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Crystal structure of *Pseudomonas aeruginosa* apo-azurin at 1.85 Å resolution

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The 3D structure of apo-azurin from *Pseudomonas aeruginosa* has been determined at 1.85 Å resolution. The crystal structure is composed of two different molecular forms of apo-azurin arranged as hetero-dimers in the tetramer of the asymmetric unit. Form 1 closely resembles the holo-protein lacking copper. Form 2 shows differences in the metal binding site region induced by the incorporation of a solvent molecule into this site. The positions of the copper ligands His^{46} and His^{117} are shifted by 0.6 Å and 1.6 Å. The His^{117} side chain adopts a position at the surface of the protein, thereby facilitating access to the copper site. The presence of two different molecular forms of apo-azurin in the crystal lattice may reflect an equilibrium between the two forms in solution. ^{1}H-NMR spectra of apo-azurin recorded as a function of pH show that at high pH the line broadening of His^{35}, His^{46} and His^{117} resonances is consistent with an interconversion between forms 1 and 2. At low pH, no broadening is observed. This may indicate that here the interconversion is fast on the NMR timescale.

Azurin; Apo-protein; Copper; Metal incorporation; Conformational change

1. INTRODUCTION

The bacterial electron transport azurin (M_r 14,000) is a very specific macromolecular ligand for copper ions. The incorporation of Cu(II) confers on it its characteristic blue colour and high redox potential [1]. Although various metals can bind to apo-azurin [2,3], copper is taken up much more rapidly than Mn(II), Co(II), Ni(II) [2] and Zn(II) [4].

Azurin is synthesized as a precursor of higher molecular weight having an additional N-terminal signal peptide of 19 amino acids [5,6] which probably serves to translocate the protein across the periplasmic membrane. Upon transport into the periplasm the preprotein is presumably processed to yield apo-azurin which subsequently takes up copper.

Copper uptake by apo-azurin has been the subject of kinetic analyses [7,8]. It has been proposed that the mechanism involves a fast complexation step which is followed by a slow rearrangement of the intermediate to form the holo-protein.

Here we report the 3D structure of apo-azurin and discuss its implications for the metal-binding mechanism.

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2. EXPERIMENTAL

2.1. *Preparation of apo-azurin*

Recombinant *Pseudomonas aeruginosa* azurin was isolated and purified as described previously [9].

Apo-azurin was prepared in solution by reduction of oxidized holo-azurin with 0.1 M ascorbate followed by dialysis against 0.1 M thiourea in 0.25 M NaCl, 0.1 M acetate (pH 5) under a nitrogen atmosphere according to [7]. The absence of residual copper was confirmed by UV/Vis spectroscopy, the absorbance of the Sγ-Cu(II) charge transfer band at 628 nm was <0.01 (<1.5% Cu(II)).

Crystals were obtained by vapour diffusion from a solution of 10 mg/ml protein in 2.0 M ammonium sulphate, 0.3 M lithium nitrate equilibrated with a reservoir containing 3.2 M ammonium sulphate, 0.5 M lithium nitrate buffered with 0.1 M acetate at pH 5.5.

2.2. *Unit cell parameters*

Apo-azurin crystals are isomorphous to holo-azurin obtained under the same crystallization conditions. However, the cell dimensions (a=57.09 Å, b=81.10 Å, c=110.97 Å, space group $P2_12_12_1$) differ slightly from those of Cu(II)-azurin crystals (a=57.65 Å, b=80.93 Å, c=110.17 Å), the maximal deviations being about 1% for the a and c axes [10]. The asymmetric unit is composed of four molecules of azurin (V_M=2.3 Å^3/Da).

2.3. *Data collection*

Diffraction intensities to 1.85 Å were collected from one crystal specimen on a Micro Vax III controlled FAST television area detector (Enraf-Nonius, Delft) at 4°C. Ni-filtered CuKα radiation from a rotating anode generator (Rigaku) operated at 5.4 kW, apparent focal spot size (0.3×0.3 mm) was used. Data were recorded in frames of 0.1° and evaluated using MADNES [11]. The structure factors were scaled and corrected for absorption effects using ABSCOR [12,13] and merged and loaded with PROTEIN [14].

