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Crystallization and initial X-ray diffraction study of the three PASTA domains of the Ser/Thr kinase Stk1 from the human pathogen *Staphylococcus aureus*

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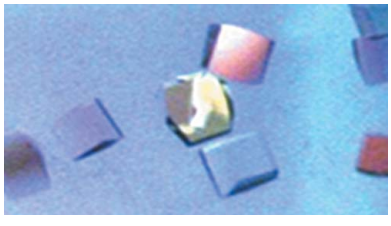

PASTA subunits (~70 amino acids) are specific to bacterial serine/threonine kinases and to penicillin-binding proteins (PBPs) and are involved in the synthesis of peptidoglycan. The human pathogen *Staphylococcus aureus* contains a serine/threonine kinase, Stk1, which plays a major role in virulence. A recombinant His-tagged portion of the extracellular domain of Stk1 containing three PASTA subunits has been crystallized using zinc sulfate as a crystallizing agent. The crystals belonged to the tetragonal space group $P4_122$, with unit-cell parameters $a = 68.0$, $b = 68.0$, $c = 158.1$ Å. Structure determination by the MAD method is now in progress.

1. Introduction

Cellular response and adaptation to various environmental stresses are essential for all organisms ranging from bacteria to humans for survival in their natural habitat. For this purpose, cells have developed numerous signal-transduction pathways involving a receptor that receives a signal that is relayed to other components inside the cell. Phosphorylation is a device of choice for these signal-transduction events (Hunter, 2000). It is accomplished by specific protein kinases and is coupled to dephosphorylation reactions carried out by protein phosphatases (Hunter, 1995). Gene pairs encoding a serine/threonine kinase adjacent to a serine/threonine phosphatase have been found in important pathogens such as *Listeria monocytogenes*, *Streptococcus agalactiae*, *S. pyogenes*, *Enterococcus faecalis*, *Mycobacterium tuberculosis* and *Yersinia pestis* (Cozzone, 2005). They have been shown to regulate various cellular functions such as growth, differentiation, pathogenicity, antibiotic resistance, stress response, biofilm formation and secondary metabolism (Zhang, 1996). However, detailed understanding of their complete signalling pathways is still lacking.

Staphylococcus aureus is a significant human pathogen that causes a number of infections ranging from skin infections to toxic shock syndrome, osteomyelitis and myocarditis (McGahee & Lowy, 2000). Recent surveys have indicated the emergence of methicillin-resistant *S. aureus* as the second most frequent pathogen in hospital-acquired pneumonia and ventilator-associated pneumonia. Of particular interest, *S. aureus* contains a Ser/Thr kinase named Stk1 (UniProtKB sequence database code Q7A5Z8) which plays a major role in virulence and resistance to the antibiotic phosphomycin (Beltramini *et al.*, 2009; Débarbouillé *et al.*, 2009). Sequence analyses show that Stk1 is composed of an intracellular Ser/Thr kinase domain, a transmembrane region and an extracellular domain containing three repeated PASTA subunits. PASTA subunits (~70 amino acids) are specific to bacterial Ser/Thr kinases and to penicillin-binding proteins (PBPs) and are involved in the synthesis of peptidoglycan (Sieradzki & Tomasz, 1999; Yeats *et al.*, 2002; Jones & Dyson, 2006; Sauvage *et al.*, 2008; Shah *et al.*, 2008). Shah and coworkers have shown that the Ser/Thr kinase PrkC from *Bacillus subtilis* is able to recognize peptidoglycan fragments and to mediate muropeptide-dependent germination (Shah *et al.*, 2008). This germination of dormant spores is blocked by the antibiotic staurosporin. Taken together, these results from *B. subtilis* and *S. aureus* suggest that the PASTA domains of Ser/Thr kinases should act as peptidoglycan sensors and represent a viable antibiotic target.

To date, only one representative structure containing PASTA subunits is available in the Protein Data Bank (PDB): that of the high-molecular-mass type II penicillin-binding protein PBP2X from *S. pneumoniae* (PDB code 1k25; Dessen *et al.*, 2001). PBP2X contains two PASTA subunits, each of which is composed of a small globular fold consisting of three β-strands and an α-helix.

The present study of Stk1 was aimed at the determination of the first atomic structure of PASTA subunits from a Ser/Thr kinase in order to understand their sensing mechanism better. The cloning, overproduction, purification, crystallization and preliminary X-ray analysis of a 24 kDa extracellular portion of Stk1 (residues 373–575), which contains the three PASTA subunits of the protein, are presented.

2. Protein overproduction and purification

2.1. Cloning of the three PASTA domains of Stk1

The SA1063 gene encoding the Ser/Thr kinase Stk1 was amplified by PCR from the genomic DNA of *S. aureus* strain N315. *Bam*HI and *Eco*RI restriction sites (shown in bold) were introduced into the oligonucleotide primers for the upstream and downstream sequences: 5′-**CGGGATCC**GGTAATAAATACGAAGAGACACCTG-3′ and 5′-**CGGAATTCTTA**ACCTTTAGAAACAACAAATGAAATCG-3′, respectively. PCR was carried out with The High Fidelity PCR Master (Roche) under the following conditions: 10 min at 368 K and 40 cycles of 1 min at 368 K, 1 min at 341 K and 1 min at 345 K, followed by 10 min at 345 K for final elongation of the products.

