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ORIGINAL PAPER

Requirement of insertion sequence IS1 for thermal adaptation of Pro-Tk-subtilisin from hyperthermophilic archaeon

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Abstract Tk-subtilisin from the hyperthermophilic archaeon *Thermococcus kodakarensis* matures from Pro-Tk-subtilisin (Pro-TKS) upon autoprocessing and degradation of propeptide. Pro-TKS contains the insertion sequence (IS1) at the N-terminus of the mature domain as compared to bacterial pro-subtilisins. To analyze the role of IS1, the Pro-TKS derivative without IS1 (ΔIS1-Pro-TKS) and its active-site mutants (ΔIS1-Pro-S324A and ΔIS1-Pro-S324C) were constructed and characterized. ΔIS1-Pro-S324A and ΔIS1-Pro-TKS represent an unautoprocessed and autoprocessed form of ΔIS1-Pro-TKS, respectively. The CD and ANS fluorescence spectra of these proteins indicate that folding of ΔIS1-Pro-TKS is not completed by binding of Ca^{2+} ions but is completed by the subsequent autoprocessing reaction. Thermal denaturation of these proteins analyzed by DSC and CD spectroscopy indicates that unautoprocessed ΔIS1-Pro-TKS is less stable than autoprocessed ΔIS1-Pro-TKS by 26.3 °C in T_m. The stability of autoprocessed ΔIS1-Pro-TKS is comparable to that of Pro-TKS, which is slightly lower than that of unautoprocessed Pro-TKS. These results suggest that ΔIS1-Pro-TKS is fully folded and greatly stabilized by autoprocessing. ΔIS1-Pro-TKS more slowly matured to ΔIS1-Tk-subtilisin than Pro-TKS did, due to a decrease in the autoprocessing rate. We propose that IS1 is required not only for hyperstabilization of Pro-TKS but also for its rapid maturation.

Keywords Subtilisin · *Thermococcus kodakarensis* · Maturation · Folding · Insertion sequence

Abbreviations

Tk-subtilisin	A subtilisin homolog from *Thermococcus kodakarensis* (Gly70-Gly398)
Pro-TKS	Tk-subtilisin in a pro-form (Gly1-Gly398)
Tkpro	Propeptide of Tk-subtilisin (Gly1-Leu69)
IS1	First insertion sequence (Gly70-Pro82)
Pro-S324A (S324C)	Pro-TKS with the Ser324 → Ala (Ser324 → Cys) mutation
ΔIS1-Pro-TKS (S324A, S324C)	Pro-TKS (S324A, S324C) derivative with IS1 deleted
Suc-AAPF-*p*NA	*N*-succinyl-Ala–Ala-Pro-Phe-*p*-nitroanilide
Tricine	*N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine
CD	Circular dichroism
TCA	Trichloroacetic acid
DSC	Differential scanning calorimetry
GdnHCl	Guanidine hydrochloride
DTT	Dithiothreitol
ANS	1-Anillino-8-napthalene sulfonic acid
CBB	Coomassie Brilliant Blue

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Introduction

Subtilisins are alkaline serine proteases and constitute one of the largest families of serine proteases (subtilase) (Siezen and Leunissen 1997). Because of the broad substrate specificity and high stability, they are widely used for industrial purposes, especially as additives of detergents (Schäfer et al. 2007). Subtilisins are synthesized as inactive prepro-subtilisins, in which a signal sequence and a propeptide are attached to the N-termini of the mature domains, and secreted into the external medium as inactive pro-subtilisins, in which a propeptide is attached to the N-termini of the mature domains (Siezen and Leunissen 1997). The subsequent maturation mechanism of subtilisins has been extensively studied using bacterial subtilisins, such as subtilisins E, BPN', and Carlsberg, as a model protein (Eder and Fersht 1995; Shinde and Inouye 1996, 2000; Bryan 2002; Chen and Inouye 2008). According to this mechanism, subtilisins mature from pro-subtilisins by three steps: (1) folding of a mature domain, (2) autoprocessing of a propeptide, and (3) degradation of a propeptide. The proteins produced by the first folding step, second autoprocessing step, and third degradation step are termed pro-subtilisins in an unautoprocessed, autoprocessed, and mature form, respectively. In an autoprocessed form, a propeptide keeps binding to its cognate subtilisin and forms an inactive stable complex with it. Propeptides function not only as an inhibitor of their cognate subtilisins but also as an intramolecular chaperone that facilitates folding of their cognate subtilisins.

Tk-subtilisin is a highly thermostable serine protease from the hyperthermophilic archaeon *Thermococcus kodakarensis* (Kannan et al. 2001; Pulido et al. 2006). It contains seven Ca^{2+} ions (Ca1–Ca7) (Tanaka et al. 2007a). The Ca1 site is conserved as the high-affinity Ca^{2+}-binding site (site 1 or A) in bacterial subtilisins, while other sites are unique in Tk-subtilisin. Because Tk-subtilisin is also highly resistant to the treatment with chemical denaturants and detergents (Foophow et al. 2010), Tk-subtilisin is a potential candidate for various biotechnological applications. Tk-subtilisin is synthesized as an inactive precursor, Prepro-Tk-subtilisin (Prepro-TKS), which consists of the signal sequence [Met (-24)-Ala(-1)], propeptide (Tkpro, Gly1-Leu69), and mature domain (Tk-subtilisin, Gly70-Gly398) (Fukui et al. 2005). It is secreted into the external medium, probably as Pro-Tk-subtilisin (Pro-TKS, Gly1-Gly398), with the assistance of the signal sequence (Takemasa et al. 2011). In vitro studies using the recombinant proteins of Pro-TKS and its derivatives indicate that the maturation mechanism of Tk-subtilisin is similar to that of bacterial subtilisins, except that the first folding step of Tk-subtilisin requires Ca^{2+} ions, instead of Tkpro, and the third degradation step of Tkpro requires high temperature (≥80 °C) (Pulido et al. 2006, 2007a, b; Tanaka et al. 2007a; b, 2008, 2009; Takeuchi et al. 2009). Tkpro is effectively degraded by Tk-subtilisin only at high temperatures, because it is too stable to be degraded by Tk-subtilisin at moderate temperatures (Pulido et al. 2006, 2007a, b). Complete degradation of Tkpro is necessary to release active Tk-subtilisin, because Tkpro keeps binding to the active-site cleft of Tk-subtilisin in a product-like manner after autoprocessing and inhibits the activity of Tk-subtilisin (Tanaka et al. 2007b). Inhibitory activity of Tkpro is necessary to prevent the self-degradation of Tk-subtilisin during the folding process (Pulido et al. 2006). Tkpro also functions as an intramolecular chaperone that accelerates folding of Tk-subtilisin, although it is not required for folding of Tk-subtilisin (Tanaka et al. 2007b, 2008, 2009).