2.4. *Structure analysis and refinement*

The crystal structure of recombinant oxidized *Pseudomonas aeruginosa* azurin has recently been solved and refined at 1.9 Å resolution

[10] providing the possibility of detecting structural changes of the order of 0.1–0.2 Å caused by metal replacement [4], site-specific mutations [15] or chemical modifications by crystallographic techniques.

A difference Fourier map of the type Fo(apo)-Fo(holo) with phases calculated from the holo-azurin model was initially used to confirm that major changes between the apo-protein and the holo-protein were present only at the metal binding site. Fig. 1 depicts this electron density map at the copper site of monomer D. Negative peaks with height – 18σ indicated the absence of copper, positive difference density at the histidine and cysteine side-chains suggested some rearrangements for the copper ligands.

The refinement of the apo-azurin structure was done using XPLOR [16]. The holo-protein coordinates (pH 5.5) were used as the input for a rigid-body refinement step with data from 6.0 to 3.0 Å. The four individual monomers of the asymmetric unit were treated as independent rigid bodies with 3 translational and 3 rotational degrees of freedom each. This was done to allow for some changes in the relative positions and orientations of the monomers manifested in the slightly altered cell constants. The crystallographic R-factor was thereby reduced from 35.3% to 24.6%. Subsequent positional and temperature factor refinement, inclusion of 166 solvent molecules and manual adjustments reduced R to 19.3% (data from 8.0 to 1.85 Å).

2.5. *^{1}H-NMR spectroscopy*

3 mM NMR samples of apo- and reduced holo-azurin in 20 mM potassium phosphate buffer in D_2O were prepared, and spectra were recorded at 300 MHz on a Bruker WM300 spectrometer at 298K as a function of *decreasing* pH, as described previously [9].

3. RESULTS

3.1. *Structure of apo-azurin*

The analysis of the crystal structure of apo-azurin reveals that there are two forms of the metal free protein present in the crystal differing mainly in the conformation of the side chains of copper ligands His^{46} and His^{117}. One form is superimposable on the copper containing protein with only minimal shifts of positions of ligand atoms towards the cavity formed upon copper removal. The other form contains a water molecule in place of the copper atom in the azurin metal binding site whose presence forces the histidine side chains to adopt significantly different positions compared to the holo-protein. Under crystallisation conditions, one monomer of form 1 and one of form 2 combine to form a 'heterodimer', the individual azurin molecules interacting via their hydrophobic patches as observed for holo-azurin [15]. The subunits A and C (B and D) of the heterodimers are related by a rotation of 176° and a translation of 1 Å causing inequivalence. The two 'heterodimers' A-C and D-B are related by a nearly exact local twofold axis and constitute the asymmetric unit of apo-azurin crystals. The tetramer thus has C_2 symmetry.

3.1.1. Apo-azurin form 1

A superposition of holo-azurin with form 1 of apo-azurin (monomers B and C) yields an overall rms. deviation of 0.2 Å for Cα atoms, no significant deviations are found for the entire molecule. The distance between the Nδ atoms of copper ligands His^{46} and His^{117} is reduced slightly to 3.15 Å in the apo-protein (3.25 Å in the Cu(II)-azurin) probably induced by protonation of His^{117} Nδ (pK_a (apo) 7.6; [9]) and the formation of a hydrogen bond between the two imidazoles (Fig. 2). The 2Fo-Fc electron density map shows no peaks in the region of the copper atom position. Some weak positive residual electron density is found, however, in the Fo-Fc map, which indicates that copper is not removed completely by the described procedure. As estimated from the peak height the copper content is less than 5%, as anticipated from the UV/Vis spectrocopic data.

