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Zinc-substituted pseudoazurin solved by S/Zn-SAD phasing

The copper(II) centre of the blue copper protein pseudoazurin from *Alcaligenes faecalis* has been substituted by zinc(II) *via* denaturing the protein, chelation and removal of copper and refolding the apoprotein, followed by the addition of an aqueous solution of ZnCl_2 . Vapour-diffusion experiments produced colourless hexagonal crystals (space group $P6_5$), which when cryocooled had unit-cell parameters $a = b = 49.01$, $c = 98.08$ Å. Diffraction data collected at 100 K using a copper sealed tube were phased by the weak anomalous signal of five S atoms and one Zn atom. The structure was fitted manually and refined to 1.6 Å resolution. The zinc-substituted protein exhibits similar overall geometry to the native structure with copper. Zn^{2+} binds more strongly to its four ligand atoms (His40 N^{δ1}, Cys78 S^γ, His81 N^{δ1} and Met86 S^δ) and retains the tetrahedral arrangement, although the structure is less distorted than the native copper protein.

1. Introduction

Pseudoazurin (PA) is a periplasmic blue copper protein (123 amino-acid residues); it is a member of the cupredoxin family and participates in the electron-transport process leading to the reduction of nitrite to mainly nitric oxide in denitrifying bacteria (Kakutani *et al.*, 1981). The electron acceptor of pseudoazurin, hereafter called Cu(II)-PA, is the copper enzyme nitrite reductase, the crystal structure of which has been determined (Murphy *et al.*, 1997), along with its solution structure in complex with Cu(II)-PA (Vlasie *et al.*, 2008). At present there are more than 30 deposited pseudoazurin crystal structures in the Protein Data Bank (Berman *et al.*, 2000), corresponding to proteins from six different bacterial species. More than a third of the known structures are of the *Alcaligenes faecalis* protein, representing structures of the native protein (Petratos *et al.*, 1988), various molecular variants containing either copper(II) or copper(I) (Libeu *et al.*, 1997), Cu(I)-PA at two pH values (Vakoufari *et al.*, 1994) and a metal-free apo PA (Petratos *et al.*, 1995). The second structurally most studied pseudoazurin comes from *Achromobacter cycloclastes* (Inoue *et al.*, 1999; Velarde *et al.*, 2007; Yanagisawa *et al.*, 2008), and exhibits 67% sequence identity to the *A. faecalis* protein. For the requirements of various spectroscopic experiments, substitution of the original copper(II) by other transition-metal ions such as cobalt(II) (Fernández *et al.*, 2003; Gessmann *et al.*, 2011), nickel(II) (Dennison & Sato, 2002) or zinc(II) (Prudêncio *et al.*, 2004) has been carried out successfully. The latter substitution also included a lanthanide-binding loop attached to the protein.

In order to examine the phasing potential of the weak anomalous signal of S and Zn atoms using our in-house diffractometer (Bruker AXS Inc.), we substituted the native Cu^{2+} ion of the protein from *A. faecalis* by Zn^{2+} ion [Zn(II)-PA] and determined its crystal structure by S/Zn SAD phasing at 2.2 Å resolution. Refinement was carried out with data extending to 1.6 Å resolution.

2. Materials and methods

2.1. Macromolecule production

The protein was purified from transformed *Escherichia coli* JM105 cells harbouring the recombinant plasmid pUB1 as described in

Table 1

Pseudoazurin-production information.

Source organism	<i>A. faecalis</i> (organism_taxid 511)
Expression vector	Plasmid PAB301
Expression host	<i>E. coli</i> (organism_taxid 562)
Complete amino-acid sequence of the construct produced	AZUP_ALCFA, P04377 (UniProt)

Yamamoto *et al.* (1987). Some details of pseudoazurin and its production are given in Table 1. The growth of the bacteria and the protein-extraction procedure are described in Vakoufari *et al.* (1994). Q-Sepharose and SP-Sepharose Fast Flow (GE Healthcare, Piscataway, New Jersey, USA) replaced the previously used chromatography materials DEAE Sephacel and CM Sepharose CL 6B, respectively. 20 g of cell pellet yields about 6 mg of essentially pure Cu(II)-PA.

