

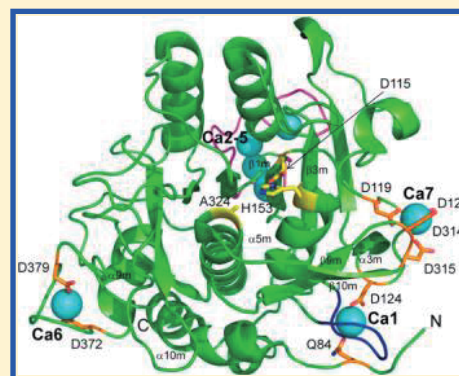
# Requirement of $\text{Ca}^{2+}$ Ions for the Hyperthermostability of Tk-Subtilisin from *Thermococcus kodakarensis*

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**ABSTRACT:** Tk-subtilisin, a hyperthermostable subtilisin-like serine protease from *Thermococcus kodakarensis*, matures from the inactive precursor, Pro-Tk-subtilisin (Pro-TKS), upon autoprocessing and degradation of the propeptide (Tkpro). It contains seven  $\text{Ca}^{2+}$  ions. Four of them (Ca2–Ca5) are responsible for folding of Tk-subtilisin. In this study, to clarify the role of the other three  $\text{Ca}^{2+}$  ions (Ca1, Ca6, and Ca7), we constructed Pro-TKS derivatives lacking the Ca1 ion (Pro-TKS/ $\Delta\text{Ca1}$ ), Ca6 ion (Pro-TKS/ $\Delta\text{Ca6}$ ), and Ca7 ion (Pro-TKS/ $\Delta\text{Ca7}$ ), and their active site mutants (Pro-S324A/ $\Delta\text{Ca1}$ , Pro-S324A/ $\Delta\text{Ca6}$ , and Pro-S324A/ $\Delta\text{Ca7}$ , respectively). Pro-TKS/ $\Delta\text{Ca6}$  and Pro-TKS/ $\Delta\text{Ca7}$  fully matured into their active forms upon incubation at 80 °C for 30 min as did Pro-TKS. The mature enzymes were as active as Tk-subtilisin at 80 °C, indicating that the Ca6 and Ca7 ions are not important for activity. In contrast, Pro-TKS/ $\Delta\text{Ca1}$  matured poorly at 80 °C because of the instability of its mature domain.

The enzymatic activity of Tk-subtilisin/ $\Delta\text{Ca1}$  was determined to be 50% of that of Tk-subtilisin using the refolded protein. This result suggests that the Ca1 ion is required for the maximal activity of Tk-subtilisin. The refolding rates of all Pro-S324A derivatives were comparable to that of Pro-S324A (active site mutant of Pro-TKS), indicating that these  $\text{Ca}^{2+}$  ions are not needed for folding of Tk-subtilisin. The stabilities of Pro-S324A/ $\Delta\text{Ca1}$  and Pro-S324A/ $\Delta\text{Ca6}$  were decreased by 26.6 and 11.7 °C, respectively, in  $T_m$  compared to that of Pro-S324A. The half-lives of Tk-subtilisin/ $\Delta\text{Ca6}$  and Tk-subtilisin/ $\Delta\text{Ca7}$  at 95 °C were 8- and 4-fold lower than that of Tk-subtilisin, respectively. These results suggest that the Ca1, Ca6, and Ca7 ions, especially the Ca1 ion, contribute to the hyperthermostabilization of Tk-subtilisin.



Subtilisins are extracellular alkaline serine proteases with a catalytic triad consisting of Ser, His, and Asp.<sup>1,2</sup> They are widely used for industrial purposes, especially as additives in detergents.<sup>3</sup> Subtilisins are synthesized as an inactive precursor, prepro-subtilisin, in which a signal sequence (presequence) and a propeptide are attached to the N-terminus of the mature domain. Upon secretion into an extracellular environment, a signal sequence is removed and the rest of the protein, pro-subtilisin, is folded with the assistance of its cognate propeptide and is subsequently processed autocatalytically.<sup>4–7</sup> The propeptide acts not only as an intramolecular chaperone, which is required for folding of the mature domain, but also as an inhibitor of the mature domain. Because the propeptide forms an inactive complex with the mature domain upon autoprocessing, complete degradation of the propeptide by the mature domain is necessary to release the active subtilisin.

Most subtilisins and subtilisin-like serine proteases, with a few exceptions,<sup>8</sup> contain one or more  $\text{Ca}^{2+}$  ions.<sup>9–24</sup> For example, bacterial subtilisins, such as subtilisins BPN,<sup>9</sup> E,<sup>13</sup> and Carlsberg,<sup>24</sup> contain two  $\text{Ca}^{2+}$  ions. One binds to the high-affinity binding site (site 1 or A), and the other binds to the low-affinity binding site (site 2 or B). Both  $\text{Ca}^{2+}$  ions at site 1<sup>25,26</sup> and site 2<sup>27</sup> contribute to protein stabilization. These  $\text{Ca}^{2+}$  ions are not involved in catalytic function<sup>28</sup> or protein folding.<sup>29</sup> Proteinase K also contains two  $\text{Ca}^{2+}$  ions, both of which are not conserved in bacterial subtilisins.<sup>11</sup> These  $\text{Ca}^{2+}$

ions also contribute to protein stabilization.<sup>30</sup> These results suggest that the stabilization mechanism via introduction of  $\text{Ca}^{2+}$  binding sites is shared by all subtilisins and subtilisin-like proteases. However, the studies of the role of the  $\text{Ca}^{2+}$  ions in these proteases are still limited.

Tk-subtilisin is a highly thermostable subtilisin-like serine protease from the hyperthermophilic archaeon *Thermococcus kodakarensis*.<sup>31,32</sup> Tk-subtilisin (Gly70–Gly398) matures from Pro-Tk-subtilisin (Pro-TKS, Gly1–Gly398) upon autoprocessing and degradation of the propeptide (Tkpro, Gly1–Leu69) as other subtilisins are.<sup>31</sup> Tkpro exhibits both chaperone function<sup>33–35</sup> and inhibitory activity.<sup>31,36,37</sup> However, Tk-subtilisin requires  $\text{Ca}^{2+}$  ions, instead of Tkpro, for folding,<sup>33</sup> although its folding rate decreases in the absence of Tkpro.<sup>34</sup> According to the crystal structures of Tk-subtilisin and its precursors,<sup>33,38</sup> Tk-subtilisin contains seven  $\text{Ca}^{2+}$  ions (Ca1–Ca7). This number is the highest among those so far reported for various subtilisins and subtilisin-like serine proteases.<sup>9–24</sup> Therefore, Tk-subtilisin is a good model for analyzing the role of the  $\text{Ca}^{2+}$  ions in the stability, activity, and folding of subtilisins. Tk-subtilisin contains a long surface loop (Gly206–Glu229) between the  $\alpha 6\text{m}$ -helix and  $\beta 5\text{m}$ -strand, both of which

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form a central  $\alpha\beta\alpha$  substructure with the  $\alpha 7$ m-helix. This loop contains a Dx[DN]xDG motif, which is known as a  $\text{Ca}^{2+}$ -binding motif.<sup>39</sup> Four of the seven  $\text{Ca}^{2+}$  ions ( $\text{Ca}2$ – $\text{Ca}5$ ) bind to this loop.<sup>33</sup> Mutational studies of these  $\text{Ca}^{2+}$ -binding sites have suggested that binding of these  $\text{Ca}^{2+}$  ions to this  $\text{Ca}^{2+}$ -binding loop is not important for the stability of Tk-subtilisin but is required to induce folding of a central  $\alpha\beta\alpha$  substructure and thereby folding of the entire protein molecule.<sup>40</sup> However, the role of other  $\text{Ca}^{2+}$  ions ( $\text{Ca}1$ ,  $\text{Ca}6$ , and  $\text{Ca}7$ ) remains to be analyzed.