3.1.2. Apo-azurin form 2

Inspection of the 2Fo-Fc electron density map of the metal binding site region of the monomers A and D in the apo-azurin structure revealed a large peak between the two imidazole side chains of His^{46} and His^{117}, but centred about 2 Å from the copper atom position in a superimposed holo-protein monomer (Fig. 3). Moreover, the superposition shows that both histidine side

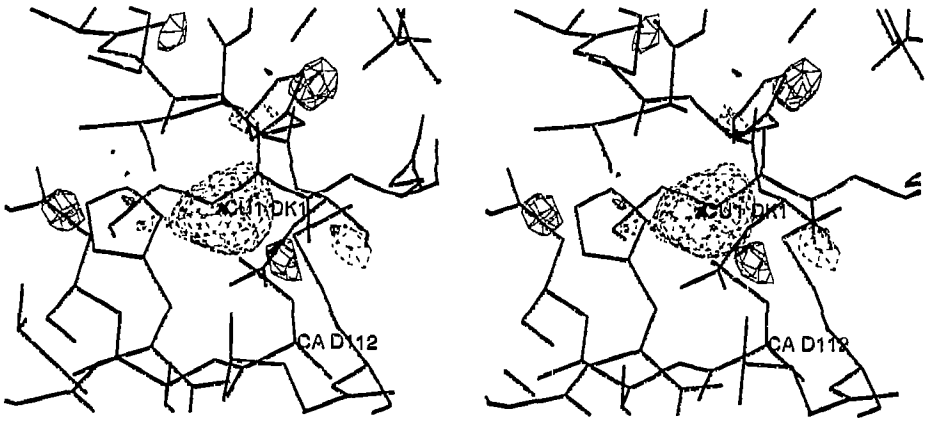

Fig. 1. Stereo view of the Fo(apo)-Fo(holo) difference electron density phased with and superimposed on the structure of oxidized azurin [10] centred at the metal binding site of monomer D (contour level 5σ). Positive electron density (solid lines) at the copper ligands His^{46} and His^{117} opposite to the Cu-Nδ bonds indicate a movement of their side chains away from the copper atom position. Negative electron density (broken lines) is centred at the metal site.

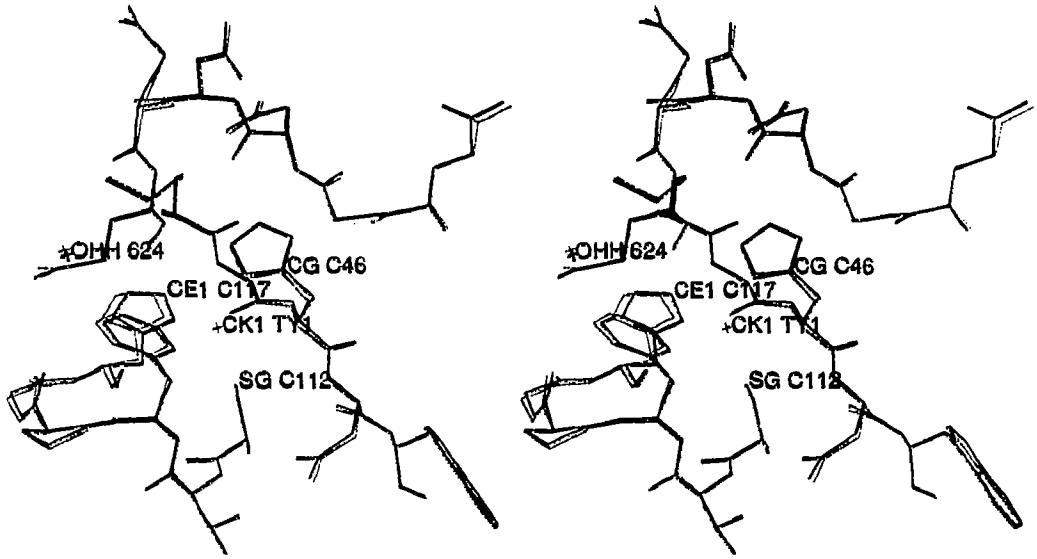

Fig. 2. Superposition of apo-azurin form 1 (monomer C, thick lines) on Cu(II) azurin (thin lines). Apart from the absence of the metal and a minor shift of the His^{117} side chain toward the cavity, the atomic models are indistinguishable.

