Increase in activation rate of Pro-Tk-subtilisin by a single nonpolar-to-polar amino acid substitution at the hydrophobic core of the propeptide domain

Kota Yuzaki,¹ Yudai Sanda,¹ Dong-Ju You,² Ryo Uehara,¹ Yuichi Koga,¹ and Shigenori Kanaya^{1*}

¹Department of Material and Life Science, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan

²Division of Electron Microscopic Research, Korea Basic Science Institute (KBSI), Yuseong-gu, Daejeon 305-333, Korea

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Abstract: Tk-subtilisin (Gly70-Gly398) is a subtilisin homolog from *Thermococcus kodakarensis*. Active Tk-subtilisin is produced from its inactive precursor, Pro-Tk-subtilisin (Gly1-Gly398), by auto-processing and degradation of the propeptide (Tk-propeptide, Gly1-Leu69). This activation process is extremely slow at moderate temperatures owing to high stability of Tk-propeptide. Tk-propeptide is stabilized by the hydrophobic core. To examine whether a single nonpolar-to-polar amino acid substitution at this core affects the activation rate of Pro-Tk-subtilisin, the Pro-Tk-subtilisin derivative with the Phe17→His mutation (Pro-F17H), Tk-propeptide derivative with the same mutation (F17H-propeptide), and two active-site mutants of Pro-F17H (Pro-F17H/S324A and Pro-F17H/S324C) were constructed. The crystal structure of Pro-F17H/S324A was nearly identical to that of Pro-S324A, indicating that the mutation does not affect the structure of Pro-Tk-subtilisin. The refolding rate of Pro-F17H/S324A and autoprocessing rate of Pro-F17H/S324C were also nearly identical to those of their parent proteins (Pro-S324A and Pro-S324C). However, the activation rate of Pro-F17H greatly increased when compared with that of Pro-Tk-subtilisin, such that Pro-F17H is efficiently activated even at 40°C. The far-UV circular dichroism spectrum of F17H-propeptide did not exhibit a broad trough at 205–230 nm, which is observed in the spectrum of Tk-propeptide. F17H-propeptide is more susceptible to chymotryptic degradation than Tk-propeptide. These results suggest that F17H-propeptide is unfolded in an isolated form and is therefore rapidly degraded by Tk-subtilisin. Thus, destabilization of the hydrophobic core of Tk-propeptide by a nonpolar-to-polar amino acid substitution is an effective way to increase the activation rate of Pro-Tk-subtilisin.

Keywords: subtilisin; *Thermococcus kodakarensis*; propeptide; activation, hydrophobic core

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*Correspondence to: Shigenori Kanaya, Department of Material and Life Science, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: kanaya@mls.eng.osaka-u.ac.jp

Introduction

Tk-subtilisin is a subtilisin-like serine protease from the hyperthermophilic archaeon *Thermococcus kodakarensis*.¹ It is a highly thermostable enzyme with optimum temperature for activity of 90°C and half-life at 100°C of 50 min. It contains seven Ca²⁺ ions.² Four of them (Ca2–Ca5) are required for folding,³

Figure 1. Activation process of Pro-Tk-subtilisin. The primary structures of the proteins are shown schematically. Filled and open boxes represent the propeptide and subtilisin domains, respectively. These domains are termed Tk-propeptide and Tk-subtilisin when they are separated with each other by autoprocessing. A gray box represents the region truncated when Tk-subtilisin is activated. The locations of Phe17, which is one of the 12 core-forming residues, Ser324, which is the catalytically essential serine residue, and the N and C-terminal residues are indicated.

whereas the other three (Ca1, Ca6, and Ca7) are required for stability.⁴ Like other subtilisins and subtilisin homologs,⁵ Tk-subtilisin (Gly70-Gly398) is synthesized as Prepro-Tk-subtilisin [Met(-24)-Gly398] with a signal peptide [Met(-24)-Ala(-1)] and a propeptide (Gly1-Leu69) at the N-terminus,⁶ secreted to the external medium as Pro-Tk-subtilisin (Gly1-Gly398),⁷ and activated by the cleavage of the propeptide.¹

The activation process of Pro-Tk-subtilisin is summarized in Figure 1. It consists of the three steps: (1) folding of the subtilisin domain, (2) autoprocessing between the propeptide and subtilisin domains, and (3) degradation of the resultant propeptide (Tk-propeptide) by Tk-subtilisin.^{1,2,8} In this last step, the N-terminal nine residues of Tk-subtilisin (Gly70-Gly78) are also truncated.² Because Tk-propeptide remains bound to Tk-subtilisin to form an inactive complex after autoprocessing, complete degradation of Tk-propeptide is necessary to release active Tk-subtilisin. This activation process is similar to that of subtilisins from mesophilic bacteria,^{9–13} except that the first step requires Ca^{2+} ions^{8,14} and is almost fully completed before autoprocessing,^{2,15} and the last step requires high temperatures ($\geq 60^\circ\text{C}$).^{1,16,17} Bacterial subtilisins require the propeptide for folding,^{9–12,18–21} are fully folded after autoprocessing,²² and efficiently degrade the propeptide even at moderate temperatures ($\sim 25^\circ\text{C}$).¹⁰ The propeptide domain of Pro-Tk-subtilisin is not required for folding of the subtilisin domain but accelerates it as an intramolecular chaperone.^{3,23}

