Cloning, expression, purification, crystallization and preliminary X-ray diffraction studies of NAD synthetase from methicillin-resistant *Staphylococcus aureus*

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Staphylococcus aureus is an important human and animal pathogen that causes a wide range of infections. The prevalence of multidrug-resistant *S. aureus* strains in both hospital and community settings makes it imperative to characterize new drug targets to combat *S. aureus* infections. In this context, enzymes involved in NAD metabolism and synthesis are significant drug targets as NAD is a central player in several cellular processes. NAD synthetase catalyzes the last step in the biosynthesis of nicotinamide adenine dinucleotide, making it a crucial intermediate enzyme linked to the biosynthesis of several amino acids, purine and pyrimidine nucleotides, coenzymes and antibiotics.

1. Introduction

Staphylococcus aureus is a dangerous and successful human pathogen of major clinical significance. It produces a range of virulence factors that contribute to its diverse pathogenicity in infections such as endocarditis, osteomyelitis and septicaemia (Kolar *et al.*, 2011). Successful therapy to deal with *S. aureus* infections is complicated by the emergence of multidrug-resistant strains (Mwangi *et al.*, 2007), thus necessitating a search for new antibacterial targets which could attenuate the virulence of, and restrict infection by, pathogenic bacteria. It has been shown by different groups that NAD(P) biosynthesis is a promising target pathway for the development of novel antimicrobial agents in the treatment of cancer, neurodegenerative and autoimmune diseases (Khan *et al.*, 2007; Magni *et al.*, 2009). NAD is the central player in several cellular processes. NAD⁺ participates in hydride transfer inside the cell in several redox reactions as well as donating ADP-ribose units as substrate to enzymes required in nonredox reactions (for example, DNA ligases and protein deacetylases), indicating its prominent role inside the cell. Thus, it is mandatory to maintain the NAD pool *via* the resynthesis and recycling of NAD degradation products. The initial steps in the NAD biosynthesis and recycling pathway differ among species, but the genes encoding the enzymes nicotinic acid mononucleotide adenyltransferase (NaMNAT), NAD synthetase and NAD kinase responsible for the downstream conversion of nicotinic acid mononucleotide (NaMN) to NAD and NAD(P) are conserved in almost all investigated bacterial genomes (Osterman & Begley, 2007; Sorci *et al.*, 2009). NAD can be synthesized in a multi-step *de novo* pathway or *via* a pyridine salvage pathway and there are significant differences between prokaryotes and eukaryotes (Fig. 1*a*; Begley *et al.*, 2001; Magni *et al.*, 1999). The key metabolite in *de novo* NAD biosynthesis in all living organisms is quinolinic acid (QA). In eukaryotes

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QA is produced *via* tryptophan degradation, while in prokaryotes QA is obtained through the condensation of iminoaspartate with dihydroxyacetone phosphate in a reaction catalyzed by the quinolinate synthetase system. QA is transformed into nicotinic acid mononucleotide (NaMN) by QA phosphoribosyltransferase, after which NaMN adenyltransferase catalyzes the adenylation of NaMN to nicotinic acid adenine dinucleotide. Finally, nicotinic acid adenine dinucleotide is converted into NAD through the reaction catalyzed by NAD synthetase (Fig. 1*b*; Begley *et al.*, 2001; Magni *et al.*, 1999). In addition, all organisms have the ability to recycle NAD through different salvage pathways, all of which converge at the level of the pyridine mononucleotide, either nicotinamide mononucleotide or NaMN (Begley *et al.*, 2001; Magni *et al.*, 2009).

In recent times, several studies have been reported to identify novel classes of inhibitors of these enzymes and to validate them as attractive drug targets (Velu *et al.*, 2003, 2005; Bonnac *et al.*, 2007; Poncet-Montange *et al.*, 2007). Effective inhibitors of NAD synthetase have been shown to inhibit the growth of Gram-positive bacteria, including antibiotic-resistant strains (Kim *et al.*, 2013). In a recent study, it has been shown that proteolytic degradation of NAD synthetase and NaMNAT depletes the NAD pool, resulting in bactericidal activity, in *Mycobacterium smegmatis* (Rodionova *et al.*, 2014).