chains have shifted significantly (0.6 Å for His^{46} and 1.6 Å for His^{117}) away from the peak in the density map increasing the distance of their Nδ atoms to the peak centre to about 2.7 Å each. We interpret this finding by the presence of a water molecule in place of the copper atom in the protein interior. This necessitates the movement of the imidazole groups in order to form hydrogen bonds with the incorporated water molecule (Fig. 4). The water molecule is in hydrogen bonding distance not only to the His Nδ atoms but also to Gly^{45} O (2.7 Å) and Cys^{112} Sγ (3.6 Å) which are ligands to the copper in the holo-protein.

In holo-azurin, the His^{117} side chain is located at the bottom of a surface depression the rim of which is built up by the hydrophobic residues Met^{13}, Met^{41}, Phe^{114}, Pro^{115} and Gly^{116} [15,17]. The His^{117} Nε atom is hydrogen bonded to another water molecule which fills up part of the surface depression.

In the apo-azurin form 2, the His^{117} side chain is moved by 1.6 Å across the depression and is placed right next to the hydrophobic residues. The surface water molecule bound to the His^{117} Nε follows this movement. The large shift of His^{117} is accompanied by shifts of the adjacent residues Phe^{114}, Pro^{115} and Gly^{116} (average atomic rms. deviation 0.6 Å) as well as a conformational change of the side chain of the nearby residue Met^{13}. The His^{46} side chain is bent by 0.6 Å (movement of Cα 0.3 Å) toward the protein interior triggering some rearrangement of its surrounding peptide structure (residues 8 to 10). The remainder of the azurin molecule is unperturbed.

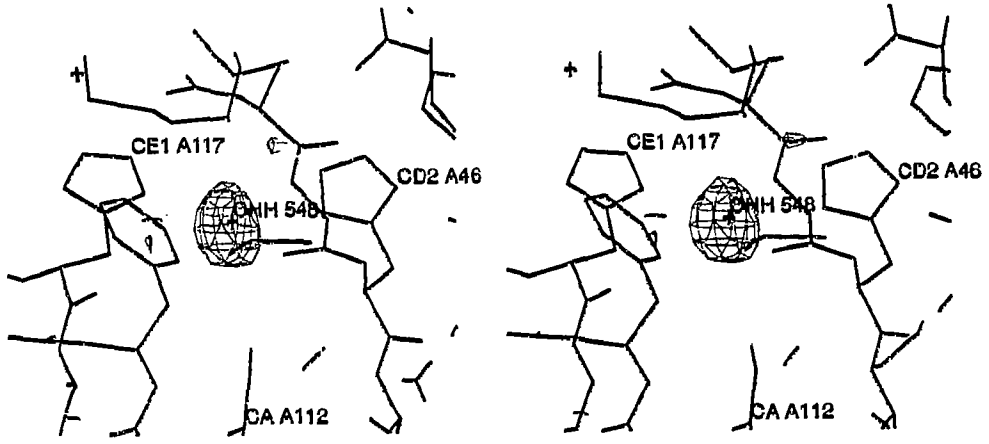

Fig. 3. Fo-Fc electron density map of apo-azurin form 2 (monomer A) computed prior to the addition of the solvent molecule OHH 548. A large positive peak (6σ) shows up between copper ligands His^{46} Nδ, His^{117} Nδ, Gly^{45} O (distance to the peak centre 2.7 Å) and Cys^{112} Sγ (3.6 Å).

