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Accelerated Refolding of Subtilisin BPN′ by Tertiary-Structure-Forming Mutants of Its Propeptide[1]

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The propeptide of subtilisin BPN′, which functions as an intramolecular chaperone and a temporary inhibitor of subtilisin, is unique in that it acquires its three-dimensional structure by formation of a complex with the cognate protease. We previously showed that the successive amino acid replacements Ala47→Phe, Gly13→Ile, and Val65→Ile in the propeptide to increase its hydrophobicity resulted in formation of a tertiary structure, accompanied by increased ability to bind to the protease and increased resistance to proteolysis. In this study, we examined the effects of these tertiary-structure-forming mutations on the intramolecular chaperone activity of the propeptide. The successive amino acid replacements mentioned above were introduced into pro-subtilisin*, possessing a Ser221→Ala mutation in the catalytic residue. Refolding experiments were started by rapid dilution of the denatured pro-subtilisin*, and formation of tertiary structure in subtilisin was monitored kinetically by increase in tryptophan fluorescence. The wild-type pro-subtilisin* was found to refold with a rate constant of 4.8×10^{-3} s^{-1} in the equation describing an intramolecular process. The Ala47→Phe replacement in the propeptide resulted in a 1.2-fold increase in the rate constant of subtilisin refolding. When the additional replacement Gly13→Ile was introduced, refolding of subtilisin was substantially accelerated, and its kinetics could be fitted to a double exponential process composed of a fast phase with a rate constant of 2.1×10^{-2} s^{-1} and a slow phase with a rate constant of 4.5×10^{-3} s^{-1}. The rate constant of the fast phase was increased slightly by a further replacement, Val65→Ile. Since the slow phase is considered to correspond to proline isomerization, we concluded that tertiary-structure-forming mutations in the propeptide produce positive effects on its intramolecular chaperone activity through acceleration of the propeptide-induced formation of the tertiary structure of subtilisin BPN′.

Key words: amino acid replacement, intramolecular chaperone, propeptide, refolding, subtilisin, tertiary-structure-forming mutant.

Many proteases have been shown to possess a propeptide region, generally located between the signal peptide and mature regions (*1*). The finding that deletion of the propeptide-coding region from protease genes produces only inactive enzymes suggested that the propeptide functions as an intramolecular chaperone that facilitates folding of the mature region of the protease (*2*). Subsequently, the activity of the propeptides to inhibit their cognate proteases was demonstrated, although the inhibition is temporary (*3–4*). The inhibitory activity of propeptides seems to be essential, since we previously demonstrated that incomplete renaturation of proteases was due to proteolytic attack by refolded molecules on refolding molecules (*5*).

Among the various propeptides, that of subtilisin BPN′ is the best characterized. X-ray crystallographic analysis of the complex with its cognate protease has been performed, clarifying the unique binding mode of the propeptide with subtilisin *via* its C-terminal region (*6, 7*). Mutational analysis using random mutagenesis and site-specific mutants of the C-terminal region identified several residues required for chaperone activity and showed a close relationship between chaperone activity and the ability of the propeptide to bind to the protease (*8–10*), although these functions were not obligatorily linked in some cases (*11*). A more surprising property of the propeptide of subtilisin BPN′ is that it does not possess a typical tertiary structure in the isolated state, but acquires a three-dimensional structure when it binds to subtilisin BPN′ (*12–14*). Attempts to introduce tertiary structure into the isolated propeptide have been carried out by Bryan's group (*15, 16*) and by us (*17*). Triple and quadruple mutants generated by Bryan *et al.* using molecular design and the phage display technique were found to acquire tertiary structure (*15, 16*). Independently, we designed three replacements that improve the hydrophobic interactions in the molecular interior and introduce tertiary structure into the propeptide. Our mutants were shown to possess tertiary structure, increased ability to bind to the protease and increased resistance to proteolysis (*17*).