In this study, we constructed three Pro-TKS derivatives lacking  $\text{Ca}1$ ,  $\text{Ca}6$ , or  $\text{Ca}7$  to examine whether the maturation of Pro-TKS is affected by the removal of these  $\text{Ca}^{2+}$  ions. We also constructed three Pro-S324A derivatives lacking the  $\text{Ca}1$ ,  $\text{Ca}6$ , or  $\text{Ca}7$  ion to examine whether the stability and folding of Pro-S324A, which is the active site mutant of Pro-TKS, are affected by the removal of these  $\text{Ca}^{2+}$  ions. On the basis of the results, we discuss the role of these  $\text{Ca}^{2+}$  ions in the stability, activity, and folding of Tk-subtilisin.

## MATERIALS AND METHODS

**Plasmid Construction.** The pET25b derivatives for overproduction of Pro-TKS/ $\Delta\text{Ca}1$ , Pro-TKS/ $\Delta\text{Ca}6$ , and Pro-TKS/ $\Delta\text{Ca}7$  were constructed using the QuickChange II Mutagenesis Kit (Stratagene, La Jolla, CA). The pET25b derivative for overproduction of Pro-TKS<sup>31</sup> was used as a template. The polymerase chain reaction (PCR) primers were designed such that the  $\text{Ca}1$  binding loop (Leu164–Gly172) is removed for Pro-TKS/ $\Delta\text{Ca}1$ , the codon for Asp372 (GAC) is changed to GCC for Ala for Pro-TKS/ $\Delta\text{Ca}6$ , and the codons for Asp121 (GAC), Asp314 (GAC), and Asp315 (GAC) are changed to GCC for Ala, GGC for Gly, and GGC for Gly, respectively, for Pro-TKS/ $\Delta\text{Ca}7$ . The pET25b derivatives for overproduction of Pro-S324A/ $\Delta\text{Ca}1$ , Pro-S324A/ $\Delta\text{Ca}6$ , and Pro-S324A/ $\Delta\text{Ca}7$  were constructed by the same methods as described above, using the pET25b derivative for overproduction of Pro-S324A<sup>33</sup> as a template. The pET25b derivative for overproduction of Tk-subtilisin/ $\Delta\text{Ca}1$  was constructed as described for that of Tk-subtilisin<sup>31</sup> using the pET25b derivative for overproduction of Pro-TKS/ $\Delta\text{Ca}1$  as a template. PCR was performed using a thermal cycler (Gene Amp PCR system 2400, Applied Biosystems, Tokyo, Japan) and *Pfu* DNA polymerase (Stratagene). All DNA oligomers for PCR were synthesized by Hokkaido System Science (Sapporo, Japan). The DNA sequence was confirmed with a Prism 310 DNA sequencer (Applied Biosystems).

**Protein Preparation.** All mutant proteins 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.<sup>33</sup> For maturation of Pro-TKS and its derivatives, the protein was dialyzed against 20 mM Tris-HCl (pH 7.0) containing 0.5 mM EDTA and 1 mM DTT for refolding into a molten globule-like structure and incubated in the same buffer containing 6 M guanidine hydrochloride (GdnHCl) at room temperature for 1 h for complete denaturation. The protein concentration was 30  $\mu\text{M}$ . Then, the protein was refolded into a native structure by 100-fold dilution with 50 mM CAPS (*N*-cyclohexyl-3-aminopropane-sulfonic acid)-NaOH (pH 9.5) containing 10 mM  $\text{CaCl}_2$  and 1 mM DTT, followed by incubation on ice for 10 min. Finally, the protein was incubated at 80 °C for 30 min for maturation. Tk-subtilisin/ $\Delta\text{Ca}1$  was refolded into a native structure by the

same procedures, except that the refolded protein was incubated at 30 °C.

MIP-Tk-subtilisin, MIP-Tk-subtilisin/ $\Delta\text{Ca}6$ , and MIP-Tk-subtilisin/ $\Delta\text{Ca}7$ , in which the active site serine residue is specifically monoisopropylphosphorylated, were prepared by the chemical modifications of Tk-subtilisin, Tk-subtilisin/ $\Delta\text{Ca}6$ , and Tk-subtilisin/ $\Delta\text{Ca}7$  that matured from their pro forms with diisopropylphosphorfluoridate (DIPF), as described previously.<sup>38</sup>

For refolding of Pro-S324A and its derivatives, the protein was dialyzed against 20 mM Tris-HCl (pH 7.0) containing 10 mM  $\text{CaCl}_2$  and 1 mM DTT at 4 °C for 5 days. The refolded protein was collected from the supernatant after centrifugation at 30000g for 30 min. The incorrectly folded protein was then digested with chymotrypsin (Sigma Chemical Co., St. Louis, MO) at 30 °C for 1 h at an enzyme:substrate ratio of 1:100 (w/w). The protein was collected from the supernatant after incubation at 80 °C for 30 min to inactivate chymotrypsin, followed by centrifugation at 30000g for 30 min, and loaded onto a Hiload 16/60 Superdex 200 pg column (GE Healthcare, Little Chalfont, Buckinghamshire, England) equilibrated with 20 mM Tris-HCl (pH 7.0) containing 10 mM  $\text{CaCl}_2$  and 50 mM NaCl. The fractions containing the protein were collected and dialyzed against 20 mM Tris-HCl (pH 7.0) containing 10 mM  $\text{CaCl}_2$ .

The purity of the protein was confirmed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)<sup>41</sup> or 12% Tricine SDS–PAGE (SDS–PAGE using the Tricine buffer),<sup>42</sup> followed by staining with Coomassie Brilliant Blue (CBB). The protein concentration was determined from UV absorption using a cell with an optical path length of 1 cm and an  $A_{280}$  value for a 0.1% (1.0 mg/mL) solution of 1.32 for Pro-S324A/ $\Delta\text{Ca}1$  and Pro-TKS/ $\Delta\text{Ca}1$  and 1.24 for all other proteins. These values were calculated by using absorption coefficients of  $1526 \text{ M}^{-1} \text{ cm}^{-1}$  for tyrosine and  $5225 \text{ M}^{-1} \text{ cm}^{-1}$  for tryptophan at 280 nm.<sup>43</sup>

**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  $\text{CaCl}_2$ . The protein concentration and optimal path length were 0.2 mg/mL and 2 mm, respectively. The mean residue ellipticity,  $[\theta]$ , which has units of degrees square centimeter per decimole, was calculated by using an average amino acid molecular mass of 110 Da.

**Differential Scanning Calorimetry.** The differential scanning calorimetry (DSC) measurement was performed using a high-sensitivity VP-capillary DSC instrument controlled by the VP Viewer software package (MicroCal Inc., Northampton, MA) at a scan rate of 1 °C/min. The temperature increased to 130 °C during the measurement. The protein (0.3 mg/mL) was dialyzed against 50 mM Tris-HCl (pH 8.5), filtered through a 0.22  $\mu\text{m}$  pore size membrane, and degassed prior to the measurement.

**Stability against Heat Denaturation.** MIP-Tk-subtilisin/ $\Delta\text{Ca}7$  and MIP-Tk-subtilisin were dialyzed against  $\text{Ca}^{2+}$ -free buffer [50 mM CAPS-NaOH (pH 9.5)] and incubated at 95 °C. At appropriate intervals, an aliquot of the protein solution was withdrawn and the thermally denatured protein was digested with chymotrypsin at 30 °C for 10 min at an enzyme:substrate ratio of 1:10 (w/w). The protein, which is kept folded upon heat treatment and is therefore resistant to chymotryptic digestion, was collected by TCA precipitation,

washed with 70% acetone twice, and analyzed by 12% SDS–PAGE. The amount of this protein was estimated from the intensity of the band visualized with CBB staining using Scion Image.