The PCR fragment was digested with *Bam*HI and *Eco*RI and subcloned into the pET-28a(+) vector (Novagen) for overproduction of the protein with a six-His tag at its N-terminus. Ligation was carried out with T4 DNA ligase (Promega) overnight at 288 K.

2.2. Overproduction and purification

Escherichia coli BL21 (DE3) bacteria transformed by heat shock with the appropriate plasmid were grown at 310 K until the A_{600} reached 0.6 in Luria–Bertani (LB) medium containing an antibiotic (50 µg ml^{-1} kanamycin). Overproduction was sustained for 90 min under shaking at the same temperature (310 K) after induction by addition of 1 m*M* IPTG. Cells were harvested by centrifugation at 3000*g* for 10 min.

The bacterial pellet (typically 15 mg) was resuspended in 40 ml 25 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl and 10% glycerol (buffer *A*) in the presence of a protease inhibitor (PMSF) and DNAse/RNAse (final concentration of 60 µg ml^{-1}). Cells were disrupted by rapid agitation (3 min at 30 Hz; TissueLyser, Qiagen) in the presence of beads or with a French press. The lysate was cleared by centrifugation (16 000*g*, 30 min at 277 K). The supernatant containing the soluble His-tagged protein was mixed with Ni–NTA resin (Qiagen) and incubated for 1 h at 277 K under rotation. The lysate/Ni–NTA–agarose mixture was washed eight times with buffer *A* containing 10 m*M* imidazole. Protein elution was carried out ten times with buffer *A* containing 200 m*M* imidazole. The eluted fractions were collected and analyzed by SDS–PAGE.

The fractions containing the protein were collected and dialyzed overnight against a buffer containing 50 m*M* Tris–HCl pH 7.5, 20 m*M* NaCl, 100 µ*M* EDTA and 1 m*M* dithiothreitol (DTT). The dialyzed fraction was loaded onto a MonoQ 5/50 Tricorn column (GE Healthcare) equilibrated in buffer *B* (50 m*M* Tris–HCl pH 7.5, 100 µ*M* EDTA, 20 m*M* NaCl) on an ÄKTA Purifier (GE Healthcare). After washing with buffer *B* until the eluent was free of protein, the elution of bound protein was carried out using a linear gradient of NaCl from 20 to 500 m*M* in buffer *C* (50 m*M* Tris–HCl pH 7.5, 100 µ*M* EDTA, 500 m*M* NaCl). Protein concentration was determined by Bradford assay using BSA as reference or by measuring the A_{280} and then concentrated to 10 mg ml^{-1} using a 10 kDa molecular-mass cutoff membrane (Vivascience).

2.3. Digestion with thrombin protease

To prepare the native protein, the His tag was cleaved by digestion with 10 units of thrombin (GE Healthcare) per milligram of Stk1 in PBS for 3 h at 310 K. The native protein was separated from the remaining His-tagged protein by mixing again with Ni–NTA resin and incubating for 45 min. The fraction containing thrombin and the native protein (flowthrough) was again loaded onto a MonoQ 5/50 Tricorn column (GE Healthcare) and purified once more by ion exchange (Fig. 1).

The protein concentration was determined by Bradford assay using BSA as reference or by measuring the A_{280} and the protein was then concentrated to 30 mg ml^{-1} using a 10 kDa molecular-mass cutoff membrane (Vivascience).

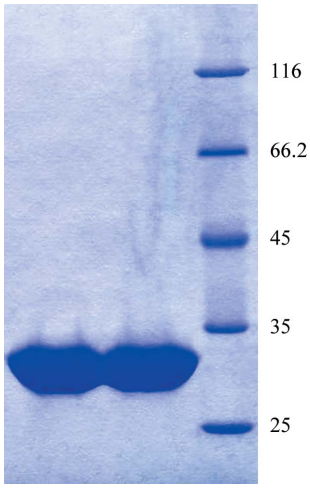

Figure 1
SDS–PAGE analysis of the final purification stage of Stk1. Lanes 1 and 2, elution fractions of Stk1 after ion-exchange chromatography carried out with a MonoQ 5/50 Tricorn column. Lane 3, molecular-mass markers (kDa). Fractions were analysed on 10% SDS–PAGE and stained with Coomassie Blue.

