Crystal structure of the ribonuclease-P-protein subunit from *Staphylococcus aureus*

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Staphylococcus aureus ribonuclease-P-protein subunit (RnpA) is a promising antimicrobial target that is a key protein component for two essential cellular processes, RNA degradation and transfer-RNA (tRNA) maturation. The first crystal structure of RnpA from the pathogenic bacterial species, *S. aureus*, is reported at 2.0 Å resolution. The structure presented maintains key similarities with previously reported RnpA structures from bacteria and archaea, including the highly conserved RNR-box region and aromatic residues in the precursor-tRNA 5'-leader-binding domain. This structure will be instrumental in the pursuit of structure-based designed inhibitors targeting RnpA-mediated RNA processing as a novel therapeutic approach for treating *S. aureus* infections.

1. Introduction

Ribonuclease P (RNase P) is a ribonucleoprotein complex that in bacteria consists of a catalytic RNA ribozyme component (P RNA) and one relatively smaller protein component known as ribonuclease P protein (RnpA) (Hartmann & Hartmann, 2003). RNase P catalyzes the cleavage of the 5'-leader sequence from all precursor-transfer-RNA (ptRNA) and both components, P RNA and RnpA, are essential *in vivo* (Schedl & Primakoff, 1973; Waugh & Pace, 1990; Evans *et al.*, 2006). Alternatively, RnpA is also a key element in the messenger RNA (mRNA) degradosome discovered in both *Staphylococcus aureus* and *Bacillus subtilis* (Roux *et al.*, 2011). Because RnpA plays a critical role in two essential cellular processes in the pathogen *S. aureus* it has been postulated that targeting RnpA-mediated RNA metabolism may represent a promising new antibiotic therapeutic strategy for treatment of *S. aureus* infections (Eidem *et al.*, 2012, 2015; Olson *et al.*, 2011; Willkomm *et al.*, 2003, 2010; Drinas, 2016; Berchanski & Lapidot, 2008). Crystal structures for P RNA, RnpA, and the full RNase P complex have been determined from non-pathogenic bacterial species such as *Thermotoga maritima* (Kazantsev *et al.*, 2003, 2005; Reiter *et al.*, 2010; Torres-Larios *et al.*, 2005) and *B. subtilis* (Daniels *et al.*, 2014; Stams *et al.*, 1998) as well as a recent NMR solution structure for *T. maritima* RnpA (Zeng *et al.*, 2018). Additionally, the NMR structure of *S. aureus* RnpA has been reported (PDB entry 1d6t, Spitzfaden *et al.*, 2000); however, the impact of the structure for drug-discovery purposes is severely limited because of the lack of a deposited NMR chemical shift data set. This lack of backbone chemical shifts

makes it impossible to map ligand-binding sites using chemical-shift perturbation unless one re-collects the data and assigns the backbone resonances to the respective residues. Additionally, as is typical of NMR structures, the result of structure determination provides an ensemble of structures that satisfy NOE-based distance criteria. Individual structures in the ensemble may show considerable variations relative to a calculated average structure. While these variations may reflect important solution-state dynamics they tend to 'smear out' atomic positions over fluctuating segments, resulting in higher uncertainties in atomic positions in these segments. For these reasons an X-ray structure of *S. aureus* RnpA would be an improved tool for structure-based drug discovery, with the NMR structure providing complementary information on dynamics. Therefore, we set out to determine the *S. aureus* RnpA crystal structure in support of structure-based drug discovery targeting RnpA-mediated RNA degradation.

