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Structures of oxidized and reduced azurin II from *Alcaligenes xylosoxidans* at 1.75 Å resolution

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Crystallographic structures of oxidized and reduced forms of azurin II are reported at 1.75 Å resolution. Data were collected using one crystal in each case and by translating the crystal after each oscillation range to minimize photoreduction. Very small differences are observed at the Cu site upon reduction and these cannot be determined with confidence at current resolution. A comparison with the three-dimensional EXAFS reveals a good correspondence for all the ligand distances except for Cu—His46, where a larger deviation of ~0.12–0.18 Å is observed, indicating that this ligand is more tightly restrained in the crystallographic refinement at the current resolution.

1. Introduction

In the denitrifying bacterium *A. xylosoxidans* subspecies *xylosoxidans* NCIMB 11015 (formerly known as *Alcaligenes sp*. NCIMB 11015, *Achromobacter xylosoxidans* or *Pseudomonas denitrificans*), the reduction of nitrite to nitric oxide is accomplished by a copper-containing nitrite reductase (Abraham *et al.*, 1993; Grossmann *et al.*, 1993; Strange *et al.*, 1995). Two classes of blue copper proteins, pseudoazurin and azurin, have so far been implicated in donating electrons to nitrite reductase; pseudoazurin to the green nitrite reductases of *A. faecalis* S-6 and *Achrom. cycloclastes* (Kakutani *et al.*, 1981; Lui *et al.*, 1986) and azurin to the blue nitrite reductases of *A. xylosoxidans* (Dodd, Hasnain, Abraham *et al.*, 1995) and *P. aureofaciens* (Zumft & Kroneck, 1987).

The X-ray crystal structures of six bacterial azurins have been reported: those from *P. aeruginosa* (AzPA; Adman & Jensen, 1981; Nar *et al.*, 1991), *A. denitrificans* (AzAD; Norris *et al.*, 1983; Baker, 1988), *P. fluorescens* (Lee *et al.*, 1997), *Methylomonas* J (Inoue *et al.*, 1999) and two from *A. xylosoxidans*, azurin I (Li *et al.*, 1998) and azurin II (Dodd, Hasnain, Hunter *et al.*, 1995). The crystallographic structure of azurin II has been determined to 1.9 Å, while the structure of azurin I has been determined at 2.45 Å resolution. Crystallographic studies comparing the oxidized and reduced copper site of AzAD (Shepard *et al.*, 1990) and poplar plastocyanin (Guss & Freeman, 1983; Guss *et al.*, 1986) have also been reported.

It has been suggested (Cotton & Wilkinson, 1988; Williams, 1985) that the copper coordination environment in the blue copper electron-transfer proteins is a compromise between the two preferred geometries of the redox states of the Cu atom, namely Cu^{I} and Cu^{II}. It has been argued that these distortions in the Cu coordination sphere result in an energized or entatic state of the Cu (Vallee & Williams, 1968; Williams, 1995). Such an environment should result in a lowering of the activation energy between the two redox states allowing rapid electron transfer to take place. To date, only

AzAD has been studied crystallographically in the two redox states and, contrary to EXAFS results (Groenveld *et al.*, 1986; Murphy *et al.*, 1990), little or no structural change was detected at the Cu site upon reduction. In order to define the upper limit of structural changes, both EXAFS and crystallographic studies have been carried out on azurin II. EXAFS results are presented in the accompanying paper (Cheung *et al.*, 2000). Here, the crystallographic structures of oxidized and reduced forms of azurin II are reported at the same high resolution of 1.75 Å. For the oxidized structure, photoreduction was kept to a minimum and data were collected by translating the crystal after each oscillation. The use of Weissenberg geometry and an off-line image-plate system (400 × 400 mm) also allowed data to be collected with speed and minimum dead time between the two oscillation images.

2. Material and methods

2.1. Crystallization and crystal reduction

Azurin II from *A. xylosoxidans* used in the crystallization was supplied by the Nitrogen Fixation Laboratory, John Innes Centre, Norwich, where it was purified by the method of Dodd, Hasnain, Abrahams *et al.* (1995). Crystals of azurin II used in the oxidized and reduced data collections were grown under the same conditions as those used for the original data collection (Dodd, Hasnain, Hunter *et al.*, 1995). The crystal used in the reduced data collection was chemically reduced with an excess of dithionite. Reduction was observed as the dithionite diffused though the drop in ~20 s. No other physical changes were observed in the crystals upon reduction. No special preparatory measures were taken with the crystals used for the oxidized data collection.

