A Covalently Trapped Folding Intermediate of Subtilisin E: Spontaneous Dimerization of a Prosubtilisin E Ser49Cys Mutant *in Vivo* and Its Autoprocessing *in Vitro*†

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Received July 26, 1993; Revised Manuscript Received November 8, 1993®

ABSTRACT: The propeptide of subtilisin E (the N-terminal 77 amino acid extension) is required for the proper folding of the nascent mature protein and is also a potent and specific inhibitor of the active enzyme. Previous studies have demonstrated that the propeptide can renature denatured mature sequence either *in cis* or *in trans* and can be considered an intramolecular chaperone, since it is not required for activity of the mature enzyme. In this paper it is shown that a prosubtilisin-S49C mutant can be expressed in *Escherichia coli* either as a monomer or as a disulfide-linked dimer, (prosubtilisin-S49C)$_2$, depending on the vector selected. Interconversion between (prosubtilisin-S49C)$_2$ and prosubtilisin-S49C could be readily achieved by reduction and oxidation in denaturing solutions, such as guanidine hydrochloride or urea. While the monomer can undergo autoprocessing *in vitro* under refolding conditions, the dimer is trapped in an intermediate state which could not be processed into active enzyme. Remarkably, the autoprocessing of this trapped intermediate could be induced readily upon reduction by dithiothreitol. This disulfide-linked (prosubtilisin-S49C)$_2$ is fairly stable, but does tend to aggregate when the ionic strength of the solution is reduced below 0.1 M. The disulfide-linked (prosubtilisin-S49C)$_2$ has far- and near-UV CD spectra revealing the presence of both secondary and tertiary structures, respectively, similar to those of the active mature monomer. Hence this autoprocessing-competent state appears to be a "late" folding intermediate, arising after the "molten globule" state formed in the absence of the prosequence, that has no discernible tertiary structure. Active mature subtilisin from the autoprocessed prosubtilisin-S49C mutant has K_m and k_{cat} values toward the synthetic substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide nearly identical to those of the wild-type enzyme. The S49C mutant could also be dimerized by mild oxidation. The dimer thus obtained is unstable and also undergoes autolysis.

Subtilisin E (Stahl & Ferrari, 1984) and BPN′ (Wells et al., 1983) are alkaline proteases consisting of single polypeptide chains of 275 amino acids and are produced from their preproproteins. The presequence functions as a signal peptide for secretion across the membrane. In previous studies from our laboratories it was shown that deletion of the prosequence of subtilisin E leads to secretion of mature but inactive subtilisin (Ikemura et al., 1987), called pHT700, suggesting that the 77 amino acid long propeptide must precede the mature subtilisin to guide the latter into an active conformation. Using an active center (D32N) mutant pHI216 it was also demonstrated that the processing of the prosubtilisin to mature active enzyme takes place by intramolecular proteolysis (Ikemura & Inouye, 1988; Ohta & Inouye, 1990). This active center mutant of prosubtilisin pHI216 was also shown to be effective in guiding the renaturation of inactive subtilisin pHT700 to active enzyme *in trans* (in an intermolecular pathway; Zhu et al., 1989). Since Asp32 is an essential part of the active center catalytic triad, pHI216 prosubtilisin could not be autoprocessed either *in vivo* (Ikemura et al., 1987) or *in vitro* (Ikemura & Inouye, 1988) to produce active subtilisin. In further work, we reported that synthetic pro-77-mer, but not pro-64-mer (corresponding to residues −1 to −77 and −1 to −64 of pre-prosubtilisin E, where the numbering is from the N-terminus of the mature sequence toward the N-terminus of the prosequence, so that position −1 is adjacent to position +1 in the mature sequence), is capable of renaturing denatured subtilisin E and inhibiting active subtilisin E in a specific fashion (Ohta et al., 1991). We also reported that the synthetic propeptides corresponding to residues −1 to −77 (pro-77-mer), −1 to −68 (pro-68-mer), and −1 to −63 (pro-63-mer) of pre-prosubtilisin BPN′ (Cash et al., 1989) can renature both acid-denatured subtilisin BPN′ and denatured subtilisin E expressed in the absence of its prosequence as well as inhibit the enzymes specifically and competitively (Zhu et al., 1992). Dramatic differences in the folding ability and inhibition efficacy are found by simple variations in the primary sequence of the propeptides. In addition, it was demonstrated that the role of various amino acids in the propeptide of pre-prosubtilisin E can be probed by random mutagenesis (Lerner et al. 1990; Kobayashi & Inouye, 1992). These results indicate that the propeptide serves as an intramolecular chaperone which is required only for the folding of subtilisin and not for its enzymatic activity (Ohta et al., 1991; Inouye, 1992).

In addition to providing an excellent model system to explore the role of the propeptide in the folding of subtilisins, subtilisins also provide a good model system for studying the effects of disulfide bonds on the stability of secreted proteins (Wells & Powers, 1986; Pantaliano et al., 1987) since there is no cysteine

† This research was supported by Grant NSF-MCB-9105293 (M.I.) and the Rutgers University Busch Fund, the Johnson and Johnson Research Discovery Fellowship, the Ciba-Geigy Corp., Ardsley, NY, and Hoffman-La Roche Inc., Nutley, NJ (F.J.).

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® Abstract published in *Advance ACS Abstracts,* January 1, 1994.