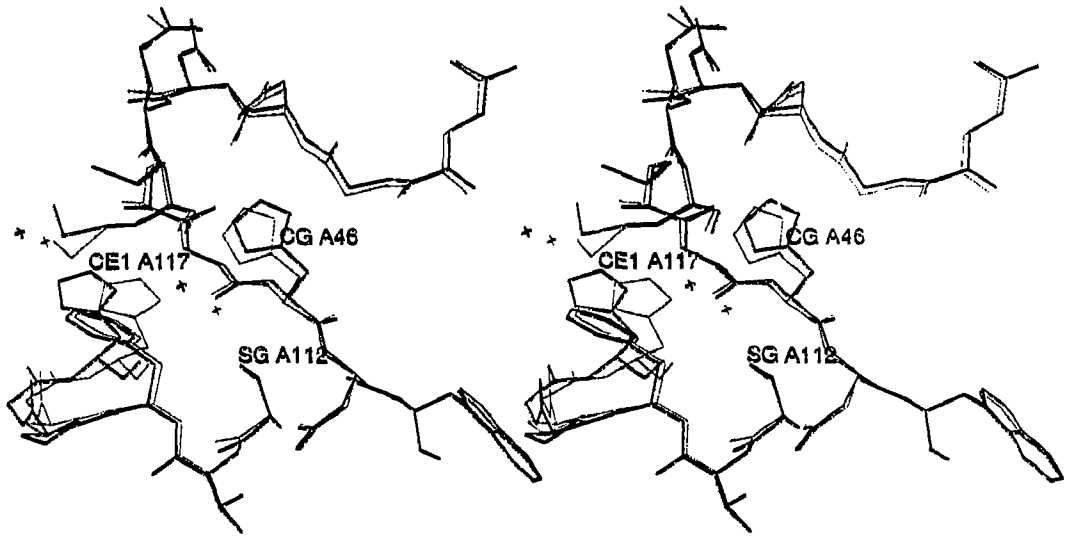

Fig. 4. Superposition of apo-azurin form 2 (monomer D, thick lines) on Cu(II) azurin (thin lines). Differences between the two structures are centred at His[46] and His[117]. The side chain of His[117] is shifted by 1.6 Å toward the protein surface without changing the orientation of the imidazole ring. The surface water molecule bound to His[117] Nε shifts accordingly. Movement of Met[13] and Phe[114] side chains provides the space necessary for the His[117] swinging motion. The His[46] side chain is moved in the opposite direction (0.6 Å). This shift results in a slight positional adjustment (0.4 Å) of the adjacent main chain of residues 8 to 10.

3.2. *^{1}H-NMR spectra of apo-azurin*

To test for the existence of the two forms of apo-azurin in solution ^{1}H-NMR spectroscopy was employed. Since the rate of the interconversion of the two molecular forms might be dependent on [H$^+$] this study was done using different pH values of the buffer medium.

In Fig. 5 the downfield region of the ^{1}H-NMR spectrum of apo-azurin is shown as a function of pH. Indicated are the resonances that have been assigned to the ligand His[46] Cδ2, His[117] Cδ2 and Cε1 protons and to the His[35] Cε1, His[83] Cδ2 and Cε1 protons [9]. Whereas the His[83] Cδ2 and Cε1 proton resonances are equally sharp at low (4.5) and high (9.5) pH, broadening is observed for the His[35] and His[117] Cε1 resonances at high pH relative to low pH. This broadening is not due to exchange between a protonated and an unprotonated species, because the pK_a's of His[35] and His[117] in apo-azurin are 6.5 and 7.6 and thus well below 9.5 [9].

Additionally, the ligand Met[121] CεH$_3$ resonance (present in the upfield region of the spectrum which is not shown here) broadens at high pH. Finally, while the His[46] Cδ2 proton resonance is detected at low pH, it disappears with increasing pH. This can be due to broadening and/or overlap.

