The complete amino acid sequence confirms the presence of pseudoazurin in *Thiosphaera pantotropha*

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The complete amino acid sequence, obtained by direct protein sequencing, of the pseudoazurin from *Thiosphaera pantotropha* is reported. It shows sequence identities varying from 46 to 66% with previously sequenced pseudoazurins. Previously identified conserved residues with key functions in pseudoazurins are found in the protein from *T. pantotropha* with the exception of glycine-39, the carbonyl group of which has been considered as a ligand to the copper, which is replaced by a serine residue. Electrospray-ionization MS (ESI-MS) has shown that pseudo-

azurin from *T. pantotropha* often contains two polypeptide species differing in molecular mass by 16 Da, presumably owing to oxidation of a methionine residue to a sulfoxide derivative. These two species have different endoproteinase Arg-C digestion patterns. Conditions for ESI-MS were identified that permitted either the retention or the loss of the single copper ion associated with the pseudoazurin. The aberrant tendency of *T. pantotropha* pseudoazurin to form a disulphide-bridged dimer on SDS/PAGE under some conditions is described.

INTRODUCTION

Type-I copper proteins, or cupredoxins, are defined by their characteristic spectroscopic properties, with intense absorption in the visible region near 600 nm and unusually small hyperfine coupling constants in the e.p.r. spectrum of the paramagnetic (oxidized) form of the protein [1,2]. All the proteins examined so far share the same Greek-key eight-stranded β -barrel protein fold [1,3]. These periplasmically located molecules function, or are presumed to function, in bacterial electron-transport reactions. For example, amicyanin mediates the oxidation of methylamine in bacteria [4], while auracyanin and plastocyanin act in photosynthetic electron transport in green bacteria and blue-green algae respectively [5,6]. Azurin and pseudoazurin (the distinction between the two was originally made on the basis of the differences in their absorption spectra [1,7,8]), have less clearly defined roles. In some bacterial species their synthesis appears to be enhanced under denitrifying conditions, and roles as electron donors to respiratory nitrite reductases [8,9] and nitrous oxide reductase [10] have been proposed. In contrast, Martinkus et al. [11] could assign no definite role to the pseudoazurin (initially called an azurin) of *Paracoccus denitrificans*, while the occurrence of pseudoazurin in the non-denitrifying organism *Methylobacterium extorquens* AM1 indicates another type of role for this class of redox protein [7].

In the case of *P. denitrificans*, which is very closely related to, but not identical with, *Thiosphaera pantotropha* [12–14], the possible role of pseudoazurin has acquired greater interest following the proposal by Moir and Ferguson [9] that it can substitute for cytochrome c_{550} , which is missing in a mutant [4] when the gene for the latter protein has been disrupted. The presence of pseudoazurin in *T. pantotropha* has been postulated on the basis of the redox properties and the visible absorption spectrum [9] of a blue protein. In the present paper the complete amino acid sequence of this blue protein and its partial charac-

terization are reported, both of which confirm its identity as a pseudoazurin.

MATERIALS AND METHODS

Bacterial strains and growth conditions

T. pantotropha wild-type and M-6 mutant [15] were grown anaerobically in 40-litre unstirred batch culture at 37 °C on minimal salt medium containing 55 mM Na_2HPO_4 , 11 mM KH_2PO_4 , 6 mM NH_4Cl and 0.4 mM MgSO_4 with 2 ml of the trace-element solution described by Vishniac and Santer [16] added per litre of growth medium. The carbon source was 10 mM potassium acetate, with 20 mM potassium nitrate present as electron acceptor. Both strains of *T. pantotropha* were also grown in a 25-litre fermenter. The same growth medium was used with slow stirring overnight without aeration or sparging with nitrogen. The cells were harvested either by centrifugation or with a membrane concentrator. The cells could be frozen at –80 °C for storage.

