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The crystal structure of pseudoazurin from *Alcaligenes faecalis* S-6 determined at 2.9 Å resolution

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The three-dimensional structure of pseudoazurin, a single copper-containing protein from *Alcaligenes faecalis* strain S-6, has been determined at 2.9 Å resolution by X-ray crystallography. The sequences of two other pseudoazurins from *Pseudomonas* AM1 and *Achromobacter cycloclastes* may also be accommodated in this structure. The structure, an eight-stranded β-barrel, resembles closely those of plastocyanin and azurin. It possesses two extra α-helices at the C-terminus, whereas azurins have an α-helical flap in the middle of their sequences.

Anaerobic respiration, Copper protein; Crystal structure; Electron transfer; Pseudoazurin

1. INTRODUCTION

The 'blue protein' from the potent denitrifying bacterium *Alcaligenes faecalis* was first isolated and studied by Kakutani et al. [1]. It is a monomeric protein of 123 residues with a tightly bound single Cu^{2+}. In its oxidised state the molecule is coloured deep blue and may be reversibly reduced to a colourless state. The copper can be detected by EPR spectroscopy [1]. This kind of copper ion in proteins is classified as 'type 1' [2]. The protein belongs to the family of blue copper proteins recently reviewed by Adman [3] who named them 'cupredoxins'. The same blue protein from another bacterium was sequenced by Ambler and Tobari [4] and named 'pseudoazurin'. We adopt the latter nomenclature. Pseudoazurins are monomeric proteins with 123 or 124 amino acids and three protein sequences are now known, from *A. faecalis* [5], *Pseudomonas* AM1 [4] and *Achromobacter cycloclastes* [6].

The function of pseudoazurins in vivo appears to be the reduction of oxidised nitrite reductase. A single electron is believed to be passed on to its electron acceptor. The ultimate oxidant in the chain is the nitrite ion (NO_2^-). The main reduction product is thought to be nitric oxide (NO) and to a lesser extent nitrous oxide (N_2O). In the presence of air the final reduction product is H_2O_2 which inactivates the enzyme ('suicide inhibition' [7]). Nitrite reductase is a tetrameric protein (subunit M_r 30000) with a single copper ion bound to each subunit. It has been crystallised in our laboratory [8] and its structure is under investigation.

The electron donor of the pseudoazurin has not yet been characterised. In vitro it can be reduced by numerous reducing agents including cysteine, ascorbate, dithionite and 2-mercaptoethanol [1].

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2. EXPERIMENTAL

2.1. *Protein purification and crystallisation*

The protein was purified according to Kakutani et al. [1] as modified in our laboratory. Crystals suitable for X-ray work were obtained by vapour diffusion [9]. A 0.1 ml aliquot containing about 1.5% protein in 50 mM potassium phosphate buffer, pH 8.0, and 1.5 M ammonium sulphate was allowed to equilibrate via vapour diffusion with 1.0 ml of 3.0 M ammonium sulphate buffered as the protein solution. The crystals belong to the hexagonal system, space group $P6_5$, with $a = b = 49.9$ Å, $c = 99.2$ Å. The same crystal form was obtained independently by Adman et al. [10]. Two different isomorphous heavy-atom derivatives were prepared when native crystals were soaked in 10 mM solutions of $UO_2(CH_3COO)_2$ or $KAu(CN)_2$ containing 50 mM CH_3COONH_4 buffer, pH 5.7, and 3.2 M $(NH_4)_2SO_4$. The buffer exchange is critical for the successful preparation of the $UO_2(CH_3COO)_2$ derivative. The relative changes in structure factor amplitudes for the uranyl crystals were about 27% [11].

2.2. *Data collection and processing*

Diffraction intensities were measured on a Nonius CAD4F-11 diffractometer using peak top scans [12]. The heavy-atom derivative crystals were measured to 2.9 Å due to weakness of diffraction and higher radiation damage. Isomorphous and anomalous differences were used in the usual way [13,14] to phase 3100 unique reflexions to an average figure of merit of 0.78. Fig.1 shows the variation of the phasing power of the derivatives with resolution. Detailed crystallographic analysis will be published elsewhere.

