The structure and dynamics in solution of Cu(I) pseudoazurin from *Paracoccus pantotrophus*

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Abstract

The solution structure and backbone dynamics of Cu(I) pseudoazurin, a 123 amino acid electron transfer protein from *Paracoccus pantotrophus*, have been determined using NMR methods. The structure was calculated to high precision, with a backbone RMS deviation for secondary structure elements of 0.35 ± 0.06 Å, using 1,498 distance and 55 torsion angle constraints. The protein has a double-wound Greek-key fold with two α -helices toward its C-terminus, similar to that of its oxidized counterpart determined by X-ray crystallography. Comparison of the Cu(I) solution structure with the X-ray structure of the Cu(II) protein shows only small differences in the positions of some of the secondary structure elements. Order parameters S^2 , measured for amide nitrogens, indicate that the backbone of the protein is rigid on the picosecond to nanosecond timescale.

Keywords: backbone dynamics; cupredoxins; electron transfer; nuclear magnetic resonance; pseudoazurin

Pseudoazurin is a small, 123 amino acid, blue copper protein that belongs to the cupredoxin family (Adman, 1991). The single copper atom can exist in either the 2+ or 1+ oxidation states so that the protein can act as an electron carrier, although its exact physiological roles have not yet been fully delineated. Pseudoazurin is frequently, but not exclusively, found in denitrifying bacteria that catalyze the sequential reduction of nitrate, nitrite, nitric oxide, and nitrous oxide to nitrogen gas (Moir et al., 1995). The location of pseudoazurin in the periplasm suggests a role in electron transport from the ubiquitous cytochrome *bc1* complex to several of the enzymes involved in the denitrification process. Indeed, it has been shown in vitro that pseudoazurin can act as an electron donor to the periplasmic nitrite (Moir & Ferguson, 1994) and nitrous (Berk et al., 1993) oxide reductases in some species of bacteria. In the particular case of *Paracoccus pantotrophus* (previously classified as *Thiosphaera pantotrophus* (Rainey et al., 1999)), it has been argued that pseudoazurin is interchangeable with a c-type cyto-

chrome in respect of this role (Moir & Ferguson, 1994). This interchangeability and the proposal that pseudoazurin can donate electrons to more than one acceptor protein raises intriguing questions about the mechanism of molecular recognition between donors and acceptors in electron transport. Thus, it has been suggested that recognition between redox partners may occur by a pseudo-specific docking of complementary hydrophobic surface patches between donor and acceptor (Williams et al., 1995).

Pseudoazurin is one of a number of electron transfer proteins that adopt the classic cupredoxin fold, characterized by an immunoglobulin-like topology (Adman, 1991) involving one four-stranded β -sheet and one five-stranded β -sheet. The periplasmic location of pseudoazurin in *P. pantotrophus* raises intriguing questions about the assembly of the protein. The *pazS* gene contains a coding region for a typical sec-dependent targeting sequence (Leung et al., 1997). This implies that the protein is translocated in an unfolded state and acquires both copper and the final three-dimensional (3D) structure in the periplasm.

Cu(II) pseudoazurin from *P. pantotrophus* crystallizes in a head-to-toe dimer and, unusually, is dimeric in solution (Williams et al., 1995). It has been suggested that the observed dimer could play a role in electron transport in vivo by reducing unproductive interactions between oxidized pseudoazurin and its promiscuous higher redox partners (Williams et al., 1995). By contrast, ultracentrifugation studies have shown that Cu(I) pseudoazurin is predominantly monomeric in solution under the same conditions of pH and temperature (Williams et al., 1995). Crystals of the Cu(II) protein crack when they are soaked with dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), suggesting

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Abbreviations: COSY, correlation spectroscopy; DQF, double-quantum filtered; HMQC, heteronuclear multiple quantum correlation spectroscopy; HSQC, heteronuclear single quantum correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; PDB, Protein Data Bank; RMSD, root-mean-square deviation; TOCSY, total correlation spectroscopy.

Here, we describe the first solution structure of a Cu(I) pseudoazurin, determined by multidimensional heteronuclear NMR methods at 750 MHz, and we compare the conformation of this monomeric Cu(I) pseudoazurin with its dimeric oxidized counterpart. In addition, we present an analysis of the backbone dynamics of Cu(I) pseudoazurin from ^{15}N relaxation measurements analyzed using the model-free approach incorporating fully anisotropic rotational diffusion.

Resonance assignments

Resonance assignments

The ^1H - ^{15}N HSQC spectrum of Cu(I) pseudoazurin at pH 6.6 and 25 °C is shown in Figure 1. Complete sequential backbone assignment was achieved using a 3D ^1H - ^{15}N NOESY-HSQC spectrum and the standard sequential assignment approach (Wüthrich, 1986) without recourse to double-labeled protein. Spin system information for backbone amides was obtained from a 3D ^1H - ^{15}N TOCSY-HMQC spectrum and from two-dimensional (2D) ^1H - ^1H COSY and TOCSY spectra. Of the 114 ^1H - ^{15}N cross peaks from backbone amides expected in the HSQC spectrum, only one, that of Lys109, is missing. Altogether 357 of the 361 expected backbone HN, ^{15}N and H α resonances, and 458 of the 483 side-chain resonances were assigned, giving an overall assignment coverage of 97%. The assignments have been deposited in the BioMagResBank (University of Wisconsin-Madison), accession number 4466.

Delineation of secondary structure

Elements of regular secondary structure in Cu(I) pseudoazurin were assigned using a number of NMR parameters including the strength of expected short and medium-range nuclear Overhauser enhancement (NOE) connectivities, backbone HN–H α coupling constants ($^3J_{\text{N}\alpha}$), the H α chemical shift index (CSI) (Wishart et al., 1992), and protection of amide hydrogens from exchange. These data are summarized in Figure 2. A number of β -strands were clearly defined by strong short-range H $\alpha(i)$ –HN($i + 1$) NOEs interspersed with HN(i)–HN($i + 1$) NOEs attributable to turns. The geometry and register of the β -sheets in Cu(I) pseudoazurin were derived from the characteristic patterns of long-range H α –H α , H α –HN, and HN–HN NOEs expected for parallel and anti-parallel β -sheets (Wüthrich, 1986). Two α -helices were clearly identified in the solution structure of Cu(I) pseudoazurin by stretches of strong sequential HN(i)–HN($i + 1$) NOEs and short-range HN(i)–HN($i + 2$), H $\alpha(i)$ –HN($i + 2$), H $\alpha(i)$ –HN($i + 3$), and H $\alpha(i)$ –H $\beta(i + 3)$ NOE connectivities; H $\alpha(i)$ –HN($i + 4$) connectivities were observed only in the second, longer helix.