Removal of the Cu²⁺ ion from Cu(II)-PA was carried out as described in Gessmann *et al.* (2011) except that the addition of an aqueous solution of CoCl₂ to the refolded apoprotein solution was replaced by ZnCl₂. The concentrated protein was colourless, as expected for a zinc-bound protein. Finally, Zn(II)-PA was transferred and concentrated into the crystallization buffer (Table 2).

2.2. Crystallization

Crystallization experiments were carried out as described in Table 2 according to the previously determined crystallization conditions for the original Cu(II)-PA. Hexagonal bipyramidal crystals (Fig. 1*a*) of varying size grew a few days after setting up the plates. The average size of the crystals obtained was 0.2 × 0.2 × 0.3 mm. Their symmetry was the same as of that of the crystals of the native blue Cu(II)-PA and the unit-cell dimensions were slightly smaller, probably owing to the cryocooling. The relevant crystal parameters are summarized in Table 3. The presence of zinc(II) was confirmed by mass-spectrometric analysis of properly washed crystals (S. A. Pergantis, private communication).

2.3. Data collection and processing

One crystal (Fig. 1*b*) was briefly immersed in a 50:50(v:v) glycerol-water mixture and subsequently cooled to 100 K in a cold nitrogen stream. It diffracted well using the focused Cu K α radiation produced by the Cu I μ S micro-source of a Bruker-AXS diffractometer. Diffraction data were collected from a single crystal to 1.6 Å resolution with moderate (eightfold) redundancy. In addition, a second

Table 2

Crystallization.

Method	Vapour diffusion, sitting drop
Plate type	Petri dishes (85 mm diameter)
Temperature (K)	292
Protein concentration (mg ml ⁻¹)	15
Buffer composition of protein solution	50 mM sodium citrate pH 5.7, 20 mM ZnCl ₂
Composition of reservoir solution	2.8 M ammonium sulfate, 50 mM sodium citrate pH 5.7, 20 mM ZnCl ₂
Volume and ratio of drop	80 μ l (3:2–2:3 protein solution:reservoir)
Volume of reservoir (ml)	10

Table 3

Data collection and processing.

Values in parentheses are for the outer shell. *PROTEUM2*, *SAINT* and *SADABS* (Bruker) were used for data collection, processing and scaling.

Diffraction source	Sealed tube, Cu anode
Wavelength (Å)	1.5418
Temperature (K)	100
Detector	PHOTON100 area detector, Bruker-AXS
Crystal-to-detector distance (mm)	70
Rotation range per image (°)	0.8
Total rotation range (°)	432
Exposure time per image (s)	20
Space group	<i>P</i> 6 ₃
<i>a</i> , <i>b</i> , <i>c</i> (Å)	49.01, 49.01, 98.08
α , β , γ (°)	90, 90, 120
Mosaicity (°)	0.58
Resolution range (Å)	21.23–1.60 (1.64–1.60)
Total No. of reflections	138568 (3476)
No. of unique reflections	17521 (1177)
Completeness (%)	99.1 (94.5)
Multiplicity	7.9 (3.0)
$\langle I/\sigma(I) \rangle$	23.1 (4.3)
$R_{p.i.m.}^\dagger$	0.0219 (0.294)
Overall <i>B</i> factor from Wilson plot (Å ²)	10.4

$^\dagger R_{p.i.m.}$ is the multiplicity-weighted precision-indicating merging *R* factor for comparing symmetry-related reflections (Weiss & Hilgenfeld, 1997).

data set was collected from the same crystal with very high redundancy (87-fold) to a resolution of 2.2 Å for SAD phasing. Details of the high-resolution data collection and processing are shown in Table 3.

2.4. Structure solution and refinement

Phasing of the Bragg reflections was carried out successfully with the weak anomalous signal of the Zn atom and the five methionine S atoms (Dauter *et al.*, 1999; Ramagopal *et al.*, 2003), which were located by *SHELXD* (Sheldrick, 2010). During the initial phasing cycles, these anomalous scatterers and only a few other residues of

Figure 1

Zinc-substituted pseudoazurin. (*a*) Colourless crystals in their mother liquor and (*b*) the mounted cooled crystal which was used for data collection. The bar indicates 200 μ m.

the structure were located, whereas in advanced SAD-phased electron-density maps 96 out of 123 partly wrongly traced residues in six chains could be detected with *SHELXE* (Sheldrick, 2010). No prior knowledge from known pseudoazurin structures was used for tracing of the chain. Reiterated restrained refinement with *REFMAC5* (Murshudov *et al.*, 2011) as incorporated in the *CCP4* suite of programs (Winn *et al.*, 2011) produced better maps after manual intervention employing *Xfit* (McRee, 1999) and *Coot* (Emsley *et al.*, 2010). All residues were identified in the final difference maps, including the three residues at the carboxyl-terminal region which are usually missing. The progress between the initial SAD phasing and

the final refinement of the structure is illustrated in Fig. 2. The relevant parameters are listed in Table 4.