The activation of Pro-Tk-subtilisin is too slow to be completed within 3 h at moderate temperatures ($\leq 50^\circ\text{C}$).^{1,16,17,24} However, Pro-Tk-subtilisin is almost fully refolded within 5 min even at 30°C .^{4,14,15} In addition, it is almost fully autoprocessed within 5 min even at 20°C .^{16,17} These results indicate that the degradation of Tk-propeptide by Tk-subtilisin is a rate-determining step of the activation process of Pro-Tk-subtilisin, as reported for bacterial prosubtilisins.^{10,22} Tk-propeptide is not efficiently degraded by Tk-subtilisin at $\leq 50^\circ\text{C}$, probably owing to high stability of Tk-propeptide, high binding affinity of Tk-propeptide to Tk-subtilisin, and low activity of Tk-subtilisin. Tk-propeptide is stabilized by the hydrophobic core, which is formed

by 12 nonpolar residues.¹⁶ This number is twice that of bacterial propeptides.^{25,26} As a result, Tk-propeptide is kept folded in an isolated form,^{16,17} whereas bacterial propeptides are mostly unfolded in this form.^{11,27,28} We have previously shown that the two single mutations, Gly56→Ser^{16,17} and Leu69→Pro,²⁴ at the propeptide domain increase the activation rate of Pro-Tk-subtilisin. The former mutation destabilizes Tk-propeptide, whereas the latter mutation decreases the binding affinity of Tk-propeptide to Tk-subtilisin. However, it remains to be determined whether the activation rate of Pro-Tk-subtilisin is significantly increased by mutating one of the hydrophobic core-forming residues to polar one.

In this study, we constructed the Pro-Tk-subtilisin derivative, Pro-F17H, in which one of the hydrophobic-core-forming residues, Phe17, is replaced by His, the active site mutants of Pro-F17H, and the Tk-propeptide derivative with the same mutation, and characterized them. We showed that the mutation does not significantly affect the structure, refolding rate, and autoprocessing rate of Pro-Tk-subtilisin, but destabilizes Tk-propeptide and thereby increases the activation rate of Pro-Tk-subtilisin.

Results

Mutant preparation

Twelve nonpolar residues form the hydrophobic core of the propeptide domain of Pro-Tk-subtilisin.⁸ They are Val8, Val10, Val12, Phe17, Val22, Ile25, Val39, Val41, Val43, Val48, Leu51, and Val57. Of them, Phe17 is the only residue with the aromatic side chain and occupies a significant fraction of the space at the hydrophobic core. To examine whether the mutation of this residue to His destabilizes Tk-propeptide and thereby increases the activation rate of Pro-Tk-subtilisin without seriously affecting the activation yield, the Pro-Tk-subtilisin derivative with this mutation (Pro-F17H) and its active-site mutants, Pro-F17H/S324A and Pro-F17H/S324C, in which the catalytically essential serine residue (Ser324) is replaced with Ala and Cys, respectively, were constructed and characterized. Their parent

Figure 2. Stereoview of the crystal structure of Pro-F17H/S324A. (A) The structure of Pro-F17H/S324A is superimposed onto that of Pro-S324A (PDB code 2E1P). For the structure of Pro-F17H/S324A, the propeptide and mature domains are colored red and green, respectively. The Ca^{2+} ions are shown as cyan spheres. His17, two active-site residues (Asp115 and His153), and Ala324, which is substituted for the catalytic serine residue, are indicated by yellow stick models, in which the oxygen and nitrogen atoms are colored red and blue, respectively. The positions of $\beta 5\text{m}$ strand and $\alpha 6\text{m}$ and $\alpha 7\text{m}$ helices that form the $\alpha\beta\alpha$ substructure are indicated. N and C represent the N- and C-termini, respectively. For the structure of Pro-S324A, the entire molecule is colored gray, the Ca^{2+} ions are shown as gray spheres, and Phe17, Asp115, His153, and Ala324 are indicated by gray stick models. (B) The propeptide domain of the Pro-F17H/S324A structure is superimposed onto that of the Pro-S324A structure. In addition to His17/Phe17, 10 residues that form the hydrophobic core are indicated by yellow stick models for Pro-F17H/S324A and gray stick models for Pro-S324A. N and C represent the N- and C-termini, respectively. Another core-forming residue (Ile25) is not shown because it is hidden. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

proteins, Pro-Tk-subtilisin, Pro-S324A, and Pro-S324C, were also characterized for comparative purposes. Pro-F17H and Pro-Tk-subtilisin were used to analyze their activation and their active site mutants were used to analyze the structure, folding, and autoprocessing. The histidine residue was

chosen as a polar residue substituted for Phe17, because it also has the aromatic side chain and is similar to Phe in size. This mutation may not cause a marked steric hindrance or create a cavity at the hydrophobic core and therefore may not seriously affect the structure of the propeptide domain of