The presence of β -sheet and α -helical regions of secondary structure was confirmed by the measured $^3J_{\text{N}\alpha}$ coupling constants. Large values for $^3J_{\text{N}\alpha}$ (>8 Hz) are consistent with the identification of β -strand structure in residues 1–8, 17–19, 21–25, 29–36, 64–68, 72–77, and 86–93. Small measured $^3J_{\text{N}\alpha}$ coupling constants (<6 Hz) confirm the presence of α -helices spanning residues 98–104 and 109–120. The H α chemical shift index method (Wishart et al., 1992) has been used to identify regions of secondary structure (Fig. 2). Despite the presence of a charged copper ion in Cu(I) pseudoazurin, the elements of secondary structure identified using the CSI method are in agreement with the assignment of these regions based on other NMR data, even in regions of the molecule close to the Cu(I) ligands (His40, Cys78, His81, and Met86). However, residues 47–49 and 56–58, which were identified as β -strands IVb and V on the basis of interstrand NOE connectivities, do not show the CSI expected for a β -strand conformation.

The secondary structure elements identified from the patterns of NOEs, $^3J_{\text{N}\alpha}$ coupling constants, and the H α CSI are also characterized, in general, by protection of amide hydrogens from exchange with solvent, as indicated in Figure 2. A large number of slowly exchanging amides are found within the β -sheet structures. The β -strands at the edges of the sheets, such as II, V, and VI, show the typical pattern of alternating slow and fast exchange resulting from the presence of interstrand hydrogen bonds on only one side of the edge strand. Internal β -strands, such as III, IV, VII, and VIII, show more extensive amide protection. The helix spanning residues 98 to 104 lacks significant amide protection; the C-terminal helix, however, shows more extensive amide protection.

Structure calculation

In the initial phase of the structure determination, trial structures were calculated with 55 torsion angle restraints and with 974 NOE restraints that had been assigned unambiguously. Restraints for the Cu ion and the Cu ligands were added, and these agreed well with the observed NOE patterns. The eight proline residues were initially assigned to the *trans* conformer. However, structure calculations performed with Pro20 fixed in this conformer showed poor convergence of the structural ensemble with a significant increase of the backbone RMSD around this residue compared with structures calculated with Pro20 in the *cis* conformer. Thus, a *trans*

conformer for Pro20 is not compatible with the remainder of the constraint set, and the *cis* conformation was assigned in subsequent calculations (in accord with its conformation in the X-ray structures of other pseudoazurins (Adman, 1991), including its oxidized counterpart (Williams et al., 1995)). Interestingly, a *cis* proline at this position in the Greek key fold has been shown to be statistically favored in forced evolution studies of the immunoglobulin fold (Spada et al., 1998), providing a short tight turn joining the middle of the two β -sheets in the β -sandwich, and thus, increasing the structural rigidity of the sandwich. Further NOEs were identified by cycles of assignment based on the calculated structures, followed by re-refinement and additional cycles of structure calculation. The final structure calculations were based on a total of 1,498 NOE restraints. As expected for a β -sheet protein with a complex topology, only $\sim 40\%$ of structure calculations converged successfully. However, as can be seen from the analytical and statistical data (Table 1), the final ensemble of structures was well converged with a low backbone RMSD against the mean of 0.54 ± 0.08 Å and a low target function energy of 209.6 ± 6.4 kcal mol $^{-1}$.

The solution structure of Cu(I) pseudoazurin

An overlay of the 25 lowest energy solution structures of Cu(I) pseudoazurin from a set of 100 starting structures is shown in Figure 3A. The ensemble of structures has a low RMSD across the whole sequence (Fig. 4). As expected, the lowest RMSD values are found in the regions containing regular secondary structure; the backbone RMSD between such regions is 0.35 ± 0.06 Å, while that calculated over all residues is 0.54 ± 0.08 Å. Two regions show higher than average RMSD values. The first of these, residues 9–16, forms an extended hook-shaped hairpin loop linking strands I and IIa of β -sheet I (Figs. 3B, 4). This region shows disorder with respect to the overall protein structure, but is ordered locally. Thus, the backbone atom RMSD for residues 9–16 calculated from a global alignment is 1.2 ± 0.4 Å, but this decreases to 0.38 ± 0.23 Å when residues 9–16 are aligned over this region alone (Table 1). The apparent disorder in this region, in part, can be traced to a lack of long-range NOEs between this region and the rest of the protein (Fig. 4). The second region of high RMSD involves residues 103–109. This region is also not well constrained by long-range NOEs, largely because of the absence of the HN resonance of Lys109 and is exacerbated by the fact that its neighboring residue, 108, is a proline. The reason for the absence of the amide resonance of Lys109 is not clear; this amide must be in rapid exchange with the solvent or broadened by a chemical exchange contribution to its T_2 value.

As shown in Figure 3, Cu(I) pseudoazurin in solution is monomeric and consists of a β -sandwich made up of a four-stranded and a five-stranded β -sheet with the expected cupredoxin topology. A pair of α -helices formed by a C-terminal extension of the β -sandwich is docked to the second of the two β -sheets. The copper binding site is composed of four residues (His40, Cys78, His81, and Met86) that form a three-sided pyramid of ligands with His81 at the top. Three of the ligands (Cys78, His81, and Met86) lie in the turn region, which joins strands VII and VIII of sheet II. The fourth ligand (His40) is located in the turn joining strand III of sheet I to strand IVa of sheet II, which is part of both of the Greek-key motifs in pseudoazurin. The copper is buried in an enclosed pocket, which shields it entirely from the solvent.

The region between residues 40–60 has less regular β -structure than other regions of the solution structure of Cu(I) pseudoazurin.

