Ca^{2+}-Dependent Maturation of Subtilisin from a Hyperthermophilic Archaeon, *Thermococcus kodakaraensis*: the Propeptide Is a Potent Inhibitor of the Mature Domain but Is Not Required for Its Folding

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Subtilisin from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 is a member of the subtilisin family. *T. kodakaraensis* subtilisin in a proform (*T. kodakaraensis* pro-subtilisin), as well as its propeptide (*T. kodakaraensis* propeptide) and mature domain (*T. kodakaraensis* mat-subtilisin), were independently overproduced in *E. coli*, purified, and biochemically characterized. *T. kodakaraensis* pro-subtilisin was inactive in the absence of Ca^{2+} but was activated upon autoprocessing and degradation of propeptide in the presence of Ca^{2+} at 80°C. This maturation process was completed within 30 min at 80°C but was bound at an intermediate stage, in which the propeptide is autoprocessed from the mature domain (*T. kodakaraensis* mat-subtilisin*) but forms an inactive complex with *T. kodakaraensis* mat-subtilisin*, at lower temperatures. At 80°C, approximately 30% of *T. kodakaraensis* pro-subtilisin was autoprocessed into *T. kodakaraensis* propeptide and *T. kodakaraensis* mat-subtilisin*, and the other 70% was completely degraded to small fragments. Likewise, *T. kodakaraensis* mat-subtilisin was inactive in the absence of Ca^{2+} but was activated upon incubation with Ca^{2+} at 80°C. The kinetic parameters and stability of the resultant activated protein were nearly identical to those of *T. kodakaraensis* mat-subtilisin*, indicating that *T. kodakaraensis* mat-subtilisin does not require *T. kodakaraensis* propeptide for folding. However, only ~5% of *T. kodakaraensis* mat-subtilisin was converted to an active form, and the other part was completely degraded to small fragments. *T. kodakaraensis* propeptide was shown to be a potent inhibitor of *T. kodakaraensis* mat-subtilisin* and noncompetitively inhibited its activity with a K_i of 25 ± 3.0 nM at 20°C. *T. kodakaraensis* propeptide may be required to prevent the degradation of the *T. kodakaraensis* mat-subtilisin molecules that are activated later by those that are activated earlier.

Subtilisin-like serine proteases (subtilases) are widely distributed in various organisms, including bacteria, archaea, and eucaryotes (48). They are divided into six families (47). Of these, the structures and functions of the subtilisin family (EC 3.4.21.108), which is represented by subtilisin E from *Bacillus subtilis* (52), subtilisin BPN′ from *Bacillus amyloliquefaciens* (61), and subtilisin Carlsberg from *Bacillus licheniformis* (25), have been most extensively studied. The crystal structures of these subtilisins have been determined (26, 54, 64). Because subtilisins are commercially valuable enzymes, extensive attempts to improve their activities and stabilities with protein-engineering technology have also been made (10, 55, 60). The subtilisin family includes subtilisins from (hyper)thermophiles (13, 27, 28, 35) and psychrophiles (3, 14, 29, 37). The crystal structures of some of them have been determined (2, 4, 50, 57). These thermostable and thermolabile subtilisins have been regarded not only as good models for studying stability-activity-structure relationships of proteins, but also as potential candidates for various biotechnological applications.

Bacterial subtilisins are synthesized in a precursor form called pre-pro-subtilisin, in which a presequence (signal peptide) and a prosequence (propeptide) are attached to the N terminus of the mature domain (47). They are secreted in a proform (pro-subtilisin) with the assistance of a signal peptide and activated upon autoprocessing and degradation of the propeptide (45). Propeptide binds to its cognate mature domain with high affinity and thereby inhibits its activity (24, 31). According to the crystal structure of the complex between the mature domain of subtilisin and propeptide (19, 26), the C-terminal tyrosine residue of the propeptide binds to the active site of the enzyme in a product-like manner. Thus, degradation of the propeptide is required to produce active subtilisin (65). It has been proposed that propeptides of bacterial subtilisins function not only as inhibitors of their cognate mature domains, but also as intramolecular (12, 15, 17, 30, 31, 40, 43, 44, 46, 58, 65–68) and intermolecular (40, 68) chaperones that facilitate folding of the mature domains. The mature domains alone are not folded into an active form but are folded into an inactive form with molten-globular structure in the absence of propeptides (15, 44). The requirement for a propeptide for maturation of its cognate mature domain has also been reported, not only for other members of the subtilase family (5, 7, 34), but also for other proteases (33, 38, 39, 49, 51, 62). However, it remains to be determined whether archaeal subtilisins mature in a similar manner.

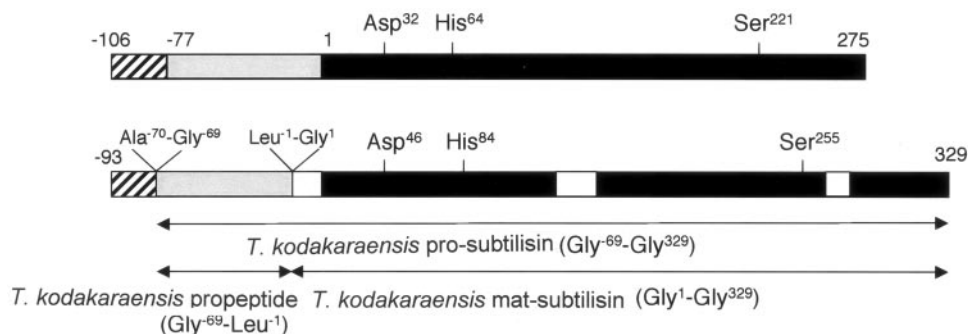*T. kodakaraensis* subtilisin from the hyperthermophilic ar-

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FIG. 1. Schematic representations of the primary structures of pre-pro-subtilisin E (top) and pre-*T. kodakaraensis* pro-subtilisin (bottom). The hatched box represents a signal peptide, the gray box represents a propeptide, the dark box represents a mature domain, and the open box represents an insertion sequence. The signal peptide, propeptide, and mature domain of pre-*T. kodakaraensis* pro-subtilisin are putative. The locations of the active-site residues and the regions of the recombinant proteins analyzed in this study are also shown.