**Determination of the Refolding Rate.** Refolding of Pro-S324A and its derivatives was kinetically analyzed by the rapid dilution method as described previously.<sup>40</sup> Briefly, these proteins were first completely denatured with 6 M Gdn-HCl. Refolding of these proteins was initiated by 100-fold dilution with 20 mM Tris-HCl (pH 8.0) containing 10 mM CaCl<sub>2</sub> and 1 mM DTT and promoted by incubation at 30 °C. With an appropriate interval, the refolding reaction was terminated by addition of 10 mM EDTA, and the incorrectly folded protein was digested with chymotrypsin at an enzyme:substrate ratio of 1:10 (w/w). Finally, the protein was collected by precipitation with 10% trichloroacetic acid (TCA), washed with 70% acetone twice, and analyzed by 12% SDS–PAGE. The amount of correctly folded protein was estimated from the intensity of the band visualized with CBB staining following 12% SDS–PAGE using Scion Image.

**Enzymatic Activity.** The enzymatic activity of the protein was determined at the temperatures indicated using azocasein (Sigma) as a substrate as described previously.<sup>31</sup> The reaction mixture (100  $\mu$ L) contained 50 mM CAPS-NaOH (pH 9.5), 1 mM CaCl<sub>2</sub>, and 2% azocasein. The enzymatic reaction was initiated by addition of an appropriate amount of the enzyme and terminated by addition of 67  $\mu$ L of 15% trichloroacetic acid. The reaction time was 20 min. After incubation on ice for 15 min, followed by centrifugation at 15000g for 15 min, an aliquot of the supernatant (80  $\mu$ L) was withdrawn, mixed with 20  $\mu$ L of 2 M NaOH, and measured for the absorption at 440 nm ( $A_{440}$ ). One unit of enzymatic activity was defined as the amount of enzyme that increases the  $A_{440}$  value of the reaction mixture by 0.1 in 1 min.

**Crystallization, Data Collection, Structure Determination, and Refinement.** Pro-S324A/ $\Delta$ Ca6 was dialyzed against 5 mM Tris-HCl (pH 7.0), concentrated to 10 mg/mL using ultrafiltration system Amicon Ultra (Millipore, Billerica, MA), and used for crystallization. Pro-S324A/ $\Delta$ Ca6 was crystallized using the sitting-drop vapor-diffusion method at 4 °C. Drops were prepared by mixing 1  $\mu$ L each of protein and reservoir solutions and were vapor-equilibrated against 100  $\mu$ L reservoir solutions. Crystals appeared after 1 week using 4.0 M sodium formate (Crystal Screen I, Hampton Research). All crystals were soaked for 5 s in cryobuffers containing 18% (v/v) ethylene glycol (Hampton Research, Alise Viejo, CA) prior to flash-freezing in a nitrogen gas stream.

X-ray diffraction data sets were collected at a wavelength of 1.0 Å on the BL38B1 station at SPring-8. The X-ray diffraction data set was collected and the structure determined by the molecular replacement method, as described previously,<sup>33</sup> 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 listed in Table 1. Figures were prepared with PyMol (<http://www.pymol.org>).

**Protein Data Bank Entry.** The coordinates and structure factors for Pro-S324A/ $\Delta$ Ca6 have been deposited in the Protein Data Bank as entry 3VHQ.

## RESULTS

**Mutant Preparation.** According to the crystal structure of Tk-subtilisin,<sup>38</sup> the Ca<sup>2+</sup> ions are heptacoordinated with Gln84 O<sup>e1</sup>, Asp124 O <sup>$\delta$ 1</sup>, Asp124 O <sup>$\delta$ 2</sup>, Leu162 O, Asn166 O <sup>$\delta$ 1</sup>, Ile168

**Table 1. Data Collection and Refinement Statistics**

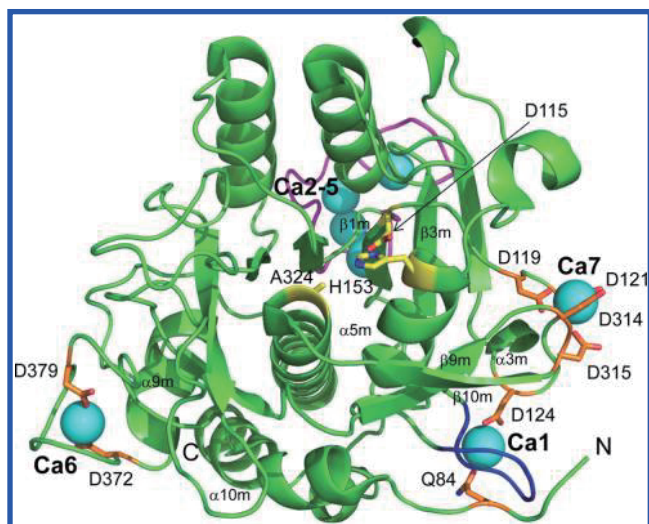
	Pro-S324A/ $\Delta$ Ca6
Crystal	
wavelength (Å)	1.0
space group	I222
cell parameters	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	73.43, 92.78, 124.28
$\alpha$ , $\beta$ , $\gamma$ (deg)	90.0, 90.0, 90.0
no. of molecules per asymmetric unit	1
resolution range (Å)	50.9–2.09
highest-resolution shell (Å)	2.16–2.09
no. of reflections measured	165887
no. of unique reflections	24074
completeness (%)	95.6 (78.1)
$R_{\text{merge}}$ (%) <sup>a</sup>	6.2 (36.5)
average $I/\sigma(I)$	30.9 (3.35)
Refinement	
resolution limits (Å)	36.1–2.15
no. of atoms	
protein	2830
water	5
Ca <sup>2+</sup>	268
$R_{\text{work}}$ (%) / $R_{\text{free}}$ (%) <sup>b</sup>	17.1/24.1
root-mean-square deviation from ideal values	
bond lengths (Å)	0.023
bond angles (deg)	1.93
average <i>B</i> factors (Å <sup>2</sup> )	
protein	35.7
water/Ca <sup>2+</sup>	46.2/36.3
Ramachandran plot (%)	
most favored regions	89.1
additional allowed regions	10.9

<sup>a</sup> $R_{\text{merge}} = \sum |I_{hkl} - \langle I_{hkl} \rangle| / \sum I_{hkl}$ , 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. <sup>b</sup> $R_{\text{free}}$  was calculated using 5% of the total reflections chosen randomly and omitted from refinement.

O, and Val170 O at the Ca1 site, hexacoordinated with Asp372 O <sup>$\delta$ 1</sup>, Leu373 O, Pro375 O, Gly377 O, Asp379 O <sup>$\delta$ 1</sup>, and one water molecule at the Ca6 site, and heptacoordinated with Asp119 O <sup>$\delta$ 1</sup>, Asp119 O <sup>$\delta$ 2</sup>, Asp121 O <sup>$\delta$ 1</sup>, Asp314 O <sup>$\delta$ 1</sup>, Asp315 O <sup>$\delta$ 1</sup>, and two water molecules at the Ca7 site (Figure 1). To analyze the role of these Ca<sup>2+</sup> ions, we created the mutant proteins of Pro-TKS and Pro-S324A, in which the amino acid residues coordinating with these Ca<sup>2+</sup> ions are deleted or mutated. The mutant proteins, from which the Ca1 binding loop (Leu164–Gly172) has been deleted, are designated Pro-TKS/ $\Delta$ Ca1 and Pro-S324A/ $\Delta$ Ca1. The mutant proteins, in which Asp372 has been replaced with Ala, are designated Pro-TKS/ $\Delta$ Ca6 and Pro-S324A/ $\Delta$ Ca6. The mutant proteins, in which Asp121, Asp314, and Asp315 have been replaced with Ala, Gly, and Gly, respectively, are designated Pro-TKS/ $\Delta$ Ca7 and Pro-S324A/ $\Delta$ Ca7. Asp314 and Asp315 were replaced with Gly, because these residues are replaced with Gly in the sequence of subtilisin E, which does not contain the Ca<sup>2+</sup> ion corresponding to the Ca7 ion of Tk-subtilisin. All mutant proteins were overproduced in *E. coli* and purified to give a single band on 12% SDS–PAGE (data not shown).

