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Expression, purification and crystallization of Dpr, a ferritin-like protein from the Gram-positive meningitis-associated bacterium *Streptococcus suis*

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Ferritin-like proteins form a novel family of bacterial proteins with diverse functions, such as DNA binding, iron storage and cell activation. A common structural feature of these proteins is their ability to form spherical dodecamers. Dpr is a ferritin-like protein from the Gram-positive bacterium *Streptococcus suis*. Full-length and truncated Dpr were expressed and purified as 6×His-tag fusion proteins. Crystals of truncated Dpr suitable for X-ray diffraction analysis were obtained after the removal of the N-terminal affinity tag by thrombin cleavage. A complete data set to 2.3 Å resolution was collected using synchrotron radiation. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 104.3$, $b = 137.6$, $c = 142.1$ Å and 12 molecules in the asymmetric unit.

1. Introduction

Ferritins are iron-storage proteins that prevent the formation of insoluble ferric hydroxide polymers and toxic free radicals in living organisms (Harrison & Arosio, 1996). They form spherical particles of ~120 Å in diameter owing to the arrangement of 24 identical or similar subunits in 432 symmetry. Ferritin-like proteins produced by bacteria form a newly emerging group of proteins characterized by diverse functions that include DNA binding, iron storage and cell activation (Grant *et al.*, 1998). A striking difference between ferritins and ferritin-like proteins is the ability of the latter to form dodecamers that obey 23 point-group symmetry. Each dodecamer resembles a spherical cage with an external diameter of ~90 Å and a large (~45 Å) hollow core. Members of the family include Dps proteins (DNA-protecting protein under starved conditions) that are expressed in *Escherichia coli* and *Bacillus subtilis* in response to oxidative stress. Accordingly, it has been suggested that Dps may protect bacterial DNA from oxidative damage by forming DNA–Dps microcrystalline assemblies (Frenkiel-Krispin *et al.*, 2001). Other family members include *Listeria innocua* Flp which exhibits iron-storage properties, while the homologous Flp from *L. monocytogenes* is believed to act as a cold-shock protein (Bozzi *et al.*, 1997; Hébraud & Guzzo, 2000). *Helicobacter pylori* neutrophil-activating protein (HP-NAP) was shown recently by electron microscopy to form hexagonal rings with a diameter of ~90 Å and to accumulate up to 500 Fe atoms per oligomer (Tonello *et al.*, 1999). Interestingly, HP-NAP binds sulfated or sialylated glycolipids and may act to increase the adhesion of neutrophils to endothelial cells during gastritis (Dundon *et al.*, 2002).

Although crystal structures of ferritin-like proteins from the Gram-positive bacteria *L. innocua* and *B. anthracis* have recently been determined (Ilari *et al.*, 2000; Papinutto *et al.*, 2002), there are no such data available for streptococcal representatives. In this report, we describe the expression, purification and crystallization of the ferritin-like protein Dpr from *Streptococcus suis*. This bacterium requires no iron for optimal growth (Niven *et al.*, 1999); it is therefore a good model to study the biological role of Dpr in cell protection under iron-mediated oxidative-stress conditions (Yamamoto *et al.*, 2002; Pulliainen *et al.*, in preparation). Moreover, *S. suis* is a causative agent of meningitis in newborn and young pigs and in adult humans and as such is a target for new antibacterial agents and vaccines.