Bryan *et al.* also investigated the chaperone activity of their mutated propeptides and reported accelerated refold-

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ing of subtilisin BPN′ by the mutated propeptide (*18, 19*). However, they used mutated subtilisin BPN′ whose refolding was enhanced by deletion of the calcium-binding site and added the mutated propeptide exogenously. To study the intramolecular chaperone activity of the mutated propeptides, pro-subtilisin forms in which the propeptide is covalently attached to the N-terminus of the mature region should be used for activity measurements. In this study, therefore, we measured the refolding rate of mutated pro-subtilisin forms in which our tertiary-structure-forming mutations had been introduced into the propeptide region, and investigated the effects of these mutations on the chaperone activity of the propeptide.

We planned to express the pro-subtilisin proteins in cells of *Escherichia coli*, because secretory production would be accompanied by removal of the propeptide region. In addition, as reported by Eder *et al.* (*13*), attempts to express the wild-type pro-subtilisin were demonstrated to result in degradation of cellular proteins, possibly due to the production of active molecules of subtilisin from the pro-subtilisin in the cells. Therefore, the catalytic residue Ser221 in the active site of subtilisin BPN′ was replaced with Ala. The DNA fragment which encodes the N-terminal portion was amplified from the plasmid pSUB-N (*20*) by PCR using a primer to introduce an *Nco*I site and an N-terminal Met residue and a primer to create a *Bam*HI site after the *Cla*I site, and was inserted after digestion with *Nco*I and *Bam*HI into the *Nco*I–*Bam*HI site of plasmid pTV119N to produce pTVSubN. To replace the catalytic residue Ser221 of the mature region of subtilisin BPN′ by Ala, an *Eco*RI-*Hin*dIII fragment that encodes the C-terminal portion of subtilisin BPN′ was inserted into the *Eco*RI–*Hin*dIII site of plasmid pTZ19U, and the plasmid thus constructed was transferred into *E. coli* CJ236. Single-stranded DNA containing uracil bases was obtained by infection with the helper phage M13KO7. Codon replacement was carried out by the method of Kunkel (*21*) with a primer of a sequence of 5′ AAC-GGT-ACG-GCA-ATG-GCA-T 3′. After mutation was verified by dideoxy sequencing, the *Nco*I–*Cla*I fragment, which encodes the N-terminal portion of wild-type pro-subtilisin, and the *Cla*I–*Bgl*II fragment, which encodes the C-terminal portion of mutated pro-subtilisin, were inserted into the *Nco*I-*Bam*HI site of plasmid pET11d.

E. coli BL21(DE3) was transformed with the constructed expression plasmid. Large-scale culture in LB medium containing 50 μg/ml ampicillin was started by inoculation of a small-scale overnight culture. When A_{600} reached 1, expression of the pro-subtilisin gene was induced by addition of isopropyl-β-D-thiogalactopyranoside to give a final concentration of 0.4 mM, followed by culture for 4 h. Cells suspended in 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0) were disrupted by ultrasonication. The pro-subtilisin with Ser221→Ala replacement in the active site was found to be expressed in the *E. coli* cells as an inclusion body. The expressed protein was initially dissolved in 5 M GdnHCl, followed by dialysis against 6 M urea, 20 mM potassium phosphate (pH 6.4). After insoluble materials had been removed by centrifugation, the pro-subtilisin-containing solution was applied to a CM-52 column (2 × 15 cm) that had been equilibrated with the same buffer. Pro-subtilisin was eluted from the column with a linear gradient from 20 mM to 250 mM potassium phosphate (pH 6.4). Fractions which contained pure pro-subtilisin were collected and dialyzed against 6 M urea, 50 mM potassium phosphate (pH 7.5). The pro-subtilisin with Ala221 in its mature region thus obtained was hereafter referred to as "wild-type pro-subtilisin*."

Then, mutated pro-subtilisins* in which amino acid replacements had been introduced into the propeptide region were generated. The replacements introduced were the same as those in the isolated propeptide described in our previous paper (*17*): Ala47→Phe, Glu13→Ile, and Val65→Ile. The plasmid pTVSubN was transferred into *E. coli* CJ236, and single-stranded DNA containing uracil bases was obtained by infection with the helper phage M13KO7. Replacement of Ala47 by Phe and the subsequent replacements Gly13→Ile and Val65→Ile to generate double and triple mutants of propeptide were carried out by the method of Kunkel (*21*), as described previously (*17*). The mutated pro-subtilisins* were expressed and purified, as described above, and subjected to refolding analysis after their purity had been checked by SDS–polyacrylamide gel electrophoresis.

