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Expression, purification, crystallization and preliminary diffraction analysis of CapF, a capsular polysaccharide-synthesis enzyme from *Staphylococcus aureus*

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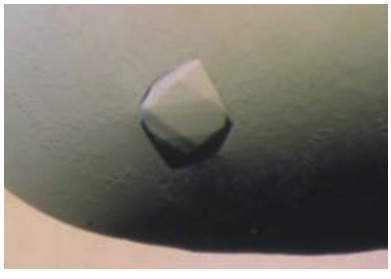


Capsular polysaccharides (CPs) are important virulence factors of *Staphylococcus aureus*. The biosynthesis of type 5 and type 8 CPs (CP5 and CP8), which are produced by most clinical isolates of *S. aureus*, is catalyzed by 16 CP-assembling proteins. One of these proteins is the enzyme CapF, which catalyzes the synthesis of UDP-*N*-acetyl-L-fucosamine, a component of both CP5 and CP8. Here, the cloning, expression, purification, crystallization and diffraction analysis of CapF are reported. Optimization of the crystallization conditions by differential scanning calorimetry afforded a crystal of selenomethionine-substituted CapF that diffracted to a resolution of 2.80 Å. The crystal belongs to space group $P3_221$, with unit-cell parameters $a = b = 119.6$, $c = 129.5$ Å.

1. Introduction

Staphylococcus aureus is a pathogenic bacterium that causes a broad spectrum of diseases including cutaneous infections, wound infections and life-threatening infections such as endocarditis and bacteraemia. *S. aureus* produces many components that affect its virulence, including extracellular capsular polysaccharides (CPs), surface-associated adhesins, exoenzymes and exotoxins (Lowy, 1998). CPs, which enhance resistance against phagocytic uptake by human polymorphonuclear leukocytes, are important virulence factors (Thakker *et al.*, 1998). CPs have been categorized into 11 serotypes. The majority of the clinically isolated strains of *S. aureus* produce either type 5 or type 8 CPs (CP5 and CP8, respectively); strains that express CP5 and CP8 account for ∼25% and 50% of clinically isolated strains, respectively (Arbeit *et al.*, 1984). CP5 and CP8 are structurally similar to each other. CP5 has the structure (→4)-3-*O*-Ac-*β*-D-ManNAcA-(1→4)-*α*-L-FucNAc-(1→3)-*β*-D-FucNAc-(1→)$_n$ and CP8 has the structure (→3)-4-*O*-Ac-*β*-D-ManNAcA-(1→3)-*α*-L-FucNAc-(1→3)-*β*-D-FucNAc-(1→)$_n$; the only differences between the two CPs are the linkages between the sugars and the sites of *O*-acetylation of the mannosaminuronic acid residues.

CapF is an essential enzyme for the synthesis of UDP-L-FucNAc (UDP-2-acetamido-2,6-dideoxy-L-galactose or UDP-*N*-acetyl-L-fucosamine), a monosaccharide derivative that is a component of CP5 and CP8. UDP-L-FucNAc is one of three key intermediates in the biosynthesis of both CP5 and CP8, the other two being UDP-D-ManNAc (UDP-2-acetamido-2-deoxy-D-mannose or UDP-*N*-acetyl-D-mannosamine) and UDP-D-FucNAc (UDP-2-acetamido-2,6-dideoxy-D-galactose or UDP-*N*-acetyl-D-fucosamine). The pathway for the synthesis of UDP-L-FucNAc from UDP-D-GlcNAc (UDP-2-acetamido-2,6-dideoxy-D-galactose or UDP-*N*-acetyl-D-fucosamine) has been extensively characterized. This pathway involves three enzymes: CapE, CapF and CapG. CapF catalyzes the reduction of UDP-2-acetamido-2,6-dideoxy-L-*xylo*-4-hexulose to UDP-2-acetoamido-2,6-dideoxy-L-talose, which is the second reaction in the pathway (Kneidinger *et al.*, 2003). Analysis of the amino-acid sequence of CapF indicates that it belongs to the short-chain dehydrogenase/reductase family, which contains more than 3000 enzymes, including the CapF homologue (WbjC) from *Pseudomonas aeruginosa*. However, no structures are known of proteins that are similar to CapF in their primary sequence, although the structures of some quite

distantly related family members have been determined, with the closest homologue having 14.5% sequence identity (PDB code 1kcl). The distant homologues of known structure also display a large divergence in their primary sequences (only 10–30% residue identity between different enzymes); however, they all display a highly similar α/β-folding pattern (Persson *et al.*, 2003).