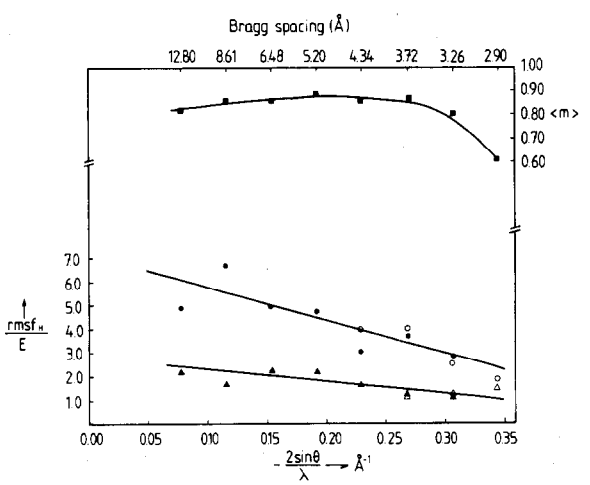

Fig.1. Variation with resolution of the ratio of the root-mean-square scattering factor of the heavy atoms ($rmsf_H$) and lack-of-closure error (E). Circles refer to the uranyl and triangles to the gold cyanide derivatives. Open and closed symbols refer to different crystals. On the top of the diagram the mean figure of merit, $\langle m \rangle$, is plotted with reference to the right-hand ordinate.

3. RESULTS

3.1. *The protein model*

A slice of the calculated electron density map at 2.9 Å is shown in fig.2. The map is of excellent quality and this enabled an unambiguous tracing of the chain, using the programme FRODO [15], even before a complete amino acid sequence was available. The folding of the protein is shown in fig.3. The molecule has approximate dimensions of 38 × 34 × 26 Å. The structure can be described as an eight-stranded β-barrel [16] consisting of two β-sheets stacked face-to-face. The first sheet, which we call β-sheet I, according to the notation of Chothia and Lesk [17], comprises strands 1, 3 and 6. The second, β-sheet II, consists of strands 2, 4, 5, 7 and 8. At the C-terminus the last 30 residues form a hairpin motif of two α-helices. The first 118 residues were placed in the density without ambiguity. However, no density could be found for the last 5 residues, or for the side chains of lysyls 24, 46, 55, 59, 109 and 117 and the glutamyls 54, 62 and 113. It is possible that some or all of them take up a well-defined conformation when the protein forms a complex with its redox partners. The preliminary results reported by Adman et al. [18] are in general agreement with those reported here.

3.2. *Comparison with other pseudoazurins*

We compared the sequence of the *A. faecalis* pseudoazurin (BLU), with those from *Pseudomonas* AM1 (PSU) and *Ac. cycloclastes* (ACCY) [19]. The sequences align exactly with no insertions or deletions for the first 123 residues (the ACCY sequence has 124 residues with the extra residue at the C-terminus). The results are shown in fig.4. The proportion of identical residues is