Figure 3. Activation of Pro-F17H and Pro-Tk-subtilisin. Pro-F17H and Pro-Tk-subtilisin were refolded in 1 mL of 50 mM CAPS-NaOH (pH 9.5) containing 10 mM CaCl_2 and 1 mM dithiothreitol (DTT) on ice for 10 min and then incubated at 40 and 80°C. After incubation for the indicated time, the protein was precipitated by trichloroacetic acid (TCA) and analyzed by 15% Tricine SDS-PAGE as described in Materials and Methods section. The sample precipitated at 0 min was refolded on ice for 10 min but was not incubated at the temperatures indicated. Lane M contained the low-molecular-weight markers (GE Healthcare, Little Chalfont, Buckinghamshire, England). The arrows indicate Pro-Tk-subtilisin (Pro-F17H), Tk-subtilisin, and Tk-propeptide (F17H-propeptide) from the top to the bottom.

Pro-Tk-subtilisin in unautoprocessed and autoprocessed forms. The propeptide domain exhibits the inhibitory activity only when it is fully folded. This inhibitory activity is required to prevent the self-degradation of Tk-subtilisin and to increase the activation yield of Pro-Tk-subtilisin.¹

All proteins were overproduced in *Escherichia coli* BL21(DE3) codon plus in inclusion bodies, solubilized with 8M urea, purified, and refolded as described in Materials and Methods section. Pro-Tk-subtilisin and its active-site mutants consist of the propeptide and subtilisin domains. These domains are termed Tk-propeptide, F17H-propeptide, Tk-subtilisin, and S324C-subtilisin, only when they are separated with each other upon autoprocessing or overproduced in *E. coli* in an isolated form.

Crystal structure of Pro-F17H/S324A

To examine whether the Phe17→His mutation affects the structure of Pro-Tk-subtilisin, the crystal structure of Pro-F17H/S324A was determined at 2.2 Å resolution. The overall structure of Pro-F17H/S324A, which represents the structure of a unautoprocessed form of Pro-Tk-subtilisin, is nearly identical with that of Pro-S324A (PDB code: 2E1P) with root mean square deviation of 0.23 Å for 395 Cα atoms from Ser4 to Gly398 (Fig. 2A). The imidazole group of His17 is well superimposed onto the phenyl group of Phe17 and the conformations of the side chains of other hydrophobic core-forming residues in the Pro-F17H/S324A structure are not seriously changed when compared with those in the Pro-S324A structure (Fig. 2B). This result indicates that the hydrophobic core of the propeptide domain struc-

turally tolerates a single nonpolar-to-polar amino acid substitution.

We have previously shown that the Gly56→Ser mutation at the surface loop alters the structure of the propeptide domain of Pro-Tk-subtilisin, such that the backbone conformation of residue 56 is changed from left- to right-handed structures.¹⁶ As a result, the hydrophobic core of this domain is partially exposed to the solvent.

Activation of Pro-F17H

The activation of Pro-F17H and Pro-Tk-subtilisin was analyzed by denaturing the protein in a Ca^{2+} -free form, refolding it on ice in the presence of Ca^{2+} ions, and incubating at 40 and 80°C. Unautoprocessed, autoprocessed, and active forms of the protein were detected by 15% Tricine SDS-PAGE. The results are shown in Figure 3. Pro-F17H and Pro-Tk-subtilisin in a unautoprocessed form migrate in the gel as a 45-kDa protein. These proteins are autoprocessed into F17H-propeptide or Tk-propeptide (8 kDa) and Tk-subtilisin (37 kDa), which form an inactive complex and migrate in the gel as separate bands. F17H-propeptide or Tk-propeptide is then degraded by Tk-subtilisin to release active Tk-subtilisin when it is dissociated from Tk-subtilisin. Thus, Tk-subtilisin migrates in the gel as a single band when the activation process of Pro-F17H or Pro-Tk-subtilisin is completed. Pro-F17H and Pro-Tk-subtilisin were refolded on ice for 10 min before the activation at 40 and 80°C. These proteins were refolded at such a low temperature to prevent rapid autoprocessing. Apparently, the autoprocessing reaction of these proteins was initiated immediately

when they were refolded in the presence of Ca^{2+} ions even on ice, but very slowly. As a result, these proteins were only partially autoprocessed in this condition (Fig. 3, lane 0 min). When the activation of Pro-F17H was compared with that of Pro-Tk-subtilisin, the activation process of Pro-F17H was almost fully completed within 2 min at 80°C and 10 min at 40°C, whereas that of Pro-Tk-subtilisin was almost fully completed within 10 min at 80°C and was not fully completed within 120 min at 40°C. Thus, Pro-F17H was activated more rapidly than Pro-Tk-subtilisin. The activation rate and activation yield of Pro-F17H at 40°C were comparable to those of Pro-Tk-subtilisin at 80°C. An autoprocessed form of Pro-F17H was not detected at 80°C, because F17H-propeptide was rapidly degraded by Tk-subtilisin within 2 min.