Pro-TKS contains three insertion sequences, IS1 (Gly70-Pro82), IS2 (Pro207-Asp226), and IS3 (Gly346-Ser358), as compared to bacterial pro-subtilisins (Pulido et al. 2006). Of them, IS2 forms the Ca^{2+}-binding loop, in which four of the seven Ca^{2+} ions (Ca2-Ca5) bind (Tanaka et al. 2007b). Binding of the Ca^{2+} ions to this loop is required to induce folding of the $\alpha\beta\alpha$ substructure of Tk-subtilisin (Takeuchi et al. 2009). In contrast, IS1 and IS3 form a long loop, in which no Ca^{2+} ions bind. IS3 may be necessary for the molecular architecture of Tk-subtilisin, while IS1 may not, because IS1 is present at the N-terminus of Tk-subtilisin and most of it (Gly70-Gly78) is truncated when Tk-subtilisin is matured from Pro-TKS (Tanaka et al. 2007a). This region is not truncated in an autoprocessed form of Pro-TKS, in which Tkpro forms a complex with Tk-subtilisin, but is truncated in a mature form of Pro-TKS. However, IS1 appears to be required for the formation of the Ca1 site of Pro-TKS in an unautoprocessed form. If IS1 is not present, one of the ligands of the Ca1 site, Gln84, which is conserved as Gln2 in bacterial subtilisins, cannot coordinate with Ca1, because there is a large distance between the Ca1 and active sites. It has been reported for bacterial subtilisins that the corresponding site (site 1 or A) is important for stability (Voordouw et al. 1976; Pantoliano et al. 1989; Bryan et al. 1992) and is only formed when the N-terminus of the mature domain of pro-subtilisin is rearranged upon autoprocessing of propeptide (Eder et al. 1993; Shinde and Inouye 1995). This site is not formed in an unautoprocessed form of pro-subtilisin, because Gln2 is located far from this site. As a result, pro-subtilisin in an unautoprocessed form is less stable than that in an autoprocessed or mature form (Yabuta et al. 2002). Therefore, it seems likely that IS1 contributes to the stabilization of Pro-TKS. However, it remains to be determined whether the deletion of IS1 seriously affects the maturation of Pro-TKS.

In this study, we constructed ΔIS1-Pro-TKS, in which IS1 of Pro-TKS is deleted, and its active-site mutants, and biochemically characterized them. Based on the results, we discuss the role of IS1 in the maturation of Pro-TKS.

Materials and methods

Plasmid construction

The pET25b derivatives for overproduction of ΔIS1-Pro-TKS, ΔIS1-Pro-S324A, and ΔIS1-Pro-S324C were constructed by QuickChange II Mutagenesis Kit (Stratagene, La Jolla, CA, USA) using the PCR primers designed to remove IS1 from Pro-TKS, Pro-S324A, or Pro-S324C. The sequences of these primers are 5′-CCAGGCGGTTCTCCTTGCA CAGACCATCCC-3′ for 5′-primer and 5′-GGGATGGT CTGTGCAAGGAGAACCGCCTGG-3′ for 3′-primer. The pET25b derivatives for overproduction of Pro-TKS, Pro-S324A, and Pro-S324C, which were constructed previously (Tanaka et al. 2007a, b), were used as a template. All DNA oligomers were synthesized by Hokkaido System Science (Sapporo, Japan). PCR was performed using a thermal cycler (Gene Amp PCR system 2400, Applied Biosystems, Tokyo, Japan) and *Pfu* DNA polymerase (Stratagene). The DNA sequences were confirmed with an ABI Prism 310 DNA sequencer (Applied Biosystems).

Protein preparation

Pro-TKS, Pro-S324A, Pro-S324C, and their derivatives without IS1 were overproduced in *Escherichia coli* BL21(DE3) codon plus in inclusion bodies, solubilized by 20 mM Tris–HCl (pH 9.0) containing 8 M urea and 5 mM EDTA, and purified in a denatured form as previously described (Pulido et al. 2006). For refolding of Pro-S324A$^+$, Pro-S324C$^+$, ΔIS1-Pro-S324A$^+$, and ΔIS1-Pro-S324C$^+$ in a Ca^{2+}-bound form, the protein was dialyzed against 20 mM Tris–HCl (pH 7.0) containing 10 mM $CaCl_2$ and 1 mM DTT at 4 °C for 5 days. Pro-S324A$^+$ and ΔIS1-Pro-S324A$^+$ were further purified by gel filtration column chromatography using a Hiload 16/60 Superdex pg column (GE Healthcare, Little Chalfont, Buckinghamshire, England) equilibrated with 20 mM Tris–HCl (pH 7.0) containing 10 mM $CaCl_2$ and 50 mM NaCl. Pro-S324C$^+$ and ΔIS1-Pro-S324C$^+$ were further purified by incubation at 80 °C for 30 min, followed by centrifugation at 30,000×*g* for 30 min to remove misfolded and unauto-processed proteins as a precipitate. All proteins in a Ca^{2+}-bound form were dialyzed against 20 mM Tris–HCl (pH 7.0) containing 10 mM $CaCl_2$. For refolding of Pro-S324A$^-$, Pro-S324C$^-$, ΔIS1-Pro-S324A$^-$, and ΔIS1-Pro-S324C$^-$ in a Ca^{2+}-free form, the protein was dialyzed against 20 mM Tris–HCl (pH 7.0) at 4 °C overnight. For refolding of Pro-TKS$^-$ and ΔIS1-Pro-TKS$^-$ in a Ca^{2+}-free form, the protein was dialyzed against 20 mM Tris–HCl (pH 7.0) containing 0.5 mM EDTA and 1 mM DTT, and concentrated to approximately 10 mg/ml using Amicon Ultra (Millipore, Billerica, MA, USA) at 4 °C. Tk-subtilisin and ΔIS1-Tk-subtilisin were prepared from Pro-TKS$^-$ and ΔIS1-Pro-TKS$^-$, respectively, by incubating these proteins in 50 mM CAPS–NaOH (pH 9.5) containing 10 mM $CaCl_2$ and 1 mM DTT at the protein concentration of 300 nM at 60 °C for 3 h. S324A-subtilisin$^+$ was prepared as described previously (Tanaka et al. 2008).

The purity of the protein was confirmed by 12 % SDS-PAGE (SDS-PAGE using a 12 % polyacrylamide gel) (Laemmli 1970) or 15 % Tricine-SDS-PAGE (SDS-PAGE using the Tricine buffer) (Schägger 2006), 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 10 mm. The A_{280} values for 0.1 % (1.0 mg/ml) solution are 1.24 for Pro-TKS, Pro-S324A, and Pro-S324C, 1.13 for ΔIS1-Pro-TKS, ΔIS1-Pro-S324A, and ΔIS1-Pro-S324C, and 1.47 for Tk-subtilisin and ΔIS1-Tk-subtilisin. These values were calculated using absorption coefficients of 1,526 M^{-1} cm^{-1} for tyrosine and 5,225 M^{-1} cm^{-1} for tryptophan at 280 nm (Goodwin and Morton 1946).

Circular dichroism (CD) spectroscopy

The far-UV CD spectrum (200–260 nm) of the protein was measured on a J-725 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) at 20 °C. The protein was dissolved in 20 mM Tris–HCl (pH 7.0) containing 10 mM $CaCl_2$ for a Ca^{2+}-bound form and 20 mM Tris–HCl (pH 7.0) for Ca^{2+}-free form. The protein concentration and optimal path length were 0.2 mg/ml and 2 mm. The mean residue ellipticity, $[\theta]$, which has the units of deg cm^2 $dmol^{-1}$, was calculated using an average amino acid molecular mass of 110 Da.