2. Materials and methods

2.1. Macromolecule production

An alternative protocol for protein production was employed compared with the NMR structure paper. The gene coding for *S. aureus* RnpA (4–117) in the pET-30 Ek/LIC::RnpA vector (Eidem *et al.*, 2015) was polymerase-chain-reaction (PCR) amplified using forward and reverse primers shown in Table 1 containing the NcoI and the XhoI restriction enzyme site (underlined), respectively. A tryptophan (W) residue was introduced by the forward primer to the N-terminus of RnpA for the convenience of monitoring protein purification with a UV detector. The subsequent PCR product was digested and ligated into the pHis-Parallel2 vector encoding a 6× His fusion tag (underlined) and a *tobacco etch virus* (TEV) protease-cleavage site (↓) (MSYYHHHHHHHDYDIPTTENLYFQ↓GAMGW-RnpA). The sequence was verified by sequencing analysis at the Purdue Genomics Core Facility and the plasmid DNA was transformed into competent *Escherichia coli* BL21 (DE3) Rosetta cells (Novagen). Expression transformants were cultured in Luria Broth (LB) medium containing 100 µg ml⁻¹ ampicillin with shaking at 225 rev min⁻¹ at 310 K to an OD_{600 nm} ≈ 0.6. Protein expression was induced with 300 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. Cells were pelleted by centrifugation at 3724g for 15 min and stored at -80°C. The pellet was resuspended in buffer A (50 mM sodium phosphate buffer, 300 mM sodium chloride, pH 7.4) and lysed by sonication the following morning. Debris was removed by centrifugation at 14 000g for 1 h, and supernatant was purified on a 5 ml Ni²⁺-NTA affinity column (GE Healthcare Bio-Sciences) with an ÄKTA-FPLC high-performance liquid chromatography system (GE Healthcare Bio-Sciences). Proteins eluted in a single peak with a linear imidazole gradient (0–500 mM) with buffer A and buffer B (1 M imidazole, 50 mM sodium phosphate buffer, 300 mM sodium chloride, pH 7.4). Fractions under the peak were analyzed by Coomassie-stained SDS-PAGE before being

Table 1

Macromolecule-production information.

Source organism	<i>Staphylococcus aureus</i> UAMS-1
DNA source	Dr Paul Dunman, University of Rochester (Eidem <i>et al.</i> , 2015)
Forward primer	AAACCATGGGATGGGAAAAAGCTTACCGA
Reverse primer	TTTCTCGAGTTACTTATCTTTT
Cloning vector	pHis-Parallel2
Expression vector	pHis-Parallel2
Expression host	<i>E. coli</i> BL21(DE3) Rosetta
Complete amino-acid sequence of the construct produced†	GAMGWEKAYRIKKNADEFQRIYKKGHSVANR QFVVYTCNNKEIDHFRLGISVSKKLGNA VLRNKIKRAIRENFKVHKSHILAKDIIIV IARQPAKDMTTLQIQNSLEHVLKIAKVF NKKIK

† The underlined sequence is the residual fusion tag after cleavage

Table 2

Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	96-well, Greiner PP opaque
Temperature (K)	298.15
Protein concentration (mg ml ⁻¹)	12
Buffer composition of protein solution	20 mM Tris pH 7.4, 200 mM NaCl
Composition of reservoir solution	0.15 M Phosphate buffer pH 6.5, 3.3 (NH ₄) ₂ SO ₄
Volume and ratio of drop (µl)	0.2:0.2
Volume of reservoir (µl)	250

pooled together, mixed with 0.5 mM EDTA and 1 mM DTT, and incubated with TEV (200:1, v:v) for 48 h at 277 K. The subsequent cleavage product was purified in the same manner as the supernatant, and eluted protein was further purified by gel filtration in buffer C (20 mM Tris pH 7.4, 200 mM sodium chloride) with a HiPrep Sephacryl S200 column (GE Healthcare Bio-Sciences) on the ÄKTA-FPLC chromatography system to remove aggregates. Fractions were analyzed by Coomassie stained SDS-PAGE, and those with >95% purity were pooled, concentrated to 1 mg ml⁻¹, and stored at 277 K for short-term use. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

The *S. aureus* RnpA protein sample was concentrated by centrifugation at 14 000g in an Amicon Ultra centrifugal filter (cutoff MW 3 kDa) to 12 mg ml⁻¹, and screened using commercial sparse-matrix crystallization screens available to our laboratory, MemGold^{TM} HT-96 and MemGold2^{TM} HT-96 (Molecular Dimensions) (Jancarik & Kim, 1991) on a TTP LabTech Mosquito Crystal crystallization robot using the hanging-drop vapour-diffusion method at room temperature. Crystals produced from the screens were flash-cooled by direct plunging into liquid nitrogen with no extra cryoprotectants. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

X-ray diffraction data were collected at the Argonne National Laboratory (Chicago, IL) and were processed using

Table 3

Data collection and processing.