To confirm that the activation yield of Pro-F17H is comparable to that of Pro-Tk-subtilisin, the enzymatic activity of Tk-subtilisin activated from Pro-F17H and Pro-Tk-subtilisin was determined at 20°C using Suc-AAPF-pNA as a substrate. The activity was determined at such a low temperature to prevent the activation of Pro-F17H during assay. The amount of Tk-subtilisin was estimated from its specific activity, which has been reported to be 13.0 U mg^{-1} .¹ Pro-F17H and Pro-Tk-subtilisin were activated at 40 and 80°C for 30 min, respectively. The amount of Tk-subtilisin activated from Pro-F17H was comparable to that activated from Pro-Tk-subtilisin (data not shown), indicating that the activation yield of Pro-F17H is similar to that of Pro-Tk-subtilisin.

Refolding of Pro-F17H/S324A

To examine whether the Phe17→His mutation affects the chaperone function of the propeptide domain of Pro-Tk-subtilisin, refolding of Pro-F17H/S324A and Pro-S324A was kinetically analyzed at 30°C by terminating the refolding reaction with appropriate intervals and determining the amount of the correctly folded protein. The spectroscopic method using circular dichroism (CD) cannot be used for this analysis, because this method cannot distinguish between refolding of the protein from a denatured state to a native state and that from a denatured state to a molten globule-like state. Pro-F17H/S324A and Pro-S324A were denatured with 6M guanidine hydrochloride (GdnHCl), diluted with the buffer containing 10 mM CaCl_2 by 100-fold for refolding, and incubated at 30°C. With appropriate intervals, the refolding reaction was terminated by the addition of ethylenediaminetetraacetic acid (EDTA) and the protein incorrectly folded or folded into a molten globule-like structure was digested with chymotrypsin. The amount of the correctly folded protein, which is resistant to chymotryptic digestion, was determined by SDS-PAGE. A plot of

Figure 4. Refolding curves of Pro-F17H/S324A and Pro-S324A. The refolding yields of Pro-F17H/S324A (closed circle) and Pro-S324A (open circle) are shown as a function of incubation time. The protein was denatured and refolded at 30°C. With appropriate intervals, the refolded protein was treated with chymotrypsin and subjected to 12% SDS-PAGE as described in Materials and Methods section. The refolding yield was calculated by estimating the amount of the correctly folded protein from the intensity of the band visualized with CBB staining following SDS-PAGE. The line represents the optimal fit to the data.

the refolding yield as a function of time is shown in Figure 4. The refolding curve of Pro-F17H/S324A is similar to that of Pro-S324A, indicating that the Phe17→His mutation does not seriously affect the chaperone function of the propeptide domain of Pro-Tk-subtilisin.

Autoprocessing of Pro-F17H/S324C

To examine whether the Phe17→His mutation affects the autoprocessing rate of Pro-Tk-subtilisin, the autoprocessing of Pro-F17H/S324C and Pro-S324C was analyzed by 12% SDS-PAGE. S324C-subtilisin (Tk-subtilisin derivative with the Ser324→Cys mutation) exhibits very weak activity, which is sufficient for the autoprocessing of Pro-S324C, but is not sufficient for the subsequent degradation of Tk-propeptide.⁸ Therefore, the effect of the Phe17→His mutation on the autoprocessing of Pro-Tk-subtilisin can be evaluated more precisely by using Pro-S324C than by using Pro-Tk-subtilisin, for which the autoprocessing and degradation steps proceed successively. As shown in Figure 5, Pro-F17H/S324C and Pro-S324C were partially autoprocessed when they were incubated at 40°C for more than 5 min. The amounts of S324C-subtilisin produced from Pro-F17H/S324C and Pro-S324C upon autoprocessing equally increased as the incubation time increased. In this figure, only the unautoprocessed and active forms of the proteins are shown. However, F17H-propeptide and Tk-propeptide were always associated with S324C-subtilisin and were not

Figure 5. Autoprocessing of Pro-F17H/S324C and Pro-S324C. Pro-F17H/S324C or Pro-S324C was denatured, refolded by 100-fold dilution with 50 mM CAPS-NaOH (pH 9.5) containing 10 mM CaCl_2 and 1 mM DTT on ice for 10 min, incubated at 40°C for the times shown at the top of the gel, precipitated with 10% (v/v) TCA, and subjected to 12% SDS-PAGE. The protein was stained with CBB. The arrow heads indicate Pro-F17H/S324C or Pro-S324C (top) and S324C-subtilisin (bottom).

significantly degraded by S324C-subtilisin upon incubation for 4 h at 40°C (data not shown). These results indicate that the Phe17→His mutation does not significantly affect the autoprocessing rate of Pro-Tk-subtilisin.