ANS fluorescence spectra

Binding of 1-anillino-8-napthalene sulfonic acid (ANS) to the protein was analyzed by measuring the fluorescence of ANS at 20 °C and pH 7.0 either in the presence or absence of 10 mM $CaCl_2$ using RF-5300PC spectrofluorophotometer (SHIMADZU, Kyoto, Japan), as described previously (Tanaka et al. 2007b). The excitation wavelength was 380 nm and the emission was measured between 400 and 600 nm.

Thermal denaturation

Thermal denaturation was analyzed by monitoring the change in CD values at 222 nm as the temperature was increased. The protein in a Ca^{2+}-bound form was dissolved in 10 mM Tris–HCl (pH 7.5) containing 6 M GdnHCl. The protein concentration and optical path length were 0.1 mg/ml and 2 mm, respectively. The linear rate for temperature

increase was approximately 1.0 °C/min. The thermal denaturation processes of all proteins examined were irreversible in this condition. However, the thermal denaturation curves of these proteins were reproducible if the protein concentration, pH, and the rate of the temperature increase were not seriously changed. The thermal denaturation curves were normalized, assuming a linear temperature dependence of the base lines of native and denatured states. The midpoints of the transition of these thermal denaturation curves, T_m, were calculated from the resultant normalized curves based on a least squares analysis.

Thermal denaturation was also analyzed by differential scanning calorimetry (DSC). DSC measurement was performed using a high-sensitivity VP-capillary DSC controlled by the VP Viewer software package (MicroCal Inc., Northampton, MA) at a scan rate of 1 °C/min. The temperature increased up to 115 °C during the measurement. The concentration of the protein was 0.3 mg/ml. All samples were dialyzed against 50 mM Tris–HCl (pH 8.5), filtered through 0.22-μm pore size membrane, and degassed prior to the measurement. Thermal denaturation of the proteins examined was irreversible in this condition.

Limited proteolysis with chymotrypsin

The protein was digested with chymotrypsin at 30 °C for 1 h in 20 mM Tris–HCl (pH 7.0) either in the presence or absence of 10 mM $CaCl_2$ at enzyme:substrate ratios of 1:10–1:10,000 (w/w). The proteolytic reaction was terminated by the addition of 120 μl of TCA to 1 ml of the reaction mixture. After incubation at −30 °C for 12 min, the precipitated proteins were collected by centrifugation, washed by 70 % acetone twice, and subjected to 12 % SDS-PAGE.

Analysis for refolding

Pro-S324A$^-$ and ΔIS1-Pro-S324A$^-$ were dissolved in 20 mM Tris–HCl (pH 7.0) containing 6 M GdnHCl at 2 mg/ml and incubated at room temperature for 1 h for complete denaturation. The refolding reaction was initiated by diluting this solution with 50 mM Tris–HCl (pH 7.0) containing 10 mM $CaCl_2$ and 1 mM DTT by 20-fold at 30 °C. The refolding reaction was followed by a CD measurement at 222 nm at this temperature, using a 1-cm path length cuvette.

Analysis for autoprocessing

Pro-S324C$^-$ and ΔIS1-Pro-S324C$^-$ were completely denatured with 6 M GdnHCl as mentioned above and refolded on ice by 100-fold dilution with 50 mM CAPS–NaOH (pH 9.5) containing 10 mM $CaCl_2$ and 1 mM DTT. The final protein concentration was 300 nM. The autoprocessing reaction was initiated by incubating the refolded protein at 60 °C. With an appropriate interval, the autoprocessing reaction was terminated by the addition of 120 μl of TCA to 1 ml of the reaction mixture. After incubation at −30 °C for 12 min, the precipitated proteins were collected by centrifugation, washed by 70 % acetone twice, and subjected to 12 % SDS-PAGE.

Enzymatic activity

The enzymatic activity was determined at pH 9.5 and various temperatures using azocasein (Sigma Chemical Co., St Louis, MO, USA) as a substrate as described previously (Pulido et al. 2006), except that the concentration of $CaCl_2$ was 1 mM. One unit of enzymatic activity was defined as the amount of the enzyme that increased the absorption of the assay reaction mixture at 440 nm (A_{440}) by 0.1 in 1 min. The specific activity was defined as the enzymatic activity per milligram of protein.

Analysis for maturation

Pro-TKS$^-$ and ΔIS1-Pro-TKS$^-$ were first dissolved in 20 mM Tris–HCl (pH 9.0) containing 6 M GdnHCl for complete denaturation, then diluted with 50 mM CAPS–NaOH (pH 9.5) containing 10 mM $CaCl_2$ and 1 mM DTT by 100-fold on ice for refolding, and finally incubated at 60 °C for maturation. The protein concentration was 300 nM. With an appropriate interval, an aliquot of the protein solution was withdrawn and its enzymatic activity was determined at 20 °C using Suc-AAPF-*p*NA (Sigma) as a substrate. The enzymatic activity was determined at such a low temperature (20 °C) to minimize the maturation of these proteins during assay. For assay, the reaction mixture (100 μl) contained 50 mM Tris–HCl (pH 8.0), 1 mM Suc-AAPF-*p*NA, and 15 nM of the enzyme. It was incubated at 20 °C for 20 min. The enzymatic reaction was terminated by the addition of 10 μl of 5 % acetic acid. The amount of *p*-nitroaniline released from the substrate was determined from the absorption at 410 nm (A_{410}) with an absorption coefficient of 8,900 M^{-1} cm^{-1}. One unit of enzymatic activity was defined as the amount of the enzyme that produced 1 μmol of *p*-nitroaniline per min. The specific activity was defined as the enzymatic activity per milligram of protein.

Results and discussion

Mutant preparation

According to the crystal structure of the active-site mutant of Pro-TKS, Pro-S324A, IS1 forms a long surface loop at

the N-terminus of the mature domain (Fig. 1a; Tanaka et al. 2007b). The scissile peptide bond between IS1 and the propeptide domain is located at the active site. To analyze the role of IS1 in maturation of Pro-TKS, the Pro-TKS derivative without IS1 (ΔIS1-Pro-TKS) and its active-site mutants, ΔIS1-Pro-S324A and ΔIS1-Pro-S324C, in which the active-site serine residue (Ser324) is replaced with Ala and Cys, respectively, were constructed and characterized. Their intact partners, Pro-TKS, Pro-S324A, and Pro-S324C, were also characterized for comparative purposes. The primary structures of these deletion mutants and their intact partners are schematically shown in Fig. 1b. ΔIS1-Pro-TKS and Pro-TKS were used to analyze their maturation and their active site mutants were used to analyze their structures, stability, and folding.