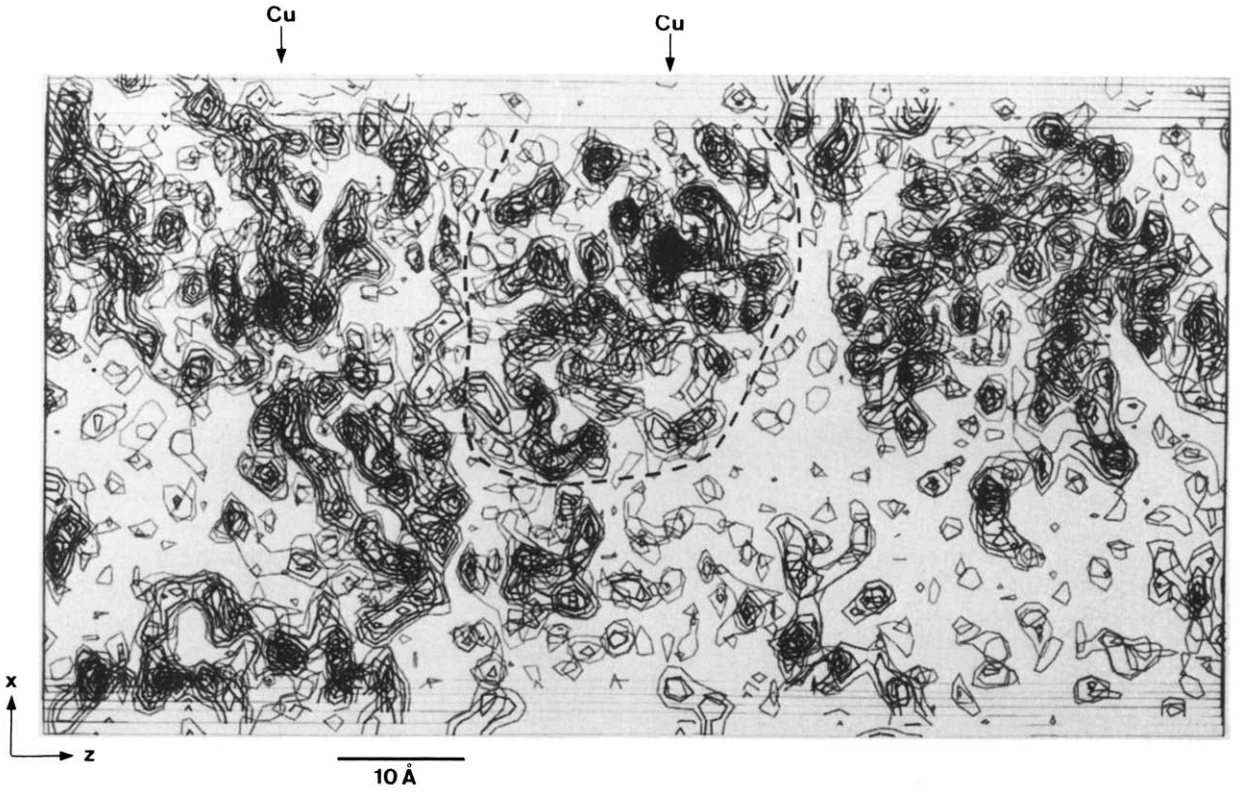

Fig.2. The pseudoazurin electron density map. A 6.6 Å slice normal to the Y-axis is shown starting with $Y = 15/45$, ending with $Y = 21/45$. Positive contours, dark lines; negative contours, light lines. The boundary of one molecule is outlined. Two copper sites belonging to two different molecules are indicated by arrows.