chaeon *Thermococcus kodakaraensis* KOD1 is a member of the subtilisin family (28). It consists of a putative signal peptide (Met^{-93}-Ala^{-70}), a propeptide (Gly^{-69}-Leu^{-1}), and a mature domain (Gly^1-Gly^{329}) (Fig. 1). The putative mature domain contains three insertion sequences compared to those of bacterial subtilisins. This domain without three insertion sequences, which consists of 271 amino acid residues, is similar to bacterial subtilisins in size and shows amino acid sequence identities of 45 to 46% to subtilisin E, subtilisin BPN′, and subtilisin Carlsberg. Nevertheless, it has been proposed that *T. kodakaraensis* subtilisin exhibits enzymatic activity in a proform, because activity staining of the gel following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated that *T. kodakaraensis* pro-subtilisin, which was overproduced in *Escherichia coli* in inclusion bodies, solubilized by 8 M urea, and refolded, exhibited enzymatic activity (28). However, it remains to be determined whether a propeptide of *T. kodakaraensis* subtilisin is removed from *T. kodakaraensis* pro-subtilisin during activity staining of the gel. If the propeptide were removed from *T. kodakaraensis* pro-subtilisin during activity staining of the gel, the mature form of the protein would exhibit activity at the position to which *T. kodakaraensis* pro-subtilisin migrates in the gel. In this report, we show that *T. kodakaraensis* pro-subtilisin, which was refolded in the absence of Ca^{2+}, was activated upon autoprocessing and degradation of its propeptide in the presence of Ca^{2+} at high temperatures. Overproduction, purification, and characterization of its propeptide and mature domain indicated that the propeptide inhibits the activity of the mature form of *T. kodakaraensis* subtilisin but is not required for its folding. Based on these results, we discuss the maturation process of *T. kodakaraensis* subtilisin.

MATERIALS AND METHODS

Cells and plasmids. *E. coli* BL21-CodonPlus(DE3)-RIL [F$^-$ *ompT hsdS*(r$_B^-$ m$_B^-$) *dcm$^+$* Tet^r *gal* λ(DE3) *endA* Hte (*argU ileY leuW* Cam^r)] was from Stratagene. Plasmid pET25b was from Novagen. Subtilisin E was kindly donated by Takara Shuzo Co., Ltd.

Overproduction and purification. *T. kodakaraensis* pro-subtilisin (Gly^{-69}-Pro^{329}) was overproduced in *E. coli* in inclusion bodies, solubilized in the presence of 8 M urea, refolded, and purified as described previously (28). The pET25b derivatives for overproduction of *T. kodakaraensis* propeptide (Gly^{-69}-Leu^{-1}) and *T. kodakaraensis* mat-subtilisin (Gly^1-Gly^{329}) were constructed as previously described for that of *T. kodakaraensis* pro-subtilisin. PCR primers

were designed so that the initiation codon for translation was attached to the 5′ termini of the genes encoding *T. kodakaraensis* propeptide and *T. kodakaraensis* mat-subtilisin. Overproducing strains were constructed by transforming *E. coli* BL21-CodonPlus(DE3)-RIL with these pET25b derivatives. For overproduction, these transformants were grown at 37°C in Luria-Bertani medium containing 50 μg/ml ampicillin. When the absorbance at 660 nm of the culture reached around 0.5, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture medium and cultivation was continued for an additional 4 h. The cells were then harvested and subjected to the following purification procedures.

For purification of *T. kodakaraensis* mat-subtilisin, cells were suspended in 20 mM Tris-HCl (pH 9.0), disrupted by sonication, and centrifuged at 15,000 × *g* for 30 min. The pellet was dissolved in 20 mM Tris-HCl (pH 9.0) containing 8 M urea, dialyzed against 20 mM Tris-HCl (pH 9.0) to remove the urea, and centrifuged at 15,000 × *g* for 30 min. The resultant supernatant was applied to a Hi-Trap Q (5 ml; Pharmacia Biotech) equilibrated with the same buffer. The refolded protein of *T. kodakaraensis* mat-subtilisin was eluted from the column as a single peak at an NaCl concentration of approximately 0.2 M by linearly increasing the NaCl concentration from 0 to 0.5 M in the same buffer.

For purification of *T. kodakaraensis* propeptide, cells were suspended in 20 mM Tris-HCl (pH 7.5), disrupted by sonication, and centrifuged at 15,000 × *g* for 30 min. The supernatant was dialyzed against 20 mM Tris-HCl (pH 7.5) and centrifuged at 15,000 × *g* for 30 min. The resultant supernatant was applied to a Hi-Trap SP HP (5 ml; Pharmacia Biotech) equilibrated with the same buffer. Proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in 20 mM Tris-HCl (pH 7.5). *T. kodakaraensis* propeptide was eluted as a single peak at an NaCl concentration of approximately 0.2 M and was pooled and dialyzed against 20 mM Tris-HCl (pH 9.0). After centrifugation at 15,000 × *g* for 30 min, the supernatant was applied to a Hi-Trap Q HP (5 ml; Pharmacia Biotech) equilibrated with 20 mM Tris-HCl (pH 9.0). A linear gradient of 0 to 1.0 M NaCl in the same buffer was used to elute the bound proteins. *T. kodakaraensis* propeptide was eluted at 0.2 M NaCl. After dialysis against 20 mM Tris-HCl (pH 9.0), followed by centrifugation, the protein was precipitated by adding 80% ammonium sulfate. The pellet was dissolved in 20 mM Tris-HCl (pH 9.0) and dialyzed against the same buffer.

The purities of the proteins were analyzed by Tricine–SDS-PAGE using a 15% polyacrylamide gel (http://hincklab.uthscsa.edu/html/protocols/tricine_sds .shtml), followed by staining with Coomassie brilliant blue (CBB). The N-terminal amino acid sequence of the protein was determined by a Procise automated sequencer (Perkin-Elmer model 491).

Protein concentration. The concentrations of the recombinant proteins were determined from the UV absorption at 280 nm with $A_{280}^{0.1\%}$ values (absorbance of a 1.0-mg/ml solution at 280 nm) of 1.25 for *T. kodakaraensis* pro-subtilisin, 1.47 for *T. kodakaraensis* mat-subtilisin, and 0.21 for *T. kodakaraensis* propeptide. These values were calculated by using absorption coefficients of 1,526 M^{-1} cm^{-1} for tyrosine and 5,225 M^{-1} cm^{-1} for tryptophan at 280 nm (20). The concentration of *T. kodakaraensis* mat-subtilisin*, which was produced from *T. kodakaraensis* pro-subtilisin upon autoprocessing, was estimated from the intensity of the band visualized with CBB staining following 15% Tricine–SDS-PAGE with the Scion Image program, using *T. kodakaraensis* mat-subtilisin purified from *E. coli* as the standard.

Autoprocessing of *T. kodakaraensis* pro-subtilisin. Autoprocessing of *T. kodakaraensis* pro-subtilisin was analyzed by 15% Tricine–SDS-PAGE. *T. kodakaraensis*

pro-subtilisin (0.3 μM) was incubated at 80°C in 1 ml of 50 mM CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid)-NaOH (pH 9.5) containing 5 mM CaCl₂, unless otherwise stated. At appropriate intervals, 112 μl of trichloroacetic acid (100% [wt/vol]) was added to this solution to precipitate the proteins. The resultant pellets were washed with 70% acetone, dissolved in the Tricine–SDS-PAGE sample buffer, and subjected to Tricine–SDS-PAGE. The amount of *T. kodakaraensis* mat-subtilisin∗ produced from *T. kodakaraensis* pro-subtilisin upon autoprocessing was estimated as described above.

