crystallization communications

Crystallization and preliminary X-ray diffraction study of an active-site mutant of pro-Tk-subtilisin from a hyperthermophilic archaeon

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Crystallization of and preliminary crystallographic studies on an active-site mutant of pro-Tk-subtilisin from the hyperthermophilic archaeon *Thermococcus kodakaraensis* were performed. The crystal was grown at 277 K by the sitting-drop vapour-diffusion method. Native X-ray diffraction data were collected to 2.3 Å resolution using synchrotron radiation from station BL41XU at SPring-8. The crystal belongs to the orthorhombic space group $I222$, with unit-cell parameters $a = 92.69$, $b = 121.78$, $c = 77.53$ Å. Assuming the presence of one molecule per asymmetric unit, the Matthews coefficient V_M was calculated to be 2.6 Å^3 Da^{-1} and the solvent content was 53.1%.

1. Introduction

Tk-subtilisin from the hyperthermophilic archaeon *Thermococcus kodakaraensis* is a member of the subtilisin family (Kannan *et al.*, 2001; Pulido *et al.*, 2006). Bacterial subtilisins such as subtilisin E, subtilisin BPN′ and subtilisin Carlsberg represent this family, which is one of six families of subtilisin-like serine proteases (subtilases; Siezen & Leunissen, 1997). Like bacterial subtilisins, Tk-subtilisin consists of a signal peptide (Met^{-93}–Ala^{-70}), propeptide (Gly^{-69}–Leu^{-1}) and mature domain (Gly^1–Gly^{329}). The mature domain of Tk-subtilisin contains three insertion sequences compared with those of bacterial subtilisins. This domain without insertion sequences is similar to those of bacterial subtilisins both in size and amino-acid sequence (amino-acid sequence identity of 45–46%).

Bacterial subtilisins are secreted into an external medium in a pro-form (pro-subtilisin) and are activated upon autoprocessing and degradation of the propeptide (Shinde & Inouye, 1996). The latter process is required to produce active subtilisin, because the propeptide remains tightly bound to the mature domain after autoprocessing and thereby inhibits its activity (Li *et al.*, 1995; Huang *et al.*, 1997; Yabuta *et al.*, 2001). It has been proposed that the propeptides of bacterial subtilisins function not only as inhibitors of their cognate mature domains but also as intramolecular chaperones that facilitate folding of the mature domains (Eder *et al.*, 1993; Shinde *et al.*, 1997; Subbian *et al.*, 2005). The mature domains alone are not folded into an active form but are folded into an inactive form with a molten globular-like structure in the absence of propeptides (Eder *et al.*, 1993; Shinde & Inouye, 1995). Requirement of a propeptide for the maturation of its cognate mature domain has also been reported not only for other members of the subtilase family (Baier *et al.*, 1996; Marie-Claire *et al.*, 2001; Basak & Lazure, 2003), but also for other proteases (Silen & Agard, 1989; Smith & Gottesman, 1989; Winther & Sorensen, 1991; O'Donohue & Beaumont, 1996; Marie-Claire *et al.*, 1999; Nirasawa *et al.*, 1999).

Subtilases contain several Ca^{2+}-binding sites, which vary in number (two to four) and location (Bode *et al.*, 1987; Betzel *et al.*, 1988; Gros *et al.*, 1988; Smith *et al.*, 1999). Subtilisin BPN′ is greatly destabilized upon removal of the high-affinity Ca^{2+}-binding site (Voordouw *et al.*, 1976). Pro-subtilisin E is folded and autoprocessed in the absence of Ca^{2+} (Yabuta *et al.*, 2002). A subtilisin BPN′ derivative without a Ca^{2+}-binding site exhibits Ca^{2+}-independent activity (Gallagher *et al.*, 1993; Strausberg *et al.*, 1995). These results suggest that Ca^{2+} is not required for activity but is required for the stability of subtilisin.
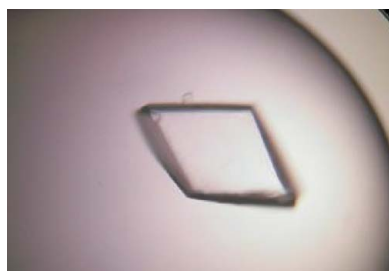
Like bacterial subtilisins, Tk-subtilisin is matured (activated) from pro-Tk-subtilisin upon autoprocessing and degradation of propeptide (Pulido *et al.*, 2006). In this process, the propeptide is first autoprocessed to produce an inactive complex between the propeptide and the mature domain. Then, the propeptide, which is simultaneously both a potent inhibitor and a good substrate of the mature domain, is degraded by the mature domain to produce active enzyme. However, unlike bacterial subtilisins, Tk-subtilisin requires Ca^{2+} for activity, probably to produce its active conformation (Pulido *et al.*, 2006). In the absence of Ca^{2+}, pro-Tk-subtilisin is not matured at all even at high temperatures. In addition, Tk-subtilisin does not require propeptide for folding of its mature domain, because the mature domain alone is refolded and exhibits Ca^{2+}-dependent activity in the absence of propeptide (Pulido *et al.*, 2006). These results suggest that the maturation process of Tk-subtilisin is different from those of bacterial subtilisins.