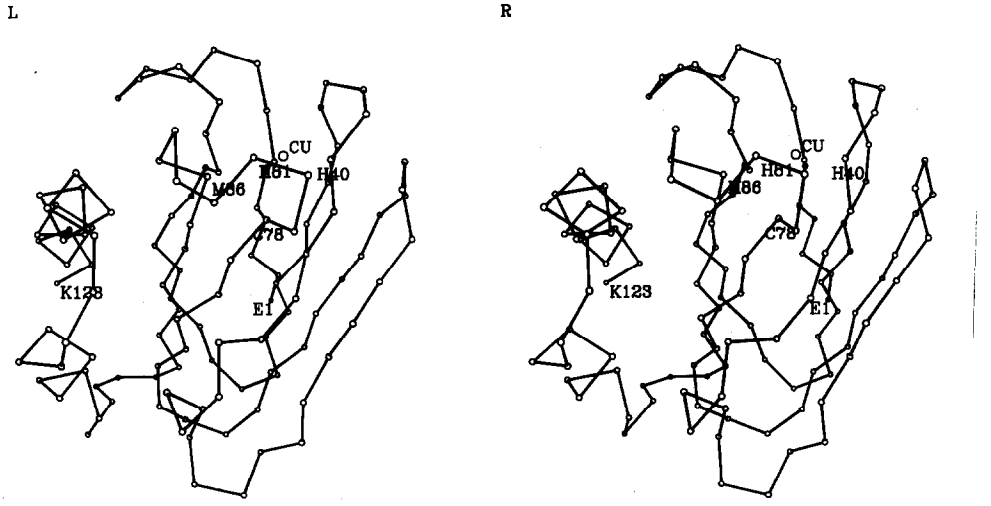

Fig.3. A stereo diagram of the Cα atoms of the pseudoazurin. E1 and K123 indicate the amino- and carboxy-termini, respectively.

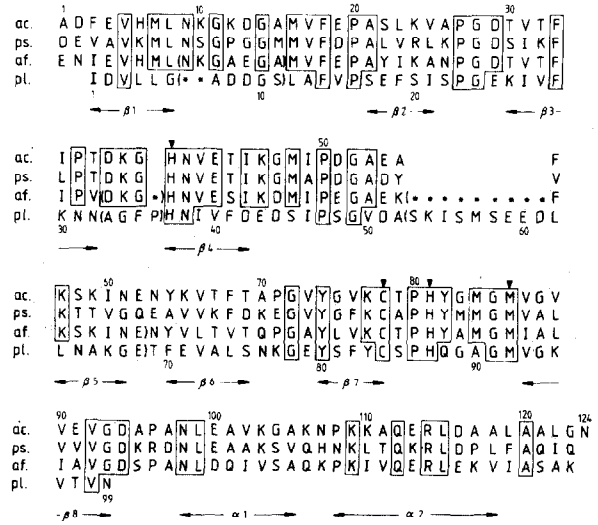

Fig.4. Alignment of the primary sequences of the pseudoazurins from *Ac. cycloclastes* (ac.), *Pseudomonas* AM1 (ps.) and *A. faecalis* (af.) and poplar leaf plastocyanin (pl.) based on the 3-D structures. The one-letter code for the amino acid residues is used. Conserved residues among all four proteins or the three pseudoazurins are boxed. Deletions are indicated with asterisks, while pieces in parentheses represent regions where the 3-D structures are sufficiently different that no precise alignment can be made. The secondary structural elements are also indicated.

BLU-ACCY 81/123 (66%), BLU-PSU 55/123 (45%) and PSU-ACCY 64/123 (52%).

This homology is high enough to accommodate the sequence of both ACCY and PSU in the BLU model. The only replacement requiring a small change in backbone conformation occurs for PSU in the short loop between the last β-strand and the first α-helix. Here, residues 95–97 which are Ser-Pro-Ala in BLU change to Lys-Arg-Asp in PSU and cannot be directly accommodated since the proline side chain points inwards. There is, however, a compensating sequence change in residues 71–73 from Pro-Gly-Ala to Glu-Gly-Val where the Val 73 side chain can replace that of Pro 96 and Glu 71 would interact with Arg 96.

A number of sequence differences on the interface between the α-helical C-terminal motif and the N-terminal β-structure indicate that the exact location of the helices with respect to the β-structure may be less conserved than the β-structure itself.

3.3. *Comparison with plastocyanin*

All the small copper-containing proteins for which the 3-D structure is known (azurins and plastocyanin) show the same folding pattern [20–22]. Our pseudoazurin structure resembles that of plastocyanin very closely, except that two