Activity staining of gel. SDS-PAGE was carried out using a 12% polyacrylamide gel containing 0.1% gelatin. Samples (0.1 μg each) were boiled for 5 min in the presence of 3 M urea before they were loaded onto the gel. After electrophoresis, the gel was washed with 25% (vol/vol) Triton X-100 at room temperature for 30 min, incubated in 50 mM CAPS-NaOH (pH 9.5) containing 5 mM CaCl₂ at 20°C for 12 h or 80°C for 4 h, and stained with CBB. Protease bands were visualized as clear zones due to the hydrolysis of gelatin.

Enzymatic activity. The enzymatic activities of *T. kodakaraensis* subtilisin (28) and subtilisin E (56) were determined under the conditions previously reported by using azocasein (Sigma) as a substrate at various temperatures. The reaction mixture (300 μl) contained 50 mM CAPS-NaOH (pH 9.5), 5 mM CaCl₂, and 2% azocasein (for *T. kodakaraensis* subtilisin) or 50 mM Tris-HCl (pH 8.5), 1 mM CaCl₂, and 2% azocasein (for subtilisin E). The enzymatic reaction was initiated by adding an appropriate amount of the enzyme and terminated by adding 200 μl of 15% trichloroacetic acid (final concentration, 6%). The reaction time was usually 20 min. After centrifugation at 15,000 × g for 15 min, an aliquot of the supernatant (40 μl) was withdrawn, mixed with 10 μl of 2 M NaOH, and measured for absorption at 440 nm (A_{440}). One unit of enzymatic activity was defined as the amount of the enzyme that increased the A_{440} value of the assay reaction mixture by 0.1 in 1 min.

The enzymatic activity was also determined by using *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-pNA) (Sigma) as a substrate at various temperatures. The reaction mixture (100 μl) contained 50 mM CAPS-NaOH (pH 9.5), 5 mM CaCl₂, and 2 mM Suc-AAPF-pNA. The amount of *p*-nitroaniline released from the substrate was determined from the absorption at 410 nm with an absorption coefficient of 8,900 M⁻¹ cm⁻¹ by automatic UV spectrophotometer (Beckman model DU640). 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. For kinetic analyses, the concentration of Suc-AAPF-pNA was varied from 0.5 to 5 mM. The enzymatic reaction followed Michaelis-Menten kinetics, and the K_m and k_{cat} values were determined from Lineweaver-Burk plots.

Nucleotide sequence accession numbers. The GenBank accession numbers of the sequences are K01988 for pre-pro-subtilisin E and AB056701 for pre-*T. kodakaraensis* pro-subtilisin.

RESULTS

Autoprocessing of *T. kodakaraensis* pro-subtilisin. Refolded *T. kodakaraensis* pro-subtilisin was incubated at 80°C in the presence of Ca²⁺. Under these conditions, *T. kodakaraensis* pro-subtilisin was rapidly degraded into two peptides with molecular masses of 38 and 10 kDa (Fig. 2). These masses were identical to those of *T. kodakaraensis* propeptide (Gly⁻⁶⁹-Leu⁻¹) and *T. kodakaraensis* mat-subtilisin (Gly¹-Gly³²⁹) separately overproduced in *E. coli* and purified (see below for details). The N-terminal amino acid sequences of the 10- and 38-kDa peptides were determined to be GEQNT and GKPSW, respectively, which were identical to those of *T. kodakaraensis* propeptide and *T. kodakaraensis* mat-subtilisin, respectively. These results indicate that *T. kodakaraensis* pro-subtilisin is autoprocessed at Leu⁻¹-Gly¹ to produce *T. kodakaraensis* propeptide∗ and *T. kodakaraensis* mat-subtilisin∗ (marked by asterisks to distinguish them from those separately overproduced in *E. coli* and purified). The amount of *T. kodakaraensis* mat-subtilisin∗ reached the maximum within ~5 min, which was estimated to be approximately 30% of that of *T. kodakaraensis* pro-subtilisin from the intensity of the band visualized with CBB staining. It was not significantly changed upon further incubation regardless of whether *T. kodakaraensis* pro-
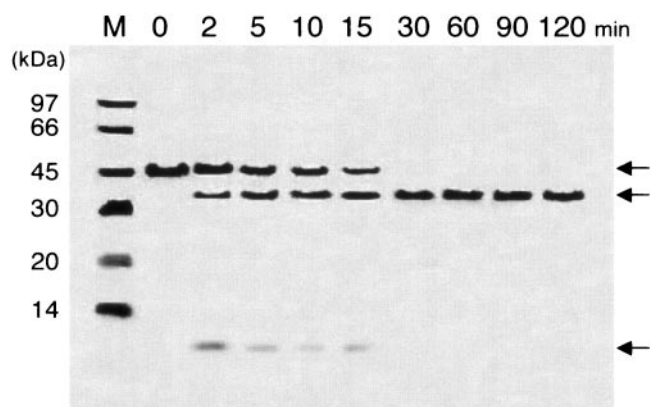

FIG. 2. Autoprocessing of *T. kodakaraensis* pro-subtilisin at 80°C. *T. kodakaraensis* pro-subtilisin (0.3 μM) was incubated in 1 ml of 50 mM CAPS-NaOH (pH 9.5) containing 5 mM CaCl₂ at 80°C for the indicated times, precipitated by adding 112 μl of trichloroacetic acid (100% [wt/vol]), and subjected to 15% Tricine–SDS-PAGE. The protein was stained with CBB. The sample precipitated at 0 min was not exposed to the buffer containing Ca²⁺. M, low-molecular-mass marker kit (Pharmacia Biotech). The arrows indicate *T. kodakaraensis* pro-subtilisin, *T. kodakaraensis* mat-subtilisin∗, and *T. kodakaraensis* propeptide∗ from the top to the bottom. The molecular mass of each standard protein is indicated beside the gel.

subtilisin was completely degraded or not, suggesting that approximately 70% of *T. kodakaraensis* pro-subtilisin was not autoprocessed into *T. kodakaraensis* propeptide∗ and *T. kodakaraensis* mat-subtilisin∗ but was fully degraded to small fragments. Moreover, *T. kodakaraensis* pro-subtilisin was not autoprocessed and remained intact when it was incubated at 80°C in the presence of 1 mM EDTA instead of 5 mM CaCl₂. This result indicates that the Ca²⁺ ion is required for autoprocessing of *T. kodakaraensis* pro-subtilisin. It is unlikely that *T. kodakaraensis* pro-subtilisin was degraded by a contaminating *E. coli* protease at such a high temperature.