The effect of data redundancy on SAD phasing was also investigated. The number of data-collection runs was gradually decreased from the original 75 (as defined by the strategy option of *PROTEUM2*) with fewer measured reflections included in the integration, scaling and phasing programs. It was thus shown that a small fraction (equivalent to six data-collection runs) of the original data collected, *i.e.* a data set with approximately eightfold redundancy, is sufficient to determine the necessary substructure for successful phase determination.

Figure 2

The same sections of Zn(II)-PA in electron-density maps calculated at two different stages of the structure determination: (a) at the initial SAD phasing cycles and (b) at an advanced stage of refinement. The electron density (blue) of the weighted $2F_o - F_c$ maps is contoured at 2.0 and 2.5σ , respectively. Protein atom colouring: carbon, yellow; oxygen, red; nitrogen, blue; sulfur, green. The zinc ion and ordered waters are shown as grey and cyan spheres, respectively. The figures showing the structures were prepared with *PyMOL* (<http://www.pymol.org>).

Figure 3

Comparison of Zn(II)-PA and Cu(II)-PA (PDB entry 1paz). A stereoview (wall-eyed stereo) of the superposed structures shown as ribbon diagrams. The four ligand side chains (His40, Cys78, His81 and Met86) are shown in stick presentation. Cu(II)-PA, its copper ion and ligand residues are uniformly coloured blue. Zn(II)-PA and its zinc ion are coloured grey. Zinc ligand side chains are coloured by element type. The electron density (green) of a weighted $2F_o - F_c$ map at the metal site is contoured at 2σ .

Table 4

Structure solution and refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	21.2–1.60 (1.64–1.60)
Completeness (%)	99.1 (94.5)
σ Cutoff	0.0
No. of reflections, working set	16645 (1110)
No. of reflections, test set	876 (67)
Final R_{cryst}	0.184 (0.248)
Final R_{free}	0.207 (0.233)
Cruickshank DPI (Å)	0.133
No. of non-H atoms	
Total	1213
Protein	1077
Ligand	11
Water	125
R.m.s. deviations	
Bonds (Å)	0.013
Angles (°)	1.7
Average B factors (Å ²)	
Overall	14.3
Protein	13.5
Ramachandran plot	
Most favoured (%)	99
Allowed (%)	1

3. Results and discussion

The final model adopts the β -sandwich fold with two α -helices in the carboxyl-terminal region (Fig. 3). The side chains of 18 residues were modelled in two or three alternate conformations. Compared with the native Cu(II)-PA structure (PDB entry 1paz; Petratos *et al.*, 1988), Zn(II)-PA shows an average r.m.s. displacement of 0.3 Å for its 480 main-chain atoms. The corresponding value for the 436 side-chain atoms was 0.8 Å. This result shows that PA can refold spontaneously back to its original structure in the absence of its 23-residue leader peptide and any metal ion. This is in agreement with previous results (Petratos *et al.*, 1995; Gessmann *et al.*, 2011), lending independent evidence to the notion that pseudoazurin is designed to have a stable metal-binding fold. The only significant deviations between the two structures are confined within the last three common residues (Val118, Ile119 and Ala120) and the long and flexible side chains that were assigned high temperature factors (>60 Å²) in the original refined structure (PDB entry 1paz).

