Purification, crystallization and X-ray crystallographic analysis of *Archaeoglobus fulgidus* neelaredoxin

Tiago M. Bandeiras, Célia V. Romão, João V. Rodrigues, Miguel Teixeira and Pedro M. Matias


Acta Crystallographica Section F: Structural Biology and Crystallization Communications is a rapid all-electronic journal, which provides a home for short communications on the crystallization and structure of biological macromolecules. It includes four categories of publication: protein structure communications; nucleic acid structure communications; structural genomics communications; and crystallization communications. Structures determined through structural genomics initiatives or from iterative studies such as those used in the pharmaceutical industry are particularly welcomed. Section F is essential for all those interested in structural biology including molecular biologists, biochemists, crystallization specialists, structural biologists, biophysicists, pharmacologists and other life scientists.

Crystallography Journals Online is available from journals.iucr.org
Purification, crystallization and X-ray crystallographic analysis of *Archaeoglobus fulgidus* neelaredoxin

Neelaredoxins are a type of superoxide reductase (SOR), which are blue 14 kDa metalloproteins with a catalytic nonhaem iron centre coordinated by four histidines and one cysteine in the ferrous form. Anaerobic organisms such as *Archaeoglobus fulgidus*, a hyperthermophilic sulfate-reducing archaeon, have developed defence mechanisms against toxic oxygen species in which superoxide reductases play a key role. SOR is responsible for scavenging toxic superoxide anion radicals ($O_2^-$), catalysing the one-electron reduction of superoxide to hydrogen peroxide. Crystals of recombinant *A. fulgidus* neelaredoxin in the oxidized form (13.7 kDa, 125 residues) were obtained using polyethylene glycol and ammonium sulfate. These crystals diffracted to 1.9 Å resolution and belonged to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = b = 75.72$, $c = 185.44$ Å. Cell-content analysis indicated the presence of a tetramer in the asymmetric unit, with a Matthews coefficient ($V_M$) of 2.36 Å$^3$ Da$^{-1}$ and an estimated solvent content of 48%. The three-dimensional structure was determined by the MAD method and is currently under refinement.

1. Introduction

Superoxide ($O_2^-$) scavenging enzymes are key antioxidant systems in living organisms as they maintain the concentration of superoxide below poisonous thresholds. This toxic species is formed in cells either continuously in the case of aerobes or transiently in the case of anaerobes and is the product of uncontrolled one-electron reduction of oxygen (Halliwell & Gutteridge, 1999). At present, two types of metal-containing enzymes are known to eliminate $O_2^-$ in an extremely efficient way: superoxide dismutases (SODs) and superoxide reductases (SORs) (Imlay, 2003; Pinto et al., 2009). These enzymes operate using distinct mechanisms; although they share a common step, $O_2^-$ reduction, SOD is also able to catalyze the oxidation of $O_2^-$ whereas SOR is not. The reasons for the different reactivities shown by these enzymes are still unclear.

Superoxide reductases are mononuclear nonhaem iron proteins and are classified according to the number of iron centres as 1Fe-SORs (neelaredoxins) or 2Fe-SORs (desulfoferredoxins) (see, for example, Pinto et al., 2009). In addition to the iron centre of the active site, 2Fe-SORs possess a second N-terminal protein domain with a desulfoferredoxin fold containing an additional iron centre $[\text{Fe(Cys)}_4]^{3+}$, the function of which is still unknown. The common iron centre responsible for $O_2^-$ reduction is pentacoordinated in the ferrous state, with the imidazole N atoms of four equatorial histidine ligands and one axial cysteine ligand in a square pyramidal-like geometry $[\text{Fe(Cys)(His)}_3]^{3+}$ (Yeh et al., 2000; Santos-Silva et al., 2006; Coelho et al., 1997; Adam et al., 2004). In the SOR resting state, when the protein is fully oxidized, a sixth glutamate ligand is present at the other axial position, yielding an octahedrally coordinated metal centre (Yeh et al., 2000; Adams et al., 2002; Berthomieu et al., 2002).
However, it should be noticed that this glutamate is not strictly
conserved among SORs (Pinto et al., 2009).