0006-2960/94/0433-562$04.50/0

in natural subtilisins. The engineered disulfide bonds impart some stabilization to all of these mutant proteins. The recombinant plasmids carrying the altered genes were expressed in *Bacillus subtilis*. All of these mutants were secreted as efficiently as was wild-type subtilisin, and disulfide bonds were formed spontaneously *in vivo* in the case of the double-cysteine mutants reported. These mutants had activities nearly equivalent to that of wild-type subtilisin with some exceptions. None of these subtilisin variants was more stable than the wild-type enzyme in terms of thermostability (T_m) and thermal inactivation rate ($t_{1/2}$). Takagi et al. (1990) made Cys substitutions at positions 61 and 98 individually or concurrently in subtilisin E. The C61/C98 double mutant subtilisin formed a disulfide bond spontaneously during the expression and gave an enzyme with protease activity equivalent to that of wild-type subtilisin and thermal stability (T_m) and autolytic stability ($t_{1/2}$) superior to those of wild-type subtilisin.

We here report that the S49C single mutant of subtilisin E forms a disulfide-linked dimer, (prosubtilisin-S49C)$_2$, spontaneously *in vivo*, thereby trapping a folding intermediate with no activity. After purification and reduction with dithiothreitol, the resulting prosubtilisin-S49C monomer was found to be capable of undergoing autoprocessing *in vitro* in renaturing medium. Conditions have been identified under which autoprocessing can proceed or is precluded. This mutant was constructed since S49 is a conserved amino acid at a distance of more than 12 Å from the active site S221 residue, on the surface of the protein according to X-ray structures of subtilisin BPN′ and subtilisin Carlsberg in the absence (Wright et al., 1969; Drenth et al., 1972; Bott et al., 1988; Neidhart & Petsko, 1988) or in the presence of inhibitors (Kraut, 1977; Matthews et al., 1977; Mitsui et al., 1979; Bode et al., 1986, 1987; McPhalen & James, 1988). While no X-ray structure is available for subtilisin E, considering the similarity of the three-dimensional structures of subtilisin BPN′ and subtilisin Carlsberg and the homology of the primary sequences (subtilisin BPN′ is 76% identical with subtilisin Carlsberg, and subtilisin E is 87% identical with subtilisin BPN′ and 72% identical with subtilisin Carlsberg), the three-dimensional structure of subtilisin E is likely to be similar to those of subtilisin BPN′ and subtilisin Carlsberg. Introduction of a Cys residue at position 49 may serve multiple purposes: to trap a disulfide-linked dimer of prosubtilisin that can serve to study the folding cascade chaperoned intramolecularly by the prosequence, to afford chemical synthesis of a disulfide-linked dimer of mature subtilisin, and finally to create a nucleophilic center on the surface of the enzyme for the introduction of a variety of molecular probes.

MATERIALS AND METHODS

Materials

All of the restriction enzymes used and the isopropyl β-D-galactopyranoside (IPTG)[1] were from US Biochemical or International Biotechnologies and were used as recommended by the suppliers. The pET expression system was from Novagen. Guanidine hydrochloride was from ICN; urea, from Fisher; succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (s-AAPF-*p*Na), from Bachem Bioscience; Macro-Prep 50CM, from Bio-Rad; Sephacryl S-300, from Pharmacia; 5,5′-dithiobis-(2-nitrobenzoic acid) and DTNB, from Eastman Kodak; phenylmethanesulfonyl fluoride (PMSF), from Sigma; 3,5-bis(trifluoromethyl)phenylboronic acid (BTFMPB), from Lancaster Synthesis.

Methods

Electrophoresis. For protein quantification and molecular weight estimation sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli (1970). Phenylmethanesulfonyl fluoride was added to a final concentration of 1 mM when samples of active subtilisins were prepared. No dithiothreitol was present during sample preparation unless otherwise noted. All protein gels were stained with Coomassie Blue or silver stain.

Mutagenesis and Cloning. Site-directed mutagenesis was done directly on plasmid pHI212 (Ikemura et al., 1987) according to Inouye and Inouye (1987). A 15-mer oligonucleotide, 5′-CGGAGCA*T*GCTTCGT, was used to change Ser49(AGC) to Cys49(TGC). This single-base mutation (indicated by an asterisk) also eliminated the *Hin*dIII site at that position (underlined) and thus greatly facilitated the screening of the mutant. The mutation was confirmed by DNA sequencing using the dideoxy sequencing method (Sanger et al., 1980) directly on the plasmids. To increase the expression level of the target protein, a T7 expression system was used in the subcloning (Studier et al., 1990). The 1.0-kb *Not*I/*Bam*HI fragment of pHI212:S49C, which contains the partial gene of subtilisin E with the S49C mutation site, was ligated respectively with the 5.8-kb *Not*I/*Bam*HI fragments of pET11a:prosubtilisin and pET11a:OmpA-pro77-subtilisin (unpublished work), which have the remainder of the gene, to give pHU514 (pET11a:prosubtilisin/S49C) and pHU810 (pET11a:OmpA-prosubtilisin/S49C) (Figure 1).

The *B. subtilis/Escherichia coli* shuttle plasmid pBS42 (ATCC37279; Band & Henner, 1984) was used as the cloning vector. An 870-bp *Eco*RI/*Nde*I fragment carrying the promoter region and the N-terminal sequence of the subtilisin E gene was obtained from pKWZ (Park et al., 1989; kindly provided by Dr. R. Doi). The C-terminal portion of the subtilisin E gene, a 960-bp *Nde*I/*Bam*HI fragment, was from pHI212/Ser49Cys. These two fragments were ligated into the *Eco*RI/*Bam*HI sites of pBS42 to form pBSE-S49C (Figure 2). Transformants of an *E. coli* strain, CL83[ara Δ(lac-proAB) rpsL φ80 lacZΔM15 recA56] (Lerner et al., 1990), were plated on an LB-agar plate supplemented with 10 μg/mL chloramphenicol. The plasmid pBSE-S49C purified from *E. coli* was then used to transform an extracellular protease deficient *B. subtilis* strain, DB104 (his nprR2 nprE18 ΔaprA3) (Kawamura et al., 1984; kindly provided by Dr. R. Doi).