2.2. Data collection

Data were collected from the oxidized and reduced azurin II crystals at beamline BL6A-2 at the Photon Factory, KEK using the Weissenberg technique (Sakabe, 1991) with Fuji image plates (200 × 400 mm; Miyahara *et al.*, 1986) and a BA100 scanning system. An X-ray wavelength of 1.0 Å was used with a 100 µm collimator defining the beam. The camera used for data collection was 2B, which holds two images, giving a total detector area of 400 × 400 mm. Camera 2B gives a crystal-to-image-plate distance of 286.5 mm. Both oxidized and reduced data sets were collected using the same parameters. Data were collected at 285 K. The oscillation per image was 4.5° with 0.2° overlap and a coupling constant of 2° mm^{-1}. A total of 12 images were exposed for a time of 135 s, giving a total oscillation range of 47.5°. Each data set was obtained from a single crystal.

The oxidized crystal was translated to a fresh point after every exposure since X-ray-induced reduction of the crystal had been observed in the past (Dodd, Hasnain, Abraham *et al.*, 1995; Debenham *et al.*, 1996). The reduced crystal was translated every other exposure in order to avoid the collection of data from radiation-damaged areas. For both oxidized and reduced data, the crystal orientation was determined manually and then refined using *WEIS* (Higashi, 1989) in conjunction with the image-display program *IMAGE*. The data were then scaled and merged using the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Data statistics are summarized in Table 1.

Table 1
Data-collection and model quality statistics.

Figures in parentheses refer to the outer shell, resolution range 1.83–1.75 Å.

	Oxidized	Reduced
Total number of reflections	34438	37754
Independent reflections	12084	11776
Overall completeness (%)	82.4 (53.6)	80.3 (50.7)
Merging R factor (%)	6.2 (20.4)	5.6 (18.2)
$I/\sigma(I)$	6.5 (3.1)	9.5 (3.8)
Wilson plot B factor (Å^2)	15.8	14.9
No. of reflections	20417	20456
No. of parameters	4080	4080
Mean B factors (Å^2)		
Main chain	20.3	19.7
Side chains	21.5	20.5
Copper	15.5	9.5
Waters	36.5	36.3
R.m.s. deviations		
Bond distances (Å)	0.008	0.008
Bond angles (°)	1.5	1.5
Dihedral angles (°)	26.1	26.0
Improper angles (°)	1.1	1.1
Ramachandran plot		
Favoured regions (%)	88.9	88.0
Additional allowed (%)	10.2	11.1
Generously allowed (%)	0.9	0.9
Residues with		
Unusual peptide orientations (%)	0.0	0.0
Non-rotamer side-chain conformation (%)	11.1	11.1
Poor density (real-space r factor > 40%) (%)	0.8	1.6
Overall G factor	+0.23	+0.23
Diffration data precision indicator (Å)	0.09	0.08

2.3. Refinement

The structures of azurin II in the oxidized and reduced form were refined with the program *X-PLOR* version 3.0 (Brünger, 1992*b*). Standard topology and parameter files derived from the Cambridge Structural Database library were used, as suggested by Engh & Huber (1991). A charge of +2 was used for the Cu atom in the oxidized refinement and +1 for the Cu atom in the reduced refinement. No restraints were applied to the copper–ligand distances or to any bond angles in the copper site. Copper was included as an anomalous scatterer with f'' = 2.282 at 1.00 Å. Prior to the refinement, the $R_{\rm free}$ (Brünger, 1992*a*) flags from the original 1.9 Å data set were transferred to the oxidized and reduced data sets. New reflection data were segregated from both data sets and 10% of these in the resolution range 50–1.75 Å were selected to add to those previously selected for cross-validation data, the test set. This gave a working set of 20 175 reflections and a test set of 2296 reflections for the oxidized data. The working set for the reduced data contained 19 664 reflections, with a test set of 2231 reflections.