Overproduction and purification of F17H-propeptide

To examine whether the Phe17→His mutation increases the degradation rate of Tk-propeptide by decreasing the stability and inhibitory activity of Tk-propeptide, the Tk-propeptide derivative with the Phe17→His mutation, F17H-propeptide, was constructed, overproduced in *E. coli*, and purified. Its production level and purification yield were comparable to those of Tk-propeptide. The far-UV CD spectrum of F17H-propeptide was considerably different from that of Tk-propeptide (Fig. 6). The far-UV CD spectrum of Tk-propeptide gave a broad trough with a minimum $[\theta]$ value of approximately -8000 at 205 nm, which was accompanied by a shoulder with a $[\theta]$ value of approximately -6000 at 220 nm. In con-

Figure 6. Far-UV CD spectra. The far-UV CD spectra of F17H-propeptide (solid line) and Tk-propeptide (broken line) were measured at 25°C and pH 9.0.

Figure 7. Susceptibilities to chymotryptic degradation. F17H-propeptide or Tk-propeptide was incubated with chymotrypsin at 37°C and pH 9.5 at an enzyme/substrate ratio of 1:100 (w/w) for the times shown at the top of the gel and subjected to 15% Tricine-SDS-PAGE. The protein was stained with CBB. The arrow head indicates F17H-propeptide or Tk-propeptide.

trast, F17H-propeptide did not give this trough. These results suggest that Tk-propeptide assumes a folded structure, whereas F17H-propeptide is almost fully unfolded. The Phe17→His mutation probably destabilizes Tk-propeptide, such that it is unfolded in an isolated form.

Stability of F17H-propeptide

F17H-propeptide is more rapidly degraded by Tk-subtilisin than Tk-propeptide (Fig. 3), probably because F17H-propeptide is less stable than Tk-propeptide and/or inhibits Tk-subtilisin less strongly than Tk-propeptide. To confirm that the Phe17→His mutation destabilizes Tk-propeptide and thereby increases the susceptibility of Tk-propeptide to proteolytic degradation, F17H-propeptide and Tk-propeptide were incubated with chymotrypsin at 37°C and pH 9.5 at an enzyme:substrate ratio of 1:100 (w/w) and subjected to 15% Tricine SDS-PAGE. It is expected that the susceptibility of Tk-propeptide to chymotryptic degradation increases as the stability of Tk-propeptide decreases. As shown in Figure 7, F17H-propeptide was rapidly degraded by chymotrypsin to produce peptides with smaller molecular masses. In contrast, Tk-propeptide was highly resistant to chymotryptic degradation and was kept intact for up to 2 h. These results indicate that F17H-propeptide is less stable than Tk-propeptide.

To gain more information on the stability of Tk-propeptide, its thermal denaturation was analyzed at pH 7.0 by CD spectroscopy. However, the CD value of the protein at 222 nm was nearly constant up to 100°C (data not shown), suggesting that Tk-propeptide is highly resistant to thermal denaturation.

Inhibitory activity of F17H-propeptide

To examine whether the Phe17→His mutation decreases the inhibitory activity of Tk-propeptide, inhibitions of the enzymatic activity of Tk-subtilisin (6 nM) with Tk-propeptide and F17H-propeptide were analyzed at 20°C using Suc-AAPF-pNA as a

Figure 8. Inhibition of Tk-subtilisin activity by Tk-propeptide and F17H-propeptide. The indicated concentration of Tk-propeptide or F17H-propeptide was premixed with the substrate (Suc-AAPF-pNA) in 50 mM Tris-HCl (pH 8.0) containing 0.01% Triton X-100 before Tk-subtilisin was added. Hydrolysis of Suc-AAPF-pNA (2 mM) by Tk-subtilisin (6 nM) was analyzed at 20°C by monitoring an increase in absorbance at 410 nm, which results from a release of *p*-nitroaniline.

substrate. The inhibitory activities of these propeptides were analyzed at such a low temperature, because F17H-propeptide is not effectively degraded by Tk-subtilisin at this temperature (data not shown). As shown in Figure 8, the enzymatic activity of Tk-subtilisin was inhibited by F17H-propeptide and Tk-propeptide in a concentration-dependent manner. Like the interactions between bacterial subtilisins and propeptides,^{19,27} the interaction between Tk-subtilisin and F17H-propeptide or Tk-propeptide also appears to be slow binding. However, 40 nM of F17H-propeptide is required to obtain an inhibition of Tk-subtilisin to a similar extent to that by 6 nM of Tk-propeptide. These results suggest that the inhibitory activity of F17H-propeptide is lower than that of Tk-propeptide by approximately sevenfold.