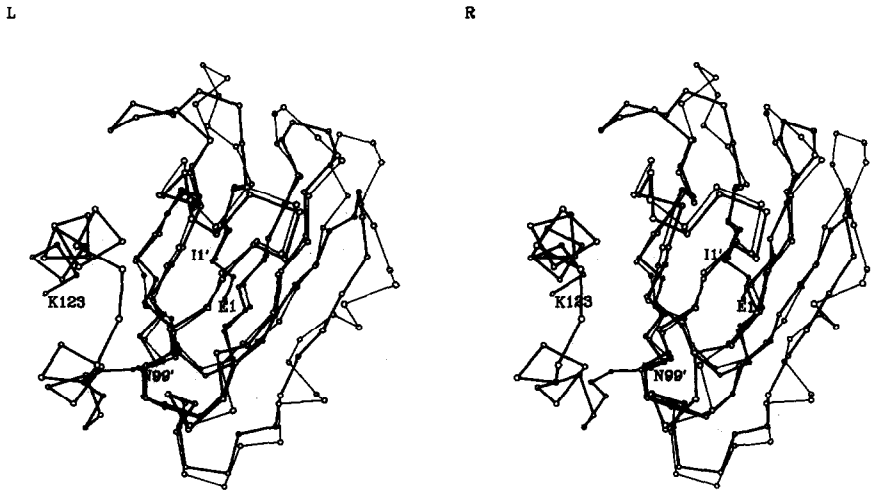

Fig.5. A stereo Cα plot showing the structures of poplar leaf plastocyanin and the *A. faecalis* pseudoazurin superimposed. Pseudoazurin is shown with thick lines, plastocyanin with thin lines. Plastocyanin residue numbers are primed.

extra α-helices are present at the C-terminus. Additional differences are found in the loop region which joins strands 1 and 2, at the turn between strands 3 and 4 and at strand 5 which is more regular in the pseudoazurin. A comparison with plastocyanin is shown in fig.5. Given that the primary sequence homology is only of the order of 20% (see also fig.4) between pseudoazurin and the plastocyanins, it is remarkable that the Cα positions of residues 3–8, 16–36, 40–55 and 63–93 of pseudoazurin can be aligned with the corresponding positions of residues 1–6, 12–32, 37–52 and 69–99 of poplar plastocyanin [23] within an r.m.s. deviation of 0.70 Å. This figure is for unrefined coordinates built without reference to the plastocyanin structure and is expected to decrease on refinement. It is worth noting that the two β-turns comprising residues 26–29 (Asn-Pro-Gly-Asp) and 50–53 (Pro-Glu-Gly-Ala) in pseudoazurin as well as the Tyr 74 loop are conserved. Proline residues 20, 27, 50 and 80, which occupy critical positions at the bends of pseudoazurins, are also conserved. Pro 20 is in the *cis* conformation as in the plastocyanin.

Plastocyanin and azurin have been compared in detail [3,17] and since pseudoazurin is so like plastocyanin the comparison with azurin will not be repeated here. We note however that the helices found in azurin and not in plastocyanin do not overlap with the two helices in pseudoazurin.

The residues that appear to be conserved throughout the plastocyanin/pseudoazurin/azurin structures are the copper ligands (two histidines, one cysteine and one methionine), the asparagine following the first histidine ligand, the tyrosine which is located four residues before the cysteine ligand and the proline before the second histidine ligand.

4. DISCUSSION

The structural similarity of pseudoazurin to plastocyanin has been demonstrated both for the *A. faecalis* protein whose structure was determined and for the similar proteins from two other bacteria. At this resolution no further insight into the functional properties of this class of molecules has been obtained.

We have recently collected a complete data set to 1.5 Å resolution from freshly prepared pseudoazurin crystals using synchrotron radiation at DESY. The refinement of the structure is under way. This will provide more detail for the Cu site (bond lengths and angles between the ligands and the metal ion) as well as for its immediate vicinity which are implied in the electron transfer. Furthermore, in order to understand more about the mechanism of action of the pseudoazurins we have reduced crystals with ascorbate and collected diffraction data from the 'white', Cu^+ crystals to 1.8 Å resolution.

At this stage we are especially fortunate in that we can crystallise the immediate electron acceptor of pseudoazurin, namely nitrite reductase, from the same species. The structure of the reductase will provide hints to the understanding of the interactions between the redox partners. Ideally, the natural interaction of these proteins should be elucidated through studies of co-crystals of the molecules. Such studies on binary systems have been successful for various proteinase/inhibitor complexes [24].

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