Activity and stability of *T. kodakaraensis* mat-subtilisin∗. To examine whether *T. kodakaraensis* mat-subtilisin∗ represents the active form of *T. kodakaraensis* subtilisin, *T. kodakaraensis* pro-subtilisin (0.3 μM) was incubated at 80°C for 90 min in the presence of Ca²⁺. Under these conditions, both *T. kodakaraensis* pro-subtilisin and *T. kodakaraensis* propeptide∗ were completely degraded (Fig. 2). The resultant solution containing approximately 0.1 μM of *T. kodakaraensis* mat-subtilisin∗ was used to analyze the activity and stability of this protein without further purification.

The temperature dependence of the enzymatic activity of *T. kodakaraensis* mat-subtilisin∗ was analyzed by measuring the activity at various temperatures ranging from 20 to 100°C using azocasein as a substrate. For comparative purposes, the temperature dependence of the enzymatic activity of subtilisin E was also analyzed. As shown in Fig. 3, *T. kodakaraensis* mat-subtilisin∗ exhibited the highest activity at 90°C. It exhibited comparable activity even at 100°C. In contrast, subtilisin E exhibited the highest activity at 60°C. The maximum specific activity of *T. kodakaraensis* mat-subtilisin∗ (3,100 ± 240 units/mg) was sevenfold higher than that of subtilisin E. *T. kodakaraensis* mat-subtilisin∗ exhibited two- to threefold-higher activity than subtilisin E even at mild temperatures (20 to 40°C).
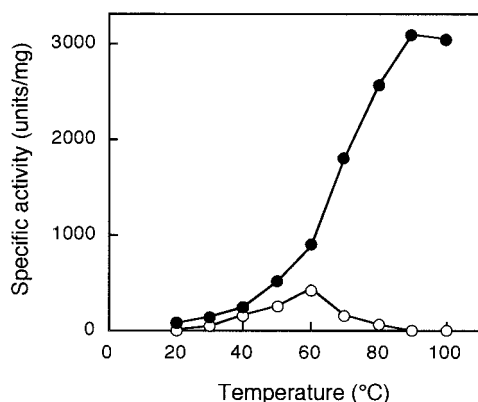
FIG. 3. Temperature dependence of enzymatic activity. The temperature dependence of the enzymatic activity of processed *T. kodakaraensis* mat-subtilisin (solid circles) is shown in comparison with that of subtilisin E (open circles). The enzymatic activities were determined at the temperatures indicated by using azocasein as a substrate, as described in Materials and Methods.

FIG. 5. Temperature dependence of maturation. *T. kodakaraensis* pro-subtilisin (0.3 μM) was incubated in 50 mM CAPS-NaOH (pH 9.5) containing 5 mM CaCl₂ at 20°C (×), 40°C (open circles), 60°C (solid circles), and 80°C (open squares). At appropriate intervals, an aliquot (10 μl) was withdrawn and the enzymatic activity at 20°C was determined using Suc-AAPF-pNA as a substrate as described in Materials and Methods. The absorbance at 410 nm, which increases as the amount of the mature protein increases, was plotted as a function of time.

The stability of *T. kodakaraensis* mat-subtilisin∗ against irreversible heat inactivation was analyzed by incubating the solution containing *T. kodakaraensis* mat-subtilisin∗ mentioned above at 80, 90, and 100°C. At appropriate intervals, an aliquot of the solution was withdrawn and the remaining activity was determined at 80°C using azocasein as a substrate. As shown in Fig. 4, *T. kodakaraensis* mat-subtilisin∗ was stable at 80°C for at least 3 h. It lost half of its activity in roughly 9 h at 90°C and in 50 min at 100°C. It has been reported that subtilisin E lost its activity even at 60°C with a half-life of 18 min (56). Thus, *T. kodakaraensis* mat-subtilisin∗ was shown to be much more stable than subtilisin E.

The kinetic constants of *T. kodakaraensis* mat-subtilisin∗ were determined using Suc-AAPF-pNA as a substrate at 20 and 80°C. The hydrolysis of this substrate with *T. kodakaraensis*
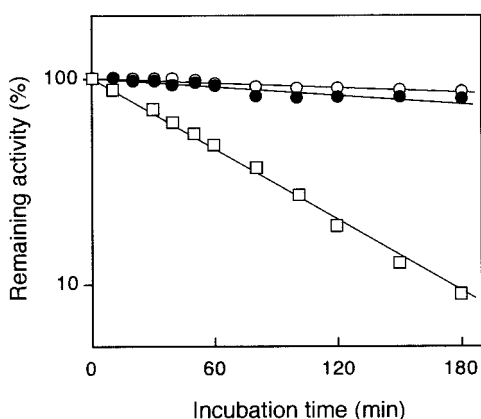
mat-subtilisin∗ followed Michaelis-Menten kinetics. The K_m and k_{cat} values were calculated to be 4.0 ± 0.5 mM and 26 ± 3.0 s⁻¹ (V_{max}, 38 ± 4.6 units/mg), respectively, at 20°C and 8.0 ± 1.0 mM and 290 ± 35 s⁻¹ (V_{max}, 420 ± 50 units/mg), respectively, at 80°C from the double-reciprocal plot $1/v$ versus $1/s$. Note that the K_m value of 8.0 ± 1.0 mM represents the apparent value, because the solubility of the substrate at pH 9.5 is too low to increase its concentration beyond 5 mM. The specific activities of *T. kodakaraensis* mat-subtilisin∗ determined at a substrate concentration of 2 mM (Suc-AAPF-pNA) were 13 ± 1.6 units/mg at 20°C and 140 ± 18 units/mg at 80°C.

Temperature dependence of maturation. To examine whether *T. kodakaraensis* pro-subtilisin matures slowly at lower temperatures, *T. kodakaraensis* pro-subtilisin was incubated at 20, 40, 60, and 80°C in the presence of Ca²⁺. The activity of the aliquot at appropriate intervals was determined at 20°C using Suc-AAPF-pNA as the substrate. The assay was done at this temperature because the maturation of the protein was expected to be too slow to proceed during assay. If the maturation of the protein did not proceed during assay, the activity of *T. kodakaraensis* pro-subtilisin relative to that determined after incubation at 80°C for 90 min, which represents the maturation yield, would increase until the maturation was completed. As shown in Fig. 5, the maturation of *T. kodakaraensis* pro-subtilisin was almost fully completed within 30 min at 80°C and 3 h at 60°C, while it was not completed within 4 h at 40°C and below. The maturation of *T. kodakaraensis* pro-subtilisin was very slow at 20°C, and only a few percent of *T. kodakaraensis* pro-subtilisin matured upon incubation at 20°C for 4 h.