Values for the outer shell are given in parentheses.

Diffraction source	Beamline 23-ID-B, APS
Wavelength (Å)	1.0
Temperature (K)	100
Detector	Dectris Eiger X 16M
Crystal-detector distance (mm)	230
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	0.5
Space group	<i>P</i> 321
<i>a</i> , <i>b</i> , <i>c</i> (Å)	72.16, 72.16, 58.96
α , β , γ (°)	90, 90, 120
Mosaicity (°)	0.4–1.0
Resolution range (Å)	50.00–2.00 (2.07–2.00)
Total No. of reflections	111835 (≥ 11411)
No. of unique reflections	12313 (1214)
Completeness (%)	100 (100)
Redundancy	9.1 (9.4)
$\langle I/\sigma(I) \rangle$	31.3 (2.34)
$CC_{1/2}$	0.999 (0.625)
$R_{\text{r.i.m.}}$	0.090 (1.3)
$R_{\text{p.i.m.}}$	0.030 (1.2)
Overall <i>B</i> factor from Wilson plot (Å ²)	39

Table 4

Structure solution and refinement.

Values for the outer shell are given in parentheses.

PDB entry	6d1r
Resolution range (Å)	31.25–2.00 (2.10–2.00)
Completeness (%)	99.0 (100)
σ cutoff	$F > 1.360\sigma(F)$
No. of reflections, working set	11086 (1125)
No. of reflections, test set	1223 (123)
Final R_{cryst}	0.214 (0.324)
Final R_{free}	0.234 (0.343)
No. of non-H atoms	
Protein	868
Ligand	0
Solvent	36
Total	904
R.m.s. deviations	
Bonds (Å)	0.004
Angles (°)	0.640
Average <i>B</i> factors (Å ²)	
Protein	60
Solvent	49
Ramachandran plot	
Most favoured (%)	99.07
Allowed (%)	0.93

HKL-2000 (Otwinowski & Minor, 1997). Data were collected from two crystals, one diffracting to 2.0 Å resolution and the other to 2.6 Å resolution. The higher resolution data is presented herein. However, an *ex post facto* analysis of the data with online server *STARANISO* (<http://staraniso.globalphasing.org>) showed it to be highly anisotropic, presumably a factor behind the relatively low $CC_{1/2}$ value in the highest resolution shell (Table 3).

2.4. Structure solution and refinement

The structure of *S. aureus* RnpA was determined by molecular replacement with *Phaser* (McCoy *et al.*, 2007) in

PHENIX using the average NMR structure of RnpA as the search model (PDB entry 1d6t). Refinement was performed with *PHENIX* (Adams *et al.*, 2010) and *Coot* (Emsley *et al.*, 2010). The coordinates have been deposited as PDB entry 6d1r, and the refinement statistics are provided in Table 4. Structure figures were prepared using the *PyMOL* molecular graphics system (Schrodinger, LLC) and the Ramachandran plot was calculated using *Coot*.

2.5. Structure alignment

The published NMR structure for RnpA (1d6t) was averaged in *PyMol* using the average three-dimensional Python script (available from the Cameron Mura laboratory, University of Virginia, http://muralab.org/~cmura/PyMOL/pymol_mainFrame.html). The average NMR structure was aligned to the crystal structure and the backbone r.m.s.d. for the alignment was recorded using *PyMol*.

