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Purification, crystallization and preliminary X-ray diffraction analysis of ThiM from *Staphylococcus aureus*

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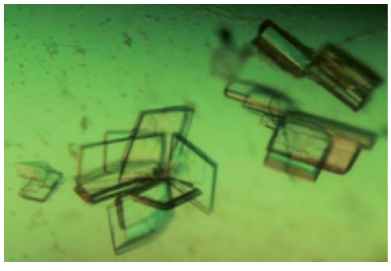

ThiM [5-(hydroxyethyl)-4-methylthiazole kinase; EC 2.7.1.50] from *Staphylococcus aureus* is an essential enzyme of thiamine or vitamin B_1 metabolism and has been crystallized by the vapour-diffusion method. The crystals belonged to the primitive space group $P1$, with unit-cell parameters a = 62.06, b = 62.40, c = 107.82 Å, α = 92.25, β = 91.37, γ = 101.48° and six protomers in the unit cell, corresponding to a packing parameter V_M of 2.3 Å^3 Da^{-1}. Diffraction data were collected to 2.1 Å resolution using synchrotron radiation. The phase problem was solved by molecular replacement.

1. Introduction

Staphylococcus aureus is a commensally existing bacterium that colonizes 20% of healthy adults permanently and up to 50% transiently. Its pathogenicity plays an important role in nosocomial infections affecting immunosuppressed patients. Symptoms caused by *S. aureus* range from superficial skin lesions to life-threatening pneumonia or endocarditis (Lowy, 1998). In 2005, *S. aureus* re-emerged as a major human pathogen owing to methicillin-resistant *S. aureus* (MRSA) strains and caused more than 18 000 deaths in the USA. Staphylococcal pneumonia contributed to more than 75% of these deaths (Klevens *et al.*, 2007; Lowy, 1998).

The active form of vitamin B_1 is thiamine pyrophosphate (TPP), which is a cofactor for several key enzymes of carbohydrate and amino-acid metabolism such as the pyruvate dehydrogenase complex, the 2-oxoglutarate dehydrogenase complex and transketolase (Pohl *et al.*, 2004; Begley *et al.*, 1999). A lack of vitamin B_1 can result in Wernicke's disease and the disease known as beriberi (Ogershok *et al.*, 2002; Platt & Lu, 1936). Current antibiotics for the treatment of *S. aureus* infections mainly target cell-wall synthesis or interfere with protein synthesis at a transcriptional or translational level (Ruhe *et al.*, 2005; Apodaca & Rakita, 2003; Nguyen & Graber, 2010). The occurrence of multidrug resistance in bacterial pathogens such as *S. aureus* necessitates novel chemotherapeutic interventions. Ideal drugs target metabolic pathways that are absent in the host organism such as vitamin B_1 biosynthesis. Recently, the vitamin B_1 metabolism of *S. aureus* has been investigated biochemically (Müller *et al.*, 2009), but structural information about the *S. aureus* enzymes involved in vitamin B_1 metabolism is so far absent. Such information is most useful for structure-based development of prodrugs. When introduced into the bacterial thiamine metabolism they lead to a toxic TPP derivative which will poison vitamin B_1-dependent enzymes and their host, the pathogen.

The synthesis of vitamin B_1 includes two branches leading to a thiazole (THZ) moiety and a pyrimidine (HMP) moiety. THZ has to be phosphorylated by the THZ kinase ThiM (Jurgenson *et al.*, 2009). This pathway is conserved in all kingdoms, whereas only lower eukaryotes and plants are able to synthesize thiamine *de novo*. *Pyrococcus horikoshii* and *Bacillus subtilis* express a ThiM analogue called ThiK to phoshorylate THZ. Both proteins show a trimeric assembly in the crystal structure (Zhang *et al.*, 1997; PDB entry 3dzv; Joint Center for Structural Genomics, unpublished work). In yeast, ThiM and the thiamine phosphate synthase ThiE reside on the bifunctional protein Thi4-p (Nosaka *et al.*, 1993).

Here, we report the crystallization and data collection of the enzyme *Sa*ThiM, which is one of five enzymes required for vitamin B_1 biosynthesis in *S. aureus*.