To examine whether slow maturation of *T. kodakaraensis* pro-subtilisin at lower temperatures was due to slow autoprocessing, *T. kodakaraensis* pro-subtilisin was incubated at 20, 40, 60, and 80°C for 30 min in the presence of Ca²⁺. The amounts of *T. kodakaraensis* pro-subtilisin, *T. kodakaraensis* mat-subtilisin∗, and *T. kodakaraensis* propeptide∗ after autoprocessing were estimated by Tricine–SDS-PAGE. As shown in Fig. 6, the

FIG. 4. Thermal stability of *T. kodakaraensis* mat-subtilisin∗. Semilog plots of the remaining activity versus incubation time are shown. *T. kodakaraensis* mat-subtilisin∗ was incubated at 80°C (open circles), 90°C (solid circles), or 100°C (open squares) in 50 mM CAPS-NaOH (pH 9.5) containing 5 mM CaCl₂. An aliquot of the solution was withdrawn at appropriate intervals, and the enzymatic activity was determined by using azocasein as a substrate. The lines were obtained by linear regression of the data.
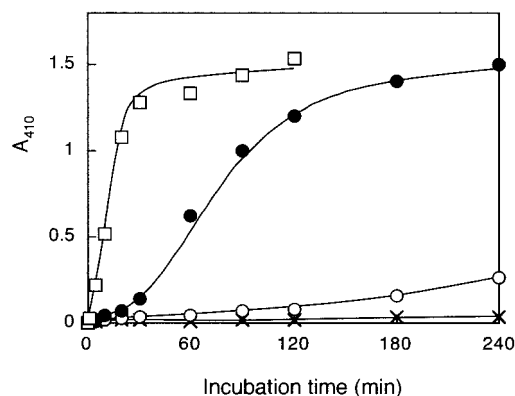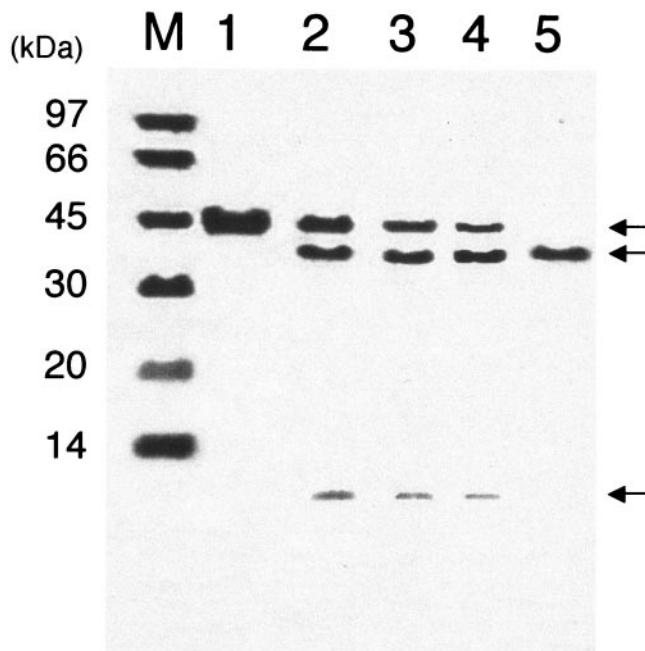
FIG. 6. Autoprocessing of *T. kodakaraensis* pro-subtilisin at various temperatures. *T. kodakaraensis* pro-subtilisin was incubated in 50 mM CAPS-NaOH (pH 9.5) containing 5 mM CaCl$_2$ at 20°C (lane 1), 40°C (lane 2), 60°C (lane 3), and 80°C (lane 4) for 30 min, and its autoprocessing was analyzed by 15% Tricine–SDS-PAGE. The protein was stained with CBB. M, low-molecular-mass marker kit (Pharmacia Biotech). The arrows indicate *T. kodakaraensis* pro-subtilisin, *T. kodakaraensis* mat-subtilisin∗, and *T. kodakaraensis* propeptide∗ from the top to the bottom. The molecular mass of each standard protein is indicated beside the gel.

amounts of *T. kodakaraensis* mat-subtilisin∗ produced at 20, 40, and 60°C were comparable to that produced at 80°C. However, as shown in Fig. 5, the maturation yields of *T. kodakaraensis* pro-subtilisin after 30 min of incubation were 0.3% at 20°C, 1.8% at 40°C, and 12% at 60°C. When the SDS-PAGE profile of *T. kodakaraensis* pro-subtilisin incubated at 80°C was compared with that at 20, 40, or 60°C, a clear difference was detected in the amounts of *T. kodakaraensis* pro-subtilisin and *T. kodakaraensis* propeptide∗. *T. kodakaraensis* pro-subtilisin and *T. kodakaraensis* propeptide∗ were completely degraded at 80°C, while they were not completely degraded at 60°C and below. These results indicate that *T. kodakaraensis* pro-subtilisin∗ requires not only autoprocessing, but also degradation of propeptide for its maturation. *T. kodakaraensis* mat-subtilisin∗ exhibits maximal activity only when *T. kodakaraensis* propeptide∗ is completely degraded. At lower temperatures, *T. kodakaraensis* propeptide∗ may continue to bind to the mature domain upon autoprocessing and thereby inhibit the activity of the mature domain.

Overproduction and purification of *T. kodakaraensis* propeptide and *T. kodakaraensis* mat-subtilisin. To analyze the role of *T. kodakaraensis* propeptide∗, strains overproducing *T. kodakaraensis* propeptide (Gly^{-69}-Leu^{-1}) and *T. kodakaraensis* mat-subtilisin (Gly^1-Gly^{329}) were constructed. *T. kodakaraensis* propeptide was overproduced in *E. coli* in a soluble form and purified to give a single band on SDS-PAGE (data not shown). *T. kodakaraensis* mat-subtilisin was overproduced in *E. coli* in
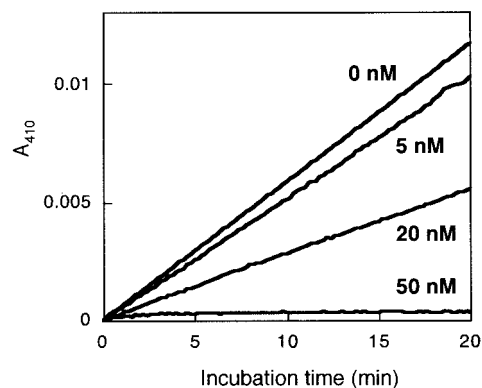

FIG. 7. Inhibition of *T. kodakaraensis* mat-subtilisin∗ by *T. kodakaraensis* propeptide. The indicated concentrations of *T. kodakaraensis* propeptide were premixed with the substrate (Suc-AAPF-pNA) before *T. kodakaraensis* mat-subtilisin∗ was added. Hydrolysis of Suc-AAPF-pNA (2 mM) by *T. kodakaraensis* mat-subtilisin∗ (0.1 nM) was analyzed at 20°C by monitoring an increase in absorbance at 410 nm, which resulted from a release of *p*-nitroaniline.

inclusion bodies, solubilized in the presence of 8 M urea, refolded in the absence of Ca^{2+}, and purified to give a single band on SDS-PAGE (see Fig. 9). Determination of the N-terminal amino acid sequences of these proteins indicated that the N-terminal methionine residue was posttranslationally removed from both proteins. The amounts of the proteins purified from 1 liter of culture were roughly 10 mg for *T. kodakaraensis* propeptide and 5 mg for *T. kodakaraensis* mat-subtilisin.