Expression and Purification. The wild-type subtilisin E and the active subtilisin-S49C mutant were purified from culture supernatants of DB104 harboring pKWZ or pBSE-S49C, respectively. *B. subtilis* was grown in a modified Schaeffer's sporulation medium (Leighton & Doi, 1971) containing 15 μg/mL chloramphenicol at 37 °C with shaking at 300 rpm. The culture supernatant was collected by centrifugation when the proteolytic activity reached a maximum (around 30 h) and was filtered through a 0.45-μm filter followed by ultradiafiltration in 20 mM Tris, pH 6.0, using a YM10 membrane at 4 °C. The concentrated retentate was loaded onto a Protein-Pak CM-15HR 10 × 100 mm column

[1] Abbreviations: IPTG, isopropyl β-D-galactopyranoside; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PMSF, phenylmethanesulfonyl fluoride; BTFMPB, 3,5-bis(trifluoromethyl)phenylboronic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; s-AAPF-*p*Na, succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide; M_{app}, the apparent molecular mass on electrophoretic gels; APCS, autoprocessing-competent state; *E. coli, Escherichia coli*; *B. subtilis, Bacillus subtilis*; propeptide, the 77 amino acid N-terminal extension of subtilisin synthesized by either genetic or solid-phase chemical methods; prosubtilisin, the mature subtilisin protein with its propeptide still covalently attached; (prosubtilisin-S49C)$_2$, the disulfide-linked dimer of prosubtilisinS49C; S49C, the Ser49Cys mutant of mature subtilisin E.

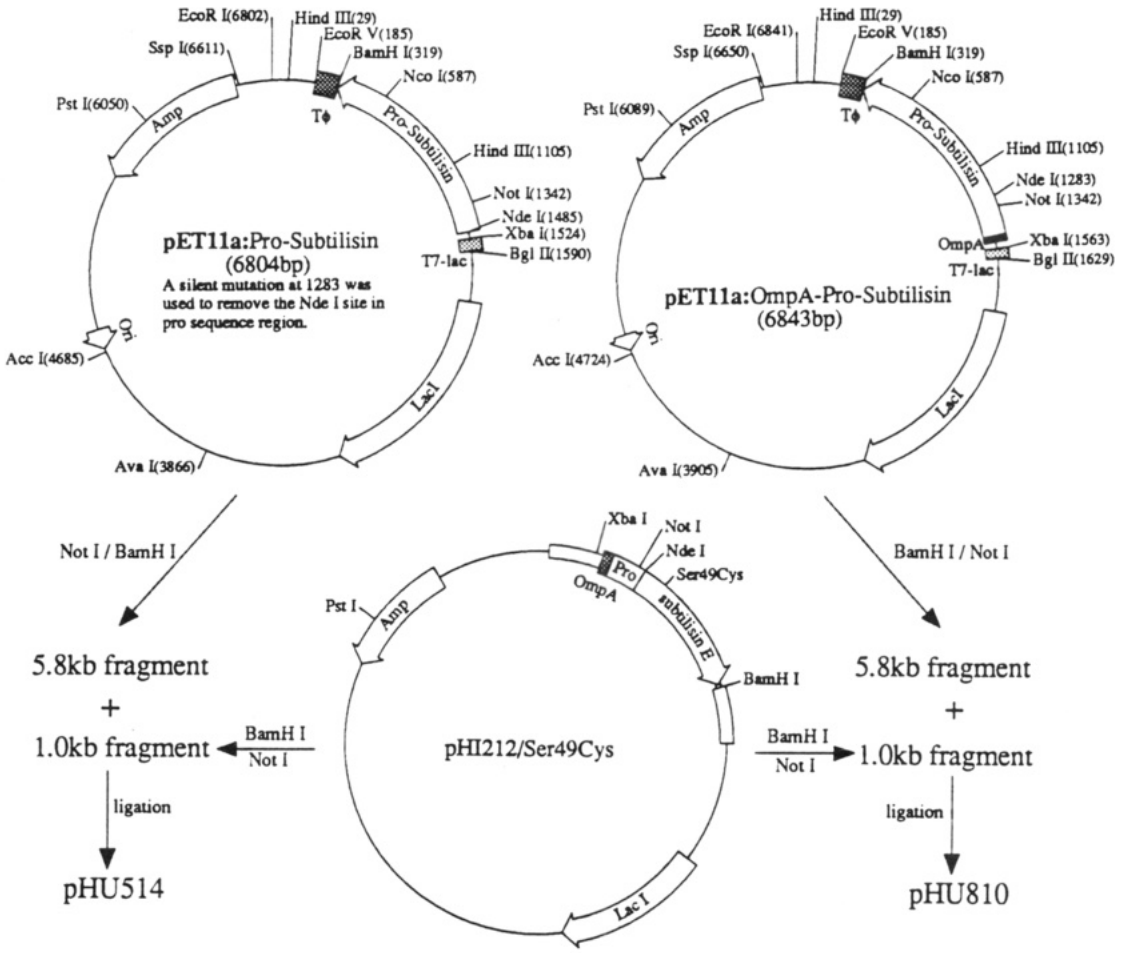


FIGURE 1: Subcloning of pHU514 and pHU810. In pET11a:prosubtilisin, the prosubtilisin gene was cloned directly at the ATG starting codon of T7 gene 10 on pET11a. The secretion plasmid, pET11a:OmpA-prosubtilisin, however, has an OmpA leader (21 amino acids long) coding sequence fused to the beginning of the prosubtilisin gene. Restriction maps of pHU514 and pHU810 correspond to those of pET11a:prosubtilisin and pET11a:OmpA-prosubtilisin, respectively, except that the *Hin*dIII site at 1105 no longer exists.