2.4. Oxidized azurin II refinement

The initial oxidized refinement with the new data used the model directly from the previous refinement at 1.9 Å (Dodd, Hasnain, Abraham *et al.*, 1995). The model was subjected to a round of refinement consisting of 40 cycles of conjugate-gradient minimization followed by individual *B*-factor refinement over 20 cycles using data in the resolution range 8.5–1.75 Å. This procedure resulted in a crystallographic *R* factor of 21.7%, with an $R_{\rm free}$ of 23.4%. The quality of the model was assessed in detail before proceeding with further refinement. In the Ramachandran plot, 89.4% of the residues fell in the most favoured regions, with two residues falling in the additionally allowed regions and a single residue, Gln2, in the generously allowed region. The electron-density maps showed significant improvement over those calculated using the 1.9 Å data at the end of the previous refinement. This allowed some rebuilding of the structure to be carried out where minor ambiguities persisted in the previous model. This rebuilding and a further round of refinement resulted in a reduction of the crystallographic *R* factor to 20.0%, with an $R_{\rm free}$ of 22.1%. The next stage of model building was to include additional waters in the model using $F_{\rm obs} - F_{\rm calc}$ difference density maps contoured at $+3\sigma$. A total of 63 peaks were examined in *O* (Jones *et al.*, 1991) against the density and for proximity to suitable hydrogen-bonding partners. Waters were only accepted where their positions were found to be coincident in the oxidized and reduced structures. This was performed to allow more meaningful comparisons between the two structures to be made. From these 63 peaks computed as potential waters, 17 were selected as water molecules for inclusion in the model. A further round of refinement lead to an *R* factor of 18.9% and an $R_{\rm free}$ of 20.5%. Finally, the model was refined against all the test data, which resulted in a final crystallographic *R* factor of 18.9% for data in the resolution range 8.5–1.75 Å.

2.5. Reduced azurin II refinement

The reduced crystal structure of azurin II was refined using the same protocol as for the oxidized structure. The initial refinement resulted in a crystallographic *R* factor of 21.3%, with an $R_{\rm free}$ of 23.4%. The Ramachandran plot was very similar to the oxidized structure, with 89.4% of the residues falling in the most favoured regions. Two residues fell in the additionally allowed regions and a single residue, again Gln2, in the generously allowed region. Rebuilding and further refinement were performed, resulting in a reduction of the crystallographic *R* factor to 20.4%, with an $R_{\rm free}$ of 22.9%. The additional 17 waters were then included in the model (47 peaks were examined in *O*). Refinement with the new waters led to an *R* factor of 19.1% and an $R_{\rm free}$ of 21.4%. Refinement of the model against all data resulted in a final crystallographic *R* factor of 19.2% for data in the resolution range 8.5–1.75 Å.

In addition, an anisotropic *B*-factor refinement of the Cu atom and the two copper-site S atoms was performed using *REFMAC* (Murshudov *et al.*, 1997). This refinement did not reveal any significant anisotropy at the copper site.

3. Results and discussion

The basic folding unit of azurin II is an eight-stranded β-barrel which can be described as two β-sheets which are sandwiched together (Fig. 1). The first, which shows a classic twisted β-sheet form, consists of strands 1, 3, 6 and the first few residues of strand 2. The second consists of strands 5, 4, 7, 8 and the majority of the residues in strand 2. Strand 2 has a pronounced kink at residues 16–17 where it ceases to be strongly associated to strand 1 in the first β-sheet and becomes more extensively hydrogen bonded to strand 8 in the second β-sheet. A flap between strands 4 and 5 consisting of ~30 residues (52–81) contains the only helical feature in the structure. This flap is separated from the main body of the molecule by an interface of non-polar residues. The flap starts with a small loop before entering an α-helical region (residues 55–67). Towards the end of the helix its character changes as it becomes more similar to a 3_{10} helix. The remainder of the flap (residues 68–81) forms an irregular loop. The molecule contains a single disulfide bridge between cysteines 3 and 26. This bridge stabilizes the N-terminus of the molecule.

