

# The crystal structure of apo-pseudoazurin from *Alcaligenes faecalis* S-6

Kyriacos Petratos<sup>a,\*</sup>, Maria Papadovasilaki<sup>a</sup>, Zbigniew Dauter<sup>b,c</sup>

<sup>a</sup>*Institute of Molecular Biology and Biotechnology (I.M.B.B.)-F.O.R.T.H., P.O. Box 1527, 711 10 Heraklion, Greece*

<sup>b</sup>*European Molecular Biology Laboratory (EMBL) c/o DESY, Notkestr. 85, 22603 Hamburg 52, Germany*

<sup>c</sup>*Department of Chemistry, University of York, Heslington York YO1 5DD, UK*

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**Abstract** The 3D structure of the apo-pseudoazurin (copper free pseudoazurin) from *Alcaligenes faecalis* strain S-6 is determined and refined at pH 6.7 using X-ray diffraction data to 1.85 Å resolution. The final crystallographic *R*-factor is 0.164. Comparing the structures of apo-pseudoazurin and the native (Cu<sup>2+</sup>) protein, we observed limited differences ranging between 0.1–0.4 Å at the vicinity of the copper site, at the loops connecting the secondary structural elements, at certain β-strands and at the amino and carboxy termini of the protein.

**Key words:** Apo-protein; Blue copper protein; Copper; Metal incorporation; Pseudoazurin; *Alcaligenes faecalis*

## 1. Introduction

Pseudoazurin is a soluble periplasmic redox protein (123 amino acid residues; MW = 13397 Da) participating in the electron transport pathway leading to the reduction of NO<sub>2</sub><sup>-</sup> to mainly NO, and to a lesser extent to N<sub>2</sub>O, in the potent denitrifying bacterium *A. faecalis* [1]. The in vivo electron donor to pseudoazurin is unknown to date. Its electron acceptor is believed to be the green copper protein nitrite reductase [2] whose structure has been determined recently [3]. The structure of the oxidised pseudoazurin at pH 6.8 has been refined at 1.55 Å resolution [4]. A closely related blue copper protein, the poplar plastocyanin, has been investigated by X-ray structure analysis in both native and metal free form [5]. The major change which had been observed in the structure of the plastocyanin upon removal of the metal, was the rotation by 180° of the imidazole ring of the solvent exposed Cu-ligand His87 around the C<sup>β</sup>–C<sup>γ</sup> bond. Similar analyses have been carried out for the azurins from *Pseudomonas aeruginosa* [6] and *Alcaligenes denitrificans* [7] as well as for amicyanin from *Paracoccus denitrificans* [8]. In the case of the apo-azurin from *P. aeruginosa* there were found two distinct conformers in its crystalline lattice. One with small differences from the native Cu-protein and a second with a clear 'opening' of the metal binding site leading to the incorporation of a water molecule [6]. These conformations are believed to represent the two states before and after metal incorporation. In the other cases of known apo-protein structures no significant changes were observed.

\*Corresponding author. Fax: (30) (81) 230469.

**Abbreviations:** rms, root-mean-square.

The coordinates and structure factors of the apo-pseudoazurin have been deposited (entry code: 1pzc) with the Protein Data Bank, Brookhaven National Laboratory, Upton NY, USA.

In order to investigate the conformational changes upon incorporation of the copper atom to the pseudoazurin from *A. faecalis*, we determined and refined the structure of the apo-protein at pH 6.7.

## 2. Experimental

The protein was extracted as described in [9]. It was crystallised in its native oxidised state [10] except that 50 mM CH<sub>3</sub>COONH<sub>4</sub> buffer pH 5.7 was used instead of 50 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> buffer pH 8.0. The average size of the crystals obtained was 0.4 mm × 0.6 mm × 1.0 mm. A blue crystal was transferred to a 3.3 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution containing 20 mM K<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> buffer pH 6.7. In contrary to the previous investigations [5–8] no reduction of the crystals was carried out prior to the addition of the metal chelating agent. The removal of the copper from the protein was done by adding solid KCN into the solution containing the crystal to a final concentration of 100 mM. A few hours later the deep blue crystal became colourless and was exposed to the X-rays for diffraction measurements during the following day. The refined unit cell parameters:  $a = b = 49.5 \text{ \AA}$ ,  $c = 99.0 \text{ \AA}$  of the hexagonal lattice remained essentially the same as for the native blue crystals [4]. The use of the image plate detector-scanner system from MARresearch (Hendrix, J. and Lentfer, A., unpublished result) in combination with the short wavelength radiation ( $\lambda = 0.87 \text{ \AA}$ ) of the wiggler beam-line BW7B (EMBL at DESY, Hamburg) made possible the collection of a 98.4% complete data set in the resolution range of 10.0–1.85 Å. A total of 73,694 Bragg reflections were collected from one crystal in about 10 hours. In order to measure the strong low-resolution reflections which were saturated in the first high resolution data set ( $d_{\min} = 1.85 \text{ \AA}$ ), a second low resolution set ( $d_{\min} = 2.6 \text{ \AA}$ ) had to be collected from the same crystal. After processing of the images with the DENZO package [11] we obtained 11,516 independent reflections for the combined data set with a merging *R*-factor of 0.077 ( $R_{\text{merge}} = \sum |I_i - \langle I \rangle| / I_i$ ). The refined model of the oxidised pseudoazurin served as the starting point for refinement with ARP [12] running in the unrestrained mode. In total 170 cycles of ARP refinement were carried out. The structure was inspected at the graphics display using the interactive molecular graphics program FRODO [13] on an Evans and Sutherland ESV10 workstation. Afterwards, we carried out 120 cycles of restrained least-squares refinement [14] using the program SFALL [15]. All computations were done using programs of the CCP4 suite [16].