Purification of pseudoazurin

Preparation of periplasmic fraction

The cell paste was resuspended in 0.5 M sucrose/0.5 mM EDTA/100 mM Tris/HCl, pH 7.3, at 4 °C. For every 20 litres of cell culture, 1 g of lysozyme was added to the cell suspension, followed by incubation at 30 °C with continuous stirring for 2 h; one equivalent volume of water at 4 °C was then added, with stirring, to the suspension. The suspension was then centrifuged at 10000 g at 4 °C for 20 min. The supernatant was collected for purification of pseudoazurin. Benzamidine and/or phenylmethanesulphonyl fluoride to a final concentration of 1 mM were added to the periplasmic fraction to inhibit proteinases.

Abbreviation used: ESI-MS, electrospray-ionization MS.

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The amino acid sequence of *Thiosphaera pantotropha* pseudoazurin will be deposited in the EMBL Data Bank under the accession number P80401.

DEAE-Sepharose chromatography

The periplasmic fraction was loaded on to a DEAE-Sepharose (CL-6B) (Pharmacia) column (2.6 cm × 40 cm). Proteins were eluted with a gradient of 0–500 mM NaCl in 100 mM Tris/HCl, pH 7.3, in a total volume of 500 ml at 4 °C. The elution of pseudoazurin was monitored by measuring the absorption at 280 and 590 nm. The fractions that contained mainly pseudoazurin were pooled and concentrated to approx. 2 ml with a membrane concentrator fitted with an Amicon YM3 membrane.

Gel-filtration chromatography

The concentrated sample was then applied to a S-200 HR (Pharmacia) gel-filtration column (1.6 cm × 100 cm) pre-equilibrated with 100 mM Tris/HCl, pH 7.3, at 4 °C. The fractions that contained pseudoazurin were pooled and concentrated as described above.

(NH₄)₂SO₄ precipitation and hydrophobic-interaction chromatography

(NH₄)₂SO₄ was added to the sample to 80% saturation. It was then mixed by rotary inversion for 30 min before harvesting the precipitate by centrifugation. The supernatant was then applied to a phenyl-Sepharose (CL-4B) (Pharmacia) hydrophobic-interaction column (1 cm × 20 cm) equilibrated with 100 mM Tris/HCl with 80% (NH₄)₂SO₄ saturation. Pseudoazurin was eluted with a linear gradient of 80–0% saturation of (NH₄)₂SO₄ in 100 mM Tris/HCl, pH 7.3, in a total volume of 50 ml at 4 °C. This set of procedures normally provided pure pseudoazurin, as judged by a single band on Coomassie Blue-stained SDS/PAGE gels at a protein loading of 5 µg of pseudoazurin per lane.

Hydroxyapatite chromatography

The above set of steps did not provide pure protein from the periplasm from the M-6 mutant strain, presumably because the protein composition of the periplasm was different from that obtained from wild-type cells. It was then necessary to use a hydroxyapatite column to complete the purification. This step was particularly useful when samples still contained a moderate proportion of unidentified cytochrome after hydrophobic chromatography. The sample was buffer-exchanged into 20 mM Hepes/NaOH, pH 7.4, at 4 °C with a Pharmacia PD-10 desalting column before loading on to the hydroxyapatite column (1 cm × 20 cm), which had been equilibrated with the same buffer. The protein was eluted with a linear gradient made by mixing 20 mM Hepes/NaOH, pH 7.4, and 100 mM potassium phosphate, pH 7.4, in a total volume of 50 ml at 4 °C, so that the latter was the final elution buffer.

Determination of purity

The purity of the protein was determined by SDS/PAGE from the profiles observed and HPLC elution. The visible absorption spectrum obtained with a Perkin-Elmer Lambda 2 spectrophotometer could also be used as an indicator of the purity of the protein.

Determination of the molecular mass and the quaternary structure of pseudoazurin

The molecular mass, and hence the native quaternary structure, of the protein was assessed by a combination of SDS/PAGE, ESI-MS and gel-filtration chromatography.

SDS/PAGE was carried out on 15% (w/v) (1.25% cross-linker) gel slabs by the Laemmli [16a] method. Samples were

prepared as indicated in the Figure legends. Gels were stained with Coomassie Blue.