2. Materials and methods

2.1. Cloning, expression and purification

The open reading frame encoding *Sa*ThiM was amplified by PCR from *S. aureus* genomic DNA using sequence-specific antisense (5′-GCGCGCGGTCTCAGCGCTTTAATGATGATGATGATGATG-GCCCTGAAAATAAAGATTCTCTTCCACCTCTTGAATGCGA-ATCCG-3′) and sense (5′-GCGCGCGGTCTCGAATGAATTAT-CTAAATAACATACGTATTG-3′) oligonucleotides. The PCR for the construct was performed using Taq polymerase (Invitrogen, USA) and the following PCR program: denaturation for 5 min at 368 K followed by 30 cycles of 45 s at 368 K, 60 s at 321 K and 60 s at 345 K. The generated PCR product was cloned *via Bsa*I restriction sites into the *Escherichia coli* expression vector pASK-IBA3 previously digested with the same enzyme, resulting in the expression construct *Sa*ThiM-IBA3. The C-terminus of the native protein is supplemented with a TEV protease cleavage site and a 6×His tag (ENLYFQGHHHHHH) that allows purification of the recombinant fusion protein using Ni–NTA agarose (Qiagen, Germany). The final construct consists of 276 amino acids and has a molecular weight of 29 744 Da. The nucleotide sequence was verified by automated sequencing (Seqlab, Germany). Nucleotide and amino-acid analyses were performed with the *Gene Runner* software (Hastings Software Inc.). *E. coli* BLR (DE3) (Stratagene, Germany) was transformed with the *Sa*ThiM-IBA3 construct. Single colonies were picked and grown overnight in Luria–Bertani medium. The bacterial culture was diluted 1:100 and grown at 310 K until the A_{600} reached 0.5. Expression was initiated with 200 ng ml^{-1} anhydrotetracycline and the cells were grown for 4 h at 310 K before being harvested. The cell pellet was resuspended in buffer *A* (100 m*M* Tris buffer pH 8, 150 m*M* NaCl) with 10 m*M* imidazole, sonicated and centrifuged at 75 000*g* for 1 h at 277 K. The soluble fraction of the lysate was applied onto an Ni–NTA column previously incubated in buffer *A* with 20 m*M* imidazole. The bound sample was washed extensively in the same buffer, eluted with buffer *A* with 250 m*M* imidazole and subsequently dialyzed overnight against buffer *A*. The purity of the sample was also assessed on SDS–PAGE (Laemmli, 1970; Fig. 1).

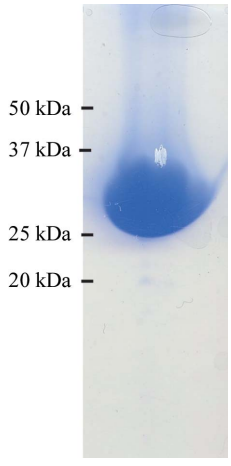

Figure 1
SDS–PAGE analysis of *Sa*ThiM after elution from the Ni–NTA column. The fraction was analysed on a 12.5% SDS gel and stained with Coomassie Blue. A single band with an approximate molecular weight of 30 kDa was observed.

Samples were centrifuged for 1 h at 277 K and 50 000*g*. The supernatant was then concentrated to 20 mg ml^{-1}. The affinity tag was not removed. The solution was monitored by dynamic light scattering (DLS) with a Spectroscatter 201 (Molecular Dimensions, UK) over a suitable period of time and a stable particle radius of approximately 3.2 nm (Fig. 2) was observed.

2.2. Crystallization of *Sa*ThiM

A total of 384 crystallization conditions were screened using a Honeybee 961 dispensing robot (Zinsser Analytic GmbH, Frankfurt, Germany) at 293 K in 96-well crystallization plates (NeXtal QIA1 µ plates, Qiagen) using the sitting-drop vapour-diffusion method, based on the commercially available JCSG+, ComPAS, Classics and Cryos Suites (NeXtal, Qiagen). A 300 nl droplet of 20 mg ml^{-1} protein solution in buffer *A* was mixed with the same volume of reservoir solution and equilibrated against 35 µl reservoir solution. Crystals appeared after 4 d in well A5 from the JCSG+ Suite, consisting of 0.2 *M* magnesium formate, 20% polyethylene glycol (PEG) 3350 in

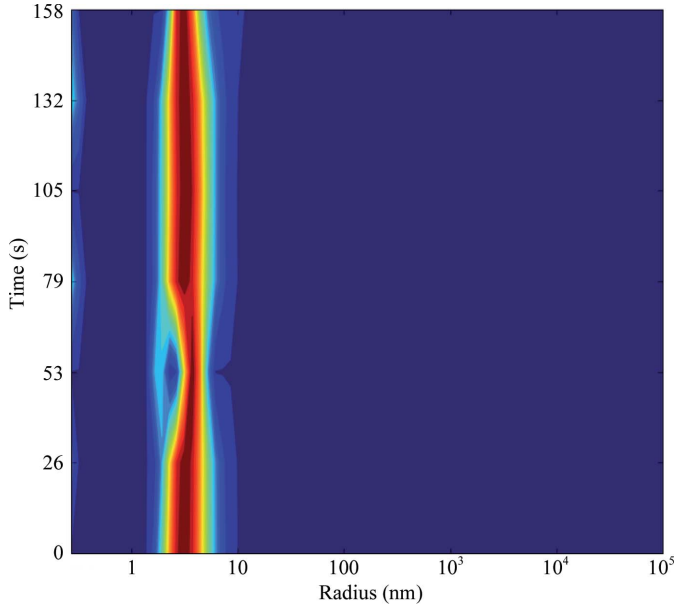

Figure 2
DLS measurement of a 20 mg ml^{-1} *Sa*ThiM solution, showing a monodisperse protein solution and a hydrodynamic radius (R_h) of approximately 3.2 nm.