The copper site of azurin II lies in what is known as the 'northern' part (Guss & Freeman, 1983) of the molecule. The Cu atom has three short ligands at ~2 Å and is bonded in a distorted trigonal planar arrangement. The ligands are His46 $N^{\delta1}$, His117 $N^{\delta1}$ and Cys112 S^{γ}. In addition to the three

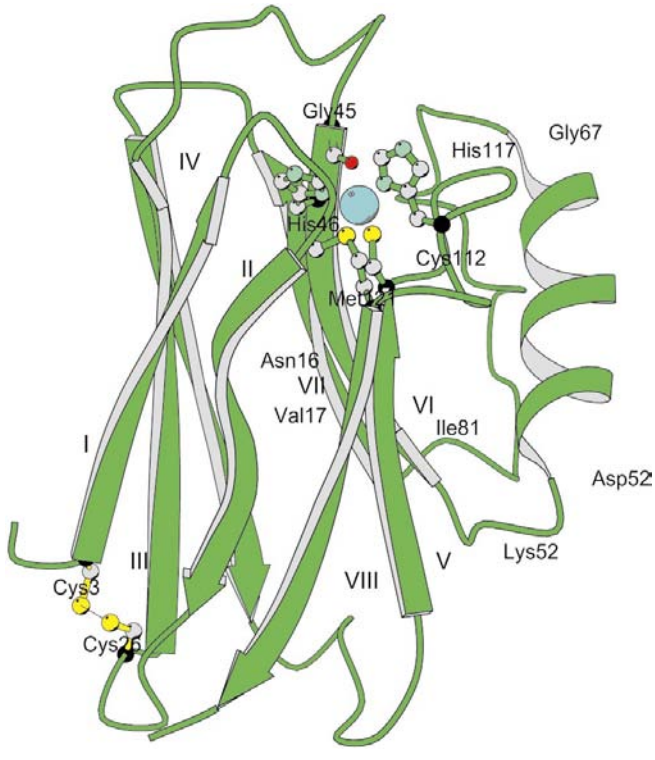

Figure 1
A ribbon diagram of azurin II showing the positions of the disulfide bridge (Cys3–Cys26) and location of the Cu centre (cyan sphere). The β-strands are labelled I to VIII. The position of the residues at the pronounced kink in strand II are labelled (Asn16, Val17), as are those delimiting the extended flap (Lys52, Ile81) and the α-helix (Asp52, Gly67).

short close planar ligands, two longer axial interactions exist. These are from Met121 S^{δ} and the carbonyl O atom of Gly45. Two of the copper-ligand side chains are stabilized by hydrogen bonds. His46 $N^{\varepsilon 2}$ is hydrogen bonded to the carbonyl O atoms of Asn10 and Cys112 S^{γ} is hydrogen bonded to the amide N atoms of Asn47 and Phe114.

3.1. Quality of the oxidized and reduced structures

The structures of oxidized and reduced azurin II have been refined to 1.75 Å resolution with moderately good *R* factors of 18.9 and 19.2%, respectively. The free *R* factors of both oxidized and reduced structures are only a little larger, being 20.5 and 21.4%, respectively. The free *R* factors (Brünger, 1992*a*) were calculated by excluding 10% of the reflections until the final round of refinement. These free *R* factors and their closeness to the *R* factors confirm the quality of the overall structure. The r.m.s. deviations of bond lengths and angles were 0.008 Å and 1.5°, respectively, for both the oxidized and reduced structures (Table 1).

In addition to the assessment of the structure through an overall quality factor, it is important to assess the accuracy and reliability of different parts of the structure (Table 1). Ramachandran plots (Ramachandran *et al.*, 1963; Ramachandran & Sasisekharan, 1968) show 89.4 and 88.5% of the residues in the core regions for the oxidized and reduced structures, respectively. In both structures, only Gln2 is in the additionally allowed regions. The main-chain and side-chain properties, as detailed in Laskowski *et al.* (1993), of these structures are in good agreement with other well refined structures. *PROCHECK* notes none of the residues in either structure as having a distorted geometry. Additional checks of the model geometry (Table 1) were made using *OOPS* (Kleywegt & Jones, 1996). A further guide to the quality of the structures in different regions is provided by an inspection of atomic *B* factors. The mean *B* factors for all the protein atoms are 20.3 and 19.3 $Å^2$, and for the waters are 36.5 and 36.3 $Å^2$, for the oxidized and reduced structures, respectively.