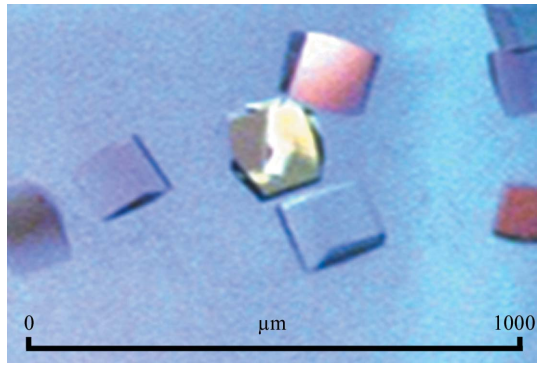

Figure 2
Crystals of the Ser/Thr kinase Stk1 (373–575) from *S. aureus* containing three PASTA subunits. Crystals were obtained by the hanging-drop vapour-diffusion method and grew to dimensions of 0.3 × 0.3 × 0.2 mm within three weeks.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

		MAD data		
	Native data	Peak	Edge	Remote
Wavelength (Å)	1.07300	1.282535	1.283145	1.275558
Resolution range (Å)	40–2.9 (2.98–2.9)	40–3.0 (3.1–3.0)	40–3.0 (3.1–3.0)	40–3.0 (3.1–3.0)
Total oscillation range (°)	180	180	180	90
Observations	118334	106557	106374	53867
Unique reflections	8748	13957	13982	12810
Completeness of data (%)	99.4 (100.0)	99.8 (100.0)	99.8 (100.0)	91.9 (94.2)
$\langle I/\sigma(I)\rangle$	25.6 (7.0)	18.4 (4.8)	25.1 (9.8)	15.5 (4.1)
Redundancy	13.5	7.6	7.6	4.2
$R_{\rm merge}$† (%)	7.4 (37.1)	9.3 (43.7)	6.2 (20.3)	7.1 (37.1)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of reflection $I(hkl)$.

3. Crystallization

About 30% of the protein was lost during the cleavage procedure with thrombin. Thus, the initial search for crystallization conditions was only carried out using the recombinant His-tagged protein. Commercial crystallization screening kits from Hampton Research, Molecular Dimensions and Qiagen were tested using the sitting-drop vapour-diffusion method and Greiner 96-well plates. Droplets composed of 0.5 µl protein solution at 10 mg ml^{-1} and an equal volume of the crystallization solution were equilibrated against 100 µl reservoir solution within a sealed well at 290 K.

Crystal hits were obtained using conditions 65 (0.1 *M* imidazole pH 7.5, 1.6 *M* zinc sulfate) and 66 (0.1 *M* imidazole pH 7.5, 0.8 *M* zinc sulfate) of the Cations Suite from Qiagen. Crystals grew to dimensions of 0.1 × 0.05 × 0.05 mm within one week.

These two similar hits, which both used zinc sulfate as a crystallizing agent, were optimized in Linbro 24-well plates using the hanging-drop vapour-diffusion method. The best crystals grew to dimensions of 0.3 × 0.3 × 0.2 mm within three weeks (Fig. 2) after mixing 1 µl protein solution and 2 µl crystallization solution 65 in the droplet and equilibrating against 500 µl crystallization solution 65 at 290 K.

Crystallization conditions 65 and 66 were also tested using the cleaved protein. The protein concentration had to be increased to 30 mg ml^{-1} to obtain crystals. These were small and formed plate-shaped aggregates. They have yet to be improved and a general screening of crystallization conditions yielded no other hits.

4. Preliminary X-ray analysis

Crystals were cryoprotected by adding 15% ethylene glycol to the hanging drop 1 min before mounting them in cryoloops and flash-cooling them in liquid nitrogen. Initial X-ray diffraction data sets were collected at 100 K on a 345 mm MAR Research image-plate system using Cu $K\alpha$ radiation from an in-house Nonius FR591 rotating-anode X-ray generator. The crystals of the uncleaved protein diffracted to 3.6 Å resolution in-house. They belonged to the tetragonal space group $P4_122$, with unit-cell parameters a = 68.0, b = 68.0, c = 158.1 Å. Assuming the presence of one or two molecules in the asymmetric unit gave acceptable $V_{\rm M}$ values of 3.5 or 1.8 Å^3 Da^{-1}, respectively, corresponding to solvent contents of 65 or 32% (Matthews, 1968). The crystals of cleaved protein diffracted X-rays poorly and were multiple. Their space group was not determined.

Synchrotron data were subsequently collected at 100 K to 2.9 Å resolution using the uncleaved protein on beamline ID14-4 at ESRF, Grenoble, France. The crystal-to-detector distance was 322.6 mm, the oscillation range was 1° and the wavelength was 1.07300 Å. The *XDS* program package was used for data reduction and scaling (Table 1). Molecular replacement was attempted with the program *AMoRe* (Navaza, 2001) using the PASTA subunits of the 2.0 Å structure of PBP2X (Dessen *et al.*, 2001) as search models. Every possible case was tested in terms of body search, assuming that our crystals may contain one or two molecules in the asymmetric unit. However, no solution was found for phasing. It should be noted that the sequence identity between the PASTA subunits of Stk1 and PBP2X is extremely low (12%), although a similar fold is expected.

The zinc ions from the crystallization solution may also be used for phasing by the multiwavelength anomalous dispersion (MAD) method (Dauter, 2002). MAD data sets were collected at the Zn K absorption edge (E = 9.659 keV) to 3.0 Å resolution on the tunable ESRF FIP-BM30 beamline. The *XDS* package was used for data reduction and scaling (Table 1). Structure determination is in progress using the MAD method.

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