In the present study, we cloned, expressed, purified and crystallized CapF from *S. aureus*. Subsequent optimization of the crystallization conditions by means of differential scanning calorimetry (DSC) improved the diffraction resolution of crystals of a selenomethionine (SeMet) derivative of CapF such that multiwavelength anomalous dispersion (MAD) data could be collected at a resolution of 2.80 Å.

2. Materials and methods

2.1. Cloning, expression and purification of CapF

The gene encoding CapF was amplified using KOD-Plus DNA polymerase (Toyobo) with *S. aureus* strain Mu50 genomic DNA as a template and the following designed primers: *Nde*I and *Xho*I sites were incorporated into the CapF-S (5′-GGAATTC**CATATG**AAT-ATTGTAATTACAGGAGCAAAAG-3′) and CapF-AS (5′-NNNN-**CTCGAG**TTATACCTCCAAGAAATACGTATCTG-3′) sequences, respectively (restriction-enzyme recognition sites are shown in bold). The polymerase chain reaction products were inserted into the *Nde*I and *Xho*I sites of the pET26b vector (Novagen). The accuracy of the DNA sequence was confirmed using an ABI 3130*xl* Genetic Analyzer (Applied Biosystems). The expressed protein has neither tags nor extra residues arising from cloning artifacts. Therefore, the expressed CapF was purified by anion-exchange chromatography and size-exclusion chromatography (see below).

Escherichia coli strain Rosetta2 (DE3) (Novagen) transformed with the expression vector encoding the gene corresponding to CapF was grown until the early stationary phase at 301 K in Luria–Bertani broth medium supplemented with 50 µg ml^{-1} kanamycin and 34 µg ml^{-1} chloramphenicol. To induce expression of the desired protein, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 m*M* and the culture was grown for 12 h at 301 K. The SeMet derivative of CapF was expressed in a methionine auxotroph, *E. coli* strain B834 (DE3), grown in M9 medium supplemented with 1 m*M* SeMet (Wako). Cells were harvested by centrifugation at 277 K and 9000*g* for 10 min, washed with a buffer containing 20 m*M* Tris–HCl pH 8.0 and then resuspended in the same buffer. The suspension was sonicated and then centrifuged at 40 000*g* for 30 min at 277 K. The supernatant was loaded onto a HiTrap Q XL column (GE Healthcare Biosciences AB) pre-equilibrated with a buffer containing 20 m*M* Tris–HCl pH 8.0. After the column had been washed with 20 ml of the same buffer, the adsorbed protein was eluted by a gradual increase in NaCl concentration to 1 *M*. Fractions containing CapF were further purified on a HiLoad 26/60 Superdex 200-pg column (GE Healthcare Biosciences AB) equilibrated with a buffer containing 50 m*M* Tris–HCl pH 8.0 and 500 m*M* NaCl. To obtain protein that was sufficiently pure for crystallization, fractions containing the desired protein were loaded onto a Resource Q column (GE Healthcare Biosciences AB) pre-equilibrated with 20 m*M* Tris–HCl pH 8.0. After the column had been washed with 30 ml of the same buffer, the adsorbed protein was eluted by a gradual increase in the NaCl concentration to 600 m*M*. The purity of the protein was confirmed by SDS–PAGE (Fig. 1; Laemmli, 1970).