In the spectrum of reduced holo-azurin (not shown) no such broadening of the His[35] Cε1H and the Met[121] CεH$_3$ resonances is observed, and the His[46] Cδ2H resonance is normally observed at low and high pH [9]. Observation of the Cδ2H and Cε1H resonances of His[117] (which does not titrate in the holo-protein) is precluded by overlap.

4. DISCUSSION

The presence of two different apo-azurin species in the crystal lattice suggests that there is an equilibrium between the different forms in solution. The crystallisation process can then be described as a co-crystallisation of those two forms. The preference for the constitution of 'hetero-dimers' packed in a unique, highly ordered fashion in the crystal lattice, rather than for an arbitrary packing resulting in cyrstalline disorder, suggests that dimer formation takes place prior to nucleation under the given crystallization conditions. Modelling of a 'homo-dimer' of molecular form 2 of apo-azurin on a graphics display shows that the favourable dimer contact, mediated by two water molecules bound to the His[117] Nε atoms of each monomer, is strongly perturbed by the changed position of His[117] and the water molecules in both monomers. As judged from the number and intimacy of contacts between the monomers, in the 'hetero-dimer' this contact is even improved over the one found in holo-protein cyrstals.

The ordered arrangement of the tetramer in the crystal is a consequence of its C_2 symmetry [15].

Interconversion of one conformer into the other is achieved by incorporation/removal of one water molecule into the copper binding site. The mechanism of this process may be akin to the mechanism of copper incorporation into apo-azurin.

In the related protein plastocyanin, the structure of the apo-protein suggested the involvement of a 180° rotation of the solvent exposed imidazole ring of His[87] about the Cβ – Cγ bond (a 'revolving door') to facilitate

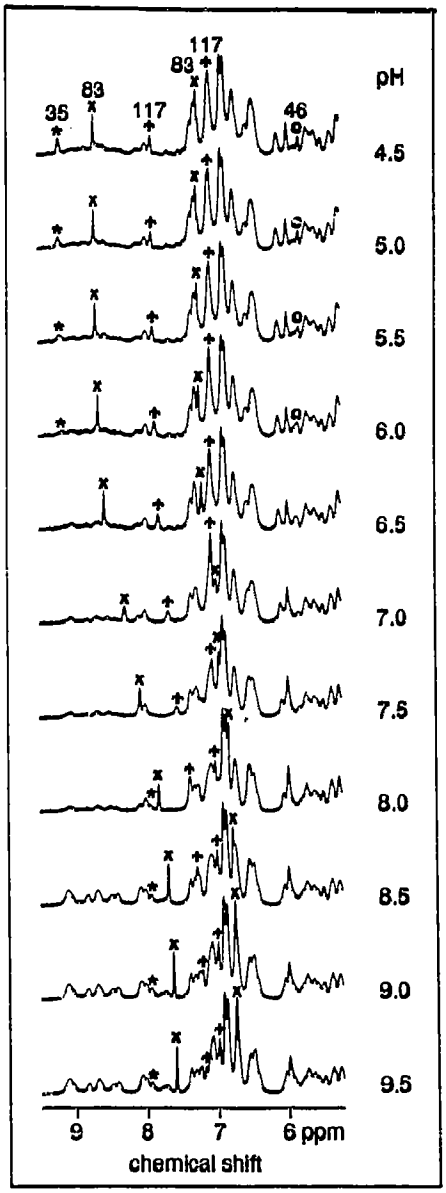

Fig. 5. Downfield region of the 300 MHz proton NMR spectrum of *Pseudomonas aeruginosa* apo-azurin as a function of pH. Indicated are the following resonances [9]: His^{35} Cε1H (*), His^{46} Cδ2H (o), His^{83} Cε1H and Cδ2H (x) and His^{117} Cε1H and Cδ2H (+).