**Crystal Structure of Pro-S324A/ $\Delta$ Ca6.** To examine whether Pro-S324A/ $\Delta$ Ca6 lacks the Ca6 ion, we determined the crystal structure of Pro-S324A/ $\Delta$ Ca6 at 2.1 Å resolution. The structure of Pro-S324A/ $\Delta$ Ca6 is nearly identical to that of





**Figure 1.** Crystal structure of S324A-subtilisin. The  $\text{Ca}^{2+}$  ions (Ca1–Ca7) are shown as cyan spheres. The  $\text{Ca}^{2+}$ -binding loop, which contains the Ca2–Ca5 ions and is required for folding of Tk-subtilisin, is colored purple. The Ca1 binding loop is colored blue. The side chains of the amino acid residues, which coordinate with the Ca1, Ca6, and Ca7 ions, are represented as orange sticks. Two active site residues (Asp115 and His153) and Ala324, which is substituted for the active site serine residue, are represented as yellow sticks. In these stick models, the oxygen and nitrogen atoms are colored red and blue, respectively. N and C represent the N- and C-termini, respectively. The positions of  $\alpha 3\text{m}$ -,  $\alpha 5\text{m}$ -,  $\alpha 9\text{m}$ -, and  $\alpha 10\text{m}$ -helices and  $\beta 1\text{m}$ -,  $\beta 3\text{m}$ -,  $\beta 9\text{m}$ -, and  $\beta 10\text{m}$ -strands are indicated.

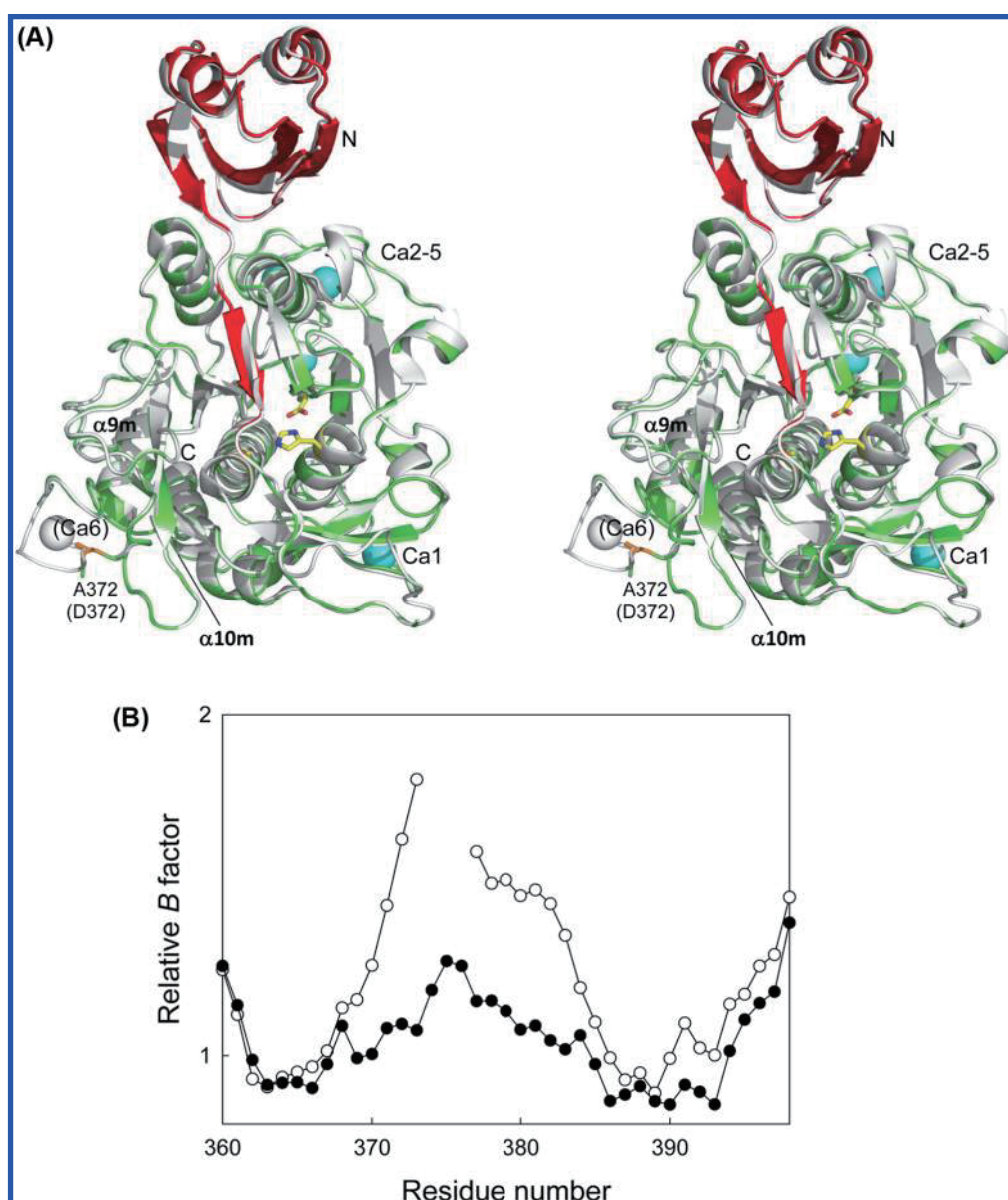
Pro-S324A, except that it does not contain the Ca6 ion, and a part of the Ca6 binding loop of Tk-subtilisin (Gly374–Thr376) and seven N-terminal residues of the mature domain (Leu75–Gln81) are disordered (Figure 2A,B). As compared to the relative *B* factors (*B* factors relative to the average of all residues) of the residues around the Ca6 site of Pro-S324A, those of the corresponding residues of Pro-S324A/ $\Delta$ Ca6 are greatly increased (Figure 2B). These results suggest that the flexibility of the Ca6 binding loop greatly increases upon removal of the Ca6 ion. Pro-S324A/ $\Delta$ Ca6 does not contain the Ca7 ion either (Figure 2A), probably because the binding affinity of the  $\text{Ca}^{2+}$  ion for the Ca7 site is much lower than those of other sites. It has been reported that the  $\text{Ca}^{2+}$  ion is dissociated from the Ca7 site upon dialysis against  $\text{Ca}^{2+}$ -free buffer while they keep binding to other sites upon this dialysis.<sup>38</sup> Hence, the  $\text{Ca}^{2+}$  ion binds to the Ca7 site of Pro-S324A very weakly regardless of whether the  $\text{Ca}^{2+}$  ion binds to the Ca6 site. Likewise, residues 75–81 of Pro-S324A are disordered regardless of whether the  $\text{Ca}^{2+}$  ion binds to the Ca6 site, because this region is disordered in the Pro-S324A/ $\Delta$ Ca2 and Pro-S324A/ $\Delta$ Ca3 structures as well.<sup>40</sup> This region may be intrinsically disordered because of its high flexibility.