The propeptide region has no tryptophan residues, whereas the mature region of subtilisin BPN′ possesses three residues, which are demonstrated to be shielded from the solvent upon folding and interaction of the propeptide and mature regions of pro-subtilisin. Therefore, folding of pro-subtilisin* was monitored kinetically at 25°C by increase in tryptophan fluorescence emission at 340 nm with excitation at 280 nm, using a Hitachi F-4000 fluorescence spectrophotometer. The pro-subtilisin proteins purified under denaturating conditions were extensively dialyzed against 6 M urea, 50 mM potassium phosphate (pH 7.5), and their concentrations were determined from their absorbance at 280 nm in this buffer with a molar absorption coefficient constant (ε_{280}) of 34,990 M^{-1} cm^{-1} (*13*), followed by adjusting the protein concentration to 100 μM. Refolding of the pro-subtilisin was started by rapidly diluting the denatured protein solution thus obtained 100-fold with the refolding buffer: 0.5 M $(NH_4)_2SO_4$, 50 mM potassium phosphate (pH 7.5). This buffer is the same as that used by Eder *et al.* (*13*). As described by Eder *et al.*, $(NH_4)_2SO_4$ was added to stabilize the tertiary structure of pro-subtilisin. Under refolding conditions in the absence of $(NH_4)_2SO_4$, pro-subtilisin* cannot form a tertiary structure, and therefore little increase in fluorescence was observed (data not shown).

Refolding experiments were repeated several times for each wild-type and mutated pro-subtilisin* species, and the fluorescence traces were averaged after correction for a small decrease of fluorescence intensity, probably resulting from instability of the tryptophan fluorescence due to prolonged exposure to the light beam. The fluorescence traces thus obtained were initially analyzed by fitting to an Eq. 1 describing a single exponential process:

$$F(t) = A \exp(-kt) + C \quad (1)$$

where $F(t)$ is the fluorescence at time t, A is the amplitude, k the rate constant of refolding, and C the offset, since the refolding of pro-subtilisins with the Ser221→Ala mutation in the mature region is an intramolecular process without peptide bond cleavage between the propeptide and mature regions. When the trace could not be fitted to this equation, it was fitted to an Eq. 2 describing the sum of several exponential processes:

$$F(t) = \Sigma A_i \exp(-k_i t) + C \quad (2)$$

As shown in Fig. 1, refolding of the wild-type pro-subtilisin* takes more than 10 min and its kinetic trace could be fitted to the single exponential equation with a rate constant of refolding of 4.8×10^{-3} s^{-1}. This value is close to that obtained by Eder *et al.* (*13*). CD spectra of the refolded sample were then measured. Intensity minima at 220 nm and the non-flatness of the spectrum around 280 nm (data not shown) were typical of the formation of secondary and tertiary structures, respectively, as described by Eder *et al.* (*13*).

When the Ala47→Phe mutation was introduced into the propeptide region, the pro-subtilisin* was found to refold more rapidly than the wild-type pro-subtilisin*, although the effect was not large. The refolding kinetic trace was fitted to the single exponential equation, and the rate constant of refolding was determined to be 5.9×10^{-3} s^{-1}, which was 1.2-fold larger than that of the wild type.