NAD synthetases are critical as they catalyze the amidation of nicotinic acid adenine dinucleotide (NaAD) to yield the enzyme cofactor NAD, which is the last step in NAD synthesis (Fig. 1*b*; Rizzi *et al.*, 1996); the enzymes have been shown to be essential in a number of pathogenic bacteria and are being pursued as an important target for antibiotic development (Velu *et al.*, 2005, 2007). To date, high-resolution crystal

Table 1

Macromolecule-production information.

Source organism	<i>S. aureus</i> COL
DNA source	Genomic DNA
Forward primer	5'-CCCCATATGAGTAAATACAAGACGTTAT-3'
Reverse primer	5'-CCAACTCGAGGGATTTGGCCACGTGTATC-3'
Cloning vector	pET-22b
Expression vector	pET-22b
Expression host	<i>E. coli</i> strain BL21 (DE3)
Complete amino-acid sequence of the construct produced	MSKLQDVIVQEMKVKKRIDSAAEIMELKQFIKNY-VQSHSFIKSLVLGISGGQDSTLVGKLVQMSVN-ELREEGIDCTFIAVKLPYGVQKDADEVEQALR-FIEPDEIVTVNIKPAVDQSVQSLKEAGIVLTD-FQKGNEKARERMKVQFSIASNRQGIIVGTDHS-AENITGFYTKYDGAADIAPIFGLNKRQGRQL-LAYLGAPKELYEKTPTADLEDDKPQLPDEDAL-GVTYEADNLYLEGKPVTPPEEQKVIENHYIRNA-HKRELAYTRYTWPKSLEHHHHHH

structures of NAD synthetases from various organisms such as *Bacillus anthracis* (PDB entry 2pzb; McDonald *et al.*, 2007), *Escherichia coli* (PDB entries 1wxf, 1wxh, 1wxi, 1wxg and 1wxg; Jauch *et al.*, 2005), *M. tuberculosis* (PDB entry 3dla; LaRonde-LeBlanc *et al.*, 2009), *B. subtilis* (PDB entry 1ee1; Devedjiev *et al.*, 2001), *Streptomyces avermitilis* (PDB entry 3n05; New York SGX Research Center for Structural Genomics, unpublished work) and *Helicobacter pylori* (PDB entries 1xng and 1xnh; Kang *et al.*, 2005) have been determined. In an effort towards the structural and functional analysis of *S. aureus* NAD synthetase (*Sa*NADS), we cloned, over-expressed, purified and crystallized this enzyme. It is anticipated that the crystal structure of this enzyme will provide a potential route to understand the catalytic mechanism from a conformational perspective as well as to molecular-docking studies to identify potential leads in compound databases.

Figure 1

(a) NAD(P)-biosynthesis pathways in bacteria. The enzymes (genes) involved in each step are abbreviated as follows: NaMNAT (*nadD*), NaMN adenyltransferase; NADS (*nadE*), NAD synthetase; NADK (*nadF*), NAD kinase. Other abbreviations: FAD, flavin adenine dinucleotide; FADH₂, reduced flavin adenine dinucleotide; DHAP, dihydroxyacetone phosphate; Asp, aspartate; Iminoasp, iminoaspartate; QA, quinolinic acid; NM, nicotinamide; NA, nicotinic acid. (b) The reaction catalyzed by NAD synthetase.

Here, we describe the cloning, purification, crystallization and preliminary crystallographic analysis of this protein.