The crystal structures of Pro-S324A/ $\Delta$ Ca1 and Pro-S324A/ $\Delta$ Ca7 were not determined, because there is no doubt that these mutant proteins do not contain the Ca1 and Ca7 ions, respectively. Pro-S324A/ $\Delta$ Ca1 lacks the Ca1 binding loop, which contains four of the six residues coordinating with the Ca1 ion, and Pro-S324A/ $\Delta$ Ca7 lacks three of the four residues, which coordinate with the Ca7 ion. The Pro-S324A structure does not contain the Ca7 ion when the protein is crystallized in the absence of the  $\text{Ca}^{2+}$  ions but contains it when the protein is crystallized in the presence of 10 mM  $\text{CaCl}_2$ .<sup>38</sup>

**CD Spectra of Pro-S324A and Its Derivatives.** The far-UV CD spectra of Pro-S324A/ $\Delta$ Ca1, Pro-S324A/ $\Delta$ Ca6, and Pro-S324A/ $\Delta$ Ca7 were similar to that of Pro-S324A (Figure 3). These results suggest that the removal of the Ca1 or Ca7 ion does not seriously affect the structure of Pro-S324A as does the removal of the Ca6 ion. It is noted that Pro-S324A/ $\Delta$ Ca1 lacks the Ca1 binding loop and Pro-S324A/ $\Delta$ Ca7 has three mutations in the Ca7 binding loops. A possibility that these mutations cause a structural change more significant than that caused by the removal of the Ca1 or Ca7 ion cannot be ruled out. This structural change may be too small to be detected by the far-UV CD spectra. However, the loops forming the Ca1 or Ca7 site, including the Ca1 binding loop, are highly destabilized or disordered upon removal of the Ca1 or Ca7 ion, like the case for the Ca6 binding loop. Deletion of these loops may not seriously affect the structure of the protein. Therefore, any structural and functional change caused by the deletion of the Ca1 binding loop or the mutations at the Ca7 site may mainly reflect that caused by the removal of the Ca1 or Ca7 ion.

**Maturation of Pro-TKS and Its Derivatives.** The maturation of Pro-TKS and its derivatives was analyzed at 80 °C by 12% Tricine SDS–PAGE. The results are summarized in Figure 4. All proteins were rapidly autoprocessed into the complex between Tkpro and the mature domain within 2 min. However, the subsequent degradation of Tkpro by the mature domain did not proceed at a similar rate for these proteins. It was completed within 30 min for Pro-TKS, Pro-TKS/ $\Delta$ Ca6, and Pro-TKS/ $\Delta$ Ca7, while it was not completed within 60 min for Pro-TKS/ $\Delta$ Ca1. When the maturation of Pro-TKS and Pro-TKS/ $\Delta$ Ca1 was analyzed at 60 °C, the degradation of Tkpro by the mature domain was completed within 4 h for Pro-TKS, while it did not occur for up to 4 h for Pro-TKS/ $\Delta$ Ca1 (data not shown). Both proteins were rapidly autoprocessed into the complex between Tkpro and the mature domain within 2 min at 60 °C. These results indicate that the removal of the  $\text{Ca}^{2+}$  ion from the Ca6 or Ca7 site does not seriously affect the maturation rate of Pro-TKS, while the removal of the Ca1 binding loop greatly decreases the rate of degradation of Tkpro and thereby decreases the rate of maturation of Pro-TKS.

**Enzymatic Activity.** To examine whether the deletion of the Ca1 binding loop and the mutations at the Ca6 and Ca7 sites affect the enzymatic activity of Tk-subtilisin, the enzymatic activities of Tk-subtilisin/ $\Delta$ Ca1 refolded in the presence of  $\text{Ca}^{2+}$  ions and Tk-subtilisin/ $\Delta$ Ca6 and Tk-subtilisin/ $\Delta$ Ca7 that matured from their pro forms were determined using azocasein as a substrate. Tk-subtilisin/ $\Delta$ Ca1 is refolded in the presence of  $\text{Ca}^{2+}$  ions and in the absence of Tkpro, because it does not mature from Pro-TKS/ $\Delta$ Ca1 at 60 or 80 °C as mentioned above. Pro-TKS/ $\Delta$ Ca1 is rapidly autoprocessed into the complex between Tkpro and Tk-subtilisin/ $\Delta$ Ca1, but the subsequent degradation of Tkpro is too slow to be completed within 1 h at 80 °C and 4 h at 60 °C. As a result, Pro-TKS/ $\Delta$ Ca1 exhibited little activity upon incubation at 80 °C for 1 h or 60 °C for 4 h for maturation. In contrast, Pro-TKS/ $\Delta$ Ca6 and Pro-TKS/ $\Delta$ Ca7, as well as Pro-TKS, exhibit maximal activities upon incubation at 80 °C for 30 min for maturation because of the release of an active mature form. Tk-subtilisin/ $\Delta$ Ca1 refolded in the presence of  $\text{Ca}^{2+}$  ions represents its active form, because the amount of protein decreased to approximately 10% of the original upon incubation at 30 °C for 30 min (data not shown). It has been reported for Tk-subtilisin that only 5% of the protein is refolded into an active form upon incubation at 80 °C for 30 min in the presence of the  $\text{Ca}^{2+}$  ions



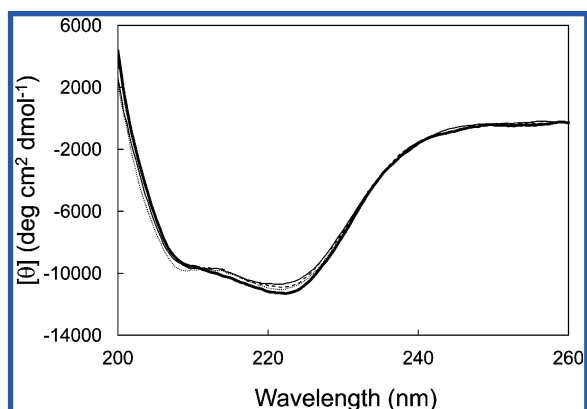
**Figure 2.** Crystal structure of Pro-S324A/ΔCa6. (A) Stereoview of the structure of Pro-S324A/ΔCa6 superimposed on that of Pro-S324A (PDB entry 2E1P). For the structure of Pro-S324A/ΔCa6, the propeptide and mature domains are colored red and green, respectively. The  $\text{Ca}^{2+}$  ions (Ca1–Ca5) are shown as cyan spheres. Two active site residues (Asp115 and His153) and Ala324 are represented as yellow sticks, in which the oxygen and nitrogen atoms are colored red and blue, respectively. N and C represent the N- and C-termini, respectively. The positions of  $\alpha 9\text{m}$ - and  $\alpha 10\text{m}$ -helices are indicated. For the structure of Pro-S324A, the entire molecule is colored gray, and the  $\text{Ca}^{2+}$  ions (Ca1–Ca6) are shown as gray spheres. (B) Relative B factors of residues 360–398 in the structures of Pro-S324A (●) and Pro-S324A/ΔCa6 (○). The relative B factor is calculated by dividing the B factor of each residue by the average of all residues.

and absence of Tkpro, because the Tk-subtilisin molecules that are activated later are rapidly degraded by those that are activated earlier.<sup>31</sup>

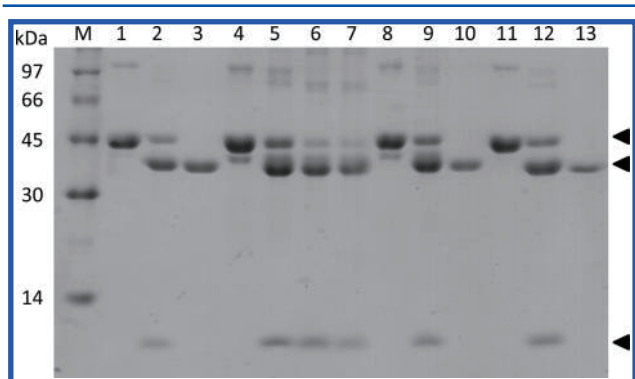
The temperature dependencies of the enzymatic activities of Tk-subtilisin and its derivatives lacking the Ca1, Ca6, or Ca7 ion were determined using azocasein as a substrate (Figure 5). The optimal temperatures for activity of the Tk-subtilisin derivatives are shifted downward by 30 °C for Tk-subtilisin/ΔCa1 and 10 °C for Tk-subtilisin/ΔCa6 and Tk-subtilisin/ΔCa7 as compared to that of Tk-subtilisin, which was previously determined to be 90 °C.<sup>31</sup> The specific activities of Tk-subtilisin/ΔCa6 ( $3700 \pm 200$  units/mg) and Tk-subtilisin/ΔCa7 ( $3300 \pm 100$  units/mg) were comparable to that of Tk-subtilisin ( $3300 \pm 150$  units/mg) at 80 °C, while the

specific activity of Tk-subtilisin/ΔCa1 ( $1000 \pm 50$  units/mg) was lower than that of Tk-subtilisin ( $2000 \pm 100$  units/mg) by 50% at 60 °C. The activities of these proteins decreased at the temperatures higher than the optimal ones probably because of the thermal denaturation of these proteins during the assay. These results indicate that all of the Tk-subtilisin derivatives, especially Tk-subtilisin/ΔCa1, are less stable than Tk-subtilisin. These results also indicate that the removal of the Ca6 or Ca7 ion does not seriously affect the enzymatic activity of Tk-subtilisin, while the removal of the Ca1 ion reduces it by approximately 50%.