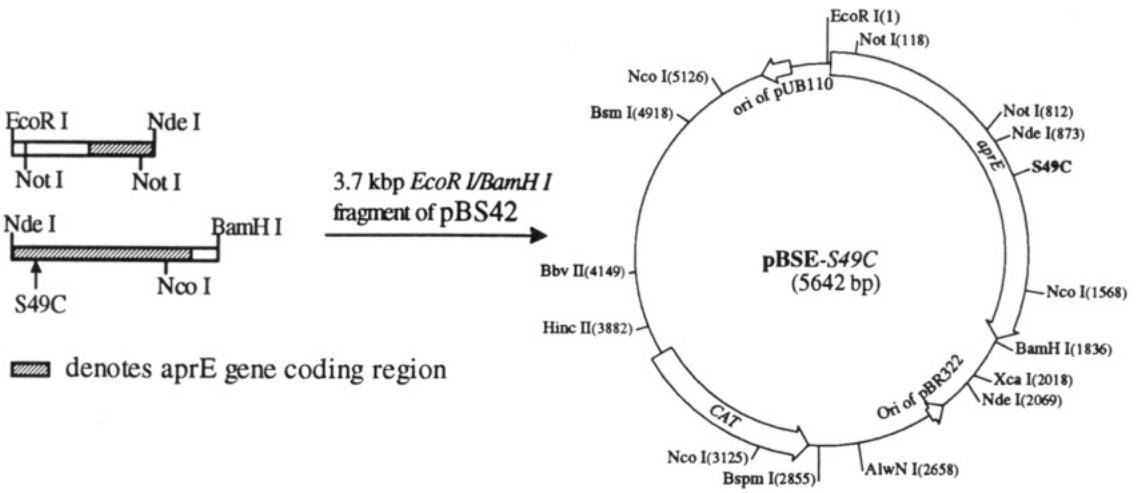


FIGURE 2: Construction of pBSE-S49C. The *Eco*RI/*Nde*I fragment is from pKWZ; the *Nde*I/*Bam*HI fragment is from pHI212/Ser49Cys. *Eco*RI and *Bam*HI are single restriction enzyme sites available on pBS42.

(Waters). Both mature subtilisin and its S49C mutant were eluted at 57 mM KCl (20 mM Tris and 1 mM DTT, pH 6.3) with a retention time of 22.6 min at room temperature. The purity was >99% according to HPLC, and both proteins gave a single band on SDS–PAGE.

E. coli BL21(DE3) (Studier & Moffatt, 1986) harboring pHU514 or pHU810 was used in the expression of monomer prosubtilisin-S49C or its dimer. The cells were grown in LB medium, containing 80 μg/mL ampicillin, 50 mM phosphate, pH 7.0, and 0.2% glucose, at 37 °C until an A_{500} = 1.2 was reached; the cells were then induced with IPTG, followed by a 2.5-h incubation at 37 °C. The cells were harvested by centrifugation and then disrupted either with a French press at 20 000 psi or by sonication on an ice bath. The cell pellets were spun down by low-speed centrifugation (10 000 rpm with a Beckman JA14 rotor) and washed twice with 50 mM Tris/2 mM EDTA, pH 7.0. The white inclusion bodies that resulted were almost free of cell debris and were solubilized in 6 M

guanidine hydrochloride containing 50 mM Tris, pH 7.0, with the aid of sonication at −10 °C. Next, the suspension was centrifuged at 90 000 rpm for 14 min using a Beckman TLA-100.2 rotor to remove insoluble materials. The supernatant was ultrafiltered (Diaflo ultrafilter from Amicon) in 8 M urea containing 50 mM Tris, pH 5.5, using Amicon YM30 and YM100 membranes (molecular mass cutoff: 30 and 100 kDa, respectively). The dialysate, which passed through YM100 and was retained on YM30, was further purified on a Macro-Prep 50CM cation-exchange column eluting with a phosphate gradient in 8 M urea, starting with 50 mM, pH 5.5, and proceeding to a final concentration of 0.2 M, pH 7.5. The protein expressed from pHU514 showed a single band on SDS PAGE, corresponding to prosubtilisin (no DTT was added to the sample treatment buffer in SDS–PAGE unless otherwise stated), while two bands corresponding to prosubtilisin monomer and dimer were observed in the case of pHU810. The higher molecular mass protein from pHU810 was separated from the monomer by gel filtration on a Sephacryl S-300 column equilibrated with 50 mM phosphate, pH 7.0, in 8 M urea.

Dimerization of Prosubtilisin-S49C in Vitro. To prosubtilisin-S49C monomer isolated from the inclusion bodies by dissolution in 6.0 M guanidine hydrochloride was added antifoam 289 (from Sigma) to a final concentration of 0.1%. Into the solution was bubbled oxygen through a fine porous HPLC filter head for 2 days at 4 °C. After chloroform extraction to remove the antifoam reagent, the solution was desalted chromatographically on Sephadex G25 (2.5 × 20 cm). The column was preequilibrated with 8.0 M urea in 25 mM phosphate buffer, pH 5.0, and the protein was eluted with the same buffer. Further purification was performed on Macro-Prep 50CM and Sephacryl S-300 according to the protocol in the preceding paragraph.

Autoprocessing of Prosubtilisin-S49C in Vitro. The renaturing buffer consisted of 50 mM phosphate, pH 7.0, containing 0.4 M $(NH_4)_2SO_4$, 2 mM $CaCl_2$, and 20 μM BTFMPB [a potent reversible inhibitor; see Philipp and Bender (1983)]. The dialysis membrane was from Spectrum with a molecular mass cutoff of 12–14 kDa. The monomer and the dimer were dialyzed against renaturing buffer overnight at 4 °C, and aliquots were taken for enzymatic activity and SDS–PAGE analysis. The $(\text{prosubtilisin-S49C})_2$ dimer was autoprocessed by dialysis against renaturing buffer for 4 h after addition of DTT to a final concentration of 2 mM.