Fig. 2. Summary of NMR data used for the identification of the secondary structure of Cu(I) pseudoazurin. The short- and medium-range backbone NOEs, slowly exchanging amide hydrogens (NH protected), HN-H α coupling constants ($^3J_{\text{N}\alpha}$), and H α chemical shift indices (CSI) (Wishart et al., 1992) are shown. The strengths of the NOEs are shown only for the $i + 1$ connectivities and are classified as strong or weak (thick or thin bars) using a cut-off of 3.1 Å. Values of $^3J_{\text{N}\alpha}$ are indicated by filled ($^3J_{\text{N}\alpha} > 8$ Hz) and open ($^3J_{\text{N}\alpha} < 6$ Hz) circles. Backbone amide hydrogens that were observed 24 h after dissolution of the sample in D₂O are marked with squares. CSI of +1, 0, and -1 are indicated by the position of the rectangular blocks. The final secondary structure assignments are derived from the solution structure using PROCHECK-NMR (Laskowski et al., 1996) and are shown below the sequence along with ideograms showing the Cu binding ligands $\bullet$, cis ζ , and trans $\sim$ proline residues.

Table 1. Structural and restraint statistics for an ensemble of 25 Cu(I) pseudoazurin structures

Number of conformational restraints		
NOE based		
Intraresidue, $ j - i = 0$		403
Sequential, $ j - i = 1$		458
Medium range, $2 \leq j - i \leq 4$		219
Long range, $ j - i \geq 5$		418
Subtotal		1,498
Hydrogen bond		76
$^3J_{N\alpha}$ backbone ϕ angles		55
$^3J_{\alpha\beta}$ χ_1 angles		1
Cu ligand		14
Total		1,644
Restraint violations ^a		
Distance restraints		
No. NOE violations $> 0.1 \text{ \AA}^b$		10
Mean length of violations per structure ($\text{\AA}$)		1.3
Dihedral restraints		
Angle violations $> 5^\circ$ ^b		0
Deviations from idealized geometry		
Angle ($^\circ$)	0.549 ± 0.003	
Bond ($\text{\AA}$)	0.002 ± 0.000	
NOE ($\text{\AA}$)	0.016 ± 0.001	
Dihedral ($^\circ$)	0.192 ± 0.047	
Improper ($^\circ$)	11.88 ± 1.248	
Energies (kcal mol^{-1}) ^{a,c}		
E_{Total}^d	209.6 ± 6.4	
E_{Repel}	9.1 ± 2.8	
E_{Angle}	157.0 ± 1.9	
E_{Bond}	6.5 ± 0.5	
E_{NOE}	19.6 ± 2.6	
E_{Dihedral}	0.13 ± 0.1	
E_{Improper}	17.3 ± 0.4	
E_{Rama}	$2,042.3 \pm 27.0$	
	Backbone atoms	Heavy atoms
RMSD of Cartesian coordinates ($\text{\AA}$)		
Backbone N, C α , and C (1–123)	0.54 ± 0.08	0.98 ± 0.09
2 $^\circ$ Structure (sheet I & II, helix I & II)	0.35 ± 0.06	0.63 ± 0.07
Sheet I (1–8, 17–17, 29–36, 64–67)	0.24 ± 0.05	0.52 ± 0.09
Sheet II (21–25, 43–45, 48–49, 56–58, 72–77, 86–93)	0.26 ± 0.07	0.61 ± 0.08
Barrel (Sheet I & Sheet II)	0.31 ± 0.06	0.60 ± 0.07
Helix I (99–104)	0.16 ± 0.05	0.58 ± 0.10
Helix II (109–120)	0.21 ± 0.06	0.91 ± 0.16
Helices I and II	0.32 ± 0.06	0.88 ± 0.12
Core (5, 18, 20–21, 23, 33, 76, 88, 89, 90)	0.22 ± 0.08	0.54 ± 0.13
All loops ^e	0.68 ± 0.12	1.23 ± 0.14
Cu binding loops (37–42, 78–87)	0.33 ± 0.01	0.82 ± 0.11
Non-Cu binding loops	0.71 ± 0.14	1.31 ± 0.18
Loop (9–16)	0.38 ± 0.23	0.96 ± 0.46
Flap region (39–60)	0.36 ± 0.10	0.74 ± 0.14
Loop (105–108)	0.65 ± 0.09	2.25 ± 0.20

^a Average over the 25 lowest energy structures taken from a starting ensemble of 100. None of the 25 structures showed restraint violations of more than $0.29 \text{ \AA}$, the largest consistent violations has $0.26 \text{ \AA}$.

^b Violated in more than 25% of structures in the final ensemble of 25 structures.

^c E_{Total} , total energy; E_{Repel} , VdW repulsive energy term; E_{NOE} , effective NOE term; E_{Dihedral} , dihedral angle restraint term; E_{Rama} , backbone dependent rotamer term.

^d The E_{Rama} term was not included in the overall final energy reported.

^e Residue ranges 9–16, 20, 26–28, 37–42, 46–47, 50–55, 59–63, 69–71, 78–85, 94–97, 105–108.

Fig. 3. The solution structure of Cu(I) pseudoazurin. **A:** Backbone representation of Cu(I) pseudoazurin. The ensemble of the 25 lowest energy structures was derived from an initial set of 100 calculations; they were fitted onto the average final backbone coordinates over all the secondary structure elements. Sheet I is colored green, sheet II is in blue, the helices are colored red, and the Cu(I) atom is white. The average NMR structure and the family of 25 structures have been deposited in the PDB (accession number: 1C71). **B:** Ribbon diagram of the average structure of Cu(I) pseudoazurin with the same colors and orientation as **A**, with the β -strands, helices, and the Cu(I) ion labeled. **C:** A superimposition of the heavy atoms from the core packing residues (Val5, Phe18, Pro20, Ala21, Val23, Phe33, Val76, Gly88, Leu89, and Val90; as identified by Williams et al., 1995) from the ensemble of 25 lowest energy structures of Cu(I) pseudoazurin. Residues are colored with the same scheme as in **A**; a ribbon diagram of the secondary structure is superimposed in grey for reference. **D:** Ribbon diagram of the least-squares superposition (Kleywegt & Jones, 1994) of the $C\alpha$ atoms of the average solution structure of Cu(I) pseudoazurin (blue) and the X-ray structure of Cu(II) pseudoazurin (green, PDB code 1ADW). The residues identified to be in the dimer interface of the Cu(II) X-ray structure (Williams et al., 1995) are shown as grey balls and sticks (His3, Glu12, Ser13, Pro20, Phe22, Val23, Arg24, Ala117, Ala120, and Gln121). Diagrams were generated using MOLSCRIPT (Kraulis, 1991) and MOLMOL (Koradi et al., 1996).