## 3. Results and discussion

In the final structure only one residue (Lys123) at the C-terminus could not be modeled as there was not enough electron density to define any limited number of conformations. For the final refined model the crystallographic reliability index *R*-factor, (where  $R\text{-factor} = \sum |F_{\text{obs}} - F_{\text{calc}}| / \sum F_{\text{obs}}$ ) including all 11,516 Bragg reflections in the 10.0–1.85 Å resolution range was 0.164, the rms-deviations from ideality for bond-lengths and angles were 0.016 Å and 3.3° respectively. The estimated standard error in bond distances based on the Luzatti plot [17] was 0.20 Å. At the last cycle of refinement, the free *R*-value [18] for a random set consisting of 10% of the data was 0.215. The final model includes 927 protein atoms and 137 ordered water molecules. The mean temperature factor for the entire structure

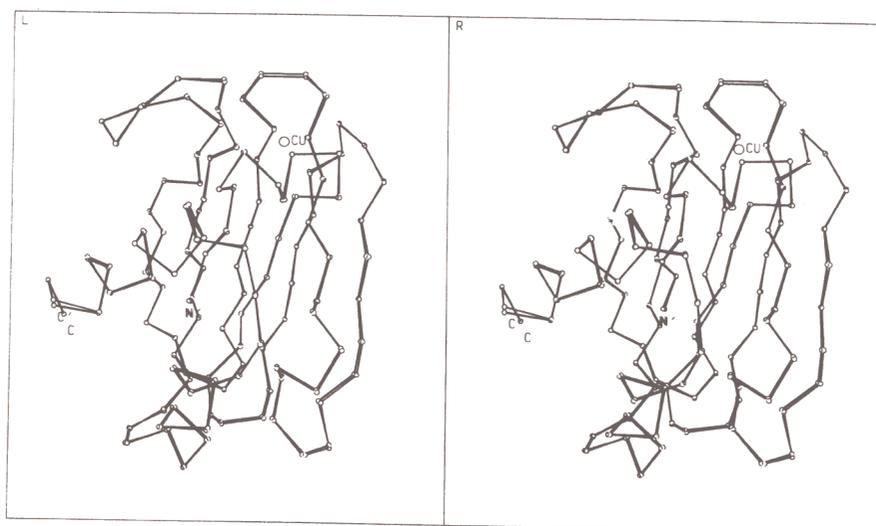


Fig. 1. Stereo diagram of the C $\alpha$  atoms of the superimposed native (thin lines) and apo-pseudoazurin (thick lines) structures. The N- and C-termini are labeled.

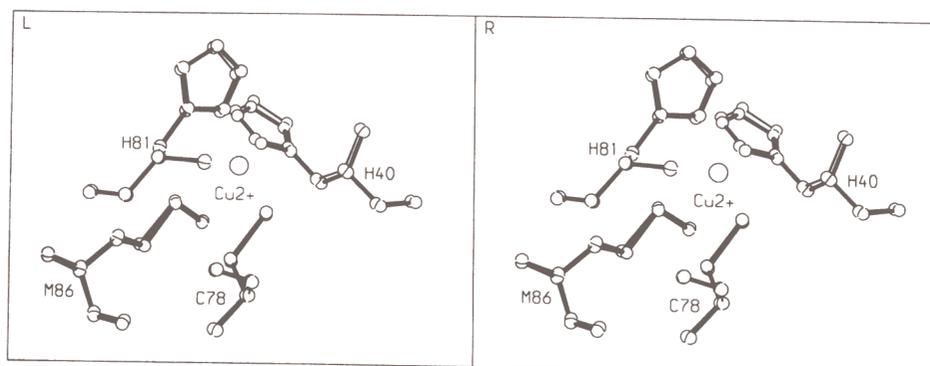


Fig. 2. Stereo diagram of the superimposed Cu-ligands of the native (thin lines) and apo-pseudoazurin (thick lines). The metal ion and the ligand residues are labeled.