**Thermal Stability.** The stabilities of Pro-S324A, Pro-S324A/ΔCa1, and Pro-S324A/ΔCa6 were analyzed using differential scanning calorimetry (DSC). It is expected that



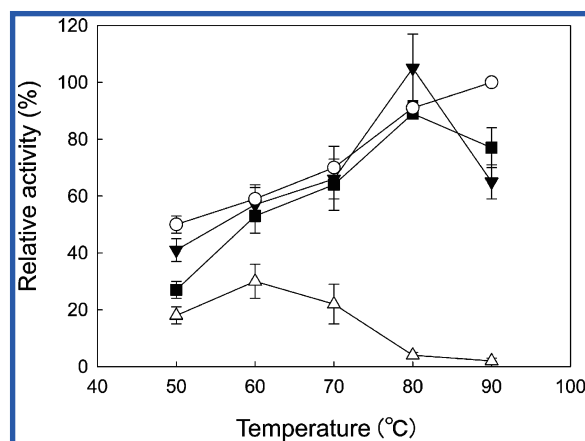
**Figure 3.** Far-UV CD spectra of Pro-S324A (thin solid line), Pro-S324A/ΔCa1 (thick solid line), Pro-S324A/ΔCa6 (dotted line), and Pro-S324A/ΔCa7 (dashed line). The spectra were recorded at 20 °C and pH 7.0 in the presence of 10 mM CaCl<sub>2</sub>.



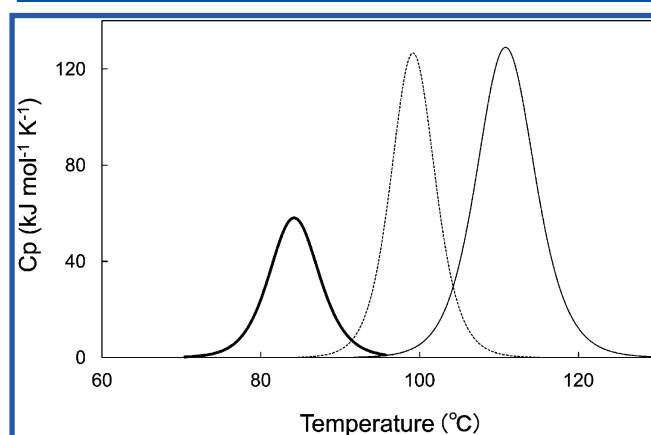
**Figure 4.** Maturation of Pro-TKS and its derivatives at 80 °C. Pro-TKS (lanes 1–3), Pro-TKS/ΔCa1 (lanes 4–7), Pro-TKS/ΔCa6 (lanes 8–10), and Pro-TKS/ΔCa7 (lanes 11–13) (0.3 μM each) were incubated in 1 mL of 50 mM CAPS-NaOH (pH 9.5) containing 10 mM CaCl<sub>2</sub> and 1 mM DTT at 80 °C for 2 min (lanes 2, 5, 9, and 12), 30 min (lanes 3, 6, 10, and 13), or 60 min (lane 7), precipitated via addition of 120 μL of trichloroacetic acid (100% w/v), and subjected to 12% Tricine SDS–PAGE. The protein was stained with CBB. Lanes 1, 4, 8, and 11 contained the sample without exposure to the buffer containing Ca<sup>2+</sup> ions. Lane M contained the low-molecular weight markers (GE Healthcare). The arrowheads indicate Pro-TKS or its derivative, Tk-subtilisin or its derivative, and Tkpro from top to bottom, respectively. The molecular mass of each standard protein is indicated along the gel.

the difference in stability between Pro-S324A and its derivative reflects that between their mature domains. The thermal unfolding curves of these proteins are shown in Figure 6. From these curves, the melting temperatures ( $T_m$ ) were determined to be 110.9 °C for Pro-S324A, 84.3 °C for Pro-S324A/ΔCa1, and 99.2 °C for Pro-S324A/ΔCa6. Thus, the stabilities of Pro-S324A/ΔCa1 and Pro-S324A/ΔCa6 were decreased by 26.6 and 11.7 °C in  $T_m$ , respectively, as compared to that of Pro-S324A. These results indicate that both of the Ca1 and Ca6 ions greatly contribute to the stabilization of Tk-subtilisin, but the Ca1 ion more significantly contributes to it than does the Ca6 ion.

The stabilities of MIP-Tk-subtilisin, MIP-Tk-subtilisin/ΔCa6, and MIP-Tk-subtilisin/ΔCa7 were analyzed by incubating the protein at 95 °C and determining the amount of the protein resistant to thermal denaturation with appropriate intervals. A plot of the amount of this protein as a function of



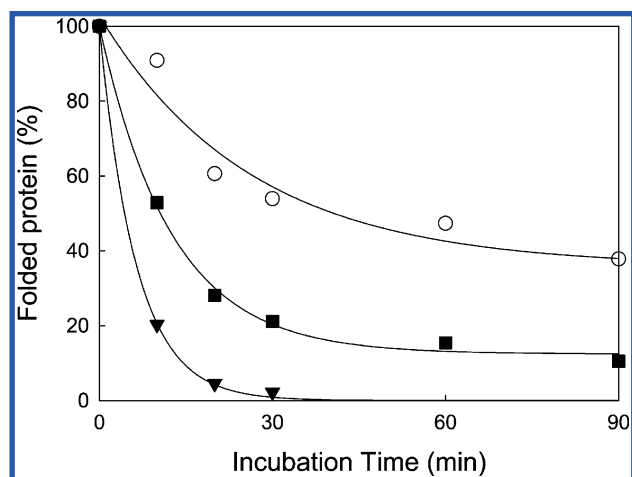
**Figure 5.** Temperature dependencies of the enzymatic activities of Tk-subtilisin (○), Tk-subtilisin/ΔCa1 (△), Tk-subtilisin/ΔCa6 (▼), and Tk-subtilisin/ΔCa7 (■) are shown. The enzymatic activities were determined at the temperatures indicated by using azocasein as a substrate, as described in Materials and Methods. The enzymatic activities relative to that of Tk-subtilisin at 90 °C are shown. The experiment was conducted at least twice, and the average values are shown, together with error bars.



**Figure 6.** DSC curves of Pro-S324A (thin solid line), Pro-S324A/ΔCa1 (thick solid line), and Pro-S324A/ΔCa6 (dashed line) were measured at a scan rate of 1 °C/min. The proteins were dissolved in 50 mM Tris-HCl (pH 8.5) at a concentration of 0.3 mg/mL.