Samples for ESI-MS were exchanged into water and concentrated to the smallest volume possible using Amicon Centricon-10 concentrators. Samples were introduced into the mass spectrometer and analysed as indicated below.

Molecular-mass estimation with gel-filtration chromatography was done using a column of Sephacryl S-200 HR (Pharmacia) resin (10 mm × 600 mm), using bovine pancreas trypsin inhibitor, horse heart cytochrome c, soya-bean trypsin inhibitor, carbonic anhydrase and BSA as molecular-mass standards. The elution of proteins was monitored at 20 °C with a UV monitor set at 280 nm.

Amino acid sequencing of pseudoazurin

Before protein-sequencing studies, further purification of the pseudoazurin was performed using reverse-phase HPLC on a Brownlee Aquapore RP-300 column (100 mm × 2 mm) (Applied Biosystems Ltd., Warrington, Cheshire, U.K.), equilibrated in 0.1% (v/v) trifluoroacetic acid, by developing a linear gradient of 2–60% (v/v) acetonitrile in 1% increments/min. Pseudoazurin was eluted as a doublet at 46% (v/v) acetonitrile.

The pseudoazurin from both peaks was reduced with dithiothreitol in 6 M guanidinium chloride/0.5 M Tris/HCl/2 mM EDTA and alkylated with 4-vinylpyridine essentially as described by Tarr et al. [17]. The reduced and alkylated protein was then rechromatographed as described above.

A 4 µg portion of the protein was then subjected to N-terminal sequencing using an Applied Biosystems 470A protein sequencer, which gave the sequence of the first 38 residues.

For further sequence analysis the pseudoazurin was digested with Endoproteinase Glu-C (Promega sequencing grade) at an enzyme/substrate ratio of 1:20 in 100 mM ammonium bicarbonate, pH 8.0, for 16 h. Sequence analysis was performed on all major peptides separated by HPLC as described below. The sequences of the C-terminal region and overlapping peptides between most fragments were obtained from Endoproteinase Arg-C-digested materials obtained at an enzyme/substrate ratio of 1:20 in 100 mM Tris/HCl, pH 8, for 6 h. All digests were separated by reverse-phase HPLC on a Brownlee Aquapore OD-300 column (100 mm × 2 mm) (Applied Biosystems) using gradient conditions identical with those used above.

MS

ESI mass spectra were obtained on two different mass spectrometers. Spectra from 1:1 acetonitrile/water + 1% (w/v) formic acid were obtained on the Fisons Instruments BioQ mass spectrometer with a source temperature of 50 °C. The spectra were calibrated with horse heart myoglobin. The spectra obtained under native conditions were recorded on a Fisons Instruments Platform mass spectrometer with 100% water at pH 5 (pH adjusted with formic acid). The source temperature was set at 20 °C, and spectra were calibrated against hen lysozyme. The sample concentration used in these experiments was 20 pmol/µl for both conditions.

RESULTS

Amino acid sequence and CD

The sequence of peptides prepared from the presumed pseudoazurin from *T. pantotropha* confirmed the identity of the protein as a pseudoazurin. The acquisition of the complete sequence was possible without either identifying the C-terminus by carboxy-

Figure 1 Complete amino acid sequence of pseudoazurin from *T. pantotropha* and its comparison with sequences of pseudoazurin from three other bacteria

The copper ligands are highlighted by shading. In the case of the *T. pantotropha* the peptides (EC, endoproteinase GluC digests; RC, endoproteinase ArgC digests) that were sequenced are indicated by the horizontal bars. Peptide RC-16 was obtained only from the form of pseudoazurin carrying an oxidized methionine residue (see the text). Genus abbreviations: *M.*, *Methylobacterium*; *Al.*, *Alcaligenes*.

Table 1 Comparison of properties of pseudoazurin from several bacteria

Genus abbreviations: *M.*, *Methylobacterium*; *Al.*, *Alcaligenes*; *A.*, *Achromobacter*; *P.*, *Paracoccus*. *M* is molecular mass.