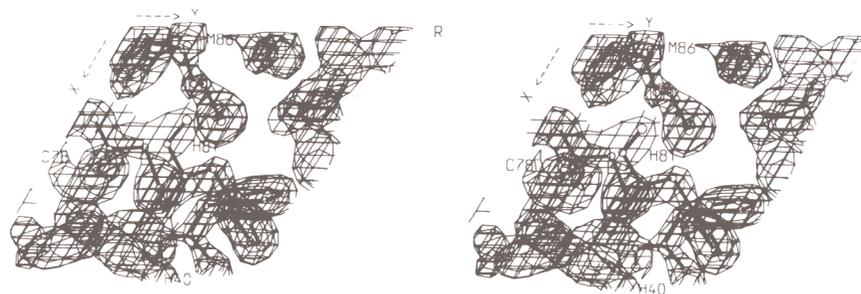


Fig. 3. Electron density map for apo-pseudoazurin computed with coefficients  $3|F_{\text{obs}}| - 2|F_{\text{calc}}|$ . The ligands of the copper in the native molecule are shown. Contouring level of the map is at  $2\sigma$ .

(including solvent) was  $29.0 \text{ \AA}^2$ , for the main chain atoms it was  $21.4 \text{ \AA}^2$ , for the side chain atoms it was  $30.4 \text{ \AA}^2$  and for the solvent it was  $51.7 \text{ \AA}^2$ . The rms-displacement was  $0.16 \text{ \AA}$  (max. =  $0.38 \text{ \AA}$ ) of the main chain atoms of the apo-protein from the native pseudoazurin. Fig. 1 is a stereo drawing of the C $\alpha$  atoms of the superimposed native and apo-protein structures. The significant structural changes (rms-displacement  $>0.2 \text{ \AA}$ ) occur at the N-terminal and at the C-terminal part of the protein, at  $\beta$ -strands 4 and 5, at the loops connecting the

$\beta$ -strands: 1 and 2, 3 and 4, 4 and 5, 6 and 7 and finally, at the loop connecting the two  $\alpha$ -helices of the structure. In particular Glu1, Val36, Asp37, and Ala120 are displaced by more than  $0.3 \text{ \AA}$  from their corresponding positions in the native molecule. The carbonyl group of Ala120 had to be turned about  $180^\circ$  in order to fit the residues Ser121 and Ala122 in the density map. The changes at the blue copper site upon removal of the metal are shown in Fig. 2. The movements at the copper site resemble those occurring in azurin from *A. denitrificans* and amicyanin

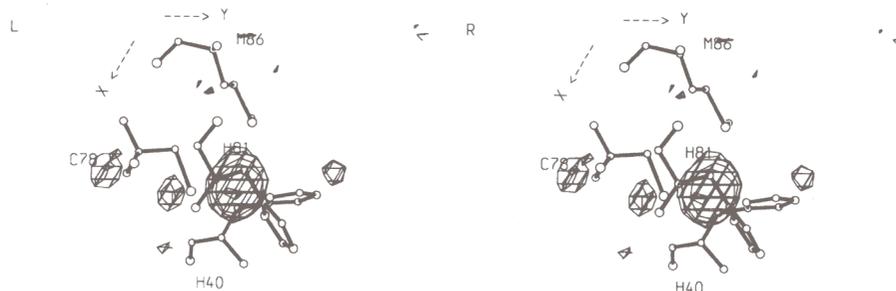


Fig. 4. Cross Difference Fourier map ( $|F_{\text{nat}}| - |F_{\text{apo}}|$ ) computed with phases from the apo-pseudoazurin structure. The copper site is shown. Contouring level of the map is at  $2\sigma$ .

from *P. denitrificans* where limited changes were observed upon removal of the metal. In apo-pseudoazurin the major movement is that of the  $\text{N}^{\delta 1}$  atom of the buried His40 which moves  $0.39 \text{ \AA}$  towards the copper position (in the native molecule). The imidazole of His40 rotates by  $9^\circ$  around its  $\text{C}^\beta\text{--C}^\gamma$  bond. The corresponding displacements of the other ligand atoms  $\text{S}^\gamma(\text{Cys78})$ ,  $\text{N}^{\delta 1}(\text{His81})$  and  $\text{S}^\delta(\text{Met86})$  are:  $0.18 \text{ \AA}$ ,  $0.19 \text{ \AA}$  and  $0.13 \text{ \AA}$  respectively. A part of the  $3|F_{\text{obs}}| - 2|F_{\text{calc}}|$  electron density map is shown in Fig. 3, where  $|F_{\text{obs}}|$  and  $|F_{\text{calc}}|$  are the observed and calculated structure factor amplitudes. In this map it is shown clearly that there is no electron density peak indicating any remaining metal in the vicinity of its potential ligands. Moreover, in computed  $|F_{\text{obs}}| - |F_{\text{calc}}|$  difference maps (data not shown) there was no peak in the vicinity at the  $2\sigma$  level. In Fig. 4 is shown the cross difference map computed with coefficients  $|F_{\text{nat}}| - |F_{\text{apo}}|$ , where  $F_{\text{nat}}$  and  $F_{\text{apo}}$  are the structure factor amplitudes of the native and apo-protein crystals respectively and phases from the apo-protein. One can clearly see that the only major difference is the occurrence of the density peak corresponding to the copper ion (peak height =  $18\sigma$ ).

The general conclusion that is to be drawn from these results is that pseudoazurin is 'designed' by nature to bind a 'blue' copper ion without large structural changes. The incorporation of the metal does not affect the protein structure which is responsible for the irregular co-ordination of the copper ion [19,20].

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