All proteins were overproduced in *E. coli* BL21(DE3) codon plus in inclusion bodies, solubilized with 8 M urea, purified, and refolded as described in "Materials and methods". The proteins with the suffix "+" and "−" represent those refolded in the presence and absence of the Ca^{2+} ions, respectively. ΔIS1-Pro-S324A^{+} and ΔIS1-Pro-S324C^{+} represent an unautoprocessed and autoprocessed form of ΔIS1-Pro-TKS, respectively, because the former gave a single band on SDS-PAGE at the position where an unautoprocessed form of the protein migrates, while the latter gave two bands on SDS-PAGE at the positions where a mature form of the protein and Tkpro migrate (Fig. S1 in the Supporting Information). Pro-S324C^{+} also represents an autoprocessed form of the protein and exists as a complex between the mature domain and Tkpro, while Pro-S324A^{+} and all proteins

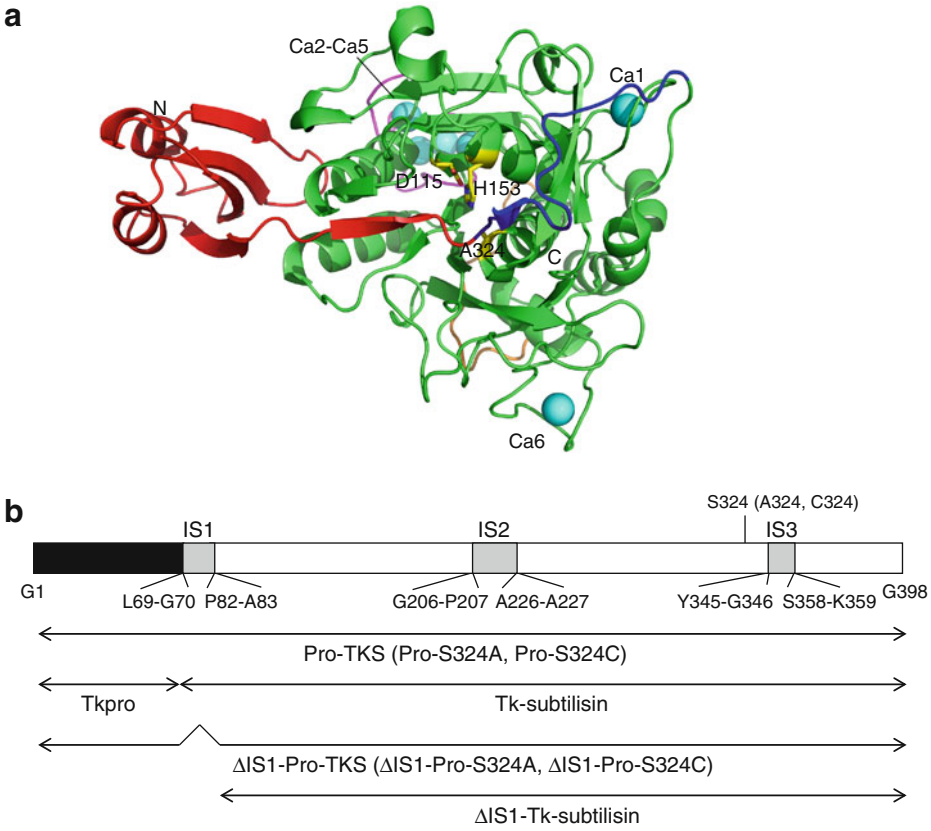

Fig. 1 **a** Three-dimensional structure of Pro-S324A^{+}. The propeptide (Asn^{4}-Lue^{69}) and mature (Gly^{70}-Gly^{398}) domains of Pro-S324A^{+} (PDB code: 2E1P) are colored *red* and *green*, respectively. The loops formed by IS1 (Gly^{70}-Pro^{82}), IS2 (Pro^{207}-Asp^{226}), and IS3 (Gly^{346}-Ser^{358}) are colored *blue*, *magenta*, and *orange*, respectively. Two active-site residues (Asp^{115} and His^{153}) and Ala^{324}, which is substituted for the active-site serine residue, are indicated by *yellow stick models*, in which the oxygen and nitrogen atoms are colored *red* and *blue*, respectively. Six Ca^{2+} ions (Ca1–Ca6) are shown in *cyan spheres*. The Ca7 ion is not observed, because it only weakly binds to the protein in a pro-form. N and C represent N- and C-termini, respectively. **b** Schematic representation of the primary structures of Pro-TKS. The *dark box* represents the propeptide domain, the *open boxes* represent the mature domain, and the *gray boxes* represent the insertion sequences (IS1–IS3). The locations of the active-site serine residue (Ser324), which is replaced by Ala in Pro-S324A or ΔIS1-Pro-S324A and Cys in ProS324C or ΔIS1-Pro-S324C, and the regions of the proteins analyzed in this study are shown. The N- and C-terminal residues of each domain and the IS1–IS3 regions are also shown. The GenBank accession number of this sequence is AB056701 (color figure online)

refolded in the absence of the Ca^{2+} ions represent an unautoprocessed form of the protein (Tanaka et al. 2007b).

CD spectra

We have previously shown that the far-UV CD spectrum of Pro-S324A$^+$ is considerably different from that of Pro-S324A$^-$, but is indistinguishable from that of Pro-S324C$^+$ (Tanaka et al. 2007b). This result suggests that folding of Pro-TKS is induced by binding of Ca^{2+} ions and is completed prior to the autoprocessing reaction. To examine whether the deletion of IS1 affects this folding process, the far-UV CD spectra of ΔIS1-Pro-S324A$^+$, ΔIS1-Pro-S324A$^-$, and ΔIS1-Pro-S324C$^+$ were measured. The spectra of Pro-S324A$^+$ and Pro-S324A$^-$ were also measured for comparative purpose. The results are shown in Fig. 2. A considerable difference was observed between the spectra of ΔIS1-Pro-S324A$^+$ and ΔIS1-Pro-S324A$^-$, although it is smaller than that between the spectra of Pro-S324A$^+$ and Pro-S324A$^-$, suggesting that folding of ΔIS1-Pro-S324A is also induced by binding of Ca^{2+} ions. However, the spectrum of ΔIS1-Pro-S324A$^+$ is slightly different from that of ΔIS1-Pro-S324C$^+$, which is similar to that of Pro-S324A$^+$, suggesting that folding of ΔIS1-Pro-TKS is not completed by binding of Ca^{2+} ions but is completed by the subsequent autoprocessing reaction.

ANS fluorescence spectra

It is known that 1-anillino-8-napthalene sulfonic acid (ANS) binds to a hydrophobic pocket of proteins and becomes highly fluorescent (Kuwajima 1989). To examine whether the deletion of IS1 affects the structure of Pro-TKS, ANS binding to ΔIS1-Pro-S324A$^+$, ΔIS1-Pro-S324A$^-$, ΔIS1-Pro-S324C$^+$, Pro-S324A$^+$, and Pro-S324A$^-$ was analyzed by measuring the fluorescence spectrum of ANS. It has been reported that ANS effectively binds to Pro-S324A$^-$ with a molten globule-like structure, but very poorly binds to Pro-S324A$^+$ with a native structure (Tanaka et al. 2007b). As shown in Fig. 3, ANS fluorescence of ΔIS1-Pro-S324A$^-$ is much higher than that of ΔIS1-Pro-S324A$^+$, suggesting that ΔIS1-Pro-S324A$^-$ assumes a molten globule-like structure on which hydrophobic pockets exist and these pockets are greatly reduced in size and number due to Ca^{2+}-induced folding. However, in contrast to Pro-S324A$^+$, which exhibits little ANS fluorescence, ΔIS1-Pro-S324A$^+$ exhibits a weak ANS fluorescence, suggesting that these hydrophobic pockets are still left in the ΔIS1-Pro-S324A$^+$ structure. These pockets are probably not left in the ΔIS1-Pro-S324C$^+$ structure, because ΔIS1-Pro-S324C$^+$ exhibits little ANS fluorescence as Pro-S324A$^+$ does. The far-UV CD spectrum and ANS fluorescence of ΔIS1-Pro-S324A$^+$ suggest that ΔIS1-Pro-TKS is not fully folded in an unautoprocessed form. ΔIS1-Pro-TKS is probably fully folded upon autoprocessing.