The refolding of the mutated pro-subtilisin* with the additional replacement Gly13→Ile was more rapid than that of the mutant with the Ala47→Phe replacement alone. As shown in Fig. 1, refolding of this mutant was essentially complete within several minutes. In particular, the initial stage of refolding within the first few minutes is dramatically accelerated by the Gly13→Ile replacement. The kinetic trace of this mutant could not be fitted by a single exponential process, but could be fitted by a double exponential process with fast and slow phases. The rate constants of refolding were determined to be 2.1×10^{-2} s^{-1} and 4.5×10^{-3} s^{-1} for the fast and slow phases, respectively. Similarly, the mutated pro-subtilisin* in which the additional Val65→Ile replacement was introduced into the propeptide region was shown to refold slightly more rapidly than the mutant with the Ala47→Phe and Gly13→Ile replacements, as shown in Fig. 1. The kinetic trace could be fitted by a double exponential process with rate constants of 2.2×10^{-2} s^{-1} for the fast phase and 5.6×10^{-3} s^{-1} for the slow phase. These values are the largest among the mutated pro-subtilisins tested in this study. The rate constants of refolding of the wild-type and mutated pro-subtilisins* thus obtained are summarized in Table I.

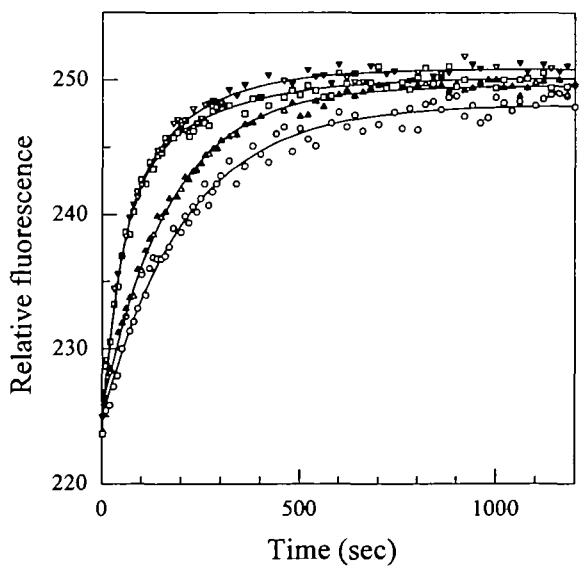

Fig. 1. **Refolding kinetic traces of wild-type and mutated pro-subtilisins*.** The wild-type (○) or mutated pro-subtilisin* at 100 μM in 6 M urea, 50 mM potassium phosphate (pH 7.5) was rapidly diluted 100-fold with 0.5 M $(NH_4)_2SO_4$, 50 mM potassium phosphate (pH 7.5) at 25°C. Refolding of pro-subtilisin* was monitored by measuring fluorescence (excitation at 280 nm and emission at 340 nm). (△), single mutant with Ala47→Phe; (□), double mutant with Ala47→Phe + Gly13→Ile; (▽), triple mutant with Ala47→Phe + Gly13→Ile + Val65→Ile.

From a structural viewpoint, the propeptide of subtilisin BPN′ is unique because it forms a tertiary structure only after formation of a complex with its cognate protease (*12–14*). This was thought to be due to weak hydrophobic interactions and the presence of unfavorable interactions. Based on this speculation, in the previous study we introduced successive amino acid replacements into the propeptide to improve the hydrophobic interactions, and succeeded in introducing a tertiary structure into the triple mutant of propeptide (*17*). We also demonstrated that the formation of the tertiary structure in the propeptide by the mutations was closely related to improvement of its function as a protease inhibitor.

In this study, we showed that the mutations that introduce a tertiary structure into the propeptide produce positive effects on another of its functions, intramolecular chaperone activity. All mutated pro-subtilisins containing the mutations examined in the previous study (*17*) in the propeptide region were found to refold more rapidly than the wild-type pro-subtilisin. The order of acceleration of refolding of subtilisin mediated by these mutated propeptides is the same as that of increases of tertiary structure formation and inhibitory activity of the mutated propeptide. By successive replacement of Ala47→Phe, Gly13→Ile, and Val65→Ile, the extent of secondary and tertiary structure of the propeptide was increased, as were its ability to bind to subtilisin BPN′ and its resistance to proteolytic degradation (*17*). These results clearly show that the improved chaperone activity of the propeptide is closely related not only to formation of the three-dimensional structure but also to its properties as a protease inhibitor. However, it seems that the inhibitory properties of the mutated propeptides are not obligatorily linked to their chaperone activity, as demonstrated recently by Fu *et al.* (*11*). They showed that, although the ability of a mutated subtilisin E possessing a Glu112→Ala replacement to bind to the propeptide was weakened 35-fold, the folding kinetics of the mutated subtilisin E by the propeptide were not altered (*11*). In our mutants, it appears that formation of a tertiary structure in the propeptide by the amino acid replacements is an important requirement for improvement of the chaperone