To understand the unique maturation process of pro-Tk-subtilisin, it is necessary to determine its crystal structure. However, pro-Tk-subtilisin is not fully stable in the presence of Ca^{2+} even at 277 K, especially when its concentration is high. Under these condition, pro-Tk-subtilisin is gradually converted to an active mature form, which is finally self-degraded. In this report, we constructed the active-site mutant of pro-Tk-subtilisin (pro-S255A), overproduced and purified the recombinant protein, crystallized it in complex with Ca^{2+} and performed preliminary X-ray crystallographic studies.

2. Experimental procedures

2.1. Plasmid construction

The gene encoding the active-site mutant of pro-Tk-subtilisin (pro-S255A) was amplified by PCR using the overlap extension method (Horton *et al.*, 1990). The mutagenic primers were designed such that the codon for Ser255 (AGC) is changed to GCC for Ala. The derivative of pET25b (Novagen) for overproduction of pro-S255A was constructed as previously described for pro-Tk-subtilisin (Kannan *et al.*, 2001). All DNA oligomers for PCR were synthesized by

Hokkaido System Science (Sapporo, Japan). PCR was performed in 25 cycles using a thermal cycler (Gene Amp PCR System 2400; Perkin–Elmer) and KOD DNA polymerase (Toyobo). The DNA sequence of the gene encoding pro-S255A was confirmed using an ABI Prism 310 DNA sequencer (Perkin–Elmer).

2.2. Overproduction and purification

Overproduction of pro-S255A in *Escherichia coli* BL21-Codon-Plus(DE3) was carried out as described previously for pro-Tk-subtilisin (Kannan *et al.*, 2001). The cells were collected by centrifugation, suspended in 20 m*M* Tris–HCl pH 9.0, disrupted by sonication on ice and centrifuged at 30 000*g* for 30 min at 277 K. The pellet was dissolved in 20 m*M* Tris–HCl pH 9.0 containing 8 *M* urea and 5 m*M* EDTA and applied onto a HiTrap Q HP column (Pharmacia/GE Healthcare) equilibrated with the same buffer. The protein was eluted from the column with a linear gradient of 0–0.3 *M* NaCl. The fractions containing denatured pro-S255A, which eluted from the column at approximately 0.1 *M* NaCl, were collected and dialyzed against 20 m*M* Tris–HCl pH 7.0 containing 10 m*M* $CaCl_2$ and 1 m*M* DTT for 5 d for refolding. The refolded pro-S255A protein was incubated at 353 K for 30 min and centrifuged at 30 000*g* for 30 min at 277 K. The resultant supernatant was finally loaded onto a Sephacryl S-200HR column (Pharmacia/GE Healthcare) equilibrated with 20 m*M* Tris–HCl pH 7.0 containing 10 m*M* $CaCl_2$ and 0.05 *M* NaCl. The fractions containing pro-S255A were collected, dialyzed against 10 m*M* Tris–HCl pH 7.0 and concentrated using a Centricon ultrafiltration system (Millipore). The protein concentration was determined from UV absorption using a cell with an optical path length of 1 cm and an A_{280} value of 1.25 for a 0.1% solution. The purity of the protein was confirmed by SDS–PAGE (Laemmli, 1970) followed by staining with Coomassie Brilliant Blue.

2.3. Crystallization

The crystallization conditions were initially screened using crystallization kits from Hampton Research (Crystal Screens I, II and Cryo) and Emerald Biostructures (Wizard I and II) with a TASCAL-1 semiautomatic protein crystallization system (Kentoku Industry Co. Ltd, Suita, Japan; Adachi *et al.*, 2004). The conditions were surveyed using the sitting-drop vapour-diffusion method at 277 and 293 K. Drops were prepared by mixing 1 µl each of the protein solutions (approximately 10 mg ml^{-1} pro-S255A in 10 m*M* Tris–HCl pH 7.0) and the reservoir solution and were vapour-equilibrated against 100 µl reservoir solution. Crystals appeared after a few weeks using Crystal Screen I solution No. 33 (4.0 *M* sodium formate). The crystallization conditions were not further optimized as cube-shaped single crystals suitable for X-ray diffraction analysis were obtained from the initial screening.

2.4. Data collection

A crystal of pro-S255A was mounted on a CryoLoop (Hampton Research), adding Paratone-N (Hampton Research) as cryoprotectant, and then flash-frozen in a nitrogen-gas stream at 100 K. X-ray diffraction data were collected at a wavelength of 1.0 Å on beamline BL41XU at SPring-8, Japan. A total of 180 images were recorded with an exposure time of 15 s per image and an oscillation angle of 1.0°. Diffraction images were indexed, integrated and scaled using the *HKL*-2000 program suite (Otwinowski & Minor, 1997).
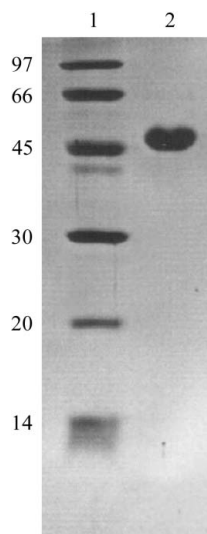

Figure 1
SDS–PAGE of pro-S255A. Samples were subjected to electrophoresis on a 15% polyacrylamide gel in the presence of SDS. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. Lane 1, low-molecular-weight marker kit (Amersham Biosciences); lane 2, purified pro-S255A. Numbers along the gel represent the molecular weights in kDa of the standard proteins.