Circular Dichroism Experiments. CD was performed on a JY Dichrographe Mark VI circular dichroism spectrophotometer at 20 °C. A 0.1-cm-path-length cell was used for far-UV CD (200–250 nm) spectra, and a 1.0-cm cell was used for the near-UV CD (250–320 nm). The wavelength increment was set at 0.5 nm, and the integration time was 1.0 s. For each spectrum eight scans (for near-UV) or four scans (for far-UV) were averaged, and spectra were corrected by subtraction of the spectrum of an appropriate buffer. CD values are expressed as mean residue ellipticity, $[\theta]_{mrw}$, calculated from

$$[\theta]_{mrw} = 3300\Delta A/(cl)\text{MRW}$$

where ΔA is the difference in absorbance of left and right circularly polarized light, c is the concentration in milligrams per milliliter, l is the path length in centimeters, and MRW is the mean residue molecular weight of the sample (MRW of prosubtilisin-S49C or prosubtilisin-S221A = 103.0; MRW of subtilisin-S49C = 100.8).

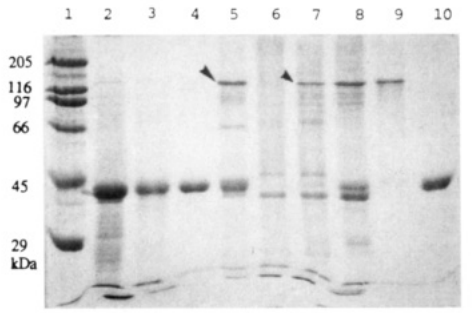


FIGURE 3: Prosubtilisin-S49C monomer and dimer expressed from pHU514 and pHU810 and their interconversion. Lane 1: molecular mass standards. Lanes 2–5 correspond to proteins from cells harboring pHU514: lane 2, total cellular protein; lane 3, pellet of the cell lysate; lane 4, purified prosubtilisin-S49C; lane 5, $(\text{prosubtilisin-S49C})_2$ (indicated by an arrowhead) resulting from chemical oxidation of the prosubtilisin-S49C shown in lane 4. Lanes 6–10 are proteins expressed from pHU810: lane 6, total cellular proteins of uninduced cells; lane 7, total cellular proteins of induced cells with a newly apparent band, indicated by an arrowhead; lane 8, pellet of cell lysate; lanes 9 and 10, purified high molecular weight protein without or with DTT in the treatment buffer, respectively.

Assay of Subtilisin Activity. Subtilisin activity was assayed spectrophotometrically by monitoring the release of *p*-nitroaniline at 410 nm (ϵ = 8900 M^{-1} cm^{-1}) due to enzymatic hydrolysis of succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (s-AAPF-*p*Na; Strongin et al., 1978) as substrate in 50 mM Tris-HCl, pH 8.0, containing 1 mM $CaCl_2$ and 1 mM DTT (DTT was used for the active C49S mutant only). 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 vs time curve was used as the initial velocity.

RESULTS

Production of Prosubtilisin-S49C and Its Dimer. In the plasmid pHU514, the prosubtilisin gene was cloned directly at the ATG starting codon of the T7 gene 10 on pET11a (Studier et al., 1990). It could be anticipated that this would provide prosubtilisin-S49C (which may contain an extra Met at the N-terminus, i.e., at position −78) and mature subtilisin, providing that autoprocessing took place. In fact, prosubtilisin-S49C comprised more than 70% of the total cellular proteins expressed from this plasmid (Figure 3, lane 2). Since this protein comprises 90% of the inclusion body that formed the pellets from the cell lysate (Figure 3, lane 3), it was readily purified (Figure 3, lane 4). The supernatant of the cell lysate of BL21(DE3) harboring pHU514 has very low enzymatic activity when tested with s-AAPF-*p*Na as substrate, indicating that only a very small amount of prosubtilisin-S49C underwent autoprocessing *in vivo*.

The secretion plasmid pHU810 is different from pHU514 only in that it has the OmpA signal peptide (21 amino acid residues) fused to the −77 position from the N-terminus of mature subtilisin (Ikemura et al., 1987). This signal peptide directs the nascent protein to the periplasmic space (Ghrayeb et al., 1984). When BL21(DE3):pHU810 was induced with IPTG ranging in concentration from 0.01 to 2 mM, the growth of the cells was greatly hindered and a new band became apparent on SDS–PAGE, with a M_{app} of 130 kDa (Figure 3; lane 7, top band). The supernatant of the cell lysate had higher enzymatic activity than that of pHU514. The new protein induced was in the cell pellet (Figure 3, lane 8). After it was purified to homogeneity (Figure 3, lane 9), it could be reduced to a form which is similar in size to that of the prosubtilisin (Figure 3, lane 10). This suggested that the top band was the dimerized $(\text{prosubtilisin-S49C})_2$. The disulfide-

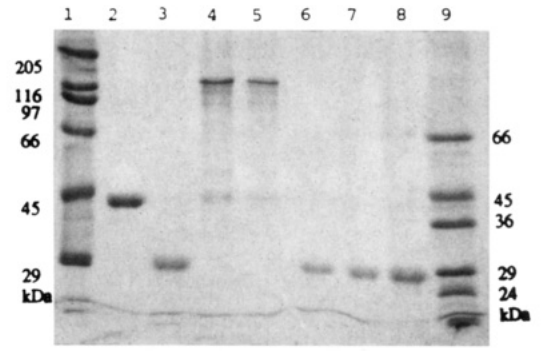


FIGURE 4: Autoprocessing of prosubtilisin-S49C. Lanes 1 and 9, molecular mass standards; lanes 2 and 3, protein purified from pHU514 and subsequently dialyzed against renaturing buffer containing DTT, respectively; lane 4, purified $(\text{prosubtilisin-S49C})_2$ dimer from pHU810; lane 5, $(\text{prosubtilisin-S49C})_2$ refolded in renaturing buffer; lane 6, refolded $(\text{prosubtilisin-S49C})_2$ dimer shown in lane 5 subjected to reduction with DTT; lane 7, $(\text{prosubtilisin-S49C})_2$ dimer refolded in renaturing buffer with DTT present; lane 8, mature subtilisin E.