According to the crystal structure of Pro-S324A$^+$, the N-terminus of the mature domain of ΔIS1-Pro-S324A$^+$ (Ala83) is located approximately 29 Å away from the C-terminus of the propeptide domain (Leu69). IS1 fills the gap between these residues with a 13-residue loop. Therefore, the deletion of IS1 must result in a significant conformational change of the mature domain of ΔIS1-Pro-S324A$^+$ especially at the N-terminal region. This may be the reason why ΔIS1-Pro-S324A$^+$ is not fully folded. ΔIS1-Pro-S324C$^+$ is fully folded, probably because the N-terminal region of the mature domain of this protein is

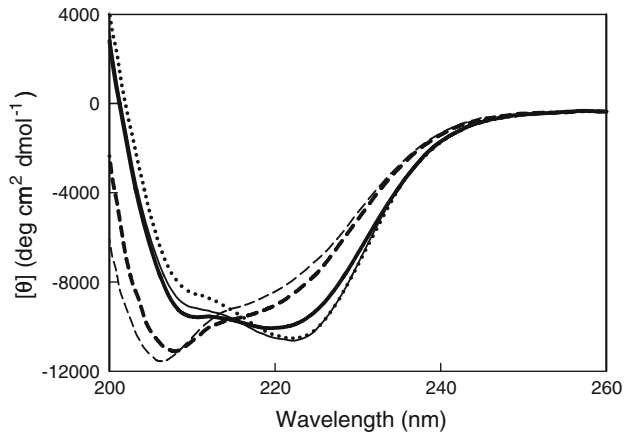

Fig. 2 Far-UV CD spectra of Pro-S324A$^-$ (*thin broken line*), Pro-S324A$^+$ (*thin line*), ΔIS1-Pro-S324A$^-$ (*thick broken line*), ΔIS1-Pro-S324A$^+$ (*thick line*), and ΔIS1-Pro-S324C$^+$ (*dotted line*). The spectra were measured at 20 °C and pH 7.0 either in the presence (for Ca^{2+}-bound form) or absence (for Ca^{2+}-free from) of 10 mM $CaCl_2$

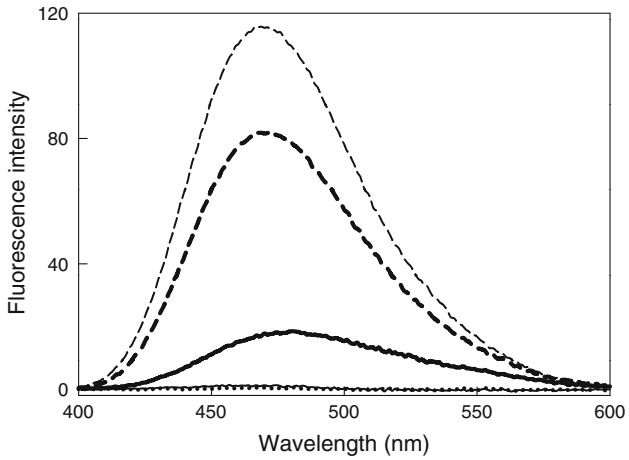

Fig. 3 ANS fluorescence spectra of Pro-S324A$^-$ (*thin broken line*), Pro-S324A$^+$ (*thin line*), ΔIS1-Pro-S324A$^-$ (*thick broken line*), ΔIS1-Pro-S324A$^+$ (*thick line*), and ΔIS1-Pro-S324C$^+$ (*dotted line*). The spectra were measured at 20 °C and pH 7.0 either in the presence (for Ca^{2+}-bound form) or absence (for Ca^{2+}-free from) of 10 mM $CaCl_2$

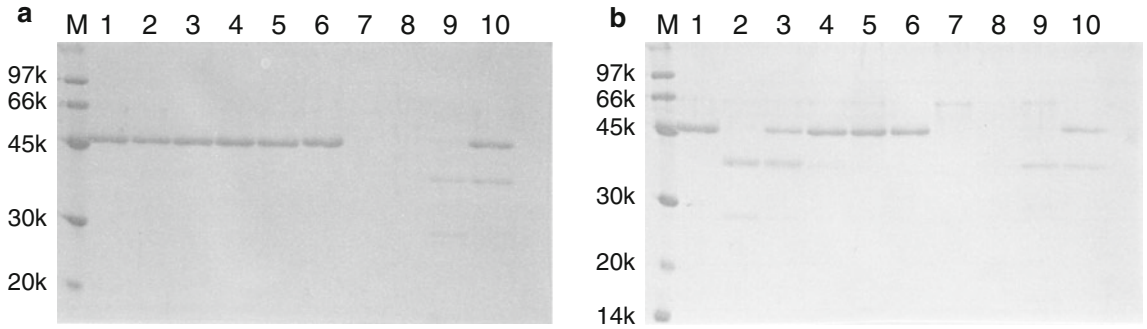

Fig. 4 Susceptibility of the proteins to chymotryptic digestion. Pro-S324A$^+$ (**a**, *lanes 1–5*), Pro-S324A$^-$ (**a**, *lanes 6–10*), ΔIS1-Pro-S324A$^+$ (**b**, *lanes 1–5*), and ΔIS1-Pro-S324A$^-$ (**b**, *lanes 6–10*) were digested with chymotrypsin at 30 °C for 1 h in 20 mM Tris–HCl (pH7.0) either in the presence (for Ca^{2+}-bound form) or absence (for Ca^{2+}-free from) of 10 mM $CaCl_2$ at enzyme:substrate ratios (by weight) of 1:10 (*lanes 2 and 7*), 1:100 (*lanes 3 and 8*), 1:1,000 (*lanes 4 and 9*), and 1:10,000 (*lanes 5 and 10*), and analyzed by 12 % SDS-PAGE. The protein was stained with CBB. *M* low molecular weight markers (GE Healthcare), *lanes 1 and 6* the undigested protein. The molecular mass of each standard protein is indicated beside the gel

released from the C-terminus of the propeptide domain by autoprocessing and the structural rearrangement of this region facilitates correct folding of the mature domain. It is unlikely that the conformation of the propeptide domain is significantly changed, such that the interface between the propeptide and mature domains is perturbed, because ΔIS1-Pro-S324C$^+$ can be autoprocessed. The structural change coincident with autoprocessing has also been reported for pro-subtilisin E, which lacks IS1 (Shinde and Inouye 1995). ANS fluorescence of pro-subtilisin E is greatly reduced when it is autoprocessed. In addition, size exclusion chromatography shows that autoprocessed pro-subtilisin E has a more compact structure than unautoprocessed pro-subtilisin E. Hence, IS1 is apparently required to facilitate complete folding of Pro-TKS prior to autoprocessing.