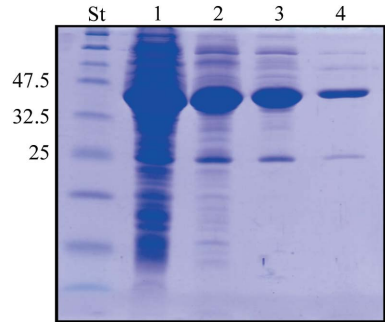
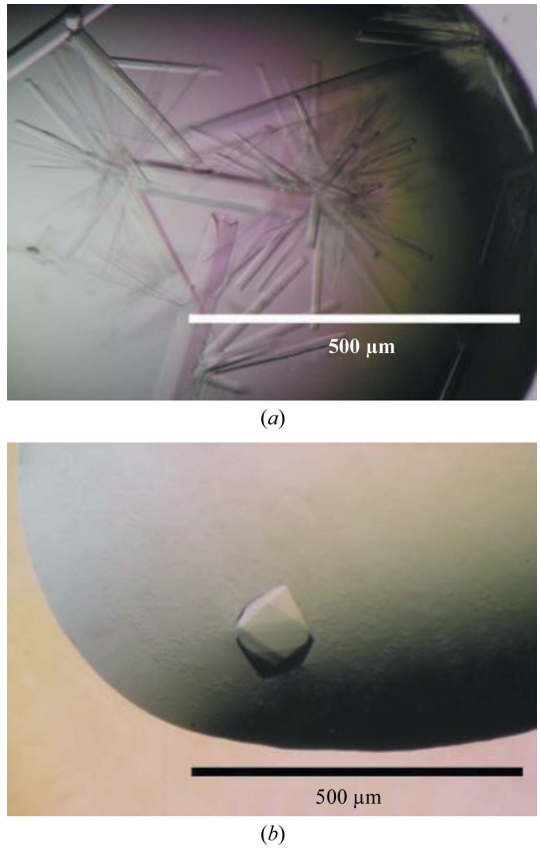


Figure 1
Purification of CapF shown as an SDS–PAGE gel (15%). Lane 1, before purification; lane 2, after HiTrap Q XL column; lane 3, after HiLoad 26/60 Superdex 200-pg column; lane 4, after Resource Q column; lane St, protein molecular-weight standards (kDa).

2.2. Initial crystallization

The purified protein was dialyzed against 10 m*M* Tris–HCl pH 8.0 and concentrated to 15 mg ml^{-1}. Initial crystallization conditions were screened by the sparse-matrix method at 293 K using Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, Crystal Screen Lite, PEG/Ion Screen, Grid Screen PEG/LiCl, Grid Screen Sodium Chloride, Grid Screen PEG 6000, Grid Screen Ammonium Sulfate and Index Screen (Hampton Research). Rod-shaped crystals were grown in buffer containing 3.5 *M* sodium formate pH 7.0 (Index Screen condition No. 25). Crystals were grown in drops containing 1 µl protein solution and 1 µl crystallization reagent using the hanging-drop vapour-diffusion method with a reservoir containing 500 µl crystallization reagent. By optimizing the buffer reagent, precipitant concentration and additive, we were able to obtain crys-



(*a*)

(*b*)

Figure 2
Crystals of CapF in space groups $P6_322$ (*a*) and $P3_221$ (*b*). The crystals diffracted to resolutions of 3.35 and 2.80 Å, respectively.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

		MAD		
	SAD	Remote	Peak	Edge
Space group	$P6_322$	$P3_221$		
Unit-cell parameters (Å)	$a = b = 229.5$, $c = 78.9$	$a = b = 119.6$, $c = 129.5$		
Wavelength (Å)	0.9791	1.0000	0.9792	0.9796
Resolution range (Å)	50–3.35 (3.47–3.35)	50–2.80 (2.90–2.80)	50–2.90 (3.00–2.90)	50–2.80 (2.90–2.80)
Total observations	513301	281357	248846	280945
Unique reflections	18155	26838	24154	26795
$I/\sigma(I)$	37.0 (11.9)	19.6 (2.77)	15.2 (2.65)	18.1 (2.78)
Completeness (%)	100 (100)	100 (100)	100 (100)	100 (100)
R_{merge} (%)	10.8 (38.4)	6.2 (34.4)	8.6 (40.0)	6.5 (34.6)

tals of sufficient size for diffraction analysis from a buffer containing 100 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.2, 25%(*w*/*v*) glycerol, 100 m*M* Li_2SO_4, 300 m*M* NaCl and 3.9 *M* sodium formate pH 6.3 (Fig. 2*a*).