Strand IVa (residues 43–45) and the very short region of β -strand between residues 48–49 (strand IVb) are separated by a two-residue bulge (Lys46 and Glu47), which is solvent exposed. The side chain of Leu48 is packed between helix I and the β -sandwich structure. Strand IVb is connected to strand V, which is also part of β -sheet II by a γ -turn and a short irregular region (residues 53–

55). Interestingly, the region between residues 40–60 shows RMSD values that are greater than those of the other, more regular, secondary structure elements in the protein such as β -sheet I, but this region is still considerably more ordered than the loops and turns in the protein. This observation of higher RMSDs fits well with the lack of secondary structure assignments for this region from

Fig. 4. Comparison of the backbone order parameters S^2 , RMSD values, and the number of restraints per residue for Cu(I) pseudoazurin. S^2 values are shown by open circles. The RMSD vs. the mean of the 25 lowest energy structures, fitted to the mean over all the secondary structure elements measured using 6d_lsqrman (Kleywegt & Jones, 1994), are shown in the center of the diagram. The number of restraints per residue is shown as a histogram, intrasidue, and short-range restraints ($H\alpha/\beta/\gamma/\delta/\epsilon/\zeta_i-HN_{i+1}$) are combined as the grey bars, and the white bars represent all other "long-range" restraints. The secondary structure of Cu(I) pseudoazurin is shown at the bottom of the graph.

PROCHECK-NMR (Laskowski et al., 1996) and the ^{15}N dynamics measurements that show an average reduction in the order parameter S^2 for this region (see below; Fig. 5).

Comparison of the structures of Cu(I) and Cu(II) pseudoazurin

The structures of Cu(I) and Cu(II) pseudoazurin are very similar with an RMSD over all backbone atoms of 1.21 Å and over all secondary structural elements of 0.89 Å (Fig. 3D); the two structures were compared using a distance difference matrix analysis. Comparison of the hydrophobic core in the two structures shows only small differences in the positions of the side chains of core residues (RMSD 1.20 Å for side-chain heavy atoms), with the largest difference being a small reorientation of Leu89. Thus, in accord with X-ray studies of Cu(I) and Cu(II) pseudoazurins from *A. faecalis* and *A. cycloclastes* (Vakoufari et al., 1994; Inoue et al., 1999), only minor conformational changes occur upon reduction. The major conformational changes predicted to explain the crack-

ing of crystals of Cu(II) pseudoazurin from *P. pantotrophus* upon reduction are thus not observed. Instead, small but critical movements in the vicinity of the subunit interface must be responsible for the observed effects of reduction on the crystal lattice. The β -sheet region involving strands IVa, IVb, and V is somewhat more regular in the crystal structure than in solution. A third helix, between residues 79 to 81, was identified in the crystal structure of Cu(II) pseudoazurin from *P. pantotrophus* as well as in pseudoazurins from other species (all obtained by X-ray crystallography (Vakoufari et al., 1994; Williams et al., 1995; Inoue et al., 1999)). This helix is not identified in Cu(I) pseudoazurin in solution, as judged by the NMR parameters including short-range NOEs, coupling constants, and chemical shifts, and is only identified in 64% of the final structures assessed by PROCHECK-NMR (Laskowski et al., 1996). Such single turn helices, however, are usually difficult to define on the basis of solution NMR parameters.

Three regions show noticeably larger RMSDs between the Cu(I) and Cu(II) structures (Fig. 3D): the extended hairpin loop between residues 9–16, the loop between helices I and II, and the three

Fig. 5. ^{15}N relaxation data plotted as a function of sequence for Cu(I) pseudoazurin. **A:** T_1/T_2 ratios. **B:** Heteronuclear NOE values. **C:** Order parameters (S^2). **D:** τ_e values in picoseconds. Exchange contributions to T_2 were calculated for five residues: 36, 78, 88, 118, and 122 ($R_{ex} = 5.5, 5.6, 7.8, 2.4$, and 0.9 Hz, respectively). These residues all show elevated values of the T_1/T_2 ratio. Regions of secondary structure are shown below the sequence.

C-terminal residues. In solution, the first two of these regions show a lack of long-range NOE constraints, resulting in a loss of structural precision in the Cu(I) solution structure. The loop between residues 9–16 and the three C-terminal residues are involved in the dimer interface region of the Cu(II) structure. Thus, the loss of specific contacts in the crystallographic dimer could result in a conformational change and the loss of structural definition in these regions.

The oligomeric state of Cu(I) pseudoazurin

Although Cu(II) pseudoazurin from *P. pantotrophus* is dimeric in solution and in the crystalline state, analysis of various NMR parameters discounts the presence of specific dimers of the form

found in the Cu(II) crystal structure in the solution structure of Cu(I) pseudoazurin. This is in accord with ultracentrifugation studies that have shown that Cu(I) pseudoazurin is predominantly monomeric in solution (Williams et al., 1995). From a total of 12 unambiguous interdimer cross peaks that would be expected for such a dimeric structure, only one candidate could be found in NOE spectra and this peak was weak in intensity. In addition, the 1,644 conformational restraints identified are consistent with a monomeric structure for pseudoazurin, and no restraints are violated consistently in the family of NMR structures. ^{15}N relaxation analysis, described in detail below, gives a correlation time of 6.2 ns for the protein in solution, consistent with a monomer of 13 kDa mass. Hydrodynamic calculations (de la Torre et al., 1994) predict a correlation time of ~ 14 ns for the dimer defined in the

Cu(II) structure. The orientation of the principal component of the rotational diffusion tensor in the monomer and dimer structures is predicted, on the basis of hydrodynamic calculations, to differ by 107° . The orientation of the principal component of the diffusion tensor determined from the NMR relaxation analysis is close to that predicted for the pseudoazurin monomer; the orientation of the diffusion tensors differs from that predicted for the monomer by only 16° . By contrast, the orientation of the measured and predicted diffusion tensors for the dimeric protein differs by 89° , essentially ruling out the possibility of a significant concentration of Cu(I) pseudoazurin dimers in solution and providing further evidence that in solution Cu(I) pseudoazurin does not adopt the dimeric structure found in the X-ray structure of the Cu(II) protein.