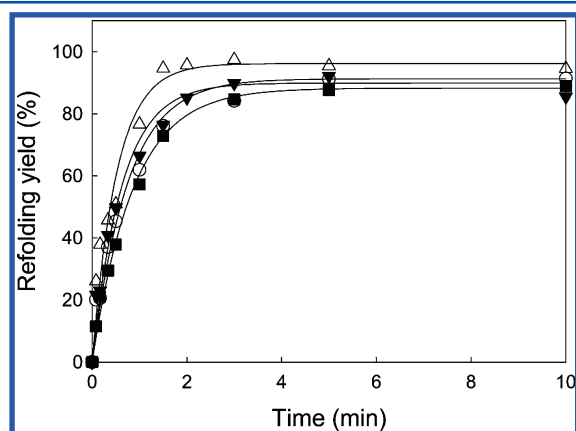
time is shown in Figure 7. From the fitting curves, the half-lives ( $T_{1/2}$ ) of Tk-subtilisin, MIP-Tk-subtilisin/ΔCa6, and Tk-subtilisin/ΔCa7 were estimated to be 40, 5, and 10 min, respectively. This result indicates that the Ca7 ion also contributes to the stabilization of Tk-subtilisin, but less significantly than does the Ca6 ion. Thus, the Ca1 ion most significantly contributes to the stabilization of Tk-subtilisin. The stability of Pro-S324A/ΔCa7 was not analyzed by DSC, because the Ca7 ion is removed from Pro-S324A upon dialysis against the Ca<sup>2+</sup>-free buffer regardless of whether the Ca7 site is mutated,<sup>33,38</sup> and all protein samples were dialyzed against the Ca<sup>2+</sup>-free buffer prior to the DSC measurement. The Ca7 ion can bind to Pro-S324A in the presence of 10 mM CaCl<sub>2</sub>.<sup>38</sup> However, the stability of Pro-S324A cannot be analyzed by DSC in the presence of 10 mM CaCl<sub>2</sub> because of the generation of a precipitate. All other Ca<sup>2+</sup> ions keep binding to Pro-S324A upon extensive dialysis against the Ca<sup>2+</sup>-free buffer.<sup>33,38</sup> The Ca7 ion tightly binds to MIP-Tk-subtilisin, such that it is not removed from the protein upon dialysis against the Ca<sup>2+</sup>-free buffer.<sup>38</sup>





**Figure 7.** Stability against heat denaturation. MIP-Tk-subtilisin (○), MIP-Tk-subtilisin/ΔCa6 (▼), and MIP-Tk-subtilisin/ΔCa7 (■) were incubated at 95 °C for heat denaturation. The amount of the protein, which is not thermally denatured and is therefore resistant to chymotryptic digestion, is shown as a function of incubation time. The amount of this protein was estimated from the intensity of the band visualized with CBB staining following SDS–PAGE at the time indicated. The line represents the optimal fit to the data.

**Refolding Rate.** Refolding of Pro-S324A and its derivatives was kinetically analyzed by terminating the refolding reaction at appropriate intervals and determining the amount of the correctly folded protein. A plot of the amount of this protein as a function of time is shown in Figure 8. The refolding curves of



**Figure 8.** Refolding curves. The refolding yields of Pro-S324A (○), Pro-S324A/ΔCa1 (△), Pro-S324A/ΔCa6 (▼), and Pro-S324A/ΔCa7 (■) are shown as a function of incubation time. These proteins were denatured, refolded with dilution, and incubated at 30 °C. With appropriate intervals, the refolded proteins were digested with chymotrypsin and subjected to 12% SDS–PAGE as described in Materials and Methods. The refolding yield was calculated by estimating the amount of correctly refolded protein from the intensity of the band visualized with CBB staining following SDS–PAGE. The line represents the optimal fit to the data.

Pro-S324A and its derivatives could be fit to a single-exponential process with rate constants of 1.3 min<sup>−1</sup> for Pro-S324A, 1.9 min<sup>−1</sup> for Pro-S324A/ΔCa1, 1.6 min<sup>−1</sup> for Pro-S324A/ΔCa6, and 1.2 min<sup>−1</sup> for Pro-S324A/ΔCa7. These results indicate that the refolding rate of Pro-S324A is not seriously changed by the removal of the Ca6 or Ca7 ion but is slightly increased by the removal of the Ca1 ion.

## DISCUSSION

In this study, we show that the Ca1, Ca6, and Ca7 sites are important for the stability of Tk-subtilisin. None of these sites is required for protein folding. The roles of the Ca1, Ca6, and Ca7 sites are individually discussed in more detail below.

**Ca1 Site.** The Ca1 site is highly conserved in various subtilisin-like serine proteases (subtilases), which are widely present in bacteria, archaea, and eukaryotes.<sup>1</sup> This site is relatively well buried inside the protein molecule and therefore allows very slow dissociation of the Ca<sup>2+</sup> ion from this site, resulting in the high-affinity Ca<sup>2+</sup>-binding site.<sup>25</sup> Upon removal of the Ca<sup>2+</sup> ion from this site, subtilisins are greatly destabilized or denatured and thereby often inactivated.<sup>4,26,28</sup> The finding that the removal of the Ca<sup>2+</sup> ion from the Ca1 site of Tk-subtilisin greatly destabilizes the protein by 26.6 °C in *T<sub>m</sub>* is consistent with these results, although the extent of the decrease in the *T<sub>m</sub>* values varies for different proteins. For example, the deletion of the Ca1 binding loop (residues 75–83) of subtilisin BPN' destabilizes the protein by only 8 °C in *T<sub>m</sub>*.<sup>28</sup> According to the crystal structure of Tk-subtilisin,<sup>33</sup> the Ca1 ion is heptacoordinated with the four residues located in the Ca1 binding loop, Asp124 (bidentate), and Gln84. Because Gln84 is located close to the N-terminus of the protein (Ser79), the Ca1 ion probably stabilizes the structure of Tk-subtilisin by anchoring the N-terminal region of the protein to the core region. It has been reported that the nine N-terminal residues (Gly70–Gly78) of the mature domain are truncated during the last step of the maturation process of Pro-TKS (degradation of Tkpro).<sup>38</sup>

The removal of the Ca<sup>2+</sup> ion from the Ca1 site seriously affects the maturation of Pro-TKS, while the removal of the Ca<sup>2+</sup> ion from the Ca6 or Ca7 site does not seriously affect it. Pro-TKS/ΔCa1 is rapidly autoprocessed into the complex between Tkpro and Tk-subtilisin/ΔCa1, but the subsequent degradation of Tkpro by Tk-subtilisin/ΔCa1 does not occur at 80 °C because of the marked instability of Tk-subtilisin/ΔCa1. At this temperature, Tk-subtilisin/ΔCa1 may be correctly folded in a pro form but thermally denatured when it is separated from Tkpro. The observation that the *T<sub>m</sub>* value of Pro-S324A/ΔCa1 is 84.3 °C (Figure 6) and Tk-subtilisin/ΔCa1 exhibits little activity at 80 °C (Figure 5) supports this proposal. The degradation of Tkpro by Tk-subtilisin/ΔCa1 does not occur even at 60 °C, at which Tk-subtilisin/ΔCa1 exhibits 50% of the activity of Tk-subtilisin, probably because this activity is not sufficient to facilitate degradation of Tkpro. Tkpro is a substrate and a potent inhibitor of Tk-subtilisin at the same time. Tkpro is probably degraded by Tk-subtilisin when it is dissociated from Tk-subtilisin. However, if the activity of Tk-subtilisin is reduced, Tkpro may not be effectively degraded by Tk-subtilisin and therefore may keep binding to Tk-subtilisin. *T. kodakarensis* grows at temperatures ranging from 60 to 100 °C, with the optimum at approximately 85 °C,<sup>44</sup> suggesting that the Ca1 site is essential for maturation of Pro-TKS at the growth temperatures of the source organism. The Ca1 site is apparently the most important Ca<sup>2+</sup>-binding site for the stability of Tk-subtilisin.