TABLE I. **Rate constants of refolding of wild-type and mutated pro-subtilisins*.** Kinetic traces of pro-subtilisin* refolding, monitored by increase in fluorescence, were analyzed by least squares fitting to the equation $F(t) = \Sigma A_i \exp(-k_i t) + C$, where $F(t)$ is the fluorescence at time t, A the amplitude, k the rate constant of folding and C the offset.

Pro-subtilisin*	Rate constant (s^{-1})	
Wild type	4.8×10^{-3}	
Ala47→Phe	5.9×10^{-3}	
	Fast phase	Slow phase
Ala47→Phe + Gly13→Ile	2.1×10^{-2}	4.5×10^{-3}
Ala47→Phe + Gly13→Ile + Val65→Ile	2.2×10^{-2}	5.6×10^{-3}

activity of the propeptide, and that the linkage of improved chaperone activity and inhibitory properties in the mutated propeptide is a consequence of such formation of a tertiary structure.

Kinetic analysis of the refolding process of pro-subtilisins* indicates that the kinetics of increase in tryptophan fluorescence of wild-type pro-subtilisin* and mutated pro-subtilisin* possessing the Ala47→Phe replacement in the propeptide region could be fitted to a single exponential equation, whereas those of mutated pro-subtilisins* possessing additional replacement(s), Gly13→Ile (and Val65→Ile), could be fitted to the sum of two exponential processes. Eder *et al.* (*13*) studied the refolding process of pro-subtilisin* by fluorescence measurements, and observed that almost all the secondary structure of subtilisin was formed in the dead-time of the experiment (about 5 s) and that increase in tryptophan fluorescence several minutes after dilution reflects the formation of the final three-dimensional structure induced by interaction with the propeptide. However, they did not exclude the possibility that proline isomerization is involved in this increase. On the other hand, Ruan *et al.* (*19*) concluded that this slow process (several minutes) is the *trans→cis* isomerization step of Pro168, which is the only Pro residue existing as a *cis* isomer in the native state of subtilisin, since refolding of denatured subtilisin was dramatically accelerated by shortening the denaturation time (*19*).

Combining these findings with the results of our study, we suggest that the slow and fast phases revealed by kinetic analysis of double (Ala47→Phe + Gly13→Ile) and triple (double + Val65→Ile) mutants of pro-subtilisin* correspond to proline isomerization and propeptide-induced formation of a tertiary structure in subtilisin, respectively. In addition, since the rate constants of the slow phase in these double and triple mutants are close to those of the single phase in wild-type pro-subtilisin* and the single (Ala47→Phe) mutant of pro-subtilisin*, it is likely that proline isomerization and propeptide-induced formation of tertiary structure proceed in parallel with very similar rate constants in the wild type and the single mutant, allowing the kinetic traces of these pro-subtilisins* to be fitted to a single exponential equation.

These arguments demonstrate that successive amino acid replacements that introduce a tertiary structure into the propeptide of subtilisin BPN′ produce positive effects on the refolding of subtilisin BPN′ by accelerating the rate of propeptide-induced formation of a tertiary structure in subtilisin, whereas effects on proline isomerization are small. We assume that the tertiary structure of the triple mutant of the propeptide is essentially the same as that formed in the wild-type propeptide by complex formation with subtilisin, and that such a tertiary structure is partly formed in the single or double mutant. In the refolding process of the mutated pro-subtilisins*, a tertiary structure will form rapidly in their propeptide region. The existence of a three-dimensional structure in the mutated propeptide without interaction with subtilisin may promote the formation of a tertiary structure in subtilisin by acting as a template for folding, whereas the tertiary structures of wild-type propeptide and subtilisin BPN′ are formed only after their interaction.

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