2. Methods and results

2.1. Cloning, expression and purification

The full-length *dpr* gene (GenBank accession No. AF319974) was cloned into vector pET30Ek/LIC (Novagen). Briefly, the *dpr* gene was amplified with primers sspns (5′-GACGACGACAAGATGATGAAACAAAAATATTATCAATCT-3′) and sspna (5′-GAGGAGAAGCCCGGTTTACAACTTAGGTGCTTGACCAAG-3′) using the *dpr* gene (cloned into vector PCR2.1, Invitrogen) as a template. PCR product was treated with T4 polymerase in the presence of dATP to generate the LIC-compatible ends for ligation into LIC vector pET30Ek. The ligation

mixture was transformed into NovaBlue cells. After sequencing the cloned gene using T7pp (Novagen T7 promoter primer) and atp1 (5′-CAGCAGAAATTGCGTCTTTC-3′) primers, the vector, designated pSSUD1, was transformed into BL21(DE3) cells harbouring the pLysS plasmid. Full-length protein was purified following the manufacturer's instructions (Novagen). However, the purified protein was found to be highly susceptible to proteolysis even in the presence of protease inhibitors. Previous work performed with *S. suis* to purify Dpr had shown that the purified protein lacked the first seven N-terminal amino acids (Tikkanen *et al.*, 1995) present in the open reading frame of the *dpr* gene. Therefore, expression of the truncated form of Dpr lacking the first seven N-terminal amino acids was undertaken for use in crystallization. The plasmid encoding the truncated *dpr* gene (*tdpr*), designated pSSUD2, was constructed by first amplifying a 5.5 kbp fragment of pSSUD1 plasmid with primers pr1 (5′-CTTGTGCTCCTGCCAGAA-3′) and pr2 (5′-CTTCCGGATTACGCGTCA-3′) using Vent polymerase. The PCR product was circularized with T4 ligase and the plasmid was transformed into NovaBlue cells. The constructed gene was sequenced to verify its correctness. The pSSUD2 plasmid was transformed into *E. coli* BL21(DE3) cells harbouring the pLysS plasmid. Protein expression was performed in Luria–Bertani medium (1.5 l) containing 30 µg ml^{-1} kanamycin and 34 µg ml^{-1} chloramphenicol. *E. coli* cells harbouring the pSSUD2 plasmid were grown to an A_{600} of 0.5 at 310 K and were induced with 1 m*M* IPTG for a further 4 h at 303 K. The cells were harvested by centrifugation and were frozen at 203 K overnight. The cell pellet was thawed on ice for 15 min and was suspended in 50 m*M* sodium phosphate buffer pH 8.0 supplemented with 10 U ml^{-1} Benzonase nuclease (Novagen) and EDTA-free Complete protease-inhibitor coctail (Roche Molecular Biochemicals). The lysate was sonicated on ice for 3 × 15 s at 2 min intervals. After adding NaCl to the lysate to a final concentration of 0.5 *M* and adding imidazole to 0.025 *M*, the insoluble cell debris was removed by centrifugation at 20 000*g* for 30 min at 277 K. The fusion protein was purified using a 5 ml Ni^{2+}–NTA column (Pharmacia). The column was first washed stepwise with 50, 100 and 250 m*M* imidazole in the presence of 0.5 *M* NaCl and 50 m*M* sodium phosphate buffer pH 8.0. The fusion protein was eluted with 0.5 *M* imidazole. Fractions containing tDpr were pooled and concentrated with YM-10 Centriprep (Amicon) and were desalted into buffer containing 25 m*M* Tris–HCl pH 8.6, 0.5 *M* NaCl using a PD-10 desalting mini-column (Pharmacia). After adding $CaCl_2$ to a final concentration of 2.5 m*M*, the fusion protein was cleaved with thrombin for 16 h at 298 K and the reaction was terminated by employing a benzamidine Sepharose affinity column (Pharmacia) to remove thrombin. The truncated protein was desalted using a PD-10 mini-column and buffer-exchanged into 10 m*M* Tris–HCl pH 7.4, 0.15 *M* NaCl. The protein was further purified with a HiPrep 26/60 Sephacryl S-300 (Pharmacia) in running buffer consisting of 10 m*M* Tris–HCl pH 7.4, 0.15 *M* NaCl. The final yield of protein was ∼3 mg as determined by the Bradford dye-binding procedure (Bio-Rad Protein assay).