Backbone dynamics of pseudoazurin

The experimental T_1 , T_2 , and NOE data collected for Cu(I) pseudoazurin at 76.02 MHz are shown in Figure 5. The data were analyzed using isotropic, axially symmetric, and fully asymmetric models of rotational diffusion (Tjandra et al., 1995, 1996; Lee et al., 1997). The fully asymmetric model was found to give a statistically significant improvement to the agreement between calculated and experimental T_1/T_2 ratios compared to isotropic or axially symmetric models. The ratio of the principal components of the diffusion tensor was found to be 1.23:1.11:1.00. This analysis gave an effective rotational correlation time of 6.2 ns, a value consistent with the presence of monomeric protein. The S^2 , R_{ex} , and τ_e values, shown in Figure 5, were derived from an analysis of the relaxation data using the model-free formalism of Lipari and Szabo (1982) incorporating fully asymmetric rotational diffusion. Full details of the analysis are given in Materials and methods.

In proteins, regions of regular secondary structure are generally found to have higher S^2 values than less structured loops. This trend is also found here for pseudoazurin although, in general, high S^2 values are found throughout the structure (Fig. 5C). Residues in the α -helical, β -sheet, and loop regions have average order parameters of 0.86, 0.83, and 0.82, respectively; a mean S^2 value of 0.83 ± 0.05 is calculated over all residues. More than 80% of the residues in Cu(I) pseudoazurin have order parameters S^2 greater than 0.8, indicating that most of the protein has a rigid structure without backbone motions of a significant amplitude on a picosecond timescale.

Residues in the loop connecting strands I and IIa (9–16) show relatively high RMSD values in the NMR structures (Fig. 4). In accord with this, residues 11–15 show a clear reduction in the heteronuclear NOE value, and Glu12 and Gly14 require a τ_e parameter in the model-free analysis. The τ_e value of ~ 100 ps for Glu12 is the highest value found for the protein. The S^2 values found for this loop are similar to the overall average for the protein. Thus, the amplitude of fast motions for these residues is not significantly larger than found for the protein as a whole but the internal correlation time for the motions is somewhat longer. The high RMSD values observed in the calculated structures for this region are likely to reflect this different dynamic behavior but are also likely to arise from a lack of NOE constraints in this region of the structure.

A number of residues, which are found in the extended loop between strands IV and VI, show increased mobility as reflected by lower heteronuclear NOEs and S^2 values, and significant τ_e contributions. Ile45, Gly52, Glu54, Ser55, Phe56, and Thr65 all have S^2 values of <0.8 . Residues in this region are designated

strands IVb and V in the crystal structure of Cu(II) pseudoazurin, where they make up the edge of β -sheet II. In the NMR structure, the assignment of these residues to β -strands is less clear, as discussed earlier. These residues form the lip of a small depression or groove in the surface of molecule between β -sheets I and II, and their enhanced dynamics could result from the lack of packing and regular structure around these residues.

Lower than average S^2 values in pseudoazurin are also found for residues 69–70, 94–95, and 106–107. These residues are located in the turn between strands VI and VII, in the turn separating the C-terminal β -strand VIII and α -helix I, and in the loop between the two α -helices, respectively. The later region also shows particularly high RMSD values in the ensemble of NMR structures.

Three residues with increased T_1/T_2 ratios, Thr36, Cys78, and Gly88, require a significant chemical exchange contribution to T_2 in the model-free analysis ($R_{ex} > 5$ Hz). Thr36, Cys78, and Gly88 are located in the loop connecting β -strands III and IVa, in the loop connecting β -strands VII and VIII, and in β -strand VIII, respectively. Cys78 is one of the Cu ligands, and the other two residues, Thr36 and Gly88, are close to the Cu(I) site at the top of the structure shown in Figure 3. In addition to an exchange contribution to T_2 , Thr36 also shows a significant reduction in the heteronuclear NOE value and a reduced S^2 value of 0.73. The loop connecting β -strands III and IVa is found to have elevated RMSD values in the family of NMR structures. Thus, Thr36 appears to be involved in motions on both the picosecond and the millisecond to microsecond timescales. This residue is discussed in more detail below.

Recently, a study of the ^{15}N backbone dynamics of azurin from *Pseudomonas aeruginosa* has been reported (Kalverda et al., 1999). Pseudoazurin and azurin are both members of the cupredoxin family; they share the same β -sheet topology but differ in the location and the number of helices in the structure (Adman, 1991). Pseudoazurin has two helices located after β -strand VIII at the C-terminus of the sequence (Fig. 3) while azurin has a single helix located in a loop between β -strands IV and V. Overall, the backbone dynamics of these two proteins are very similar. Average order parameters of 0.86 ± 0.05 and 0.83 ± 0.05 were obtained for azurin and pseudoazurin, respectively; both proteins are rigid with only limited backbone internal motions on a picosecond timescale.

In the analysis of the pseudoazurin data, a τ_e contribution was required for 17 residues compared to only five residues in azurin (Kalverda et al., 1999). The τ_e contribution, which indicates enhanced backbone dynamics on a fast timescale, is generally required when the observed heteronuclear NOE is more than two standard deviations below the theoretical maximum NOE value. In azurin, relatively high values were observed for the NOE, and a number of residues gave values above the predicted maximum value. This was attributed to the effects of fast amide exchange with solvent (Kalverda et al., 1999). In pseudoazurin lower values of the heteronuclear NOE were observed requiring additional τ_e terms. Several of the residues requiring τ_e terms and having lower S^2 values in both azurin and pseudoazurin are found in homologous regions of the two structures. Residues 105 and 107, in azurin, and residues 69 and 70, in pseudoazurin, are located in the loop region connecting β -strands VI and VII. Residue 74 is located in the loop region of azurin that links the single helix to strand V. In pseudoazurin, loop residues that connect strand VIII to helix I and helix I to helix II also require a τ_e term. The two C-terminal residues in both proteins have lower order parameters indicating enhanced backbone mobility. A difference in backbone dynamics

between azurin and pseudoazurin is found in the region of the structure including β -strands IVb and V and the loops following these strands. This region appears to be more mobile in pseudoazurin than in azurin. This difference may result from the difference in topology of the proteins in this region. In azurin, the single helix is inserted between strands IV and V, whereas in pseudoazurin a short loop connects these regions.