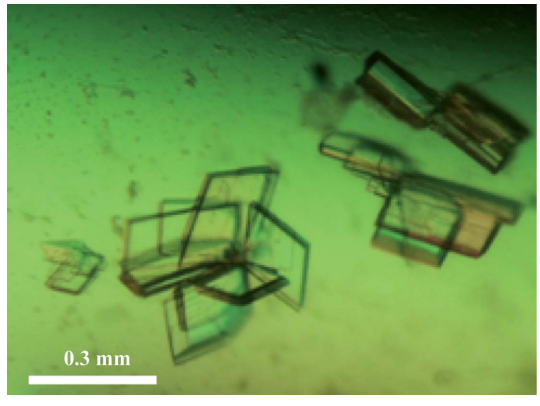

Figure 3
*Sa*ThiM crystals grew to maximum dimensions after 5 d. A single lamella (0.3 × 0.2 × 0.02 mm) was prepared by separating the bundle of crystals with microtools prior to data collection.

Table 1
Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell.

Data-collection parameters	
Wavelength (Å)	0.8081
Temperature (K)	100
Oscillation range (°)	0.5
Crystal-to-detector distance (mm)	195.02
Data-integration statistics	
Space group	*P*1
Unit-cell parameters (Å, °)	a = 62.06, b = 62.40, c = 107.82, α = 92.25, β = 91.37, γ = 101.48
Resolution limits (Å)	50–2.15
Total No. of reflections	1271580
No. of unique reflections	84534
Multiplicity	3.6 (3.4)
Completeness (%)	98.7 (97.8)
$R_{\rm merge}$†	0.076 (0.353)
Mean $I/\sigma(I)$	13.4 (2.8)
Molecules in the unit cell	6
$V_{\rm M}$ (Å^3 Da^{-1})	2.3
Solvent content (%)	46.3

† $R_{\rm merge}$ is defined as $\sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity from multiple observations.

the reservoir. This condition was further optimized and lamella-shaped crystals were reproducibly obtained using 0.2 *M* magnesium formate, 18% PEG 3350 and 5% 2-propanol in 24-well Linbro plates (ICN Biomedicals, USA) sealed with 20 × 20 mm siliconized cover slips (Marienfeld, Germany) applying the hanging-drop technique (Fig. 3). A 1 µl droplet of 20 mg ml^{-1} protein solution in buffer *A* was mixed with the same volume of reservoir solution and equilibrated against 1 ml reservoir solution at 293 K. Crystals grew to maximum dimensions of approximately 0.3 × 0.2 × 0.02 mm after 5 d (Fig. 3). The crystals were separated with microtools (Hampton Research, USA) prior to data collection and were harvested in nylon loops.

2.3. Diffraction experiment

Diffraction data were collected to a resolution of 2.1 Å from a flash-frozen crystal at 100 K on the consortium's fixed-wavelength beamline X13 (HASYLAB/DESY) in Hamburg, Germany at a wavelength of 0.8081 Å using a MAR CCD detector system. Addition of cryoprotectant to the crystal was not required since the PEG concentration in the crystallization solution was sufficient to protect the crystal from cryogenic damage. The oscillation angle was 0.5° and the exposure time was 45 s per frame. Initial crystal characterization and space-group assignment were performed using the *DENZO* software (Otwinowski, 1993) and scaling was performed using *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

*Sa*ThiM was cloned with an affinity tag and expressed in *E. coli* BLR (DE3) cells. The protein consists of 276 amino acids with a molecular weight of 29 744 Da as calculated from the amino-acid sequence. The purified protein showed a single band of 30 kDa on SDS–PAGE and the crystals grew to dimensions of 0.3 × 0.2 × 0.02 mm after 5 d (Fig. 3). The crystals belonged to the triclinic space group *P*1, with unit-cell parameters a = 62.06, b = 62.40, c = 107.82 Å, α = 92.25, β = 91.37, γ = 101.48°. A Matthews coefficient of 2.3 Å^3 Da^{-1} and a corresponding solvent content of 46.3% were calculated assuming the presence of six molecules in the unit cell. A native data set consisting of 84 534 unique reflections with a completeness of 98.7% was collected in the resolution range 50.0–2.15 Å (Table 1).

The structure of ThiM from *B. subtilis* (*Bs*ThiK; PDB entry 1c3q; Campobasso *et al.*, 2000) was used as a search model for initial molecular-replacement calculations. The sequence identity between the two proteins is 39%. A protomer of *Bs*ThiK was successfully placed into the unit cell six times using the program *Phaser* (McCoy *et al.*, 2005), with a corresponding final Z score of 15.5 and a log-likelihood gain value of 849.7. The six protomers appear to assemble into two trimers in the unit cell. Model building and refinement are in progress.

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