Inhibition of *T. kodakaraensis* mat-subtilisin∗ activity by propeptide. Inhibition of the enzymatic activity of *T. kodakaraensis* mat-subtilisin∗ (0.1 nM) with *T. kodakaraensis* propeptide was analyzed at 20°C using Suc-AAPF-pNA as a substrate. At this temperature, the concentration of *T. kodakaraensis* propeptide is not significantly changed during the assay. This is because *T. kodakaraensis* propeptide is not effectively cleaved by *T. kodakaraensis* mat-subtilisin∗. As shown in Fig. 7, the enzymatic activity of *T. kodakaraensis* mat-subtilisin∗ was inhibited by *T. kodakaraensis* propeptide in a concentration-dependent manner and was almost completely inhibited by 50 nM *T. kodakaraensis* propeptide. To determine the K_i value of *T. kodakaraensis* propeptide, the enzymatic activity of *T. kodakaraensis* mat-subtilisin∗ was kinetically analyzed in the presence or absence of 20 nM *T. kodakaraensis* propeptide. The kinetic constants were determined from the double-reciprocal plot $1/v$ versus $1/s$ as shown in Fig. 8. Compared to the kinetic constants of *T. kodakaraensis* mat-subtilisin∗ in the absence of *T. kodakaraensis* propeptide, the K_m value was not significantly changed (4.0 ± 0.5 mM), while the k_{cat} value was decreased from 26 ± 3.0 to 14 ± 1.8 s^{-1} in the presence of 20 nM *T. kodakaraensis* propeptide. These results indicate that *T. kodakaraensis* propeptide noncompetitively inhibits the enzymatic activity of *T. kodakaraensis* mat-subtilisin∗ with a K_i value of 25 ± 3.0 nM. A similar result was obtained when the enzymatic activity of *T. kodakaraensis* mat-subtilisin∗ was determined in the presence of 10 nM *T. kodakaraensis* propeptide (data not shown).
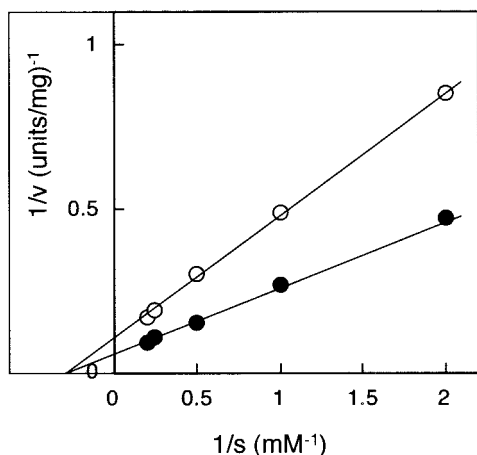
FIG. 8. Lineweaver-Burk plot. Suc-AAPF-pNA was hydrolyzed by 0.1 nM *T. kodakaraensis* mat-subtilisin∗ in the absence (solid circles) or presence (open circles) of 20 nM *T. kodakaraensis* propeptide as described in Materials and Methods, and $1/v$ was plotted versus $1/s$.

Enzymatic activity of *T. kodakaraensis* mat-subtilisin. The specific activity of refolded *T. kodakaraensis* mat-subtilisin was determined to be 130 ± 14 units/mg at 80°C using azocasein as a substrate. This value was approximately 5% of that of *T. kodakaraensis* mat-subtilisin∗. However, SDS-PAGE analysis indicated that the amount of *T. kodakaraensis* mat-subtilisin decreased to approximately 5% upon incubation with Ca^{2+} at 80°C for 30 min (Fig. 9, lane 2). This amount was not significantly changed upon further incubation for up to 2 h (data not shown). These results suggest that only 5% of the refolded
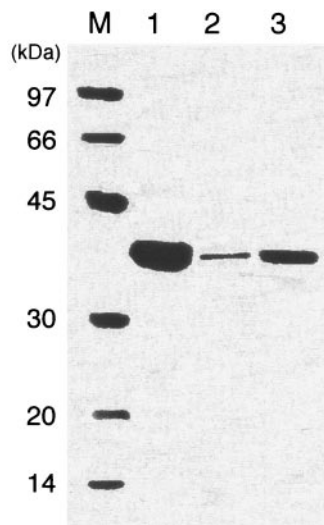

FIG. 10. Activity staining of gel. Samples (0.1 μg) were subjected to 12% SDS-PAGE using a gel containing 0.1% gelatin. After electrophoresis, the gel was washed with 25% (vol/vol) Triton X-100 at room temperature for 30 min, incubated in 50 mM CAPS-NaOH (pH 9.5) containing 5 mM $CaCl_2$ at 80°C for 4 h (lanes 1 and 2) or 20°C for 12 h (lanes 3 and 4), and stained with CBB. Protease bands were visualized as clear zones due to the hydrolysis of gelatin. Lane M, low-molecular-mass marker kit (Pharmacia Biotech); lanes 1 and 3, *T. kodakaraensis* pro-subtilisin; lanes 2 and 4, *T. kodakaraensis* mat-subtilisin. The arrows indicate *T. kodakaraensis* pro-subtilisin (top) and *T. kodakaraensis* mat-subtilisin (bottom). The molecular mass of each standard protein is indicated beside the gel.