linked dimeric structure was further confirmed by oxidizing the prosubtilisin-S49C isolated from pHU514 with oxygen: a higher M_{app} protein was generated (Figure 3, lane 5, top band), which had the same size as the dimer from pHU810 (Figure 3, lane 9). Since induction by IPTG ranging in concentration from 0.1 to 1 mM gave almost the same expression level, 0.1 mM IPTG was used for further experiments in the production of prosubtilisin dimer.

Under its own promoter in the case of pBSE-S49C, active subtilisin-S49C mutant was secreted into culture medium at a reduced level ($\sim$0.3 mg/mL) compared to that of wild type ($\sim$1.0 mg/mL). The culture supernatant gave rise, in addition to the S49C mutant, to a prominent band (molecular mass = 21 kDa) on SDS gel, which is very faint during expression of the wild-type enzyme (results not shown). We believe that it was the major fragment product resulting from autolysis of the mutant during culturing. On the other hand, wild-type subtilisin E itself is quite resistant to autolysis (Zukowski et al., 1990).

Autoprocessing of Prosubtilisin-S49C. On dialysis against renaturing buffer, prosubtilisin-S49C monomer expressed from either pHU514 or pHU810 could be autoprocessed into active enzyme with the size of mature subtilisin E (lanes 3 and 7, respectively, in Figure 4). Autoprocessing was complete in less than 4 h at 4 °C judging from the SDS–PAGE.

By contrast, the $(\text{prosubtilisin-S49C})_2$ dimer did not undergo autoprocessing when dialyzed against renaturing buffer (Figure 4, lane 5), but yielded a relatively stable solution. This transient state, trapped by a disulfide bond between two prosubtilisin-S49C molecules, was found to exist in an autoprocessing-competent state (designated as APCS). Upon reduction with DTT, the APCS is readily autoprocessed into active mature subtilisin (Figure 4, lane 6).

Dimerization of mature subtilisin-S49C could also be accomplished by reacting unmodified S49C mutant with S49C modified with 5-mercapto-2-nitrobenzoic acid. A band at 55 kDa was detected on SDS–PAGE, which is in good agreement with the molecular weight of the subtilisin dimer (not shown). The dimer was found to be unstable and was proteolyzed to smaller fragments; one of the major proteolytic products had an apparent molecular mass of 23–24 kDa. The amino-terminal sequence of this fragment was ADSVPYGISQ IKAPALH-, which coincides with the amino-terminal sequence of the mature subtilisin E, indicating that this large proteolytic product was derived from the amino-terminal end

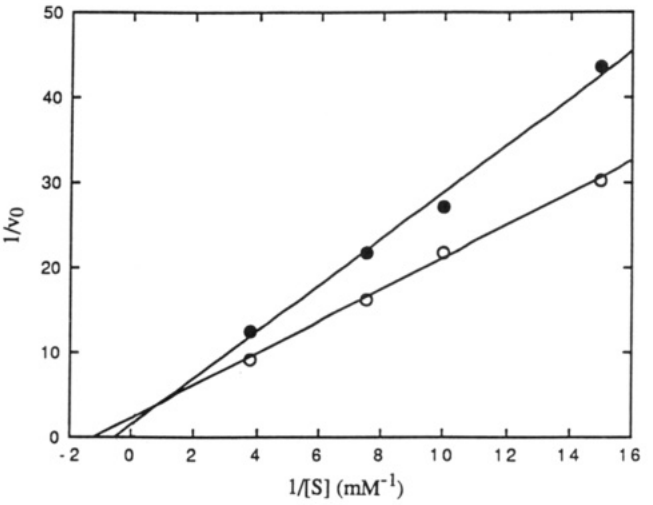


FIGURE 5: Comparison of the S49C mutant treated with Ellman's reagent (○) with untreated enzyme (●): double-reciprocal plot of initial velocity, v_o, versus concentration of substrate [S]. Assays were carried out in 50 mM Tris-HCl, pH 8.5, containing 1 mM $CaCl_2$ at 25 °C using succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate.

of subtilisin E. According to a three-dimensional model of subtilisin, the central α-helix (amino acid residues 220–239) is proximal to C49 and the C-terminal sequence emanating from the end of this helix may be removed during autolysis.

Mature subtilisin-S49C modified with 5-mercapto-2-nitrobenzoic acid was active with a larger K_m and a smaller k_{cat} than the unmodified enzyme (Figure 5). The sulfhydryl-modified subtilisin mutant was also unstable, as was the dimer of the mutant subtilisin-S49C. All SH-modified derivatives were shown to undergo the proteolytic fragmentation reported in the previous paragraph. A possible reason for the fragmentation might be the steric strain introduced by the modifier. It is relevant in this regard that SH modification with iodoacetamide, a small neutral molecule, also led to proteolysis of the mutant. In all of the SH-modified S49C mutants the same major proteolytic product is observed.