2.6. Sequence alignment

The sequence of *S. aureus* RnpA was aligned with RnpA's from eight other bacteria, four Gram-positive (*B. subtilis*, *T. maritima*, *Enterococcus faecalis*, *Streptococcus pneumoniae*) and four Gram-negative (*E. coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*), using the *Clustal Omega* webserver (Sievers *et al.*, 2011) and the alignment graphic (Fig. 5) was prepared using *ESPrnt* 3.0 (Robert & Gouet, 2014).

3. Results and discussion

3.1. Crystallization of *S. aureus* RnpA

The *S. aureus* RnpA protein was crystallized using hanging-drop vapour diffusion with a protein solution in 20 mM Tris pH 7.4, 200 mM NaCl at a concentration of 12 mg ml^{−1} combined with a reservoir solution of 0.15 M phosphate buffer pH 6.5, 3.3 M (NH₄)₂SO₄ at a 0.2:0.2 µl ratio (Table 1). Two crystals (one shown in Fig. 1) were harvested and flash-cooled for data collection.

Figure 1

Crystal of *S. aureus* RnpA in hanging-drop well prior to harvest.

3.2. Structure of *S. aureus* RnpA

The *S. aureus* RnpA-protein subunit crystallized in space group *P*321. The structure was determined at 2.0 Å resolution (PDB entry 6d1r) (Table 3). The asymmetric unit of the crystal contained a single monomer of the protein. A total of 107 out

Figure 2
S. aureus RnpA crystal structure at 2.0 Å resolution. The secondary structures are labelled. The figure on the right is generated by counter-clockwise rotation of the one on the left by 90° around the indicated axis.

Figure 3
Representations of *S. aureus* RnpA crystal structures. (a) RnpA with residues involved in ptRNA substrate binding shown as sticks. Phe15, Tyr19, Ser24, Tyr33, Ser48 are shown in magenta. Lys51 and Lys52 are shown in green. (b) RnpA RNR-box residues on α_2 involved in binding to P-RNA-catalytic subunit shown in salmon. (c) Surface representation of RnpA with the same regions highlighted in (a) and (b). The black arrow indicates the ptRNA 5'-leader-sequence-binding channel.

of 117 of the residues were modelled. The final modelled residues contained 99% of residues in the favoured region and 1% in the allowed region with no outliers. The six N-terminal residues (GAMGWE) and four C-terminal residues (KKIK) were not modelled because of a lack of continuous density. Regions 12–16, 37–41 and 74–79 represent real-space *R*-value *Z*-score (RSRZ) outliers, however, these regions were somewhat disordered with relatively weaker electron density.

The overall architecture of *S. aureus* RnpA is similar to that of previous RnpA structures. The secondary structure is laid out in an α_1 - β_1 - β_2 - β_3 - α_2 - β_4 - η_1 - α_3 sequence (Figs. 2 and 5). The four β -sheets are arranged next to one another β_1 - β_2 - β_4 - β_3 from right to left as observed previously (Fig. 2, magenta) (Spitzfaden *et al.*, 2000). Strands β_1 - β_2 - β_4 orient antiparallel to each other while the β_4 - β_3 are arranged in parallel. This creates a β -sheet surface that is solvent exposed on one face with hydrophobic contacts on the opposite face. This surface is flanked by three α -helices (Fig. 2, grey), one on the front-bottom running perpendicular (α_1 , residues 12–21) and two behind (α_2 , residues 56–73, and α_3 , residues 95–109) along with one 3_{10} -helix (η_1 , residues 88–92). The hydrophobic face of the β -sheets packs against the α_2 and α_3 helices to create a characteristic $\alpha\beta$ -fold conserved in ribonucleoprotein RNA-binding modules (Leulliot & Varani, 2001; Varani, 1997).