FIG. 9. Comparison of the yields of *T. kodakaraensis* mat-subtilisin∗ in the presence and absence of *T. kodakaraensis* propeptide. Refolded *T. kodakaraensis* mat-subtilisin (0.3 μM) was incubated in 50 mM CAPS-NaOH (pH 9.5) containing 5 mM $CaCl_2$ at 80°C for 30 min in the absence (lane 2) or presence (lane 3) of *T. kodakaraensis* propeptide (0.3 μM) and subjected to 15% Tricine–SDS-PAGE. The protein was stained with CBB. Lane 1, refolded *T. kodakaraensis* mat-subtilisin without exposure to the buffer containing Ca^{2+}; M, low-molecular-mass marker kit (Pharmacia Biotech). The molecular mass of each standard protein is indicated beside the gel.
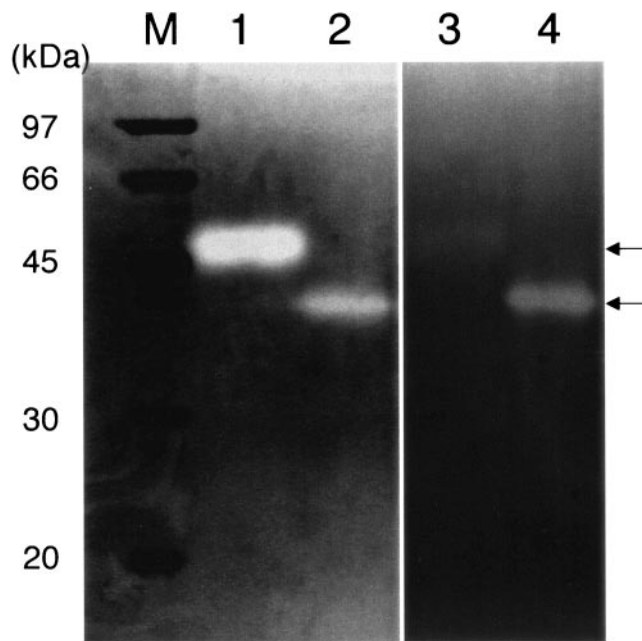
protein of *T. kodakaraensis* mat-subtilisin is converted to the active form and the other part is completely degraded to small fragments upon Ca^{2+} binding at 80°C. Therefore, refolded *T. kodakaraensis* mat-subtilisin was first incubated with Ca^{2+} at 80°C for 30 min for activation, and then the enzymatic activities of the resultant activated protein were determined at various temperatures using azocasein as a substrate. The concentration of the protein was estimated from SDS-PAGE. The protein exhibited the highest activity at 90°C, like *T. kodakaraensis* mat-subtilisin∗ (data not shown). The specific activity of the protein was determined to be $2,700 \pm 220$ units/mg at 80°C, which was comparable to that of *T. kodakaraensis* mat-subtilisin∗ ($2,600 \pm 210$ units/mg). The K_m and k_{cat} values of the protein determined at 20 and 80°C using Suc-AAPF-pNA as a substrate were nearly identical to those of *T. kodakaraensis* mat-subtilisin∗. These results indicate that the mature domain of *T. kodakaraensis* subtilisin does not require propeptide for its correct folding. This domain can be converted to *T. kodakaraensis* mat-subtilisin∗ in the absence of *T. kodakaraensis* propeptide. However, the yield of *T. kodakaraensis* mat-subtilisin∗ from refolded *T. kodakaraensis* mat-subtilisin increased by approximately five times in the presence of an equal molar concentration of *T. kodakaraensis* propeptide (Fig. 9, lane 3). The resultant yield (~25%) was slightly lower than but comparable to that from *T. kodakaraensis* pro-subtilisin (~30%). This result indicates that *T. kodakaraensis* propeptide increases

the maturation yield of *T. kodakaraensis* mat-subtilisin regardless of whether *T. kodakaraensis* propeptide is added in an isolated form or is present as an N-terminal domain of the refolded protein (*T. kodakaraensis* pro-subtilisin).

To confirm that *T. kodakaraensis* mat-subtilisin refolded in the absence of propeptide exhibits Ca^{2+}-dependent activity, *T. kodakaraensis* mat-subtilisin and *T. kodakaraensis* pro-subtilisin were subjected to SDS-PAGE, and their protease activities were analyzed by activity staining of the gel at 20 and 80°C. Both proteins exhibited activity, at the positions to which they migrated, only in the presence of Ca^{2+} (Fig. 10) and not in the absence of Ca^{2+} (data not shown). However, *T. kodakaraensis* pro-subtilisin exhibited higher and lower activities than refolded *T. kodakaraensis* mat-subtilisin at 80 and 20°C, respectively. As mentioned above, *T. kodakaraensis* pro-subtilisin exhibited activity, because it matured during activity staining of the gel. *T. kodakaraensis* pro-subtilisin exhibited very weak activity at 20°C, probably because the protein matures very slowly at this temperature. Likewise, as mentioned above, the maximal yield of *T. kodakaraensis* mat-subtilisin* produced from *T. kodakaraensis* pro-subtilisin upon incubation at 80°C (~30%) is much higher than that produced from refolded *T. kodakaraensis* mat-subtilisin (~5%). This may be the reason why *T. kodakaraensis* pro-subtilisin exhibited higher activity than refolded *T. kodakaraensis* mat-subtilisin at 80°C in activity staining of the gel.

DISCUSSION

Enzymatic properties of *T. kodakaraensis* mat-subtilisin*. *T. kodakaraensis* mat-subtilisin* is a highly stable enzyme with half-lives of 9 h at 90°C and 50 min at 100°C. However, the half-lives of the protein have previously been reported to be 20 min at 90°C and 7 min at 100°C (28). This discrepancy is probably caused by differences in the concentrations of the protein analyzed for thermal stability. In the previous work, *T. kodakaraensis* pro-subtilisin, instead of *T. kodakaraensis* mat-subtilisin*, was incubated at a concentration of 42 μg/ml (approximately 1 μM) in the presence of Ca^{2+} and analyzed for remaining activity at 80°C. In the present work, *T. kodakaraensis* mat-subtilisin* was incubated at a concentration of 4 μg/ml (0.1 μM). The susceptibility of *T. kodakaraensis* mat-subtilisin* to self-degradation may increase, and thus its stability apparently decreases, as the concentration of *T. kodakaraensis* mat-subtilisin* increases.

The K_m and k_{cat} values of *T. kodakaraensis* mat-subtilisin* were determined to be 4.0 ± 0.5 mM and 26 ± 3.0 s^{-1}, respectively, at 20°C using Suc-AAPF-pNA as a substrate. These values were comparable to those of subtilisin E at 37°C for the same substrate (K_m, 1.9 mM, and k_{cat}, 21 s^{-1}) (56). Both of these values increased at 80°C compared to those at 20°C. This indicates that the hydrolysis rate of the protein increases while the substrate-binding affinity decreases at high temperatures. The specific activity of *T. kodakaraensis* mat-subtilisin* for Suc-AAPF-pNA at 80°C was previously reported to be 3,900 units/mg (3.9 units/mg according to the definition of the present work) (28), while in the present work, it was determined to be 140 ± 18 units/mg. This discrepancy is due to a difference in the substrate concentration, which was previ-

ously 0.1 mM and is 2 mM here. The enzymatic activity decreases as the substrate concentration decreases below the K_m.

Role of Ca^{2+}. Crystallographic studies indicated that subtilisin BPN′ (36, 41) and subtilisin Carlsberg (9, 36) have two Ca^{2+} binding sites, site 1 (site A) and site 2 (site B), both of which are located far from the active site. Sites 1 and 2 represent high- and low-affinity binding sites, respectively. The enzyme is greatly destabilized with respect to both thermal denaturation and autodegradation and is thereby inactivated upon removal of Ca^{2+} from site 1 (11, 42, 59). However, pro-subtilisin E has been reported to be folded and autoprocessed in the absence of Ca^{2+} (66). In addition, deletion of the loop forming site 1 of subtilisin BPN′, followed by directed evolution and selection for increased stability, resulted in a Ca^{2+}-independent subtilisin mutant with native-like activity (11, 18, 53). These results suggest that Ca^{2+} is not required for activity of subtilisin but is required for stability.