Catalytic Properties and Thermal Stability of Ser49Cys Subtilisin E. Against s-AAPF-*p*Na as substrate, the K_m values (mM) are 1.05 and 0.81, the k_{cat} values (s^{-1}) are 18 and 13, and the k_{cat}/K_m values (s^{-1} mM^{-1}) are 17 and 16 for wild-type (S49) and mutant enzyme (S49C), respectively, at 25 °C, 20 mM Tris, and pH 8.0 with 0.1 M KCl, 1 mM DTT, and 1 mM $CaCl_2$. The k_{cat}/K_m ratios of S49C subtilisin E and wild-type subtilisin E (which reflect their specificity toward s-AAPF-*p*Na), are identical within experimental error. The heat stability of the purified enzymes at 60 °C was analyzed in the presence of 1 mM $CaCl_2$. The S49C mutant was less stable than the wild-type enzyme at 60 °C in 1 mM $CaCl_2$: the half-life for thermal inactivation was 7 min for the wild-type enzyme and 5 min for the S49C mutant (data not shown).

Circular dichroism (CD) spectra of the various species were recorded in both the near- and the far-UV regions as indicators of the presence of tertiary and secondary structure, respectively (Figure 6).

DISCUSSION

The data presented show that most of the prosubtilisin E S49C mutant expressed could not be autoprocessed to mature subtilisin *in vivo*. Instead, it is mainly secreted in *E. coli* as the prosubtilisin dimer bonded via a disulfide bond when a secretion vector, pHU810, is used. As the dimer could also be produced from monomer prosubtilisin-S49C by means of

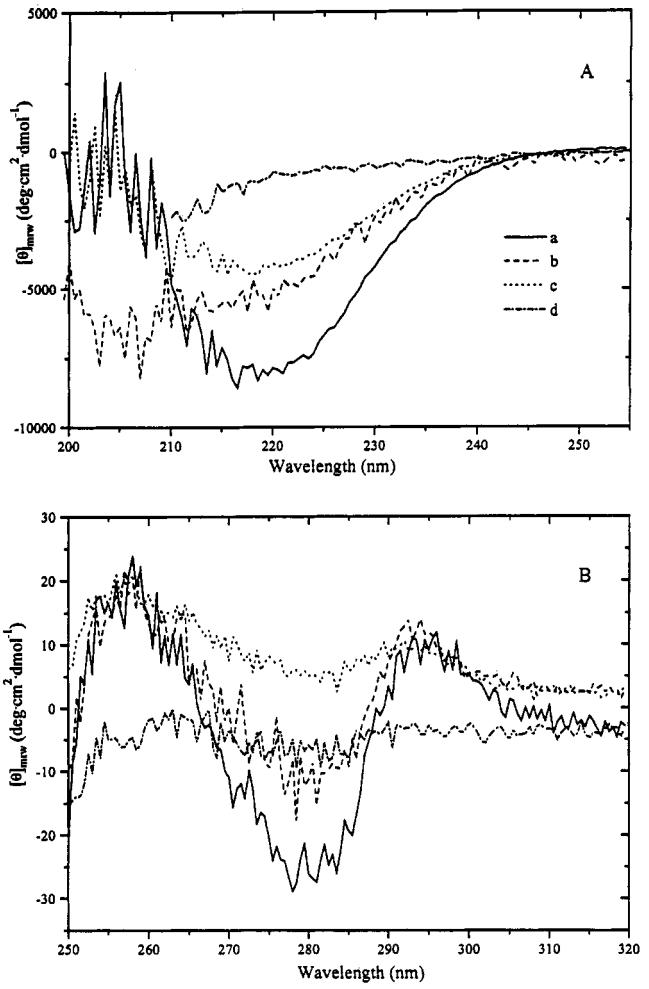


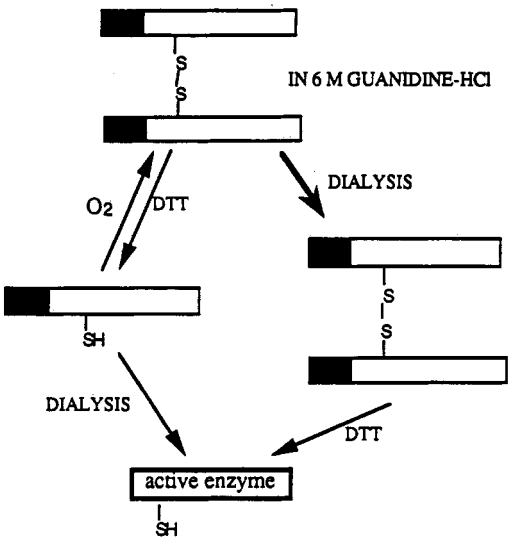
FIGURE 6: CD spectra of different conformations of subtilisin E at 25 °C. Protein concentrations were from 4 to 30 μM. (A) Far-UV spectra were the average of 4 scans in a 0.1-cm cell. (B) Near-UV spectra were the average of 8 scans in a 1.0-cm cell. (a) Refolded active subtilisin-S49C; (b) refolded prosubtilisin-S221A; (c) refolded (prosubtilisin-S49C)$_2$ dimer; (d) unfolded (prosubtilisin-S49C)$_2$ dimer in 8.0 M urea.

chemical oxidation, the spontaneous dimerization process *in vivo* observed above is probably just a consequence of the environment to which the nascent protein is exposed. In cells harboring pHU514, the expressed protein remains in a reducing environment, in the cytoplasm. However, prosubtilisin-S49C was secreted into the periplasmic space in the case of pHU810. Since pHU514 produces solely monomer prosubtilisin-S49C, it would appear that dimer formation takes place in the periplasmic space. As it was difficult to oxidize the prosubtilisin-S49C to dimer directly in the inclusion body *in vitro*, we could assume that the prosubtilisin-S49C existed in its soluble form long enough to be oxidized in the cell. Hence, the folding process must be much slower than the oxidative dimerization. Otherwise, the monomer might have undergone refolding and autoprocessing prior to these events being blocked by oxidation.

Subtilisin-S49C possesses protease activity nearly identical to that of the wild-type subtilisin E. This is not unexpected, since this residue is presumed to be on the surface of subtilisin and far from the catalytic triad.