Discussion

Folding of propeptide domain of Pro-F17H

The Phe17→His mutation greatly destabilizes Tk-propeptide, such that it is unfolded in an isolated form (Fig. 6). This result agrees with the general concept that hydrophobic effect in the protein core is the major stabilizing factor in protein folding^{29,30} and the stability of the protein greatly decreases when one of the hydrophobic core-forming residue is replaced by a polar residue.^{31,32} Nevertheless, the propeptide domain of Pro-F17H is fully folded (Fig. 2A), indicating that this domain requires the subtilisin domain for folding. Because the propeptide domain of Pro-F17H interacts with the $\alpha\beta\alpha$ substructure of the subtilisin domain, which is formed by $\beta 5$ m-strand and $\alpha 6$ m- and $\alpha 7$ m-helices (Fig. 2A), and because folding of the subtilisin domain is initi-

ated by the formation of this substructure, which is induced by binding of the Ca^{2+} ions to the Ca^{2+} -binding loop between $\alpha 6$ m-helix and $\beta 5$ m-strand,¹⁴ folding of the propeptide domain of Pro-F17H is probably initiated immediately when folding of the subtilisin domain is initiated by binding of the Ca^{2+} ions. Once the propeptide domain is folded, it is probably kept folded after autoprocessing and keeps forming the inactive complex with Tk-subtilisin.

It is noted that the solution structure of *Pleurotus ostreatus* proteinase A inhibitor 1 (POIA1), which is a homolog of propeptide of subtilisin, is similar to the crystal structure of the propeptide in complex with bacterial subtilisin.³³ This protein is unfolded by the single Phe→Ala mutation that destabilizes the hydrophobic core.³⁴ These results suggest that Tk-propeptide assumes a native structure in an isolated form.

Mechanism for rapid activation of Pro-F17H

In this study, we showed that the Phe17→His mutation increases the activation rate of Pro-Tk-subtilisin. This mutation does not significantly affect folding of the subtilisin domain and autoprocessing of Pro-Tk-subtilisin, but increases the susceptibility of Tk-propeptide to degradation with Tk-subtilisin. Two factors affect the susceptibility of Tk-propeptide to degradation with Tk-subtilisin. One is the stability of Tk-propeptide and the other is the inhibitory activity of Tk-propeptide. Because Tk-propeptide forms an inactive complex with Tk-subtilisin after autoprocessing, it is degraded by Tk-subtilisin only when it is dissociated from Tk-subtilisin. Therefore, as the stability and/or inhibitory activities of Tk-propeptide decrease, its susceptibility to degradation with Tk-subtilisin increases. The inhibitory activity

of F17H-propeptide was shown to be lower than that of Tk-propeptide by sevenfold. This result is consistent with the previous results that Tk-propeptide derivative lacking in an ability to fold in an isolated form exhibits lower inhibitory activity than that of Tk-propeptide,¹⁷ and the bacterial propeptide derivatives with increased secondary structure in an isolated form exhibit a higher inhibitory activity than that of the parent one.^{19,35,36} Thus, Pro-F17H is more rapidly activated than Pro-Tk-subtilisin, because F17H-propeptide is more rapidly degraded by Tk-subtilisin than Tk-propeptide owing to its decreased stability and decreased inhibitory activity.

Advantage of Pro-F17H for production of Tk-subtilisin

Tk-subtilisin is highly resistant not only to thermal denaturation but also to the treatment with chemical denaturants and detergents.³⁷ We have recently shown that Tk-subtilisin efficiently degrades abnormal prion protein accumulated in the mouse brain infected with scrapie in a harsh condition.³⁸ Abnormal prion protein is a causative agent of a variety of fatal neurodegenerative diseases, such as scrapie of sheep, bovine spongiform encephalopathy, and Creutzfeldt-Jakob disease of human, and is highly resistant to proteolytic degradation.³⁹ Thus, Tk-subtilisin is a promising candidate of a detergent additive that can be used to degrade persistent proteins, including abnormal prion protein. However, to use Tk-subtilisin for practical purpose, it is necessary to improve the activation process of Pro-Tk-subtilisin. To prevent the self-degradation of Tk-subtilisin during the activation process at 80°C and to obtain Tk-subtilisin in high yield, it is necessary to activate Pro-Tk-subtilisin at very low concentration (0.3 μ M).¹ This is disadvantageous for bulk production of Tk-subtilisin, because dilution and heating are costly and time-consuming. Requirement of high temperatures ($\geq 60^\circ\text{C}$) for efficient activation of Pro-Tk-subtilisin is one of the reasons why Pro-Tk-subtilisin is activated in high yield only at such a low concentration. Because the activity of Tk-subtilisin decreases as the temperature decreases below 60°C, the self-degradation of Tk-subtilisin during the activation process of Pro-Tk-subtilisin would be greatly suppressed at mild temperatures. In fact, Tk-subtilisin was activated from Pro-F17H at 40°C with a yield of 70% even when the protein concentration was increased by 10-fold (data not shown). Therefore, Pro-F17H is more suitable than Pro-Tk-subtilisin for bulk production of Tk-subtilisin.