	Residue no.	<i>M</i> (Da)	pI	<i>E</i> ⁰ (mV)	Charge at pH 7.4	Reference
<i>T. pantotropha</i>	123	13344	5.37	+ 230	— 5	The present study
<i>A. cycloclastes</i>	124	13017	6.79	N/A	— 1	[25]
<i>Al. faecalis</i> S-6	123	13366	7.65	+ 270	— 1	[26]
			6.35			
<i>M. extorquens</i> AM1	123	13392	8.41	N/A	1	[7]
<i>Pa. denitrificans</i>	123	13790	4.6	+ 230	N/A	[11]

peptidase treatment or obtaining a peptide that overlapped with the endopeptidase fragment EC-13 (Figure 1). The lack of information from these sources was overcome by the correspondence between the calculated molecular mass for the protein (13341.29 Da) and that obtained experimentally by ESI-MS (13344 ± 0.5 Da; [8] and see below). The similarity between the sequence obtained for *T. pantotropha* pseudoazurin and other pseudoazurins (Figure 1) also suggested that there were no residues missed on either side of EC-13.

The far-UV CD spectrum of pseudoazurin in 20 mM potas-

sium phosphate buffer, pH 7.0, had a negative band at about 220 nm and a large positive ellipticity below 200 nm. These observations indicate that pseudoazurin from *T. pantotropha* is predominately β -sheet in structure, consistent with a presumed Greek-key topology.

The pI of pseudoazurin obtained by isoelectric-focusing electrophoresis was 5.37, which is similar to the value estimated from the amino acid sequence of 5.31 using programme pepstats from the University of Wisconsin Genetics Computer Group software package release 7.0 [18] (Table 1).

Figure 2 Behaviour on SDS/PAGE of pseudoazurin from *T. pantotropha* under various conditions

Lane 1, pseudoazurin sample without boiling and reductant; lane 2, pseudoazurin sample with reductant without boiling; lane 3, boiled (100 °C for 5 min) pseudoazurin sample without reductant; lane 4, boiled pseudoazurin sample with reductant (100 °C for 5 min). Each lane was loaded with 5 µg of pseudoazurin; where present, the reductant was 800 mM 2-mercaptoethanol. Abbreviation: *M*, molecular mass.

Molecular-mass analysis by SDS/PAGE and gel filtration

Moir et al. [8] reported that SDS/PAGE analysis indicated a molecular mass of approx. 13 000 Da for the pseudoazurin from *T. pantotropha*. In the present work it was frequently observed that the purified protein ran on SDS/PAGE as if it were a mixture of species with approx. molecular masses of 13 000 Da and 30 000 Da (Figure 2, lanes 1 and 3). This observation raised the possibility of a dimeric state of the protein. On further examination it was found that the behaviour on SDS/PAGE was critically dependent on the presence or the absence of reductant, especially during the boiling stage of sample preparation. The presence of a reductant such as β-mercaptoethanol (approx. 800 mM) resulted in a single species with an electrophoretic mobility consistent with a molecular mass of approx. 13 000 Da (Figure 2, lanes 2 and 4). These observations raised the possibility that the protein may exist as a dimer with a disulphide bridge linking two monomers. However, the presence of only one cysteine residue in the polypeptide chain (Figure 1), which in the protein from *Alcaligenes faecalis* S6 is known to be involved in copper binding [19], suggested that the S-S-linked dimer is not the functional native form.

The possibility that *T. pantotropha* pseudoazurin might exist as a homodimer in its native state was further explored by molecular-mass determination by gel-filtration chromatography. A pseudoazurin sample of 0.5 mg/ml was loaded on to an

Figure 3 (a) ESI-MS of pseudoazurin under 'standard' ESI conditions from 600 to 2000 Da, (b) maximum-entropy solution of the spectrum in (a), (c) ESI-MS of pseudoazurin under 'native conditions' from 600 to 2000 Da and (d) maximum-entropy solution of the spectrum in (c)

Figure 4 Resolution of the two forms of pseudoazurin by HPLC

S-200 HR column at room temperature at either pH 5 or pH 7. The molecular masses determined at pH 5 and 7 were 15 500 and 17 600 Da respectively, demonstrating that, at this concentration and under the tested conditions, pseudoazurin is predominantly monomeric.