2. Materials and methods

2.1. Cloning, expression and purification of SaNADS

The gene encoding the enzyme was PCR-amplified from the genomic DNA of *S. aureus* strain COL using the primers 5'-CCCCCATATGAGTAAATTACAAGACGTTAT-3' and 5'-CCAACTCGAGGGATTTTGGCCACGTGTATC-3'. The PCR product was subsequently cloned between the NdeI and XhoI sites of expression vector pET-22b (Novagen) in such a way that Leu and Glu followed by a hexahistidine tag (LEHHHHHH) were added from the vector sequence at the C-terminal end of the recombinant protein (Table 1). The integrity of the clones was confirmed by DNA sequencing. The expression clones were transformed into *E. coli* BL21(DE3) competent cells. The transformed cells were grown at 310 K to an optical density of 0.4 at 600 nm in Luria–Bertani medium supplemented with 100 mg ml⁻¹ ampicillin. Expression of the recombinant protein was induced by the addition of 0.2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Post-induction, the cells were grown at 289 K for 14 h. The cells were harvested by centrifugation at 6500 rev min⁻¹ for 10 min at 277 K. The harvested cells were resuspended in ice-cold lysis buffer A (20 mM Tris pH 8.0, 200 mM NaCl) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) to prevent nonspecific proteolysis and lysed using a Vibra-Cell (Sonics & Materials, USA) instrument with a medium-size probe at 20% output power, 50% duty cycle with a pulse time of 30 s. The lysate was then centrifuged at 14 000 rev min⁻¹ in a Kubota 7780 centrifuge for 30 min at 277 K to remove cell debris.

The clear supernatant was incubated with nickel-affinity beads (Ni-IDA; GE Healthcare) for 30 min at 277 K and the incubated slurry was loaded into a column. The column was subsequently washed with five column volumes of buffer A and then five column volumes of wash buffer B (20 mM Tris

Table 2
Crystallization.

Method	Microbatch-under-oil; hanging-drop vapour diffusion
Plate type	72-well flat bottom type; 24-well plates
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM Tris–HCl pH 8.5, 200 mM NaCl, 5% glycerol
Composition of reservoir solution	200 mM CaCl ₂ , 100 mM Tris–HCl pH 7.5, 22% PEG 4000
Volume and ratio of drop	2 μ l (1:1); 4 μ l (1:1)
Volume of reservoir (μ l)	0 (microbatch); 400 (vapour diffusion)

pH 8.0, 200 mM NaCl, 20 mM imidazole). The bound protein containing the hexahistidine tag was then eluted using a linear gradient of 20–200 mM imidazole in buffer B. The purity of the protein was monitored by running samples on a 12% SDS–PAGE gel (Fig. 2*a*). The gel was stained with Coomassie Brilliant Blue R-250. The purified fractions were pooled and concentrated using a Centricon concentrator (10 kDa cutoff; Amicon). The concentrated protein was further purified by gel-filtration chromatography using a Superdex S-200 HR10/300 column pre-equilibrated with buffer consisting of 20 mM Tris pH 8.5, 200 mM NaCl, 5% (v/v) glycerol and mounted on an ÄKTA FPLC system (GE Healthcare) (Fig. 2*b*). The purity was confirmed by running a 12% SDS–PAGE gel (Fig. 2). The column was calibrated using standard molecular-weight protein markers for gel filtration obtained from GE Healthcare. Purified protein samples (10 mg ml⁻¹) were flash-frozen with liquid nitrogen and then stored at –80°C. To determine the protein concentration after each purification step, the Bradford assay was used (Bradford, 1976).

2.2. Crystallization

Initial crystallization trials with the recombinant protein (with a polyhistidine tag at the C-terminus) were performed using the microbatch-under-oil method (Chayen *et al.*, 1992), in which 1 μ l protein solution was mixed with 1 μ l crystal-