Susceptibility to chymotryptic digestion

To examine whether the deletion of IS1 affects the stability of Pro-TKS in an unautoprocessed and autoprocessed form, the susceptibilities of ΔIS1-Pro-S324A$^{+/-}$, ΔIS1-Pro-S324C$^{+/-}$, and Pro-S324A$^{+/-}$ to chymotryptic digestion were analyzed. As shown in Fig. 4, ΔIS1-Pro-S324A$^-$ and Pro-S324A$^-$ were highly susceptible to chymotryptic digestion and were almost fully digested by chymotrypsin at an enzyme:substrate ratio of 1:1,000. The susceptibility of ΔIS1-Pro-S324A$^+$ to chymotryptic digestion decreased as compared to that of ΔIS1-Pro-S324A$^-$, as that of Pro-S324A$^+$ did, but less significantly than that of Pro-S324A$^+$. Pro-S324A$^+$ was not significantly digested by chymotrypsin even at an enzyme:substrate ratio of 1:10, while ΔIS1-Pro-S324A$^+$ was almost fully digested by chymotrypsin at this ratio. The susceptibilities of ΔIS1-Pro-S324C$^{+/-}$ to chymotryptic digestion were nearly identical to those of Pro-S324A$^{+/-}$ (data not shown). These results suggest that the deletion of IS1 considerably decreases the stability of unautoprocessed Pro-TKS, but does not significantly affect the stability of autoprocessed Pro-TKS.

Thermal stability

Thermal denaturation of Pro-S324A$^+$, Pro-S324C$^+$, ΔIS1-Pro-S324C$^+$, and S324A-subtilisin$^+$ was analyzed by CD spectroscopy in the presence of 6 M GdnHCl. ΔIS1-Pro-S324A$^+$ was too unstable to be folded in this condition even at 25 °C. The thermal denaturation curves of these proteins are shown in Fig. 5a. The midpoints of the transition of these thermal denaturation curves, T_m, are summarized in Table 1. The T_m value of ΔIS1-Pro-S324C$^+$ (73.3 °C) is comparable to that of Pro-S324C$^+$ (74.4 °C), indicating that the deletion of IS1 does not seriously affect the stability of Pro-TKS in an autoprocessed form. The T_m value of Pro-S324C$^+$ is lower than that of Pro-S324A$^+$ by 2.7 °C. Likewise, the T_m value of S324A-subtilisin$^+$ is lower than that of Pro-S324C$^+$ by 2.8 °C. These results suggest that the stabilities of unautoprocessed Pro-TKS, autoprocessed Pro-TKS, and Tk-subtilisin gradually decrease in this order.

Thermal denaturation of ΔIS1-Pro-S324A$^+$ and ΔIS1-Pro-S324C$^+$ was analyzed by differential scanning calorimetry (DSC). The thermal denaturation curves of these proteins are shown in Fig. 5b. The T_m values determined from these curves are 66.3 °C for ΔIS1-Pro-S324A$^+$ and 92.6 °C for ΔIS1-Pro-S324C$^+$ (Table 1), indicating that ΔIS1-Pro-S324C$^+$ is more stable than ΔIS1-Pro-S324A$^+$ by 26.3 °C. Because the deletion of IS1 alters the conformation of the mature domain of Pro-S324A$^+$, such that one of the ligands of Ca1, Gln84, changes its position from the Ca1 to active sites, it is highly likely that ΔIS1-Pro-S324A$^+$ does not contain Ca1. In contrast, ΔIS1-Pro-

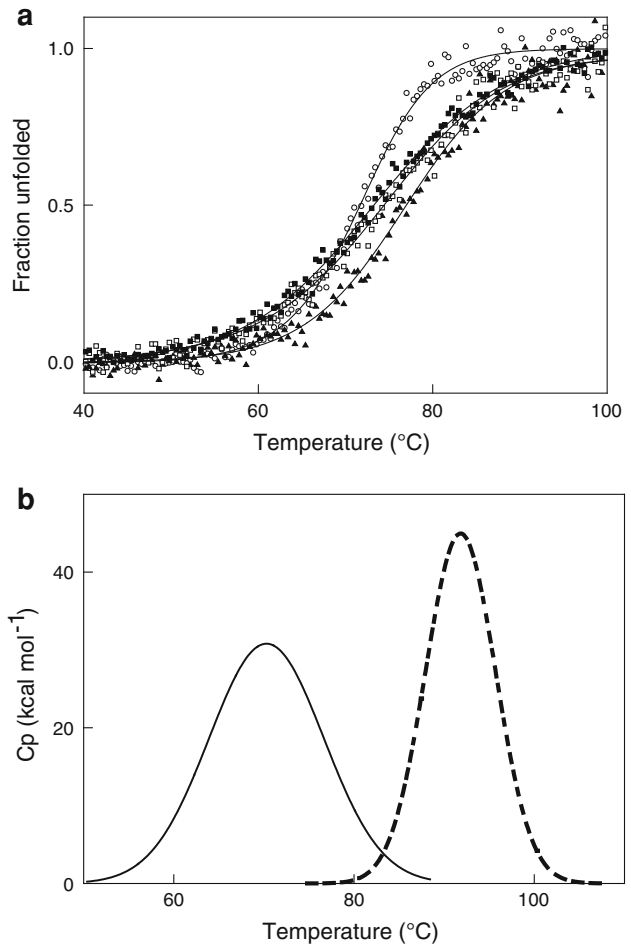

Fig. 5 Thermal denaturation curves of the proteins. **a** The thermal denaturation curves of Pro-S324A$^+$ (*closed triangle*), Pro-S324C$^+$ (*open square*), ΔIS1-Pro-S324C$^+$ (*closed square*), and S324A-subtilisin$^+$ (*open circle*) measured by monitoring the change in CD values at 222 nm at a scan rate of 1 °C/min are shown. The proteins were dissolved in 10 mM Tris–HCl (pH 7.5) containing 6 M GdnHCl at 0.1 mg/ml. **b** The DSC curves of ΔIS1-Pro-S324A$^+$ (*thin line*) and ΔIS1-Pro-S324C$^+$ (*thick broken line*) measured at a scan rate of 1 °C/min are shown. The proteins were dissolved in 50 mM Tris–HCl (pH 8.5) at 0.3 mg/ml

S324C$^+$ probably contains this calcium ion, because the structure of the N-terminal region of its mature domain is rearranged by autoprocessing, such that Gln84 is located at the Ca1 site. We have recently shown that Ca1 contributes to the stabilization of Pro-S324A$^+$ by 26.6 °C in T_m (Uehara et al. 2012). This value is comparable to the difference in the T_m values between ΔIS1-Pro-S324A$^+$ and ΔIS1-Pro-S324C$^+$, suggesting that unautoprocessed ΔIS1-Pro-TKS is stabilized by autoprocessing due to Ca1 binding. The T_m value of Pro-S324A$^+$ determined in the presence of 6 M GdnHCl by CD is higher than that of ΔIS1-Pro-S324C$^+$ by 3.8 °C in T_m, while the T_m value of ΔIS1-Pro-S324A$^+$ determined in the absence of GdnHCl by DSC is lower than that of ΔIS1-Pro-S324C$^+$ by 26.3 °C in T_m. These results suggest that ΔIS1-Pro-S324A$^+$ is less stable than Pro-S324A$^+$ by 30.1 °C in T_m. Thus, the stability of Pro-S324A$^+$ is decreased by the deletion of IS1 mainly due to the loss of Ca1.