ESI-MS

ESI mass spectra were recorded under two different conditions and the results are presented in Figure 3. The spectrum in Figure 3(a) was recorded under 'standard' ESI conditions [acetonitrile/water, 1:1 (v/v), containing 1% formic acid]. The charge state series extends from +11 to +19, and the split nature of the peaks (not shown under this mass range) indicates the presence of two components with masses 13343.0 ± 1.34 Da and 13356.4 ± 2.25 Da. Maximum entropy was used to further resolve this splitting (Figure 3b), demonstrating that the mass difference was 16.8 Da, presumably corresponding to the oxidation of one of the six methionine residues. The degree of oxidation was found to be sample dependent and ranged from 0 to 50% of the sample. The molecular mass of 13341.3 Da determined by the maximum-entropy process is in excellent agreement with that (13341.29 Da) calculated for the amino acid sequence determined here. When spectra were obtained under 'native conditions' from a solution containing 100% water at pH 5.0 and at 20 °C, species at molecular masses 13403.1 ± 3.7 Da and 13415.8 ± 2.6 Da were observed (Figure 3c). Maximum entropy was applied to this spectrum to resolve further these species, giving masses of 13402.4 Da and 13418.3 Da (Figure 3d). These species correspond to the addition of one copper ion to both the oxidized and non-oxidized species with removal of two hydrogen ions from each of the species observed in formic acid/acetonitrile solution, demonstrating that pseudoazurin from *T. pantotropha* binds a single copper ion. Under 100% 'native' conditions,

100% copper occupancy was observed. In addition, a significant change in the charge distribution was observed from +11 to +8 under 100%-aqueous conditions. Such changes in charge-state distribution have been observed in other protein studies when spectra are obtained from solutions where the protein is known to exist in denatured and native-like conformations [20].

When pseudoazurin was subjected to a final purification step of HPLC before sequencing, it was observed that, for some samples, two peaks were eluted closely together in a ratio that reached as high as 1:1 (Figure 4). Material in these two peaks was separately reinjected on to the HPLC column. Single-peak elution profiles were obtained in each case, but with the same difference in retention times that was observed during the first step of HPLC. This finding shows that the two species do not arise from equilibrium isoforms. Subsequent analysis by ESI-MS showed that the separately eluting samples of pseudoazurin differed in molecular mass by the same 16 Da that had been observed in other samples of pseudoazurin (Figure 3) analysed by ESI-MS, the later-eluted peak being the oxidized form. Material in the two pseudoazurin fractions had the same sequence, and the origin of the extra 16 Da is presumed to be a methionine sulphoxide residue, which, on sequencing, cannot be distinguished from methionine. As a form of pseudoazurin with an extra 32 Da was never observed, it is probable that one particular methionine residue within the sequence is susceptible to oxidation. An unexpected occurrence in the present work was the additional cleavage site, between lysine-77 and the sole cysteine residue, for endoproteinase Arg-C that was found in the protein carrying the extra oxygen atom. The basis for this additional cleavage site is not clear. The cysteine itself could not have been carrying the extra oxygen atom, as it could be carboxymethylated in the +16 Da form of the enzyme. Thus we surmise that an oxygen atom on nearby methionine-84 altered the propensity of the proteinase to cleave between lysine-77 and cysteine-78. It is unlikely that methionine-86 is oxidized, because pseudoazurin containing copper and bearing the extra 16 Da could be identified by ESI-MS. This residue is a ligand to the copper, and it is improbable that an extra oxygen could be tolerated, especially as no perturbation in the visible absorption spectrum was observed.