The superoxide-reduction mechanisms of the different SOR
families have been studied by pulse radiolysis and stopped-flow
spectroscopy, and it seems clear that SORs from different sources
show striking differences in their kinetics upon reaction with O2
(Niviére et al., 2004; Emerson et al., 2003; Rodrigues et al., 2006, 2007,
2008). It has previously been shown that both 1Fe-SOR and 2Fe-SOR
from the hyperthermophilic archaean Archaeoglobus fulgidus react
with O2 at diffusion-limited rates of \( \sim 10^9 M^{-1} s^{-1} \) to form an Fe\( ^{3+}\)-
(hydro)peroxide transient that decays to an intermediate in which a
solvent molecule is initially bound to the iron and is then displaced
by a glutamate residue (Rodrigues et al., 2006, 2007). In contrast,
although a similar Fe\(^{3+}\)-(hydro)peroxide transient is formed in the
case of the 2Fe-SOR from the mesophile Desulfovibrio vulgaris,
in this enzyme this intermediate decays directly to the final Fe\(^{3+}\)–
glutamate resting state (Emerson et al., 2003; Huang et al., 2007).

Crystal structures have been solved of 2Fe-SORs from the bacteria
Desulfovibrio desulfuricans (Coelho et al., 1997) and Desulfoarculus
baarsii (Adam et al., 2004) and of 1Fe-SORs from the archaea
Pyrococcus furiosus (Yeh et al., 2000) and P. horikoshii and the bacteria
Thermotoga maritima and Treponema pallidum (Santos-Silva et al., 2006);
the T. pallidum enzyme has an additional N-terminal domain but lacks the second iron centre [Fe(Cys)]3. All
these SOR molecules from different sources present similar archi-
tectural folds; the oligomerization states (homodimers or tetramers)
appear to be correlated with the absence or presence of the extra
N-terminal domain, respectively.

The superoxide reductase isolated from A. fulgidus, a hyperther-
mophilic sulfate-reducing archaean, was isolated as a functional homotetramer of four 14 kDa subunits (Abreu et al., 2000). The crystal structure of A. fulgidus SOR will be the second single crystal structure of a 1Fe-SOR, also known as nearedoxin (Nlr), from an archaean and the first example of a fully oxidized 1Fe-SOR. Comparison with known three-dimensional SOR structures, together with detailed analysis of the iron active site, will contribute to clarification of the remaining questions regarding the superoxide-reduction mechanism.

2. Experimental procedures and results

2.1. Protein expression and purification

The overexpression and purification of A. fulgidus nearedoxin
(Af Nlr) were performed as described previously (Rodrigues et al.,
2006) with some modifications. For the overexpression of A. fulgidus
nearedoxin, cultures of BL21-Gold (DE3) cells (Stratagene) con-
taining the plasmid pT7AfNlr (Abreu et al., 2000) were grown aerobic-
ly at 303 K in M9 minimal medium supplemented with
100 \( \mu \)g ml\(^{-1} \) ampicillin and 100 \( \mu \)M FeSO\(_4\) in a 10 l fermenter. When
the culture reached a cell density of \( A_{600} = 0.3 \), 0.1 \( m \)M isopropyl
\( \beta \)-D-1-thiogalactopyranoside was added and after 6 h induction
the cells were harvested by centrifugation (10 000 \( g \), 10 min) and washed
with 10 mM Tris–HCl pH 7.6. All subsequent purification steps
were performed at 277 K. The cells were broken in a mini-cell French
press at 6.2 MPa and the soluble fraction was obtained by ultra-
centrifugation (10 000 \( g \), 4 h). After overnight dialysis against 10 mM
Tris–HCl pH 7.6, the sample was degassed and incubated for \( \sim 1 \) h
at 277 K with 1 mM dithiothreitol, 10 mM ascorbate and 0.1 mM FeSO\(_4\)
under anaerobic conditions by purging with N2. The sample was then
loaded on to a Q-Sepharose column (Pharmacia) previously equili-
brated with Tris–HCl pH 7.6. A linear gradient of 0–0.5 M NaCl was
applied and the fraction containing Af Nlr was eluted at 0.25 M NaCl.
This fraction was dialyzed against 10 mM Tris–HCl pH 7.6 and loaded
onto a Fractogel EMD TMAE column (Merck) equilibrated with the
same buffer. A linear gradient of 0–0.5 M NaCl was applied and the
fraction containing Af Nlr was eluted at 0.25 M NaCl. The sample
was concentrated by ultrafiltration (Millipore) and loaded onto a
Superdex 200 column (Pharmacia) equilibrated with 20 mM Tris–HCl
pH 7.6 and 150 mM NaCl. This last step allows effective separation
of the most abundant tetrameric form of nearedoxin from other
higher oligomeric states of the protein, as described by Rodrigues et al.
(2006). Protein purity was tested by SDS–PAGE (Fig. 1) and the
Bicinchoninic Acid Protein Assay Kit (Pierce; Smith et al., 1985) was
used to determine the protein concentration. The total iron content
was determined using the 2,4,6-tripyridyl-s-triazine (TPTZ) method
(Fisher & Price, 1964). The oxidized protein was prepared by incuba-
tion with a slight excess of K2IrCl6 and the oxidant was removed by
repeated dilutions and concentrations in a Centricon device.