2.3. Optimization of crystallization conditions by DSC

To improve the crystals of SeMet-substituted CapF, we used DSC to screen for crystallization conditions that stabilized the structure of CapF (Privalov & Khechinashvili, 1974). The DSC measurements were carried out with a VP-Capillary DSC System (MicroCal, Northampton, Massachusetts, USA). The pH of the MES and the cations against sulfate ion were adjusted because the most suitable conditions for these variable elements could not be determined in the initial optimization. Substitution of MES by citrate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid sodium salt (HEPES) or Tris–HCl, which are used as near-neutral buffer reagents, did not improve the crystals in the primary crystallization screening; therefore, we used MES, which has a useful pH range of 5.2–7.2, as the buffer reagent. Proteins were dialyzed against 10 m*M* MES pH 5.5, 6.0, 6.5 or 7.0 (Fig. 3*a*) with 100 m*M* Na_2SO_4, Li_2SO_4 or $(NH_4)_2SO_4$ (Fig. 3*b*). The buffers used for dialysis were used as reference solutions for the DSC measurements. Protein samples at 1.0 mg ml^{-1} were heated from 283 to 373 K at a scanning rate of 1 K min^{-1}. For crystallization, it is desirable that all molecules have identical structure, so the temperatures at which CapF began to denature were compared. We chose 100 m*M* MES pH 7.0 with $(NH_4)_2SO_4$ as the most suitable buffer for the stabilization of CapF. The pH range above 7.0 was not explored in this study, but may allow further improvement in the future.

After optimizing the crystallization conditions based on the DSC results, SeMet-substituted CapF crystals with a different shape were obtained from 100 m*M* MES pH 7.2, 25%(*w*/*v*) glycerol, 100 m*M* $(NH_4)_2SO_4$, 300 m*M* NaCl and 3.9 *M* sodium formate pH 6.3 (Fig. 1*b*).

2.4. X-ray diffraction data analysis

All X-ray diffraction data were obtained on beamline BL17-A at the Photon Factory (Tsukuba, Japan) under cryogenic conditions (100 K) using a Quantum 4R CCD detector (ADSC). Since the crystallization buffer already contained 25% glycerol, no cryoprotectant was added to the mother solution before the crystals were mounted. The rod-shaped crystal diffracted anisotropically and was sensitive to radiation damage (Table 1). For single-wavelength anomalous diffraction (SAD) phasing, a wavelength was chosen based on the fluorescence spectrum of the Se *K* absorption edge that corresponded to the maximum f'' (peak, 0.9791 Å). The crystal diffracted to a resolution of 2.98 Å. Although diffraction data were collected to 2.98 Å resolution, data higher than 3.35 Å resolution