Figure 2
(*a*) 12% SDS–PAGE gel of expressed *S. aureus* NAD synthetase. Lane M, molecular-weight markers (labelled in kDa); lane 1, Ni²⁺-affinity purified protein; lane 2, purified protein after size-exclusion chromatography. (*b*) Gel-filtration profile of *S. aureus* NAD synthetase from a Superdex S200 HR 10/300 column. The total volume of the column is 25 ml, while the void volume of the column is 8.17 ml. The protein eluted at 14.9 ml, which is consistent with a dimeric association in solution.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Rigaku MicroMax-007 HF rotating anode
Wavelength (Å)	1.54
Temperature (K)	100
Detector	MAR 345
Crystal-to-detector distance (mm)	175
Rotation range per image (°)	1
Total rotation range (°)	200
Exposure time per image (s)	600
Space group	$P2_1$
a, b, c (Å)	51.00, 107.74, 94.00
α, β, γ (°)	90.00, 96.72, 90.00
Mosaicity (°)	0.42
Resolution range (Å)	53.87–2.03
Total No. of reflections	254853 (31569)
No. of unique reflections	62920 (8177)
Completeness (%)	96.9 (86.9)
Multiplicity	4.1
$\langle I/\sigma(I) \rangle$	9.3 (5.1)
$R_{\text{merge}}^\dagger$ (%)	11.0 (25.2)
Overall B factor from Wilson plot (Å ²)	20.97

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity.

lization solution and overlaid with 50 μ l Al's oil [1:1(v:v) silicone oil:mineral oil] in 72-well flat-bottom plates, and the hanging-drop vapour-diffusion method using commercially available crystallization screens (Hampton Research, USA) in 24-well crystallization plates using 2 μ l protein solution mixed with 2 μ l reservoir solution and equilibrated against 400 μ l reservoir solution. Crystallization trials were set up using a variety of protein concentrations (6, 10 and 12 mg ml⁻¹). After one week, microcrystals appeared in 100 mM CaCl₂, 100 mM

Figure 3

Crystals of *S. aureus* NAD synthetase grown using the microbatch-under-oil method. The crystals have typical dimensions of 0.4 × 0.1 × 0.1 mm

Tris-HCl pH 8.5, 20%(w/v) polyethylene glycol 3350 at 293 K. The conditions were further optimized by using several variations of the composition of the initial crystallization conditions to obtain diffraction-quality crystals with rod-shaped morphology. These crystals appeared in about 2–3 d (Fig. 3) and grew to maximum size in 5–6 d. The approximate dimensions of the crystals were 0.4 × 0.1 × 0.1 mm. These crystals are also being incubated with different ligands in co-crystallization trials to obtain crystals of protein–ligand complexes. Crystallization details are summarized in Table 2.

2.3. Data collection and processing

Crystals of SaNADS were transferred into a cryoprotectant consisting of 10% glycerol and immediately flash-cooled in liquid nitrogen. X-ray diffraction data were collected using a crystal grown through the microbatch method in a condition consisting of 200 mM CaCl₂, 100 mM Tris-HCl pH 7.5, 22%(w/v) polyethylene glycol 4000. Diffraction data were collected to 2.0 Å resolution at 100 K using a MAR 345 image-plate detector with a Rigaku MicroMax-007 HF rotating-anode X-ray generator operated at 40 kV and 30 mA (Fig. 4). The data were collected using a 1° oscillation per image with a crystal-to-detector distance of 175 mm. The crystals were exposed to X-rays for 600 s per image and a total of 200 frames were recorded. The diffraction data were integrated using *iMosflm* (Battye *et al.*, 2011) and scaled using *SCALA* (Evans, 2006). Data-collection and processing statistics are summarized in Table 3.

Figure 4

A snapshot of the diffraction pattern of an SaNADS crystal. The image corresponds to 1° oscillation with 10 min exposure time and a crystal-to-detector distance of 175 mm. The edge of the circle represents 2.03 Å resolution.

2.4. Sequence alignment of bacterial NAD synthetases

A multiple protein sequence alignment was performed between NAD synthetase from *E. coli* strain K12 (P18843) and those from four additional bacterial species [*B. subtilis* strain 168 (P08164), *S. aureus* strain COL (Q5HEK9), *B. anthracis* (Q81RP3) and *Salmonella typhi* (Q8Z6G6)] along with *Homo sapiens* (Q6IA69) and *Dictyostelium discoideum* (Q54ML1) (extracted from <http://www.uniprot.org>; protein accession numbers are given in parentheses). This alignment was performed using *ClustalW* (<http://www.ebi.ac.uk/clustalw/>; Fig. 5).