2.2. Crystallization and cryoprotection

Preliminary crystallization trials were performed with protein
centrifuged at 15 mg ml\(^{-1} \) in 20 mM phosphate buffer pH 7.5,
150 mM NaCl using the vapour-diffusion technique. Nanolitre-scale
drops were prepared with the commercially available Classics I kit
(Qiagen) using a Cartesian Crystallization Robot Dispensing System
(Genomics Solutions) with round-bottom 96-well Greiner Crystal-
Quick plates (Greiner Bio-One). Three different drop proportions
were prepared per condition screened, using (i) 100 nl reservoir
solution and 100 nl protein solution, (ii) 100 nl reservoir solution and
200 nl protein solution and (iii) 200 nl reservoir solution and 100 nl
protein solution. The drops were equilibrated against 100 \( \mu \)l reservoir
solution. Blue crystalline forms were observed using condition No. 85
of the Classics I screen, consisting of 0.2 \( M \) ammonium sulfate, 0.1 \( M 

\text{NaCl was applied and the fraction containing Af Nlr was eluted at 0.25 M NaCl. This fraction was dialyzed against 10 mM Tris–HCl pH 7.6 and loaded onto a Fractogel EMD TMAE column (Merck) equilibrated with the same buffer. A linear gradient of 0–0.5 M NaCl was applied and the fraction containing Af Nlr was eluted at 0.25 M NaCl. The sample was concentrated by ultrafiltration (Millipore) and loaded onto a Superdex 200 column (Pharmacia) equilibrated with 20 mM Tris–HCl pH 7.6 and 150 mM NaCl. This last step allows effective separation of the most abundant tetrameric form of nearedoxin from other higher oligomeric states of the protein, as described by Rodrigues et al. (2006). Protein purity was tested by SDS–PAGE (Fig. 1) and the Bicinchoninic Acid Protein Assay Kit (Pierce; Smith et al., 1985) was used to determine the protein concentration. The total iron content was determined using the 2,4,6-tripyridyl-s-triazine (TPTZ) method (Fisher & Price, 1964). The oxidized protein was prepared by incubation with a slight excess of K2IrCl6 and the oxidant was removed by repeated dilutions and concentrations in a Centricon device.}

**Figure 1**

10% SDS–PAGE gel of pure recombinant Af Nlr. Lane 1, Bio-Rad unstained protein markers (product No. 161-0363). Lane 2, Af Nlr monomer migrating according to a molecular mass of 13.7 kDa.
tion containing 0.2 M ammonium sulfate, 0.1 M sodium acetate pH 4.6, 15% PEG 4000 with 1.5 μl protein solution and equilibrating against 500 μl reservoir solution. Quadrangular prismatic blue crystals grew for 10 d and reached final dimensions of 0.3–2 mm in the longest axis (Fig. 2). AF Nlr crystals were cryoprotected using reservoir solution supplemented with 25% glycerol prior to flash-cooling in liquid nitrogen.

2.3. Data collection and crystallographic analysis

The vast majority of the AF Nlr crystals tested for X-ray diffraction were highly anisotropic and their diffraction images could not be indexed. However, the diffraction pattern of one AF SOR crystal could be indexed and a three-wavelength MAD data set was collected at 100 K from this crystal using a MAR 225 CCD detector on the tunable-wavelength beamline BM14 at ESRF, Grenoble. An X-ray fluorescence spectrum near the Fe K absorption edge was measured in order to select the monochromator settings for the peak (λ₁ = 1.740 Å, maximum Δf°) and the inflection-point (λ₂ = 1.741 Å, minimum Δf°) wavelengths using CHOOCH (Evans & Pettifer, 2001). The high-energy remote wavelength λ₃ was chosen as 0.9537 Å.