The Cu atom has *B* factors of 15.5 and 9.5 $Å^2$ in the oxidized and reduced structures, respectively. In the oxidized structure, the *B* factors of the copper ligands are 17.3, 11.7, 11.5, 12.5 and 15.5 $Å^2$ for Gly45 O, His46 $N^{\delta 1}$, Cys112 S^{γ}, His117 $N^{\delta 1}$ and Met121 S^{δ}, respectively. In the reduced structure, the *B* factors were 13.9, 9.6, 14.2, 12.6 and 12.7 $Å^2$ for Gly45 O, His46 $N^{\delta 1}$, Cys112 S^{γ}, His117 $N^{\delta 1}$ and Met121 S^{δ}, respectively. The estimated uncertainties for the *B* factors were 2.7 and 2.5 $Å^2$ for the oxidized and reduced structures, respectively.

Table 2
A detailed comparison of the oxidized and reduced copper sites of azurin II and AzAD.

	Azurin II oxidized	Azurin II reduced	AzAD oxidized†	AzAD reduced‡
Distances (Å)				
Gly45 O—Cu	2.72	2.75	3.13	3.22
His46 $N^{\delta 1}$—Cu	2.04	2.03	2.08	2.13
Cys112 S^{γ}—Cu	2.14	2.16	2.15	2.26
His117 $N^{\delta 1}$—Cu	1.99	2.02	2.00	2.05
Met121 S^{δ}—Cu	3.26	3.26	3.11	3.23
Angles (°)				
Gly45 O—Cu—His46 $N^{\delta 1}$	78	76	74	68
Gly45 O—Cu—Cys112 S^{γ}	104	105	104	104
Gly45 O—Cu—His117 $N^{\delta 1}$	86	86	80	83
Gly45 O—Cu—Met121 S^{δ}	148	147	147	143
His46 $N^{\delta 1}$—Cu—Cys112 S^{γ}	133	133	135	132
His46 $N^{\delta 1}$—Cu—His117 $N^{\delta 1}$	106	106	105	104
His46 $N^{\delta 1}$—Cu—Met121 S^{δ}	74	75	77	78
Cys112 S^{γ}—Cu—His117 $N^{\delta 1}$	122	121	119	123
Cys112 S^{γ}—Cu—Met121 S^{δ}	105	105	107	109
His117 $N^{\delta 1}$—Cu—Met121 S^{δ}	88	89	96	92

† AzAD 1.8 Å oxidized structure (Baker, 1988). ‡ AzAD 1.9 Å reduced structure (Shepard *et al.*, 1990).

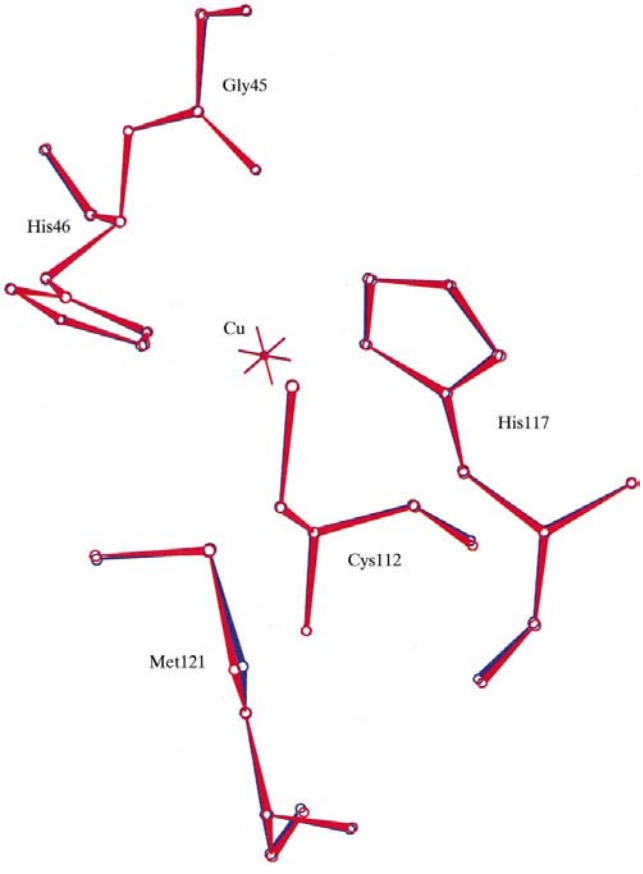

Figure 2
A superposition of the oxidized (blue) and reduced (red) Cu sites of azurin II showing the minimal differences in the geometry.