3. Results and discussion

As a first step to obtain insight into its molecular mechanisms, *Sa*NADS was successfully cloned in pET-22b expression vector, overexpressed in *E. coli* BL21(DE3) cells and purified to homogeneity using a two-step protocol consisting of affinity and size-exclusion chromatography (Table 2, Fig. 2). The molecular weight of 31.8 kDa for a subunit of His₆-tagged protein was confirmed by 12% SDS-PAGE. Size-exclusion experiments are in agreement with a dimeric association of the protein in solution. A multiple protein sequence alignment was performed between NAD synthetase from *S. aureus* strain COL and four additional bacterial species (*B. subtilis* strain 168, *E. coli* strain K12, *B. anthracis* and *S. typhi*) along with *H. sapiens* and *D. discoideum* (Fig. 5). This was carried out to investigate the homology of NAD synthetase in *S. aureus* strain COL to NAD synthetases from representative Gram-positive and Gram-negative bacterial species and from eukaryotic species. The primary sequence of *Sa*NADS shares 50% identity with *B. subtilis* NAD synthetase (*Bsu*NADS), 59% identity with *E. coli* NAD synthetase (*Eco*NADS) and 49% sequence identity with *B. anthracis* NAD synthetase, for which crystal structures are available. Loop P1, the function of which is to close the ATP-binding site upon the addition of a nucleotide (Jauch *et al.*, 2005), seems to be a conserved flexible element. Similarly, loop P2 and other motifs such as those important for magnesium coordination are conserved (Fig. 5). This homology illustrates the importance of these residues and domains to the structure and function of the enzymes. The subtle differences in the NAAD/NAD-binding sites, such as Trp256 in *Eco*NADS and His253 in *Sa*NADS, may not affect the binding of the natural substrate but could be of relevance for rational inhibitor design. In contrast to bacterial NADS, in which many catalytic residues are invariant, *D. discoideum* NADS1 and human NADS1 contain several amino-acid substitutions and peptide insertions at the ATP-binding and NAAD/NAD-binding sites (Fig. 5). These differences confirm the suitability of the NADS enzymes for the design of antibiotics, because it should be possible to alleviate side effects in humans.

*Sa*NADS crystals suitable for X-ray analysis were obtained by the microbatch-under-oil method as well as the hanging-drop vapour-diffusion method in 200 mM CaCl₂, 100 mM Tris-HCl pH 7.5, 22%(w/v) polyethylene glycol 4000 (Fig. 3). The

crystals diffracted to 2.0 Å resolution (Fig. 4) and belonged to space group *P*2₁, with unit-cell parameters *a* = 51, *b* = 107.74, *c* = 94 Å. The crystal mosaicity was around 0.4°, with an overall data completeness of 97%. Assuming that the asymmetric unit contains a dimer, the calculated Matthews coefficient is 2.14 Å³ Da⁻¹ (Matthews, 1968), corresponding to 43% solvent content. *Bsu*NADS (Rizzi *et al.*, 1996; PDB entry 1nsy), which shares 50% sequence identity with *Sa*NADS, was used as a search model for molecular replacement. A total of 5% of the reflections were used for the calculation of *R*_{free} (Brünger, 1992) during refinement. Initial rigid-body refinement with *REFMAC5* (Murshudov *et al.*, 2011) using the model output by *Phaser* gave an *R*_{work} of 36% and an *R*_{free} of 42%. Examination of the crystal symmetry revealed that the two subunits in the asymmetric unit associate to form a dimer broadly similar to those of the *E. coli* and *B. subtilis* NADS. Further structural refinement and model building are currently under way.

In summary, we present the cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of NAD synthetase from methicillin-resistant *S. aureus* subspecies COL. Understanding the structural biology of NAD synthetase in pathogenic species of bacteria such as methicillin-resistant *S. aureus* will provide us with the preliminary information necessary for future antimicrobial development.

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