In the analysis of the azurin data, an exchange contribution to T_2 , R_{ex} , was included for 25 residues (Kalverda et al., 1999). For pseudoazurin R_{ex} terms were required for only five residues. The larger number of R_{ex} terms used for azurin is likely to be the result of the isotropic rotational diffusion model used in the analysis (Kalverda et al., 1999). This model assumes a single T_1/T_2 ratio for the protein and residues showing an elevated value of this ratio will require an R_{ex} term. It is interesting to note that several of the residues in azurin that require an R_{ex} term are located in the single α -helix (Kalverda et al., 1999). This helix is oriented nearly parallel to the long axis of the azurin molecule; therefore, the N-H vectors for these helical residues will be parallel to the principal component of the diffusion tensor of azurin. Residues with this orientation are expected to have a higher T_1/T_2 value than residues with N-H vectors oriented perpendicular to the principal component of the diffusion tensor, such as the majority of the β -sheet residues, and will require an R_{ex} term in the fitting procedure. In the present analysis for pseudoazurin, a fully asymmetric rotational diffusion model that takes orientational dependent variations in T_1/T_2 into account has been used (Tjandra et al., 1995, 1996; Lee et al., 1997).

An exchange term R_{ex} is required for the homologous residues Thr36, in pseudoazurin, and Gly37, in azurin. These residues are located in the loop connecting β -strands III and IV adjacent to the Cu(I) site. In azurin this exchange broadening is caused by the protonation/deprotonation equilibrium of His35, which leads to a flip in the peptide bond of Pro36/Gly37 and a change in the hydrogen bonding patterns of residues 35–37 (Nar et al., 1991; Kalverda et al., 1999). This conformational exchange leads to broadening of residues in several adjacent loops in azurin, notably in the I–IIa, V–VI, and VII–VIII loops. The exchange broadening of Thr36 in pseudoazurin must arise from a different mechanism since the residue preceding Pro35 is a valine, which cannot undergo a protonation/deprotonation equilibrium. Interestingly, Thr36 has been found to be involved in a concerted peptide bond flip in the X-ray structures of the oxidized and reduced forms of the homologous pseudoazurin from *A. cycloclastes* (Inoue et al., 1999). The change observed in the X-ray structures of this pseudoazurin around Thr36 is accompanied by changes in the hydrogen bonding pattern involving the side chains of Glu4, His6, and Thr36. These residues are conserved in pseudoazurin from *P. pantotrophus* and the observed exchange broadening of Thr36 may arise from a similar conformational equilibrium. The definition of the side-chain conformations of the solvent exposed residues Glu4, His6, and Thr36, however, is not of sufficient resolution in the solution structure of Cu(I) pseudoazurin to determine whether there is a structural difference in this region between the oxidized and reduced proteins as observed for the protein from *A. cycloclastes* (Inoue et al., 1999). Further NMR studies, utilizing ^{13}C labeled pseudoazurin, may provide insight into the conformational equilibrium responsible for the observed exchange broadening in this region. It is interesting, however, that the same residue, located in close proximity to the Cu site, is implicated in a conformational equilibrium in three members of the cupredoxin family that may have implications for the redox behavior of these proteins.

Conclusion

In this paper, we report the solution structure of Cu(I) pseudoazurin from *P. pantotrophus*, determined to high precision (backbone RMSD 0.35 ± 0.06 Å in secondary structure elements). The structure of the Cu(II) form of this protein had been determined previously by X-ray crystallography, and its structure was found to be dimeric (Williams et al., 1995). Attempts to reduce the Cu(II) pseudoazurin in situ resulted in cracking of the crystals; this raised the intriguing and unexpected possibility that the structure of the Cu(I) protein might show significant structural changes compared with its Cu(II) counterpart. Comparison of the solution structure of Cu(I) pseudoazurin determined here and the previous X-ray structure of the Cu(II) protein shows only very small changes in conformation (RMSD over backbone heavy atoms 1.2 Å). Therefore, the crystal cracking observed is not due to a major conformational change but must result from more subtle effects perhaps related to changes in the electrostatic environment in the crystals or small changes at the dimer interface. ^{15}N relaxation measurements show that the backbone of Cu(I) pseudoazurin in solution is relatively rigid throughout the sequence and only a few residues show dynamics of significant amplitude on a picosecond time-scale. These results support the currently held view that the cupredoxin fold acts as a rigid framework holding the copper in an “entatic” state to facilitate electron transfer (Williams et al., 1995). A comparison of the ^{15}N dynamics results presented here with similar data for azurin (Kalverda et al., 1999) and with the X-ray structures of oxidized and reduced pseudoazurin from *A. cycloclastes* (Inoue et al., 1999) indicates that the same residue located in close proximity to the copper site, Thr36 in pseudoazurin and Gly37 in azurin, is implicated in a conformational equilibrium that may have implications for the redox behavior of these proteins.

The structure of Cu(I) pseudoazurin described here leaves open the question of the relevance of the change in protein oligomeric state as a potential redox control point in the bacterial periplasm. Clearly, the concentration of the dimeric form of Cu(II) pseudoazurin determined in vitro is relatively low suggesting that if a similar K_d is found in vivo, a significant proportion of pseudoazurin molecules could function as electron carriers without the need to switch oligomeric state. Such a view would be consistent with the characteristics of the large number of cupredoxins for which structural information about the Cu(II) and Cu(I) states is currently available (Adman, 1991). Nevertheless, the possibility remains that the conditions of the bacterial periplasm may favor the dimeric form of Cu(II) pseudoazurin providing a fascinating control point for electron transfer not currently explored. The periplasm is a crowded environment and thus in vivo concentrations of pseudoazurin might be high, leading to significant amounts of dimer even if the in vitro K_d remains unaltered. Such dimerization may provide a novel route for controlling electron transfer, for instance by the dimer having a high affinity for a redox partner and thus blocking the arrival of electrons from other potential electron donors. Whether or not this is the case will have to await further experiments on bacterial electron transfer in vivo.