Helix α_1 is positioned perpendicularly across the bottom of the solvent-exposed side of the β -sheet face. This creates a groove lined with aromatic and/or hydroxyl-containing residues (Phe15, Tyr19, Ser24, Tyr33 and Ser48; Fig. 3a, magenta sticks) that probably facilitate base-stacking and hydrogen-bonding interactions with single-stranded RNA substrates (Spitzfaden *et al.*, 2000; Reiter *et al.*, 2010, 2012). Two highly conserved lysine residues, Lys51 and Lys52 (Fig. 3a, green sticks), flank the opening of the RNA-binding groove opposite Phe15 and Ser48 to provide stabilizing electrostatic interactions with the substrate RNA backbone as previously described (Reiter *et al.*, 2010, 2012). The motif of highest phylogenetic conservation in the RnpA proteins is the RNR box motif represented by residues 59–67 (Fig. 3b, salmon sticks). This positive-charged sequence is responsible for

Figure 4
The *S. aureus* RnpA crystal structure (grey) overlaid onto the average of 20 solution-state NMR structures from *S. aureus* RnpA (green, PDB: 1d6t) with a measured r.m.s.d. = 1.2 Å. β -sheets 1 and 2 are labelled.

Figure 5

Sequence alignment of *S. aureus* RnpA with eight other bacterial RnpA's, four Gram-positive (top bracket) and four Gram-negative (bottom bracket). Sequence similarity depicted by shaded boxes: Red box-white character (100% identity), red character (>70% residue similarity), blue box (>70% residue similarity across group). Secondary structure sequences: helices (curly line) and sheets (arrows). 5'-leader-ptRNA substrate-binding residues indicated by * and RNR-box motif indicated by ^.

Figure 6

S. aureus RnpA (grey, transparent surface) overlaid with the phosphate backbone of 5'-leader sequence of ptRNA substrate (orange sticks, PDB entry 3q1r).

facilitating electrostatic contacts with the catalytic P-RNA portion of the complex. These residues reside at the top half of α_2 and are solvent exposed, poised to form the required interactions with P RNA.

The crystal structure was aligned with the average solution-state NMR structure (Fig. 4). This indicated a root-mean-square deviation (r.m.s.d.) of 1.2 Å between backbone atoms. In particular, β_1 and β_2 were more ordered in the crystal structure compared with the NMR structure. Additionally, the overall position of the α -helices was also shifted toward the

centre of the molecule compared with the average NMR structure, giving the crystal structure a slightly more compact appearance. This difference between the crystal structure and the average NMR structure perhaps reflects local protein dynamics, as can be seen from higher-than-average *B* factor in this helix (see Fig. S2 in the supporting information).

Sequence alignment of *S. aureus* RnpA with eight other bacterial RnpAs demonstrates the importance of the substrate and P-RNA-binding RNR-box (Fig. 5). These residues are highly conserved across all species and necessary for the proper functioning of the RNase-P holoenzyme in bacterial cells. The high phylogenetic and structural conservation of these basic amino acids explains the ability for RnpA subunits of bacterial species to be used interchangeably with P RNA of other bacterial species (Guerrier-Takada *et al.*, 1983).

The surface representation displays the RNA-binding groove that is formed from the β -sheet face meeting α_1 (Fig. 3c). An overlay of the 5'-leader-sequence ptRNA from the RNase-P-ribonucleoprotein complex structure from *T. maritima* (PDB entry 3q1r, Reiter *et al.* 2010) with the *S. aureus* RnpA (Fig. 6) shows the phosphate backbone of the 5'-leader sequence (orange sticks) fitting into the channel created by the β -sheet face with α_1 . While we do not have a 5'-leader-sequence-bound structure to depict the structural dynamics upon substrate binding, the *B*-factor measurements for the crystal structure clearly indicate there is flexibility along the substrate-binding site (see Fig. S2 in the supporting

information). Higher-than-average *B*-factor values are calculated, α_1 indicating a degree of flexibility to accept and conform to leader-sequence binding. The rest of the ordered secondary structures appear to have medium-to-low *B*-factor values suggesting the rest of the protein is fairly rigid in this structural representation.

To summarize, this communication describes the crystallization conditions and X-ray structure for *S. aureus* RnpA. This work will provide an alternative approach to determine ligand-bound structural data in support of future structure-based drug discovery. This structure may also begin to shed light on the newly hypothesized role *S. aureus* RnpA plays in RNA degradation in addition to pRNA maturation.

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