3.2. Structural comparison of oxidized and reduced azurin II

The structures of oxidized and reduced azurin II are very similar indeed. A superposition of all the protein atoms and the Cu atom gives an r.m.s. fit of 0.08 Å. Superposition of the water molecules in each model gives an r.m.s. fit of 0.17 Å. When superimposed on the 1.9 Å structure of azurin II, both oxidized and reduced models have an r.m.s. fit of 0.10 Å.

3.2.1. The copper sites. Comparison of the copper sites of the oxidized and reduced models of azurin II show there to be very little difference between the structures (Fig. 2, Table 2). The copper geometry consists of three strong planar ligands (namely His46 $N^{\delta1}$, His117 $N^{\delta1}$ and Cys112 S^{γ}, the NNS plane), which are arranged in a distorted trigonal plane about the Cu atom. Two other atoms interact with the copper, Gly45 O and Met121 S^{δ}. These are in axial positions with respect to the trigonal plane. The transition from the oxidized to the reduced form results in only small changes in the positions of the ligands which are well within the experimental errors. The His46 $N^{\delta1}$ copper bond is shortened by 0.01 Å in the reduced structure, with the His117 $N^{\delta1}$ and Cys112 S^{γ} bond lengths increasing by 0.03 and 0.02 Å, respectively. The bond length to Gly45 O increases by 0.03 Å, while the Met121 S^{δ} distance remains unchanged. In both refinements, the position of the Cu atom is essentially in the NNS plane, the deviation being 0.01 Å. The only notable difference between the oxidized and reduced copper sites is the change in B factor of the Cu atom from 15.5 to 9.5 $Å^2$, given respective estimated uncertainties in B factors of 2.7 and 2.5 $Å^2$ for the two structures. The reduction in the B factor may partially be a consequence of a slightly tighter position of the Cu atom in the reduced state copper (I) compared with the oxidized state copper (II). The higher B for Cu^{II} may indicate a slightly delocalized nature of Cu in the oxidized state.

These minimal changes are consistent with the requirements for fast electron transfer (Marcus & Sutin, 1985). Similar small changes have been observed between the oxidized and reduced forms of AzAD (Shepard *et al.*, 1990), which have been studied at 1.8 and 1.9 Å, respectively (Table 2). These structures support the view that the protein structure surrounding the Cu atom provides an environment which is optimized for the function of biological electron transfer. The rigidity of the azurin II structure, with the copper site being constrained by hydrogen bonding and van der Waals interactions, allows little movement upon change in redox state of the Cu atom. Thus, the Cu atom is held in a geometry which is an intermediate between the trigonal planar favoured by copper (I) and the square planar or tetrahedral favoured by copper (II). Such a state is said to be entatic, in which the Cu atom is bound in an energized conformation (Vallee & Williams, 1968; Williams, 1995). However, calculations of the energy state of other blue copper sites have revealed there to be little strain, casting doubt upon the idea of the copper binding being entatic (Ryde *et al.*, 1996). Qualitatively, this the lack of an energized binding state for copper is explained by the NNS plane binding the copper so that two of the Cu^{2+} $d(x^2 - y^2)$ electron-orbital lobes interact with the two N atoms as σ bonds and the remaining two Cu^{2+} $d(x^2 - y^2)$ lobes interact with the two lobes of the Cys112 S^{γ} π orbital, completing an undistorted square-planar bonding arrangement for Cu^{2+} (Solomon *et al.*, 1998; Malmström & Leckner, 1998). In the reduced Cu site, the interaction between the Cys112 S^{γ} and the Cu^{+} is weakened (Guckert *et al.*, 1995). The increased stability of oxidized *versus* reduced azurin against guanidine hydrochloride denaturation suggests that the oxidized form is not bound in an energized state (Leckner *et al.*, 1997; Wittung-Stafshede *et al.*, 1998), supporting the hypothesis of 'rack' induced bonding (Malmström, 1994).