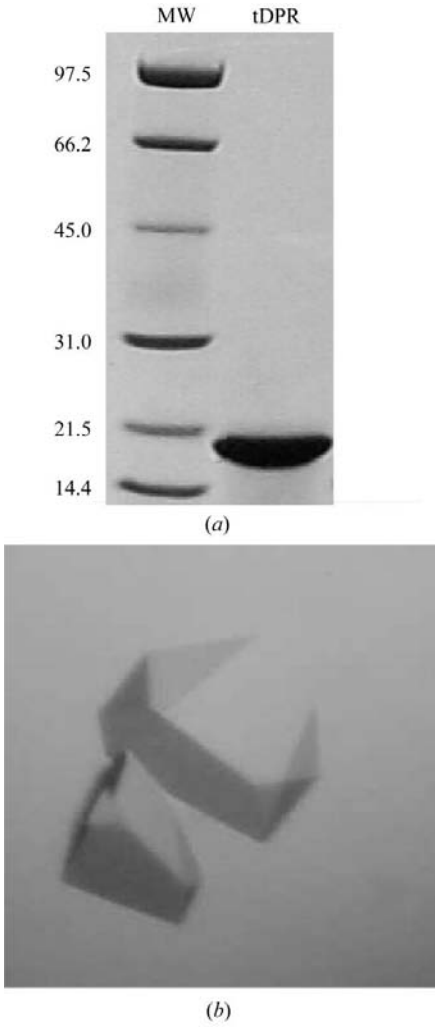

Figure 1
(*a*) SDS–PAGE (BioRad 4–15% gradient gel) showing the purity of tDpr before crystallization. Molecular-weight markers are shown (values in kDa). (*b*) Single crystals of tDpr grown after optimization of the crystallization conditions. The approximate dimensions of these crystals are 0.1–0.2 mm.

2.2. Protein characterization

The construction of pSSUD2 generated tDpr fusion protein linked to an N-terminal 6×His tag through a thrombin-cleavage site. Thrombin digestion produced tDpr with N-terminal sequence Gly-Ser-Pro-Ala-Glu-Ile-Ala-Ser-Phe (Gly in position 1 replaces Gln owing to the engineering of the thrombin-cleavage site) as expected. The purity of the protein was assessed by SDS–PAGE, which showed a single band at the expected MW range (∼19 kDa; Fig. 1*a*). Precise measurement of the MW was achieved by MALDI–TOF analysis (Applied Biosystems Voyager DE PRO), which revealed a single peak of molecular weight 18 449 Da in accordance with the sequence-estimated MW. Purified tDpr protein shows an apparent MW of ∼235 kDa when analyzed by gel filtration (data not shown), suggesting that the protein forms oligomers (dodecamers).

2.3. Crystallization

Prior to crystallization, the protein was concentrated to 10 mg ml^{-1} using YM-10 Centricon filters (Amicon). Initial crystallization trials of tDpr with the 6×His tag present were set up using the sparse-matrix (Hampton) screen (Jancarik & Kim, 1991) and the hanging-drop vapour-diffusion method. Inspection of the drops after 2 d revealed amorphous precipitate in most of the conditions, providing no clear clues to potential crystallization conditions. It was therefore decided to remove the 6×His tag by thrombin cleavage. Initial crystallization trials at 289 K using the 6×His-free tDpr produced microcrystals in condition 14 of the sparse-matrix screen. Further optimization of condition 14 produced crystals suitable for X-ray diffraction experiments by mixing 2.0 µl of protein (10 mg ml^{-1}) with 2.0 µl of a reservoir solution consisting of 27–30%(*v*/*v*) PEG 400, 0.2 *M* $CaCl_2$, 0.1 *M* HEPES–NaOH pH 7.2–7.4. Single crystals appeared overnight and reached their maximum size (0.1–0.2 mm) within 2 d (Fig. 1*b*).