The removal of the Ca1 binding loop decreases the enzymatic activity of Tk-subtilisin by 50%. This loop is located between the α5m-helix and the β3m-strand. In addition, the loop containing Asp124, which is one of the ligands of the Ca1 ion, is located between the β1m-strand and the α3m-helix. Because the α5m-helix and β1m-strand contain the catalytically

essential aspartic acid (Asp115) and histidine (His154) residues, respectively, the conformations of these active site residues may be slightly altered by the deletion of the Ca1 binding loop such that they are not optimal for activity. It has been reported for the active site mutant of subtilisin BPN', in which the catalytically essential serine residue is replaced with Cys, that the deletion of the corresponding loop does not seriously affect the activity.<sup>28</sup> However, the enzymatic activity of this mutant protein is only 0.003% of that of the wild-type protein, which may not be sufficient to determine the difference in activity between the parent and mutant proteins.

It is noted that the refolding rate of Pro-S324A/ $\Delta$ Ca1 significantly increases compared to that of Pro-S324A (Figure 8). A similar phenomenon is observed for subtilisin BPN'.<sup>25,28,45</sup> The refolding rate of subtilisin BPN' is significantly accelerated by the removal of the Ca1 ion. As a result, the mutant protein lacking the Ca1 ion can be folded even in the absence of its cognate propeptide. Because the Ca1 ion is relatively well buried inside the protein molecule of subtilisin, the dissociation and association of this ion appear to require a transient disruption of interactions in the proximity of the Ca1 site, such as hydrogen bonds between the N-terminal loop and the Ca1 binding loop, resulting in the slow kinetics of Ca1 binding. These interactions are mostly conserved in the structure of Tk-subtilisin. Hence, the Ca1 site is probably important for the kinetic stability of Tk-subtilisin, and the removal of the  $\text{Ca}^{2+}$  ion from this site may decrease the kinetic barrier for folding at the cost of thermal stability.

**Ca6 Site.** The removal of the  $\text{Ca}^{2+}$  ion from the Ca6 site destabilizes the protein by 11.7 °C in  $T_m$ . Unlike the Ca1 ion, the Ca6 ion is coordinated by the amino acid residues located in the single surface loop between  $\alpha 9m$ - and  $\alpha 10m$ -helices. Bacterial subtilisins also have a surface loop at the same position. However, these loops do not contain the  $\text{Ca}^{2+}$  ions. Introduction of two aspartic acid residues corresponding to Asp372 and Asp379 of Tk-subtilisin and insertion of three residues corresponding to Thr376–Trp378 of Tk-subtilisin into this loop seem to be responsible for binding of the  $\text{Ca}^{2+}$  ion. According to the crystal structure of Pro-S324A/ $\Delta$ Ca6, the removal of the Ca6 ion increases the flexibility of the Ca6 binding loop, suggesting that the Ca6 ion contributes to the stabilization of Tk-subtilisin by decreasing the flexibility of the Ca6 binding loop. Interestingly, subtilisin sphericase (Sph) from a mesophile and subtilisin S41 from a psychrophile also have the corresponding  $\text{Ca}^{2+}$ -binding sites.<sup>15,21</sup> These proteases have four additional  $\text{Ca}^{2+}$ -binding sites, which are conserved between them but are not conserved in Tk-subtilisin. The roles of these  $\text{Ca}^{2+}$ -binding sites remain unknown. However, the  $\text{Ca}^{2+}$  ion bound to the site corresponding to the Ca6 site of Tk-subtilisin probably contributes to the stabilization of Sph and subtilisin S41 and thereby decreases their sensitivity to autolysis by decreasing the flexibility of the loop, as does the Ca6 ion of Tk-subtilisin.

**Ca7 Site.** The removal of the  $\text{Ca}^{2+}$  ion from the Ca7 site decreases the half-life of Tk-subtilisin at 95 °C by 4-fold, indicating that the Ca7 ion contributes to the stabilization of Tk-subtilisin. The Ca7 site is unstable in the un-autoprocessed form of Pro-TKS but is greatly stabilized upon autoprocessing of Pro-TKS.<sup>38</sup> Formation of the hydrogen bond between Asp315 O and Gln81 N<sup>e2</sup> appears to contribute to this stabilization, because Asp315 O <sup>$\delta 1$</sup>  is one of the ligands of the Ca7 ion, and because this hydrogen bond is not formed in the un-autoprocessed form of Pro-TKS, in which the side chain of

Gln81 faces in the opposite direction of Asp315 O. When the Ca7 ion is forced to bind to the un-autoprocessed form of Pro-TKS, the N-terminal loop of the mature domain (Gly70–Thr80) is disordered. On the basis of these results, it has been proposed that the Ca7 ion is required to promote the autoprocessing reaction. However, the autoprocessing rate of Pro-TKS/ $\Delta$ Ca7 seems to be comparable to that of Pro-TKS (Figure 4). Thus, the Ca7 ion specifically stabilizes the autoprocessed and mature forms of Pro-TKS but does not promote the autoprocessing reaction. The Ca7 site is located close to the Ca1 site and shares the same surface loop, in which the ligands for the Ca1 ion (Asp121) and Ca7 ion (Asp124) are present. Therefore, binding of the  $\text{Ca}^{2+}$  ion to the Ca7 site may stabilize the Ca1 site and thereby makes the association and dissociation rates of the Ca1 ion slow, resulting in a high kinetic barrier for folding and unfolding of Tk-subtilisin.

## CONCLUSION

Our finding that three of the seven  $\text{Ca}^{2+}$  ions (Ca1, Ca6, and Ca7) are not required for folding but are required for the stability of Tk-subtilisin, together with the previous finding that four other  $\text{Ca}^{2+}$  ions (Ca2–Ca5) are not required for stability but are required for folding of Tk-subtilisin,<sup>40</sup> indicates that each  $\text{Ca}^{2+}$  ion contributes either to the stabilization of the protein or to the increase in the folding rate of the protein. The source organism of Tk-subtilisin, *T. kodakarensis*, was isolated from sediments and seawater samples from a solfatara,<sup>44</sup> suggesting that the concentration of the  $\text{Ca}^{2+}$  ion in its growing environment is similar to that in seawater (10 mM). Because of the abundance of this ion, Tk-subtilisin probably utilizes the  $\text{Ca}^{2+}$  ions to reduce the high kinetic barrier for folding and at the same time to acquire hyperthermostability, and thereby to adapt to the high-temperature environment. In fact, the Tk-subtilisin derivative lacking the Ca1 ion does not mature from its pro form at the growth temperatures of *T. kodakarensis* because of its instability and decreased activity.

## ASSOCIATED CONTENT

### Accession Codes

Coordinates of Pro-S324A/ $\Delta$ Ca6 were deposited as Protein Data Bank entry 3VHQ.

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### Notes

The authors declare no competing financial interest.



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## ■ ABBREVIATIONS

Tk-subtilisin, subtilisin homologue from *T. kodakarensis* (Gly70–Gly398); Pro-TKS, Tk-subtilisin in a pro form (Gly1–Gly398); Pro-S324A, Pro-TKS with the Ser324 → Ala mutation; Pro-TKS/ΔCa1 (Pro-S324A/ΔCa1), Pro-TKS (Pro-S324A) with the deletion of the Ca1 binding loop (Leu164–Gly172); Pro-TKS/ΔCa6 (Pro-S324A/ΔCa6), Pro-TKS (Pro-S324A) with the Asp372 → Ala mutation; Pro-TKS/ΔCa7 (Pro-S324A/ΔCa7), Pro-TKS (Pro-S324A) with the Asp121 → Ala, Asp314 → Gly, and Asp315 → Gly mutations; MIP-Tk-subtilisin, monoisopropylphospho-Tk-subtilisin; Tkpro, propeptide of Tk-subtilisin (Gly1–Leu69); Suc-AAPF-pNA, N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide; CD, circular dichroism; TCA, trichloroacetic acid; DSC, differential scanning calorimetry; PDB, Protein Data Bank.

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