In this report, however, it was shown that *T. kodakaraensis* pro-subtilisin requires Ca^{2+} for maturation (autoprocessing and degradation of propeptide). It was also shown that *T. kodakaraensis* mat-subtilisin requires Ca^{2+} for activity even at mild temperatures, at which this hyperthermophilic protein should be stable. These results suggest that Ca^{2+} is required to make the conformation of the mature domain of *T. kodakaraensis* subtilisin active. Various subtilases, such as sphericase from *Bacillus sphaericus* (1, 2), cell envelope proteinase from *Lactococcus lactis* (16), and psychrophilic subtilisins (14, 37), have also been reported to exhibit Ca^{2+}-dependent activities. These enzymes may also assume an active conformation only when the Ca^{2+} ions bind to the enzymes. It remains to be determined, however, whether *T. kodakaraensis* subtilisin has two or more Ca^{2+} binding sites and whether its conformation is altered upon Ca^{2+} binding. Binding of the Ca^{2+} ions has been suggested to trigger sequential concerted movements that exert long-range effects on the geometry of the catalytic site for proteinase K (6), but not for thermitase (22) and subtilisin BPN′ (41). The number and location of Ca^{2+} binding sites vary greatly for different subtilases. For example, proteinase K (8), thermitase (21), and *Bacillus* sp. strain Ak. 1 protease (50) contain four, three, and four Ca^{2+} binding sites, respectively.

Role of *T. kodakaraensis* propeptide. Unlike propeptides of bacterial subtilisins, *T. kodakaraensis* propeptide is not required for folding of the mature domain of *T. kodakaraensis* subtilisin, because the mature domain alone is refolded and exhibits Ca^{2+}-dependent activity in the absence of *T. kodakaraensis* propeptide. Refolding of the mature domain without the assistance of propeptide has been reported for subtilisin BPN′ covalently attached to agarose beads (23), a Ca^{2+}-independent subtilisin mutant (11), and a thermolysin-like neutral protease (TLP-ste) from *Bacillus stearothermophilus* (32). In the case of TLP-ste, the refolding yield of the active mature TLP-ste is not significantly changed in the presence or absence of its cognate propeptide. However, the amount of *T. kodakaraensis* mat-subtilisin* produced from *T. kodakaraensis* pro-subtilisin was approximately six times higher than that produced from refolded *T. kodakaraensis* mat-subtilisin when 0.3 μM of the refolded proteins was incubated at 80°C in the presence of Ca^{2+}. The latter amount increased by five times when *T. kodakaraensis* propeptide was added in an isolated form (Fig. 9). In addition, Ca^{2+}-free *T. kodakaraensis* mat-

subtilisin is rapidly degraded to small fragments by *T. kodakaraensis* mat-subtilisin∗ under conditions in which *T. kodakaraensis* mat-subtilisin∗ is not self-degraded (S. Tanaka, unpublished data). These results suggest that *T. kodakaraensis* propeptide is required to allow the mature domain of *T. kodakaraensis* pro-subtilisin or *T. kodakaraensis* mat-subtilisin to complete its Ca^{2+}-induced folding. In the absence of *T. kodakaraensis* propeptide, the *T. kodakaraensis* mat-subtilisin molecules, which are converted to active form earlier, may rapidly degrade those that are converted to active form later. However, the possibility that *T. kodakaraensis* propeptide is not required for folding of the mature domain but assists its effective folding cannot be ruled out.

Inhibition mode of *T. kodakaraensis* propeptide. Like the propeptides of bacterial subtilisins, *T. kodakaraensis* propeptide inhibits the activity of *T. kodakaraensis* mat-subtilisin∗ when added in *trans*. However, its inhibition mode is different from those of bacterial propeptides. The propeptides of bacterial subtilisins have been reported to be slow and tight binding inhibitors of the mature cognate, with inhibition constants (K_i) of 1.0 to 6.1 nM (24, 31). The progress curves for the inhibition of these subtilisins by their cognate propeptides reveal a hyperbolic pattern. In contrast, *T. kodakaraensis* propeptide did not exhibit slow binding inhibition at 20°C (Fig. 7). The enzymatic reaction of *T. kodakaraensis* mat-subtilisin∗ followed Michaelis-Menten kinetics in the presence of *T. kodakaraensis* propeptide. A Lineweaver-Burk plot revealed noncompetitive inhibition, suggesting that the major binding site of *T. kodakaraensis* propeptide in *T. kodakaraensis* mat-subtilisin∗ is different from the substrate-binding site. The K_i value of *T. kodakaraensis* propeptide (25 ± 3.0 nM) is comparable to that of the propeptide of thermolysin. The propeptide of thermolysin has been reported to act as a mixed, noncompetitive inhibitor of the mature cognate with a 50% inhibitory concentration of 14 nM and a K_i value of 6 to 20 nM (39). It has been noted that propeptides of bacterial subtilisins bind to the mature cognates more tightly than *T. kodakaraensis* propeptide, but the concentrations of these propeptides sufficient for complete inhibition of the mature cognates are 0.5 to 5 μM, which are at least 10 times higher than that of *T. kodakaraensis* propeptide, because of their slow binding properties. It remains to be determined whether the enzymatic activity of *T. kodakaraensis* mat-subtilisin∗ is inhibited by *T. kodakaraensis* propeptide in a slow-binding mode at higher temperatures close to the optimum, because *T. kodakaraensis* propeptide is rapidly degraded by *T. kodakaraensis* mat-subtilisin∗ at temperatures higher than 80°C. Construction of the active-site mutants may facilitate quantitative analysis of the interaction between *T. kodakaraensis* propeptide and *T. kodakaraensis* mat-subtilisin∗ at higher temperatures.

Adaptation to high temperature. The maturation process of *T. kodakaraensis* pro-subtilisin is bound at an intermediate stage of autoprocessing and degradation of propeptide at temperatures below 60°C. At high temperatures close to the optimum growth temperature of the source organism (90°C), however, *T. kodakaraensis* propeptide∗ is rapidly degraded by *T. kodakaraensis* mat-subtilisin∗. Therefore the maturation process of *T. kodakaraensis* pro-subtilisin is not bound at this intermediate stage. At high temperatures, the activity of *T. kodakaraensis* mat-subtilisin∗ increases, the stability of *T.* *kodakaraensis* propeptide∗ decreases, and the binding affinity of *T. kodakaraensis* propeptide∗ to *T. kodakaraensis* mat-subtilisin∗ probably also decreases. The combination of these effects may facilitate rapid degradation of *T. kodakaraensis* propeptide∗ and thereby facilitate rapid maturation of *T. kodakaraensis* pro-subtilisin at high temperatures. In this regard, *T. kodakaraensis* subtilisin is well adapted to a hyperthermic environment. Phylogenetic analyses have suggested that hyperthermophilic archaea and bacteria retain a trace of early life forms and produce enzymes which may represent prototypes in the same protein family (63). Therefore, *T. kodakaraensis* subtilisin may represent a prototype of subtilisin in which a propeptide functions as a potent inhibitor but does not exhibit a chaperoning property. A similar function of propeptide has been reported for a thermostable subtilisin homologue, aqualysin I from *Thermus aquaticus* (34).

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