2.4. Data collection and processing

Preliminary characterization of the crystals was carried out in the laboratory using a MAR345 imaging-plate detector mounted on a Rigaku Rotaflex RU-200 rotating-anode generator equipped with Osmic focusing mirrors. Owing to the small size of the crystals and their weak diffraction properties in-house, X-ray synchrotron radiation was necessary. A complete data set to 2.3 Å resolution was collected from a

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.37–2.30 Å).

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 104.3, b = 137.6, c = 142.1
No. of crystals	1
Temperature (K)	100
No. of measurements	1522974
No. of unique reflections	90491
Resolution range (Å)	20.0–2.3
Mosaicity (°)	0.45
$R_{\rm merge}$† (%)	9.2 (23.6)
Completeness (%)	98.8 (88.4)
$I/\sigma(I)$	13.1 (2.4)
B factor‡ (Å^2)	30.2

† $R_{\rm merge} = \sum(|I_j - \langle I\rangle|)/\sum\langle I\rangle$, where I_j is the observed intensity of reflection j and $\langle I\rangle$ is the average intensity of multiple observations. ‡ Wilson plot.

single crystal at the XRD1 beamline, Elettra Synchrotron Light Laboratory, Trieste, Italy. The crystal was flash-cooled to 100 K in a nitrogen-gas cold stream (Oxford Cryosystems Cryostream Cooler). The concentration of PEG 400 in the crystallization medium was sufficient for cryoprotection of the crystals; therefore, no cryoprotectant was added. Diffraction data were recorded on a MAR CCD detector (diameter 165 mm). The exposure time was 10–15 s per image (dose mode), the oscillation range was 0.5° and the crystal-to-detector distance was set to 172 mm. A total of 201 images were collected at a wavelength of 1.0 Å. All data were processed, scaled and merged with the *HKL* package (Otwinowski & Minor, 1997). Owing to poor completeness in the low-resolution shells, a second pass was necessary to record low-resolution reflections. The exposure time used for the low-resolution pass was one third of that used for the recording of the high-resolution reflections. A total of 131 images were collected on the second run and when merged with the previously recorded 201 images gave a completeness of 99.7% in the resolution shell 20.0–5.2 Å. The crystals belong to the orthorhombic space group $P2_12_12_1$, as revealed by analysis of the systematic absences along the $hk0$, $h0l$ and $0kl$ zones. The unit-cell parameters are $a = 104.3, b = 137.6, c = 142.1$ Å, $\alpha = \beta = \gamma = 90°$. Complete data-collection statistics are shown in Table 1. Assuming 12 molecules in the asymmetric unit, the Matthews coefficient (Matthews, 1968) is 2.3 Å^3 Da^{-1}, which corresponds to a solvent content of ∼44%. The presence of a dodecamer in the crystal is in agreement with the results obtained by gel filtration.

3. Conclusions

Full-length and truncated Dpr from *S. suis* was cloned, expressed and purified. Full-length protein was prone to proteolysis and hence was not suitable for crystallization attempts. Truncated Dpr lacking the first seven N-terminal residues of the full-length protein was stable against proteolysis and was therefore used in crystallization. However, protein carrying the 6×His tag did not crystallize and enzymatic cleavage was used to remove the hexahistidine affinity tag in an effort to reduce flexibility in the protein. Crystals suitable for X-ray diffraction experiments were obtained. Owing to weak diffraction of the crystals in the home source, synchrotron radiation was used to collect a complete data set to 2.3 Å resolution. The structure will be determined either by the MAD method (72 methionines in total for the entire dodecamer) or molecular-replacement techniques (Navaza, 1994). In the latter approach, the structure of *L. innocua* ferritin (Ilari *et al.*, 2000) will be initially used as a search model (sequence homology $\simeq$ 60%). Dpr is the first ferritin-like protein from the streptococcal family that has been crystallized. Structure determination will provide further insights into the structural basis of the diverse functions observed in ferritin-like proteins from different bacteria.

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