Materials and Methods

Protein preparation

The pET25b derivatives for overproduction of Pro-F17H, Pro-F17H/S324C, Pro-F17H/S324A, and

F17H-propeptide were constructed by KOD mutagenesis kit (Toyobo, Kyoto, Japan). The pET25b derivatives for overproduction of Pro-Tk-subtilisin,⁶ Pro-S324C,⁸ Pro-S324A,⁴⁰ and Tk-propeptide¹ were used as a template. The PCR primers were designed such that the codon for Phe17 (UUC) is changed to CAC for His for all mutants. All DNA oligomers were synthesized by Hokkaido System Science (Sapporo, Japan). PCR was performed with a thermal cycler (Gene Amp PCR system 2400, Applied Biosystems, Tokyo, Japan). The DNA sequence was confirmed by a Prism 310 DNA sequencer (Applied Biosystems).

For overproduction of the proteins, *E. coli* BL21(DE3) codon plus was used as a host strain. Pro-F17H, Pro-F17H/S324C, Pro-F17H/S324A, Pro-Tk-subtilisin, Pro-S324C, and Pro-S324A were overproduced in inclusion bodies, purified in a urea-denatured form, and refolded in a Ca^{2+} -free form, as described previously.⁸ F17H-propeptide was also overproduced in inclusion bodies, purified in a urea-denatured form, and refolded, as described for G56S-propeptide.¹⁷ Tk-propeptide was overproduced in a soluble form and purified as described previously.¹

The purity of the protein was confirmed by 12% SDS-PAGE (SDS-PAGE using a 12% polyacrylamide gel)⁴¹ or 15% Tricine SDS-PAGE (SDS-PAGE using the Tricine buffer),⁴² followed by staining with Coomassie Brilliant Blue (CBB). The protein concentration was determined from UV absorption at 280 nm (A_{280}) using a cell with an optical path length of 1 cm and an A_{280} values for 0.1% (1.0 mg mL⁻¹) solution are 1.24 for Pro-F17H, Pro-F17H/S324C, Pro-F17H/S324A, Pro-Tk-subtilisin, Pro-S324C, and Pro-S324A, and 0.21 for F17H-propeptide and Tk-propeptide. These values were calculated by using absorption coefficient of 1526 M⁻¹ cm⁻¹ for tyrosine and 5225 M⁻¹ cm⁻¹ for tryptophan at 280 nm.⁴³

Crystallization, data collection, structure determination, and refinement

Pro-F17H/S324A was refolded in a Ca^{2+} -bound form, treated with chymotrypsin to digest incorrectly folded protein, and purified by gel filtration chromatography, as described previously.⁴ The protein was dialyzed against 5 mM Tris-HCl (pH 7.0), concentrated to 10 mg mL⁻¹ using ultrafiltration system Amicon Ultra (Millipore, Billerica, MA), and used for crystallization. The protein was crystallized using the sitting-drop vapor-diffusion method at 4°C. Drops were prepared by mixing 1 μ L each of protein and reservoir solutions and were vapor-equilibrated against 100 μ L reservoir solutions. Crystals appeared after 1 week using 4.0M sodium formate (Crystal Screen I, Hampton Research). All crystals were flash-frozen in a nitrogen-gas stream.

X-ray diffraction datasets were collected at a wavelength of 0.9 Å on the BL44XU station at

SPRING-8 (Hyogo, Japan). X-ray diffraction dataset was collected and the structure was solved by the molecular replacement method, as described previously,⁸ except that the 2.3 Å structure of Pro-S324A (Protein Data Bank entry 2E1P) was used as a starting model. The data collection statistics and refinement statistics are summarized in Table I. Figures were prepared by PyMol (<http://www.pymol.org>).

Protein data bank accession number

The coordinates and structure factors of Pro-F17H/S324A have been deposited in the RCSB Protein Data Bank under ID code 4JP8.

CD spectrum measurement

The far-UV (200–260 nm) CD spectrum of the protein was measured on a J-725 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) at 25°C. The protein was dissolved in 20 mM Tris-HCl (pH 9.0). The protein concentration and optimal path length were 0.2 mg mL⁻¹ and 2 mm. The mean residue ellipticity, θ , which has the units of deg cm² dmol⁻¹, was calculated by using an average amino acid molecular mass of 110 Da.