Refolding rate

To examine whether the deletion of IS1 affects the refolding rate of Pro-TKS, the refolding rates of Pro-S324A and ΔIS1-Pro-S324A were analyzed at 30 °C. The refolding curves of these proteins are shown in Fig. 6. These curves can be fitted to a single-exponential process with a rate constant of 1.07 min^{-1} for ΔIS1-Pro-S324A$^+$ and 0.98 min^{-1} for Pro-S324A$^+$. This result indicates that the refolding rate of Pro-TKS is not significantly changed by the deletion of IS1.

Enzymatic activity

Most part of IS1 (Gly70-Gly78) is autocatalytically removed when Pro-TKS matures to Tk-subtilisin (Tanaka et al. 2007a), indicating that ΔIS1-Tk-subtilisin lacks the N-terminal four residues (Ser79-Pro82) of Tk-subtilisin. To examine whether the removal of these N-terminal residues

Table 1 T_m values of Pro-TKS derivatives

Protein	Method	GdnHCl (M)	T_m[a] (°C)	ΔT_m1[b] (°C)	ΔT_m2[c] (°C)
Pro-S324A$^+$	CD	6	77.1 ± 0.3	–	
Pro-S324C$^+$	CD	6	74.4 ± 0.3	−2.7	
S324A-subtilisin$^+$	CD	6	71.6 ± 0.1	−5.5	
ΔIS1-Pro-S324C$^+$	CD	6	73.3 ± 0.2	−3.8	
	DSC	0	92.6		+26.3
ΔIS1-Pro-S324A$^+$	DSC	0	66.3		–

[a] The melting temperature (T_m), which is the temperature of the midpoint of the transition, was determined from the thermal denaturation curves shown in Fig. 5a, b

[b] ΔT_m1 is the difference in T_m between Pro-S324A$^+$ and Pro-S324C$^+$, S324A-subtilisin$^+$, or ΔIS1-Pro-S324C$^+$ in the presence of 6 M GdnHCl and is calculated as T_m (Pro-S324C$^+$, S324A-subtilisin$^+$, or ΔIS1-Pro-S324C$^+$) − T_m (Pro-S324A$^+$)

[c] ΔT_m2 is the difference in T_m between ΔIS1-Pro-S324A$^+$ and ΔIS1-Pro-S324C$^+$ in the absence of GdnHCl and is calculated as T_m (ΔIS1-Pro-S324C$^+$) − T_m (ΔIS1-Pro-S324A$^+$)

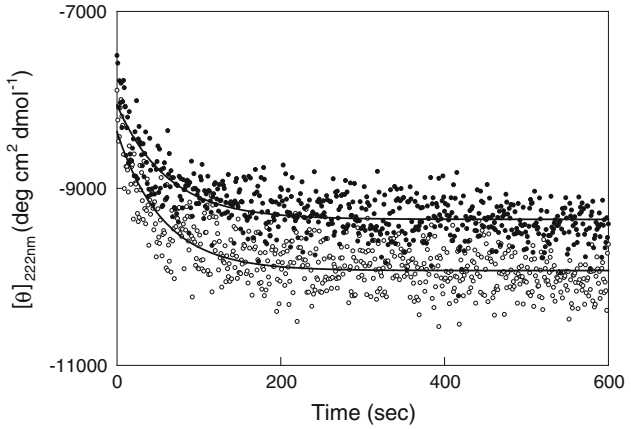

Fig. 6 Refolding kinetics of Pro-S324A$^+$ and ΔIS1-Pro-S324A$^+$. Pro-S324A$^-$ (*open circle*) and ΔIS1-Pro-S324A$^-$ (*closed circle*) were denatured by 6 M GdnHCl and refolded by 20-fold dilution with 50 mM Tris–HCl (pH 7.0) containing 10 mM $CaCl_2$ and 1 mM DTT. The CD values of these proteins are shown as a function of time. The *line* represents a single exponential fit

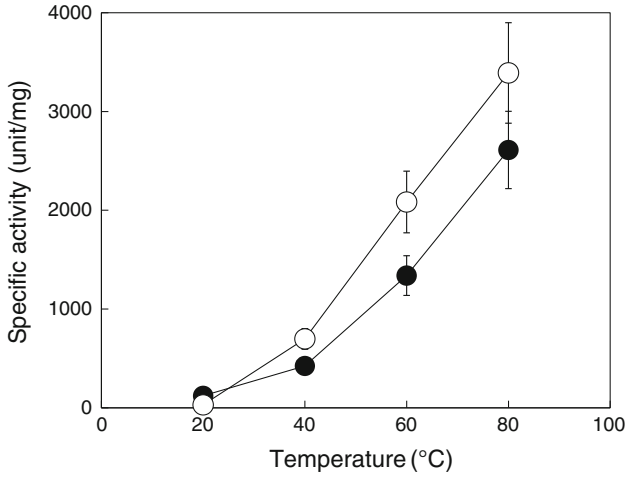

Fig. 7 Temperature dependencies of enzymatic activities of Tk-subtilisin (*closed circle*) and ΔIS1-Tk-subtilisin (*open circle*). The enzymatic activities of these proteins were determined at pH 9.5 in the presence of 1 mM $CaCl_2$ at the temperatures indicated using azocasein as a substrate

affects the activity and stability of Tk-subtilisin, the enzymatic activities of ΔIS1-Tk-subtilisin and Tk-subtilisin matured from ΔIS1-Pro-TKS and Pro-TKS, respectively, were measured at various temperatures using azocasein as a substrate. The results are shown in Fig. 7. The enzymatic activity of ΔIS1-Tk-subtilisin was slightly higher than but comparable to that of Tk-subtilisin at 40–80 °C. Both activities increased as the temperature increased up to 80 °C. These results suggest that neither the activity nor the stability of Tk-subtilisin is significantly affected by the removal of the N-terminal four residues.

Maturation rate

To examine whether the deletion of IS1 affects the maturation rate of Pro-TKS, the maturation rates of Pro-TKS and ΔIS1-Pro-TKS were analyzed at 60 °C. As shown in Fig. 8, the maturation of Pro-TKS and ΔIS1-Pro-TKS was almost fully completed within 3 h. Both proteins exhibited comparable maximum activities upon maturation, indicating that the maturation yields of both proteins are comparable to each other, because the specific activity of ΔIS1-Tk-subtilisin is similar to that of Tk-subtilisin. However, the maturation rate of ΔIS1-Pro-TKS was significantly decreased as compared to that of Pro-TKS. Thus, IS1 is required to increase the maturation rate of Pro-TKS.