Zn(II)-PA also exhibits a similar active-site geometry to the recently determined cobalt-substituted variant [Co(II)-PA; PDB entry 3nyk; Gessmann *et al.*, 2011] as well as to the original structure containing copper (Fig. 3). After superposition of the latter structure onto Zn(II)-PA, the respective coordinating atoms and the metal ions deviate by 0.1–0.2 Å. The only prominent differences are the tighter coordination of zinc(II) by the axial Met86 S^δ (the bond distance decreased by 0.2 Å) and the more ‘canonical’ values for the two angles His40 N^{δ1}–Zn–Cys78 S^γ and His40 N^{δ1}–Zn–Met86 S^δ of 127 and 91°, respectively, compared with the ideal tetrahedral value of 109.5°. The corresponding angles in the Cu(II)-PA structure are 136 and 87°, respectively, leading to the highly distorted (‘perturbed’) type 1 site. The latter classification is based on the different spectroscopic fingerprints of copper-containing proteins (Randall *et al.*, 2000). In addition, zinc(II) and the plane of imidazole of His40 have moved so that the metal ion lies closer (0.2 Å) to the plane of the strong ligands (His40 N^{δ1}, Cys78 S^γ and His81 N^{δ1}). Selected geometrical values for the metal site of the protein compared with Cu(II)-PA are shown in Table 5.

Finally, the structure of an engineered double variant (E51C, E54C) of pseudoazurin has also been determined previously from twinned crystals to 2.0 Å resolution (PDB entry 1py0; Prudêncio *et al.*, 2004). This protein was also zinc-substituted and contained a

Table 5

Comparison of the coordination geometries of Cu(II)-PA and Zn(II)-PA.

Distance† (Zn ²⁺ /Cu ²⁺) (Å)	
His40 N ^{δ1}	2.1/2.2
Cys78 S ^γ	2.2/2.2
His81 N ^{δ1}	2.0/2.1
Met86 S ^δ	2.6/2.8
Gly39 O‡	3.8/3.8
Cys78 S ^γ –Asn41 N§	3.6/3.6
His40 N ^{ε2} –Asn9 O ^{δ1} §	2.8/2.7
His81 N ^{ε2} –Ow§	2.8/2.8
Distances of ions from planes (Zn ²⁺ /Cu ²⁺) (Å)	
Imidazole ring (His40)	0.2/0.1
Imidazole ring (His81)	0.0/0.0
His40 N ^{δ1} , Cys78 S ^γ , His81 N ^{δ1}	0.2/0.4
His40 N ^{δ1} , Cys78 S ^γ , Met86 S ^δ	0.7/0.7
His40 N ^{δ1} , His81 N ^{δ1} , Met86 S ^δ	1.0/1.1
Cys78 S ^γ , His81 N ^{δ1} , Met86 S ^δ	0.7/0.7
Angles¶ (Zn ²⁺ /Cu ²⁺) (°)	
His40 N ^{δ1} –M††–Cys78 S ^γ	127/136
His40 N ^{δ1} –M–His81 N ^{δ1}	104/100
His40 N ^{δ1} –M–Met86 S ^δ	91/87
Cys78 S ^γ –M–His81 N ^{δ1}	111/112
Cys78 S ^γ –M–Met86 S ^δ	112/108
His81 N ^{δ1} –M–Met86 S ^δ	110/112
M–Cys78 S ^γ –Cys78 C ^β ‡‡	101/105

† The estimated standard error in the reported distances is less than 0.1 Å. ‡ The carbonyl O atom of Gly39 remains distant from the metal as in the native structure (PDB entry 1paz). § These atoms participate in hydrogen bonds to the metal ligand S^γ or to members (N^{ε2}) of the metal-interacting imidazole rings. ¶ The estimated error in the bond angles is ~2°. †† M denotes either Zn²⁺ or Cu²⁺. ‡‡ This angle is implicated in the multiple mode of binding of the thiolate S atom and the copper ion (Holm *et al.*, 1996).

lanthanide carrier group chemically attached to the cysteine residues. The comparison of the latter structure to that presented here yields an average r.m.s. displacement of 0.5 and 1.3 Å for the common main-chain and side-chain atoms, respectively.

In view of the above and other results, zinc(II) binds at least as well to pseudoazurin as copper(II). Hence, it remains unclear why pseudoazurin prefers to bind copper over the generally more abundant zinc under native conditions. A possible answer could be that copper ions are required for the redox activity of the protein as they readily convert between the copper(II) and copper(I) oxidation states. In contrast, zinc ions are redox-inactive as they only assume the zinc(II) oxidation state. However, more detailed experiments are required in order to thoroughly examine the metal-binding preference of the protein *in vivo*.

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