Materials and methods

Uniformly ^{15}N labeled Cu(II) pseudoazurin

Cu(II) pseudoazurin uniformly labeled with ^{15}N was prepared by the expression of the *pazS* gene in *Escherichia coli* XL1-Blue

grown on M9 minimal medium (Sambrook et al., 1989) containing $^{15}\text{NH}_4\text{Cl}$ (1 g/L), D-glucose (12 g/L) MgSO_4 , (0.002 M), CaCl_2 (0.1 mM), thiamine-HCl (3 μM), CuSO_4 (0.01 mM), ampicillin (100 $\mu\text{g/mL}$), and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (18.5 μM). The cells were grown at 37 °C and expression of pseudoazurin induced at an OD_{600} of 1.0 by the addition of 0.3 mM isopropyl- β -D-thiogalactoside (IPTG). The protein was purified as described by Leung et al. (1997). The overall yield of pure ^{15}N -Cu(II) pseudoazurin was 11 mg of pure protein per liter of culture.

NMR spectroscopy

NMR samples of Cu(I) pseudoazurin contained 600 μL of 3 mM pseudoazurin dissolved in either 95% H_2O /5% D_2O , or 100% D_2O containing 20 mM PO_4^{2-} at pH 6.6. Reduction of the Cu(II) protein to the Cu(I) form was achieved by addition of 4 μL of freshly prepared 1 M $\text{Na}_2\text{S}_2\text{O}_4$. The sample was then sealed in the NMR tube using parafilm after passing O_2 -free N_2 gas over it for 2 min.

NMR experiments were carried out at ^1H frequencies of 500 and 750 MHz on home-built spectrometers in the Oxford Center for Molecular Sciences and at 500 MHz on a Varian Unity 500 spectrometer in the Faculty of Biological Sciences at the University of Leeds. ^1H - ^{15}N NOESY-HSQC (Fesik & Zuiderweg, 1988) (200 ms mixing time), ^1H - ^{15}N TOCSY-HMQC (Marion et al., 1989) (31 ms spin-lock time), and ^1H - ^{15}N HMQC-J (Kay & Bax, 1990) experiments were collected in 95% H_2O /5% D_2O at 25 °C using an ^{15}N labeled sample. In addition, several ^1H homonuclear experiments were carried out with ^{15}N decoupling during the t_1 and t_2 periods: NOESY (Kumar et al., 1980) (200 ms mixing time), pre-TOCSY-COSY (Otting & Wüthrich, 1987), and TOCSY (Davis & Bax, 1985) (60 ms spin-lock period). The following spectra were collected using D_2O samples; DQF-COSY (Rance et al., 1983), NOESY (Kumar et al., 1980) (100 and 200 ms mixing times), and TOCSY (Davis & Bax, 1985) (60 ms spin-lock period). The $\text{HN-H}\alpha$ coupling constants from the HMQC-J spectrum were calculated using the fitting procedure described by Redfield et al. (1991). Details of data collection and processing parameters have been deposited, with the resonance assignments, in the BioMagResBank (accession number 4466).

^{15}N backbone dynamics

^{15}N T_1 , T_2 , and ^1H - ^{15}N steady-state NOE measurements were carried out at a ^{15}N frequency of 76.02 MHz at 25 °C. Data were collected with sensitivity-enhanced pulse sequences employing gradients (Kay et al., 1989, 1992a, 1992b; Boyd et al., 1990; Palmer et al., 1992). Recycle delays of 2.5, 1.6, and 5.0 s were used in the T_1 , T_2 , and NOE experiments, respectively. For the T_1 measurements, nine experiments with relaxation delays of 0.02, 0.06, 0.10, 0.20, 0.40, 0.60, 0.80, 1.10, and 1.50 s were collected. For the T_2 measurements, nine experiments with relaxation delays of 0.006, 0.018, 0.030, 0.048, 0.060, 0.078, 0.097, 0.145, and 0.194 s were collected. Two spectra were collected for the NOE measurement; in the first experiment, 225° ^1H pulses were applied at 5 ms intervals during the 5 s recycle delay to saturate the ^1H spectrum while in the second experiment no saturation was applied. The heteronuclear NOE effect was calculated as the ratio of peak heights in spectra recorded with and without ^1H saturation. Relaxation rates $1/T_1$ and $1/T_2$ were fitted as single exponential decays to peak heights, using the pro-

gram Model-Free 3.1 (kindly provided by Prof. A.G. Palmer III); uncertainties were estimated from baseline noise (Palmer et al., 1991). The errors in the peak heights were taken as 2, 3, and 2 standard deviations of base-plane noise in the T_1 , T_2 , and NOE experiments, respectively. Relaxation parameters were measured for 111 of the 116 nonproline residues of pseudoazurin.

Calculation of ^{15}N amide backbone order parameters

Hydrodynamic calculations performed with the program HYDRO (de la Torre et al., 1994) indicate that the average structure of pseudoazurin in solution is slightly asymmetric with a ratio of the principal components of the diffusion tensor of 1.14:1.05:1.00. It has been demonstrated for ubiquitin that even a relatively small degree of anisotropic rotational diffusion has detectable effects on measured T_1/T_2 ratios (Tjandra et al., 1995). These effects are more pronounced at higher magnetic fields, and the relatively small anisotropy of pseudoazurin is found to have a statistically significant effect on the fitting of the experimental T_1/T_2 ratios obtained at 76 MHz.

T_1/T_2 ratios for 99 of the 111 residues for which data are available have been analyzed using isotropic, axially symmetric and fully asymmetric models for rotational diffusion; residues 12, 36, 55, 56, 69, 70, and 123, with heteronuclear NOEs of <0.7, and residues 36, 37, 42, 78, 88, and 118, with elevated T_1/T_2 ratios, are removed from the analysis. Application of an axially symmetric model is found to give a statistically significant improvement to the agreement between calculated and experimental T_1/T_2 ratios compared to an isotropic model (p-value = 2.34×10^{-5} , >99.99% statistical significance). Furthermore, use of a fully asymmetric model gives a statistically significant improvement compared to an axially symmetric model (p-value = 1.46×10^{-2} , >98.5% significance) (Devore, 1982; Tjandra et al., 1995).