Unautoprocessed ΔIS1-Pro-TKS is highly less stable than unautoprocessed Pro-TKS and must be more rapidly degraded by its mature form than unautoprocessed Pro-TKS is. Nevertheless, the maturation yield of ΔIS1-Pro-TKS is comparable to that of Pro-TKS, probably because Tkpro inhibits the activity of ΔIS1-Tk-subtilisin until the

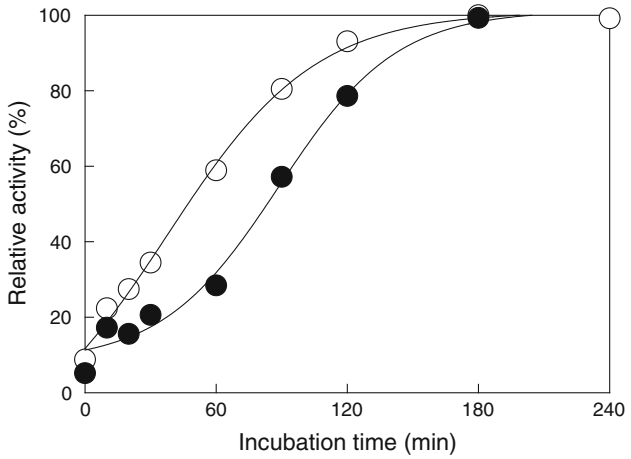

Fig. 8 Maturation of Pro-TKS (*open circle*) and ΔIS1-Pro-TKS (*closed circle*). The proteins were refolded at pH 9.5 in the presence of 10 mM $CaCl_2$ at 4 °C and incubated at 60 °C. With an appropriate interval, an aliquot was withdrawn and the enzymatic activity was determined at 20 °C using Suc-AAPF-*p*NA as a substrate. The activity relative to the maximal one was plotted as a function of time. The *line* represents the optimal fit to the data

unstable unautoprocessed form of ΔIS1-Pro-TKS is converted to the stable autoprocessed form.

Autoprocessing rate

To examine whether the deletion of IS1 affects the autoprocessing rate of Pro-TKS, the autoprocessing rates of Pro-S324C and ΔIS1-Pro-S324C were analyzed at 60 °C. The enzymatic activity of S324C-subtilisin, which is greatly reduced as compared to that of Tk-subtilisin, is

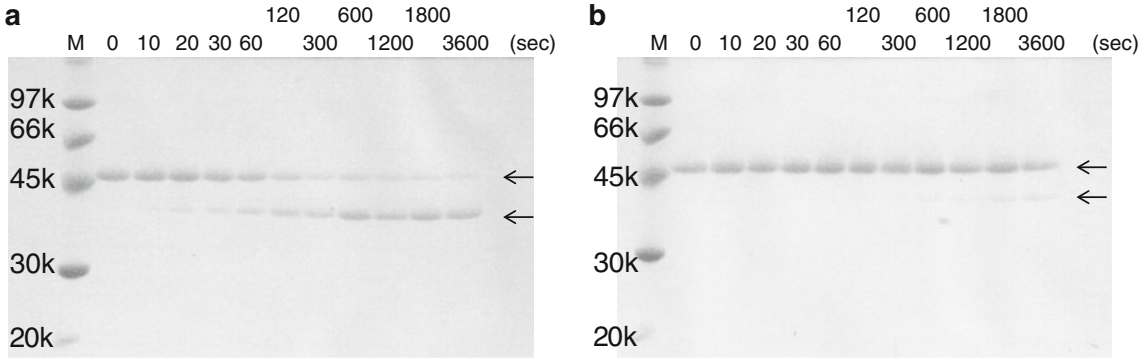

Fig. 9 Autoprocessing of Pro-S324C$^+$ (**a**) and ΔIS1-Pro-S324C$^+$ (**b**) at 60 °C. The protein was denatured by 6 M GdnHCl, refolded by 100-fold dilution with 50 mM CAPS–NaOH (pH 9.5) containing 10 mM $CaCl_2$ and 1 mM DTT at 4 °C, incubated at 60 °C for the indicated times, precipitated by 10 % (v/v) TCA, and subjected to 12 % SDS-PAGE. The protein was stained with CBB. *M* low molecular weight markers (GE Healthcare). The *arrows* indicate Pro-S324C or ΔIS1-Pro-S324C (*top*) and S324C-subtilisin or ΔIS1-S324C-subtilisin (*bottom*)

sufficient to promote autoprocessing, but is not sufficient to promote degradation of Tkpro that forms a complex with S324C-subtilisin (Tanaka et al. 2007b). As shown in Fig. 9, ΔIS1-Pro-S324C$^+$ was only partially autoprocessed even after incubation for 60 min, whereas Pro-S324C$^+$ was almost fully autoprocessed within 30 min. Because ΔIS1-Tk-subtilisin exhibits a comparable activity to that of Tk-subtilisin, it should degrade Tkpro with a similar rate as does Tk-subtilisin. Hence, the slow maturation of ΔIS1-Pro-TKS is probably due to the slow autoprocessing. Unautoprocessed ΔIS1-Pro-TKS is not fully folded although the subsequent autoprocessing and degradation of Tkpro can occur. The structure around the scissile peptide bond between the propeptide and mature domains may be changed due to a strain caused by the connection of Ala83 to Leu69. This structural change may be responsible for slow autoprocessing and therefore for slow maturation of ΔIS1-Pro-TKS.

Role of IS1

In this study, we showed that IS1 is required not only for the hyperstabilization of unautoprocessed Pro-TKS but also for its effective maturation. IS1 is required for the stabilization of unautoprocessed Pro-TKS, because it is required to facilitate binding of Ca1. IS1 is also required to increase the maturation rate of Pro-TKS, because it is required to increase the autoprocessing rate of Pro-TKS by facilitating complete folding of unautoprocessed Pro-TKS. Unautoprocessed pro-subtilisin E from a mesophilic bacterium is less stable than autoprocessed pro-subtilisin E, because the former does not contain Ca1 while the latter contains it (Yabuta et al. 2002). Thus, Pro-TKS requires IS1 to adapt to high-temperature environment. The source organism of Pro-TKS, *T. kodakarensis*, grows most optimally at 85 °C (Atomi et al. 2004). Pro-TKS may not be folded and mature at this temperature unless it contains IS1. In fact, ΔIS1-Pro-TKS cannot mature at 80 °C.

BLAST searches indicate that subtilases from hyperthermophilic archaea, such as *T. onnurineus* NA1 (accession no. YP_002308296), *Ferroglobus placidus* DSM 10642 (accession no. YP_003436500), *Pyrolobus fumarii* 1A (accession no. YP_004781243), and *Aeropyrum pernix* K1 (accession no. NP_147093), also contain an insertion sequence corresponding to IS1 of Pro-TKS. None of these subtilases, except for *A. pernix* K1 subtilase, termed pernisine (Catara et al. 2003), has been biochemically characterized. However, it is plausible to speculate that these insertion sequences play a similar role as that of IS1 of Tk-subtilisin, and therefore they are required for these subtilases to adapt to high-temperature environment where their source organisms grow.

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