3.3. Accuracy and precision

3.3.1. Global accuracy and precision. The average positional error, Δr, in the atomic coordinates of a structure can be estimated in several ways. A plot of R factor against resolution calculated by the method of Luzzati (1952) suggests an average coordinate error of 0.15 Å for both the oxidized and reduced structures. This method, however, has a number of shortcomings in terms of its approximations. Luzzati assumes that there are no errors in the experimentally determined structure factors, which is clearly not the case, and that all reflections are present. It is also assumed the only errors in the calculated structure factors come from coordinate errors in the model and that these errors are the same for all atoms. Since the scattering power of atoms decreases significantly with B factor, the errors determined by this method will at best give an estimate of the errors in the best determined regions. A σ_A plot (Read, 1986) of $\ln(\sigma_A)$ as a function of $\sin^2\theta/\lambda^2$ indicates an average error of 0.28 and 0.24 Å for the oxidized and reduced structures, respectively. However, this method also assumes that there is a normal distribution of errors. It also assumes that any atoms missing from the model are of the same type and have the same overall B factor as those of atoms present. This is clearly a poor assumption, as those missing atoms tend to be the less well ordered solvent.

The diffraction data-precision indicator (DPI; Cruickshank, 1996) gives a 'quick and rough guide' for the diffraction data only error for an atom with the same B factor as that determined by the Wilson plot. This has been extended to incorporate the free R factor as the expected value of the crystallographic residual (Murshudov & Dodson, 1997). This approach also takes into account the numbers of observations and atoms, the resolution and the completeness of the data used in the refinement. The DPIs for the refinement of oxidized and reduced azurin II at 1.75 Å are 0.09 and 0.08 Å, respectively.

3.3.2. Accuracy and precision at the copper site. It remains problematic to know what precision can be claimed for the atomic positions in a correctly determined and competently refined structure at modest resolution. The average coordinate error can be estimated by the DPI, but this does not provide an accurate assessment for the accuracy of a particular part of the structure, *e.g.* the metal site in a metalloprotein. In several cases, the differences in the crystallographically independent molecules in the asymmetric unit have been used to provide an assessment of accuracy of metal–ligand bond distances. For AzAD (Baker, 1988), an average 'maximum error' of 0.04 Å was estimated for copper–ligand distances, while for AzPA (Nar *et al.*, 1991) a precision ranging from 0.01 to 0.11 Å was calculated from the four molecules in the asymmetric unit. Guss *et al.* (1992) pointed out that as the structures of the molecules in the asymmetric unit are not independently determined, the error estimates based on internal self-

Table 3
A comparison of the copper sites of azurin II and AzAD with the three-dimensional EXAFS metrical data from Cheung *et al.* (2000) for azurin II.

For comparison, crystallographic data for plastocyanin (Pc) is included to show the effect of resolution on the Cu ligands.

Cu ligand (azurin)	Azurin II oxidized crystal	Azurin II reduced crystal	Azurin II oxidized EXAFS†	Azurin II reduced EXAFS†	AzAD oxidized crystal‡	AzAD reduced crystal§
His46 $N^{\delta1}$	2.04	2.03	1.86	1.91	2.08	2.13
His117 $N^{\delta1}$	1.99	2.02	1.94	2.01	2.00	2.05
Cys112 S^{γ}	2.14	2.16	2.12	2.19	2.15	2.26
Met121 S^{δ}	3.26	3.26	3.39	3.35	3.11	3.23
Gly45 O	2.72	2.75	2.82	2.98	3.13	3.22
Resolution (Å)	1.75	1.75			1.80	1.90

Cu ligand (plastocyanin)	Pc crystal, 1.6 Å¶	Pc crystal, 1.33 Å††
His37 $N^{\delta1}$	2.04	1.91
His87 $N^{\delta1}$	2.10	2.06
Cys84 S^{γ}	2.13	2.07
Met92 S^{δ}	2.90	2.82

† Cheung *et al.* (2000). ‡ Baker (1988). § Shepard *et al.* (1990). ¶ Guss & Freeman (1983). †† Guss *et al.* (1992).

