would lead to a lag-phase in the autoprocessing, as is indeed observed in the development of activity that results from the folding cascade potentiated by the reduction of the (prosubtilisin-S49C)₂ disulfide-linked dimer (16).

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