The reduction-potentiated autoprocessing/folding cascade offers an excellent method for elucidation of the role of the prosequence *in cis* in the folding of the mature sequence to an active confirmation. Scheme 1 summarizes the various pathways available to the mutant for activation.

Scheme 1: Autoprocessing of Prosubtilisin S49C Mutant



The far-UV CD spectrum of (prosubtilisin-S49C)$_2$ in renaturing buffer (Figure 6A) shows that its autoprocessing-competent state (APCS) has a well-defined secondary structure, similar to that of active center mutant prosubtilisin-S221A and to that of the refolded native mutant enzyme S49C, but different from that of the denatured state of (prosubtilisin-S49C)$_2$ in 8.0 M urea. These APCS secondary structures are also similar to that of mature subtilisin BPN′ refolded in the absence of the prosequence (Eder et al., 1993). According to the near-UV CD, the APCS has a significant amount of tertiary structure, nearly the same as that of the active enzyme (Figure 6B). It should be noted that the prosubtilisin CD spectra represent the sum of the spectral features of the pro and mature sequences—compare the CD spectra of prosubtilisin-S221A and subtilisin-S49C, for example. The minimum in ellipticity at 278 nm indicates that the aromatic amino acid residues are in an asymmetric environment, hence suggesting a defined 3° structure. In this respect, the APCS structures here revealed are distinct from the subtilisin BPN′ refolded in the absence of the prosequence that exhibited essentially no tertiary structural features (Eder et al., 1993) and that was suggested to exist as a "molten globule" state.

It should also be mentioned that a third folding model of subtilisins has been developed recently, in which the Ca^{2+}-binding loop (amino acids 75–83) encompassing the crucial Asn78 side chain was excised (Bryan et al., 1992; Gallagher et al., 1993). This modified subtilisin BPN′, with an active center mutation that rendered it inactive, was claimed to undergo facile refolding according to a fluorescence spectroscopic criterion and X-ray evidence even in the absence of its propeptide.

To facilitate further progress in this admittedly already complicated subject, we are using as a working hypothesis the qualitative potential energy diagrams shown in Figure 7. In the ensuing discussion, A is misfolded or aggregated protein; U is unfolded or denatured protein; pro is the propeptide; and N is the mature, active sequence that is assumed to be thermodynamically favored.

Mature subtilisin refolded in the absence of the propeptide exists as the molten globule (MG) intermediate and was shown to be stable in renaturing buffer (Eder et al., 1993). But this MG intermediate is folding-incompetent; hence a high barrier to conversion to active conformation results (see diagram I, Figure 7). In this MG intermediate there is evidence for

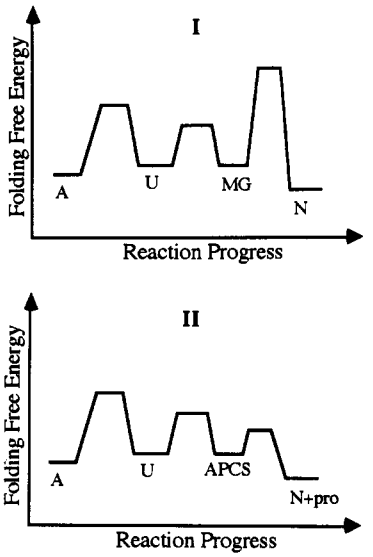


FIGURE 7: Two models representing the folding pathways of subtilisin in the presence and absence of its propeptide: A, misfolded; U, unfolded or denatured; MG, molten globule state; APCS, autoprocessing-competent state; N, native enzyme. In diagram I, the protein is trapped in the MG state in the absence of propeptide. In diagram II, the APCS closely resembles the native enzyme but is thermodynamically unstable due to the covalently attached propeptide. The equilibrium is shifted to the side of folded enzyme only when autoprocessing occurs.

extensive secondary, but essentially no tertiary, structure (Eder et al.,1993).

The wild-type pre-prosubtilisin is autoprocessed with a low kinetic barrier; the propeptide is in essence a single-turnover catalyst, single turnover because the catalyst is also an excellent inhibitor (diagram II, Figure 7). In the folding-competent but autoprocessing-incompetent (prosubtilisin-S49C)$_2$ disulfide-linked dimer the kinetic barriers are diminished, and there is already considerable tertiary structure present, in addition to secondary structure.

Although the APCS, such as the (prosubtilisin-S49C)$_2$ dimer, is fairly stable in renaturing buffer, it tends to form aggregates when the ionic strength is reduced lower than 0.1 M. Therefore, the APCS which has native-enzyme-like structure is kinetically unstable when it has the propeptide covalently attached to its N-terminus. It is commonly accepted that the native structure of a protein is its thermodynamically most stable conformation. Furthermore, the subtilisin denatured by 6.0 M guanidine hydrochloride plus HCl at pH 2.0 could be refolded without the presence of propeptide to a limited extent (<1%; Shindo et al., 1993). We therefore speculate that the prosequence acts as a folding catalyst which dramatically reduces the activation energy barrier between the folded and the unfolded conformations. At the same time, there has evolved a mechanism to inhibit unfolding of the native enzyme, simply by proteolyzing the propeptide, whose absence no longer allows the equilibration to the unfolded conformation. In other words, the propeptide as a reversible catalyst could catalyze the folding and the unfolding (see diagram II, Figure 7). The inhibitory properties of the propeptide account for the observation that while the propeptide lowers the kinetic barrier to refolding, it is a single-turnover catalyst, since it is not released from the active enzyme in the absence of proteolysis.

ACKNOWLEDGMENT

We thank Ms. Yuyun Li for constructing the T7 expression system for prosubtilisin E. We thank Professor Roy Doi of the University of California, Davis, for generously providing the DB104 strain of *B. subtilis* and the plasmid pKWZ.

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