consistency without allowance for systematic effects are likely to be too low. They suggest that metal–ligand distances in a well refined structure of a blue copper protein have uncertainties of 0.04, 0.05 and 0.07 Å, respectively, at 1.3, 1.6 and at 1.8 Å resolution. From these criteria, the larger deviation for His46 observed here with respect to EXAFS is outside the range. We note that in the 1.33 Å refined structure of plastocyanin (Guss *et al.*, 1992), a good agreement has been found with the metrical information obtained from EXAFS data of solution (Murphy *et al.*, 1990) and single-crystal (Scott *et al.*, 1982) plastocyanin. For plastocyanin, all of the ligand distances had shortened somewhat in going from 1.6 to 1.33 Å, but Cu–His37, which is equivalent to Cu–His46 in azurin, had shortened by 0.13 Å (Table 3). Thus, it is likely that different ligands are affected to a different extent by the improvement in resolution as the effects of restraints are reduced. We note that His46 (His37 of plastocyanin) is from a separate chain compared with the other three residues (Cys112, His117 and Met121), which are in close proximity at the C-terminus.

It is clear that the overall geometry of the copper site in azurin II is maintained in the two redox states. A cursory glance at the metrical data would suggest that there are no detectable differences between the two structures. However, we know from the EXAFS studies of azurin II (Cheung *et al.*, 2000) and of other azurins that a significant change at the copper site takes place. A detailed comparison of the crystal structures with the EXAFS of oxidized and reduced azurin II (Cheung *et al.*, 2000) reveals a number of interesting features. The value of the Cu—S(Cys) distance is very similar in both techniques for both redox states of the protein, *i.e.* 2.14 Å (PX) *versus* 2.12 Å (EXAFS) in the oxidized state and 2.16 Å (PX) *versus* 2.19 Å (EXAFS) for the reduced state. Even though the agreement for the individual distances is excellent, *i.e.* within 0.03 Å, metrical information from the two crystallographic structures at 1.75 Å would be best interpreted as evidence for no structural change, while EXAFS data would be interpreted as showing an increase of 0.07 Å for the Cu—S(Cys) bond confirming a weakening of this interaction upon reduction. We note that for AzAD (Shepard *et al.*, 1990), a difference of 0.11 Å was found between the oxidized (1.8 Å) and reduced (1.9 Å) structures. A similar change is apparent for the two histidines from the EXAFS data; a change of 0.07 Å for His117 and a change of 0.05 Å for His46. In the EXAFS analyses of oxidized and reduced azurin II, one of the histidines (His117) is found to be close to the crystallographic distance (within 0.05 Å), while the other (His46) is significantly shorter; 0.18 Å in the oxidized structure and 0.12 Å in the reduced structure (Table 3; Cheung *et al.*, 2000). The larger difference observed for His46 is somewhat outside the expected uncertainty for a crystallographic refinement.

A further consideration in the positional accuracy with which atomic positions can be determined is the *B* factors of those atoms. The *B* factor of an atom is related to the atomic displacement amplitude, u, by $B = 8\pi^2\langle u^2\rangle$. Thus, for an atom with a *B* factor of 10 $Å^2$, close to the minimum for either oxidized or reduced structures, the r.m.s. displacement will be 0.36 Å. At the average *B* factor for the structures, ~20 $Å^2$, the r.m.s. displacement increases to 0.50 Å. At the upper limit, around 45 $Å^2$, the r.m.s. displacement is 0.75 Å. However, this does not give us useful information concerning the accuracy to which bond lengths can be determined, as the *B* factors relate to an atom's mobility and not its positional accuracy. In general, the bond lengths are also restrained by the geometric information used in the refinement.

4. Conclusions

The slight expansion in the copper site and lowering in *B* factor of the Cu atom upon the reduction of the Cu atom from copper (II) to copper (I) results in minimal geometric changes at the copper site. These small geometric changes are consistent with the requirement for rapid electron transfer, *i.e.* there is a minimal energy barrier between the oxidation states of copper. The accurate determination of the copper–ligand distances in order to quantify the changes upon the oxidation–reduction step will require even higher resolution structure determinations than the current 1.75 Å studies. At the current resolution, the positional errors in the model are too great for ligand distances to be determined with the accuracy required. A comparison with the three-dimensional EXAFS

suggest that the ligand distances change by ∼0.07 Å upon reduction.

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