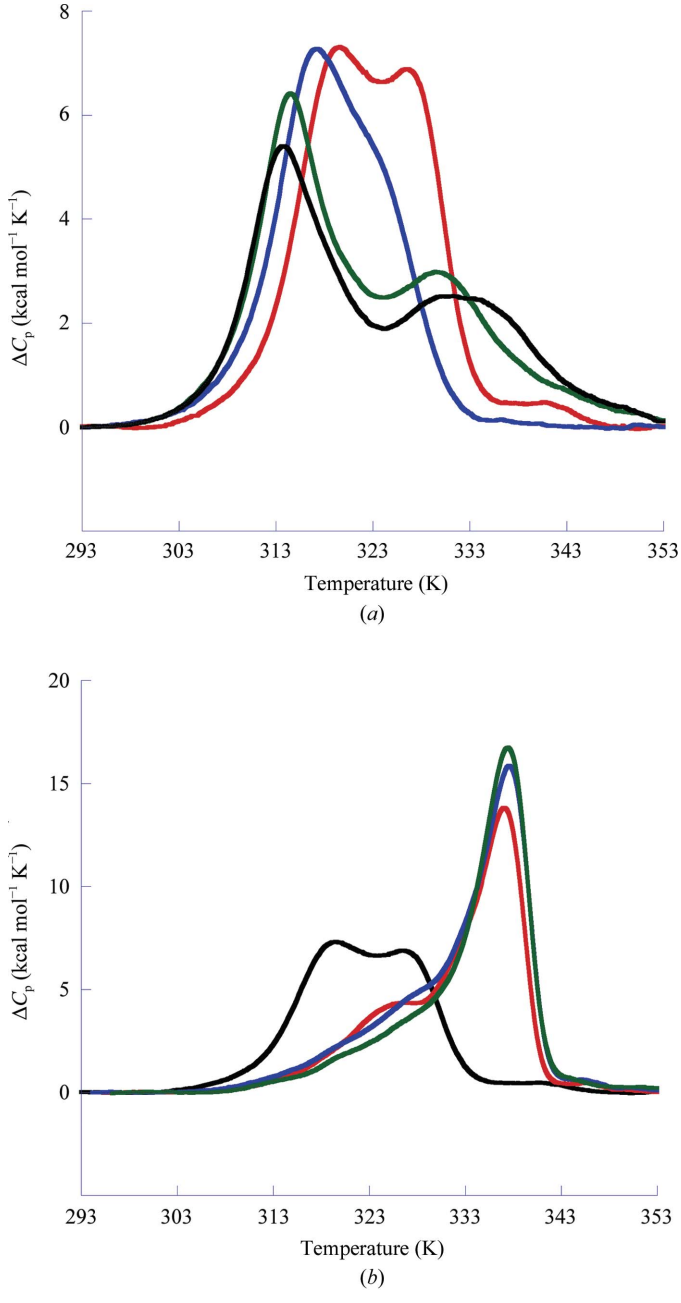


Figure 3
Heat-capacity curves of CapF. (*a*) pH-dependence: 10 m*M* MES pH 5.5 (black), 6.0 (green), 6.5 (blue) and 7.0 (red). (*b*) Effects of salt: no salt (black), 100 m*M* Li_2SO_4 (green), 100 m*M* Na_2SO_4 (blue) and 100 m*M* $(NH_4)_2SO_4$ (red). 1 kcal = 4.186 kJ.

were not used because of the rapidly increasing value of $R_{\rm merge}$ beyond this resolution, even though $I/\sigma(I)$ remained reasonable. This was most likely to be the consequence of a combination of anisotropy and, in particular, radiation damage. The data to 3.35 Å resolution were scaled using the *HKL*-2000 program package (Otwinowski & Minor, 1997). The data-collection strategy consisted of the collection of 300 frames of data with an oscillation of 0.80° and an exposure time of 20 s. The rod-shaped crystal of SeMet-substituted CapF belongs to space group $P6_322$, with unit-cell parameters $a = b = 229.5$, $c = 78.9$ Å.

The optimized crystal form diffracted well and was sufficiently resistant to radiation damage (Table 1). Three wavelengths were chosen on the basis of the fluorescence spectrum of the Se *K* absorption edge, corresponding to the maximum f'' (peak, 0.9792 Å), the minimum f' (edge, 0.9796 Å) and a reference point (remote, 1.0000 Å). The peak data were collected to a resolution of 2.90 Å and the edge and remote data sets to 2.80 Å resolution. The data-collection strategy consisted of the collection of 180 frames of data with an oscillation of 1.0° and an exposure time of 20 s. The optimized crystal is of a different crystal form, with space group $P3_221$, as determined by preliminary solution of the structure, and unit-cell parameters $a = b = 119.6$, $c = 129.5$ Å.

3. Discussion

Using conventional screening methods, *S. aureus* CapF was crystallized in a hexagonal crystal form that diffracted to 3.35 Å resolution. DSC was used to find stabilizing buffer conditions for CapF, which led to the formation of a trigonal crystal form that diffracted to 2.80 Å resolution. These results suggest that DSC measurements are useful for improving crystallization conditions. Some reports have suggested that optimization of the protein-solution condition with DLS is also helpful for crystallization (Habel *et al.*, 2001). Combining these measurements might provide a new systematic method for improving crystallization conditions.

The Matthews volumes (Matthews, 1968) for the hexagonal and trigonal crystals, assuming the presence of a monomer in the asymmetric unit, are 7.14 and 6.32 Å^3 Da^{-1}, respectively. This would allow the presence of more than one monomer per asymmetric unit, but self-rotation function maps do not clearly define noncrystallographic symmetry. Structure determination is in progress and should resolve the asymmetric unit content.

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