All data sets were integrated with MOSFLM (Leslie, 1992) and scaled with SCALA from the CCP4 suite (Collaborative Computational Project, Number 4, 1994). The data-collection and processing statistics are summarized in Table 1. The AF Nlr crystal belonged to one of the enantiomorphic tetragonal space groups P4₁2₁2₁ or P4₃2₁2, with unit-cell parameters a = b = 75.72, c = 185.44 Å. The Matthews coefficient was determined as Vm = 2.36 Å³Da⁻¹, suggesting the presence of four SOR molecules in the asymmetric unit with a predicted solvent content of 48%.

2.4. Structure determination

The three-dimensional structure of AF Nlr was solved using the MAD method. Matthews coefficient calculations suggested the presence of one Nlr tetramer in the asymmetric unit, hence four Fe sites were expected. Using the HKL2MAP graphical user interface (Pape & Schneider, 2004), the MAD data set was analysed with SHELX, the heavy-atoms substructure was determined with SHELXD and the phase problem was solved with SHELXE (Sheldrick, 2008). SHELXD found at least one possible solution out of 200 trials, the best of which had a correlation coefficient of 51.36% and contained four possible iron sites in the crystal asymmetric unit with occupancy factors ranging from 0.85 to 1.0. SHELXE gave a clear discrimination between the correct and inverted substructures and resolved the space-group ambiguity in favour of P4₁2₁2₁.

Automated model building was performed with Buccaneer (Cowtan, 2006) using the 1.9 Å remote-wavelength data set, which generated a complete 500-residue model with R = 0.361 and Rmerge = 0.381 and a mean figure of merit of 0.642. Rmerge was based on a randomly chosen 5% subset of the measured data. The Fe atoms in the SOR active sites were modelled using Coot (Emsley & Cowtan, 2004) and the structure is currently under refinement.

The authors would like to acknowledge José A. Brito for assistance with data processing and phasing at IFOB. JR is the recipient of an FCT-MCTES (Portugal) fellowship (SFRH/BPD/34763/2007). The work was supported by Fundação para a Ciência e Tecnologia Project PTDC/BIA-PRO/67263/2006. We would like to thank the EMBL Grenoble Outstation for providing support for measurements on the ESRF EMBL–CRG BM14 beamline under the European Community Access to Research Infrastructure Action FP6 Programme ‘Structuring the European Research Area Specific Program’ with contract No. RI3-CT-2004-506008. The help and advice given by Martin A. Walsh and Hassan Belrhali (EMBL, Grenoble) during the crystallographic analysis

Table 1  Data collection and processing statistics. Values in parentheses are for the last resolution shell.

| Wavelength (Å) | 1.740 | 1.741 | 0.9537 |
|Resolution range (Å) | 46.4–2.4 (2.5–2.4) | 46.4–2.2 (2.3–2.2) | 37.1–1.9 (2.0–1.9) |
|Completeness, overall (%) | 100 (100) | 99.9 (99.5) | 100 (100) |
|Completeness, anomalous (%) | 100 (100) | 99.9 (99.1) | 100 (99.9) |
|No. of observations | 156599 (22337) | 190247 (21348) | 297205 (38951) |
|No. of unique reflections | 25725 (3219) | 28388 (4020) | 42230 (6054) |
|Multiplicity, overall | 6.9 (6.9) | 7.0 (6.1) | 6.9 (6.9) |
|Multiplicity, anomalous | 3.6 (3.5) | 3.6 (3.6) | 3.6 (3.6) |
|Rmerge † (%) | 0.071 (0.270) | 0.065 (0.318) | 0.071 (0.384) |
|Rmerge ‡ (%) | 0.047 (0.122) | 0.036 (0.167) | 0.026 (0.176) |
|Rmerge (for I) | 6.3 (1.9) | 8.2 (2.0) | 7.2 (2.0) |

† Rmerge = ∑_hkl |I(hkl)−⟨I(hkl)⟩|/∑_hkl I(hkl), ‡ Rmerge = ∑_hkl |⟨I(hkl)⟩|/∑_hkl I(hkl), where ⟨I(hkl)⟩ is the observed intensity. ⟨I(hkl)⟩ is the average intensity of multiple observations from symmetry-related reflections and N is their redundancy.

Figure 2
Crystals of A. fulgidus superoxide reductase (neelaredoxin) grown in 0.2 M ammonium sulfate, 0.1 M sodium acetate pH 4.6, 15% (v/v) PEG 4000. The crystal dimensions are ~0.6 × 0.2 × 0.15 mm.

References