The detection of a species of pseudoazurin from *T. pantotropha* with molecular mass 16 Da greater than expected parallels a previous finding with the cytochrome c_{550} from this organism [14]. Whereas in the latter case it was possible to identify the specific methionine residue that underwent oxidation to a sulphoxide, attempts to obtain the same information for pseudoazurin have so far failed. Consideration was given to the possibility that the oxidized form of pseudoazurin was formed only following growth of *T. pantotropha* M6 cells in the presence of chlorate [15], which provides an oxidizing environment. However, such a correlation was not found. The basis for a variation in the occurrence of the +16 Da species remains to be studied in future work.

DISCUSSION

The amino acid sequence of pseudoazurin was acquired rapidly by direct protein sequencing of a small amount of material. The need for sequencing of a complete set of overlapping peptides was obviated because the ordering of the polypeptides could be assigned by comparison with previously known sequences. The accurately determined molecular-mass value from the ESI-MS provided confirmation that none of the sequence, including the C-terminus, had been missed.

The amino acid sequence of pseudoazurin from *T. pantotropha* shows many identities with, and considerable overall similarity

to, the previously described sequences of this type of protein. Therefore, there is no doubt that Moir et al. [8] were correct in assigning this protein as a pseudoazurin. Of the residues that differ in the sequence of the *T. pantotropha* pseudoazurin compared with other pseudoazurins (Figure 1), the following are most notable. At position 39 a serine residue is found rather than a glycine (Figure 1). The main-chain carbonyl group of this glycine residue, by analogy with azurins, has been considered as a possible ligand to the copper [19,21]. However, the oxygen–Cu bond length in pseudoazurin would be significantly longer than in azurin, and too long to be considered formally as a bond. Although under the concept of rack-induced bonding [2] such a small bonding contribution might contribute to the unusual spectral properties of pseudoazurin, its significance is not established. Thus the presence of serine residue at position 39 in the *T. pantotropha* protein may not affect copper ligation. Adman et al. [19] identified a set of 12 residues whose side chains are involved in interactions with main-chain atoms or other side-chain atoms and are involved in maintenance of the copper-binding site. Of these residues, glutamine-112, seen in other pseudoazurins, shows a significant change, being replaced by arginine in the *T. pantotropha* protein. As expected, the residues known to provide side-chain ligands to the copper in pseudoazurins from structural studies [19] are fully conserved in the sequence from *T. pantotropha*.

Pseudoazurin from *T. pantotropha* is, as expected, similar to those from other species in many respects (Table 1). They all have a similar mass and redox potential. However, they have different pI values, ranging from acidic (4.6) to alkaline (8.4). Pseudoazurin from *T. pantotropha* differs from others in that it is more negatively charged at pH 7.4. The pI of pseudoazurin from *T. pantotropha* is smaller than the pI of pseudoazurin from *Alcaligenes faecalis* of 7.65.

An unexpected aspect of the present work was the finding that pseudoazurin ran as an apparent dimer on SDS/PAGE under some conditions. The possible explanation for this behaviour is that loss of the copper atom from the denatured protein permits the single-cysteine-residue thiol group, which in native pseudoazurin is a ligand to the copper, to form an S–S bridge to a second polypeptide chain. A similar phenomenon has recently been reported for amicyanin from *P. denitrificans* [22] and may account for the dimeric forms of other blue copper proteins, including auracyanins from *Chloroflexus auranticans* [5,23] and halocyanin from *Natronobacterium pharaonis* [24] observed under certain conditions.

The characterization of pseudoazurin reported here provides a firm basis on which to test whether this electron-transport protein can act as an electron donor to reductases for electron acceptors such as nitrite or nitric oxide [9] and as a model for further structural and functional studies. We have demonstrated the enormous potential of ESI-MS to examine proteins in their native states, under which conditions the single copper ion is

retained in the pseudoazurin monomer. In addition, the combination of amino-acid-sequence data with the high mass accuracy available by ESI-MS even under 'native' conditions, obviates the requirement for overlapping peptides for complete sequence determination.

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