X-ray wavelength (Å)	1.0
Temperature (K)	100
Space group	$I222$
Unit-cell parameters (Å)	$a = 92.69$, $b = 121.78$, $c = 77.53$
Resolution range (Å)	30.0–2.30
No. of measured reflections	124455
No. of unique reflections	18765
R_{merge}† (%)	4.8 (9.6)
Data completeness (%)	94.0 (77.2)
Average $I/\sigma(I)$	38.04 (13.47)

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

3. Results and discussion

3.1. Overproduction, purification and characterization

Comparison of the amino-acid sequence of pro-Tk-subtilisin with those of various subtilases indicates that Asp46, His84 and Ser255 form the catalytic triad of Tk-subtilisin. Therefore, the active-site mutant of pro-Tk-subtilisin (pro-S255A) was designed such that Ser255 is replaced by Ala. Upon induction for overproduction, pro-S255A accumulated in the cells in inclusion bodies like pro-Tk-subtilisin. It was solubilized in 8 M urea, purified in the presence of 8 M urea and refolded by removing urea in the presence of Ca^{2+}. The protein was refolded in the presence of Ca^{2+} because it has been suggested that the mature domain of pro-Tk-subtilisin requires Ca^{2+} to attain its active conformation (Pulido *et al.*, 2006). The refolded protein was further purified by gel-filtration column chromatography to give a single band on SDS–PAGE (Fig. 1). The amount of the protein purified from 1 l culture was typically 10 mg.

The molecular weight of pro-S255A estimated from gel-filtration column chromatography (45 kDa) was nearly identical to that estimated from SDS–PAGE, indicating that pro-S255A exists in a monomeric form. Unlike pro-Tk-subtilisin, which is rapidly maturated in the presence of Ca^{2+} at 353 K, pro-S255A was not auto-processed at all but remained intact under these conditions (data not shown), indicating that Tk-subtilisin is completely inactivated by the mutation. Pro-Tk-subtilisin was refolded in the presence of Ca^{2+}, dialyzed against Ca^{2+}-free buffer and crystallized. However, the number of the Ca^{2+} ions bound to the protein used for crystallization was determined to be six by atomic absorption spectrometry (Jarrel-

Figure 2
Cube-shaped crystal of the Ca^{2+}-bound form of pro-S255A.

Ash A-8500 Mark II), indicating that at least six Ca^{2+} ions bind to pro-S255A too tightly to be removed by dialysis.

3.2. Preliminary X-ray diffraction analysis

Crystals appeared after a few weeks and grew to maximum dimensions of $0.43 \times 0.35 \times 0.30$ mm after one month (Fig. 2). The crystals diffracted to 2.3 Å. A total of 124 455 measured reflections were merged into 18 765 unique reflections with an R_{merge} of 4.8%. The crystals belong to the primitive orthorhombic space group $I222$, with unit-cell parameters $a = 92.69$, $b = 121.78$, $c = 77.53$ Å. Table 1 summarizes the data-collection statistics. Based on the molecular weight and the space group, it was assumed that the crystal contains one protein molecule per asymmetric unit, giving a V_M value of 2.6 Å^3 Da^{-1} and a solvent content of 53.1%. These values are within the ranges frequently observed for protein crystals (Matthews, 1968), suggesting that this crystal is suitable for structural determination. It has also been found that six calcium ions bind to pro-S255A at the loop regions. We are currently solving this structure.

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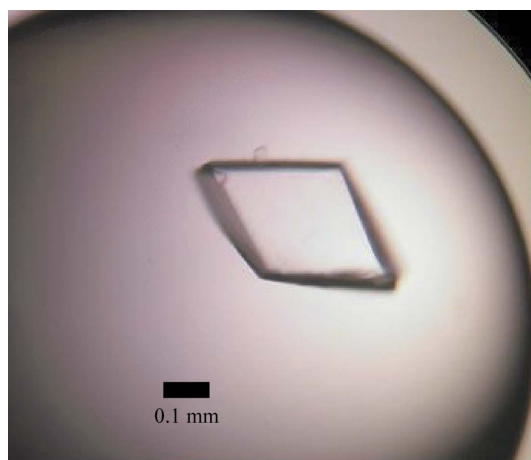Smith, C. A., Toogood, H. S., Baker, H. M., Daniel, R. M. & Baker, E. N. (1999). *J. Mol. Biol.* **294**, 1027–1040.

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