The relaxation data for pseudoazurin were analyzed using the model-free formalism of Lipari and Szabo (1982) incorporating fully asymmetric rotational diffusion using in-house software. Errors in S^2 , τ_e , and R_{ex} were assessed using Monte Carlo simulations (Palmer et al., 1991). The values of the diffusion constants D_x , D_y , and D_z and the orientation of the diffusion tensor were determined by fitting to experimental T_1/T_2 ratios using standard procedures (Tjandra et al., 1995) and the average solution structure. The values of D_x , D_y , and D_z were 2.66×10^7 , 2.40×10^7 , and 2.95×10^7 , respectively. These correspond to an effective rotational correlation time of 6.2 ns. The S^2 values were calculated using a fixed NH bond length of 0.102 nm and a ^{15}N chemical shift anisotropy ($\Delta\sigma$) of -170 ppm (Tjandra et al., 1996). A simple model requiring only a single parameter S^2 (the order parameter) was found to give good agreement between experimental and calculated T_1 , T_2 , and NOE values for 91 of the 111 residues analyzed. These residues have an experimental NOE value that is within two standard deviations of the theoretical maximum of 0.84 indicating that they do not experience backbone motions of significant amplitude on a picosecond timescale. A more complex model including S^2 and an exchange contribution to T_2 , (R_{ex}), was used for three residues (78, 88, and 118); these are all found to have very high T_1/T_2 ratios. A model including S^2 and τ_e , the correlation time for internal motion, was used for 15 of the 111 residues (12–14, 54–56, 60, 63, 65, 69, 70, 94, 101, 120, and 123); these residues all have a heteronuclear NOE that is more than two standard deviations below the theoretical maximum value of 0.84 indicating significant backbone motions on a picosecond time-

scale. Finally, a model requiring S^2 , R_{ex} , and τ_e was used for two residues (36 and 122); both residues had an elevated value of the T_1/T_2 ratio and a low value for the NOE.

Collection of distance constraints

Distance constraints were derived from a 3D ^1H - ^{15}N NOESY-HSQC spectrum and from NOESY spectra acquired in D_2O (100 and 200 ms mixing times). NOEs were classified according to intensity and assigned to upper limits of 2.5, 3.0, 4.0, 5.0, and 6.0 Å. For the ^1H - ^{15}N NOESY-HSQC experiment distances were calibrated against the $\text{H}\alpha$ -HN intensities in helical regions of the molecule. For the ^1H - ^1H NOE experiments, calibrations were derived from the $\text{H}\delta$ - $\text{H}\epsilon$ NOEs in tyrosine and phenylalanine and the $\text{H}\epsilon$ - $\text{H}\zeta$ NOEs in phenylalanines. Allowance was made for the differential relaxation of methyl protons by adding 0.5 Å to the upper distance limits in 100 ms mixing time spectra and 1.0 Å in 200 ms spectra. Two empirical corrections were made to the 200 ms mixing time NOE constraints. It was found that using a $1/r^4$ relationship between NOE intensity and distance gave better convergence (Guntert et al., 1991), and 20% was added to the upper limits for the restraints.

The Cu(I) atom was positioned in the structure using a network of distance restraints between the heavy atoms of its ligands and the Cu atom. These constraints were derived from the crystal structure of Cu(I) pseudoazurin from *Alcaligenes faecalis* S6 [(Vakoufari et al., 1994), PDB code 1PZA] at pH 7.0, following a method previously described by Bagby et al. (1994). Distance restraints involving the copper ion and the four ligand residues, His40, Cys78, His81, and Met86, were applied to define the copper binding site. These restraints used a square-well potential with a well size of ± 0.2 Å, and a force constant of $1 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$. The parameter σ_{Cu} , which controls nonbonded Cu interactions, was set to 1.467 Å, which was derived from a search of high-resolution crystal structures of Cu containing proteins with a resolution better than 1.8 Å. This value was calculated using the standard σ values for C and O provided by the XPLOR NMR parameters PARALLHDG.PRO (Brünger, 1993).

Torsion angle restraints for ϕ were based on measured $^3J_{\text{N}\alpha}$ values. Residues with a coupling constant of $>8 \text{ Hz}$ were assigned a ϕ restraint of $-120^\circ \pm 40^\circ$. Residues with a coupling constant of $<6 \text{ Hz}$ and NOEs characteristic of helical structure were assigned a ϕ restraint of $-60^\circ \pm 30^\circ$. A total of 38 hydrogen bond constraints were used in the calculations, each hydrogen bond was constrained using two distances ($d_{\text{HN}\dots\text{O}} = 1.8\text{--}2.0 \text{ \AA}$ and $d_{\text{N}\dots\text{O}} = 2.7\text{--}3.0 \text{ \AA}$). Hydrogen bonds were located in regions of regular secondary structure and were based on hydrogen exchange protection in a DQF-COSY of a fully protonated sample after dissolution in D_2O for 1 day.

Structure calculations

Structures were calculated using X-PLOR (Brünger, 1993). The calculations used an *ab initio* simulated annealing strategy (YASAP 3.0) (Nilges et al., 1988, 1991). Sum averaging was used for the distance restraints of methylene and methyl hydrogens where there was no stereochemical assignment (Brünger et al., 1986). Assignment of prochiral groups was achieved by floating assignment and swapping of prochiral groups (Holak et al., 1989; Folmer et al., 1997). A reduced set of nonbonded interactions was used during the conformational search phase (Nilges, 1993). Initial structures

were calculated by randomization of the backbone ϕ and ψ angles of a template structure. The Cu(I) moiety was incorporated throughout structure calculation from template generation onward, as was the ionized Cys residue. The 40 lowest energy structures from the initial calculation were then refined by simulated annealing with slow cooling. Ramachandran refinement was used in the final stage of structure calculation using a torsion angle database (Kuszewski et al., 1996, 1997). This increased the number of ϕ and ψ angles in the most favored region of the Ramachandran plot, as measured by PROCHECK-NMR (Laskowski et al., 1996), by $\sim 8\%$ without changing the RMSD or target function energy significantly. Further improvement in the RMSD of the ensemble of structures was achieved by using very long simulated annealing and quenching sequences in the refinement protocol. These refinement steps reduced the overall RMSD within the regions of secondary structure by $\sim 0.1 \text{ \AA}$. The Ramachandran refinement was followed by energy minimization without the Ramachandran potential. The 25 lowest energy structures were selected to represent the final solution structure; these were overlaid using 6d_lsqman (Kleywegt & Jones, 1994). The geometry of the final structures was analyzed using PROCHECK NMR (Laskowski et al., 1996); 79.1, 19.5, 1.2, and 0.2% of the residues, in the family of 25 structures, were found in the most favored, allowed, generously allowed, and disallowed regions of Ramachandran space, respectively (the residues in the disallowed regions are Asn41 and Lys106).

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