Determination of enzymatic activity

The enzymatic activity was determined at 20°C using *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-*p*NA) (Sigma Chemical, St Louis, MO) as a substrate, as described previously, except that 50 mM Tris-HCl (pH 8.0) was used as a buffer.¹

Activation of Pro-F17H and Pro-Tk-subtilisin

Pro-F17H and Pro-Tk-subtilisin were denatured by dissolving in 20 mM Tris-HCl (pH 9.0) containing 6M GdnHCl at 30 μM and refolded by 100-fold dilution with 50 mM CAPS-NaOH (pH 9.5) containing 10 mM CaCl₂ and 1 mM DTT on ice. The refolded protein was then incubated at the temperatures indicated for activation. With an appropriate interval, the protein was precipitated by 10% (v/v) TCA, washed by 70% acetone twice, and subjected to 15% Tricine SDS-PAGE. Unautoprocessed, autoprocessed, and active forms of the protein were detected by CBB staining.

Refolding of Pro-F17H/S324A and Pro-S324A

Pro-F17H/S324A and Pro-S324A were denatured by dissolving in 20 mM Tris-HCl (pH 7.0) containing 6M GdnHCl at 15 μM and refolded by 100-fold dilution with 50 mM Tris-HCl (pH 7.0) containing 10 mM CaCl₂ and 1 mM DTT at 30°C. With an appropriate interval, the refolding reaction was terminated by the addition of 10 mM EDTA, and the protein with a non-native structure was digested with chymotrypsin at an enzyme to substrate ratio of 1:10 (w/w) at 30°C for 10 min. The refolded protein was then precipitated by 10% (v/v) TCA, washed

Table I. Data Collection and Refinement Statistics

Crystal	F17H-propeptide: S324A-subtilisin
Wavelength (Å)	0.9
Space group	I222
Cell parameters	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	72.32, 92.50, 122.22
α , β , γ (°)	90.0, 90.0, 90.0
Resolution range (Å)	50.0–2.2
Highest resolution shell (Å)	2.20–2.24
Reflections measured	393349
Unique reflections	22172
Completeness (%)	99.5 (99.5) ^a
<i>R</i> _{merge} (%) ^b	9.7 (48.9) ^a
Average <i>I</i> /σ (<i>I</i>)	37.7 (5.04) ^a
Refinement statistics	
Resolution limits (Å)	46.2–2.2
No. of atoms	
Protein/water/Ca ²⁺	2893/79/6
<i>R</i> _{work} (%)/ <i>R</i> _{free} (%) ^c	23.0/28.0
RMS deviations from ideal values	
Bond lengths (Å)	0.008
Bond angles (°)	0.98
Average <i>B</i> -factors (Å ²)	
Protein	44.6
Water/Ca ²⁺	42.8/51.8
Ramachandran plot (%)	
Most favored regions	97.2
Additional allowed regions	2.8

^a Values in parentheses are for the highest resolution cell.

^b $R_{\text{merge}} = \sum |I_{hkl} - \langle I_{hkl} \rangle| / \sum I_{hkl}$, where I_{hkl} is an intensity measurement for reflection with indices hkl and $\langle I_{hkl} \rangle$ is the mean intensity for multiply recorded reflections.

^c R_{free} was calculated using 5% of the total reflections chosen randomly and omitted from refinement.

with 70% acetone twice, and subjected to 12% SDS-PAGE. The amount of the protein was estimated from the intensity of the band visualized with CBB staining following 12% SDS-PAGE using the Scion Image program. The refolding yield was calculated by dividing the amount of the refolded protein precipitated after chymotryptic digestion by that before chymotryptic digestion.

Autoprocessing of Pro-F17H/S324C and Pro-S324C

Pro-F17H/S324C and Pro-S324C were denatured by dissolving in 20 mM Tris-HCl (pH 7.0) containing 6M GdnHCl at 30 μM and refolded by 100-fold dilution with 50 mM CAPS-NaOH (pH 9.5) containing 10 mM CaCl₂ and 1 mM DTT on ice. The refolded protein was then incubated at 40°C for autoprocessing. With an appropriate interval, the protein was precipitated by 10% (v/v) TCA, washed by 70% acetone twice, and subjected to 12% SDS-PAGE.

Chymotryptic degradation of F17H-propeptide and Tk-propeptide

F17H-propeptide and Tk-propeptide (0.2 mg mL⁻¹) were incubated with chymotrypsin (Wako Pure

Chemical Industries, Osaka, Japan) in 50 mM CAPS-NaOH (pH 9.5) at an enzyme to substrate ratio of 1:100 (w/w) at 37°C. With an appropriate interval, the proteolytic reaction was terminated by the addition of 2 mM diisopropyl fluorophosphate (DFP) and the reaction mixture was subjected to 15% Tricine SDS-PAGE. The protein was stained with CBB.

Thermal denaturation

Thermal denaturation of Tk-propeptide was analyzed by monitoring the change in CD values at 222 nm as the temperature was increased. The protein was dissolved in 50 mM sodium phosphate (pH 7.0). The protein concentration and optical path length were 0.3 mg mL⁻¹ and 2 mm, respectively. The temperature of the protein solution was linearly increased by 1.0°C min⁻¹.

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