Table I

Data collection statistics, final model and refinement results

Space Group		$P2_12_12_1$
Unit Cell Constants	a	57.09 Å
	b	81.10 Å
	c	110.97 Å
Crystal Mosaicity		0.19°
Measurements ($I>2.5\sigma(I)$)		141499
unique reflections		39493
nonrejected unique reflection		36725
data completeness:	∞–1.85 Å	81.1%
	1.90–1.85 Å	20.3%
reflecting averaging:	R_{merge}*	8.9%
	R_F**	5.1%
agreement between data sets: Apo-Azu vs. Holo-azurin***		30.7%
Number of atoms	all non-hydrogen atoms	4062
	non-hydrogen protein atoms	3896
	solvent	166
rms deviation from standard geometries:	bonds	0.012 Å
	angles	2.94°
Resolution range		8.0–1.85 Å
Number of reflections		34350
Number of parameters		16248
$R = \Sigma \mid F_o - F_c \mid / \Sigma \mid F_o \mid$	8.0–1.85 Å	19.3%
	1.9–1.85 Å	35.6%

*$R_{merge} = \Sigma \mid I(k) - \langle I \rangle \mid / \Sigma I(k)$, where $I(k)$ and $\langle I \rangle$ are the intensity values of individual measurements and of the corresponding mean values, the summation is over all measurements
**$R_F = R_{merge}$ after independent averaging of Friedel pairs
***agreement factor: $R = \langle \mid F_{Apo\text{-}Azu} - F_{Holo\text{-}azu} \mid \rangle / \langle \mid F_{Holo\text{-}azu} \mid \rangle$

access to the copper binding site. Otherwise the structure closely resembles that of the holo-protein [18]. In the structure of apo-azurin from *Alcaligenes denitrificans*, the removal of the metal causes no change in the ligand positions [19]. By analogy to plastocyanin, for *Pseudomonas aeruginosa* apo-azurin we suggest a 'swinging door' mechanism of metal incorporation.

In azurin, His^{117} is buried much deeper in the protein interior compared to the analogous His^{87} in plastocyanin. Thus, it can not inverse the orientation of its imidazole ring because of steric hindrance by the neighbouring side chains Phe^{114} and Met^{13}. In fact, in apo-azurin forms 1 and 2, the orientation of the imidazole is conversed as judged by the presence of the water molecule bound to the His^{117} Nε. Instead, as can be seen in Fig. 4, access to the azurin copper binding site is provided by the motion of the His^{117} side chain hinged at the main chain segment 116 to 118. At the point of maximal displacement of His^{117} from the position it occupies in holo-azurin, a copper ion may bind to the imidazole ring and be carried into the protein interior by a closing motion.

Kinetic studies of the reconstitution of holo-azurin from Cu(II) and the apo-protein have been performed in weakly [8] and strongly [7] complexing media. Both analyses yielded reaction schemes that involved a fast pH-dependent complexation step leading to at least one intermediate which then slowly rearranges to form native azurin, again in a pH-dependent manner.

On the basis of the 3D structure of the apo-protein, the pH dependence of the complexation step is consistent with deprotonation of the His^{117} Nδ. The slow rearrangement is identified with the motion of the His^{117} which might be dependent upon Cys^{112} or His^{46} depro-

tonation or both. From the structural data available no definite conclusions about the nature of the putative intermediates can be drawn.

In form 1 of apo-azurin, only one of the histidines should be protonated (pH of crystallization 5.5), since a favourable geometry for the formation of a hydrogen bond between the Nδ atoms is found (distance Nδ...Nδ 3.15 Å, angle Nδ–H...Nδ 130°). In form 2, protonation of two or three residues (His^{46}, His^{117} and Cys^{112}) is compatible with a possible hydrogen bonding pattern involving the intercalated water molecule.

The proposed interconversion between form 1 and 2 of apo-azurin has several aspects in common with the situation encountered in reduced amicyanin and plastocyanin. In these proteins, the protonation of the histidine ligand which is the equivalent of His^{117} in azurin is coupled to a conformational change of this residue. Protonation and subsequent rotation of the His^{87} in plastocyanin has been followed by X-ray crystallography [20]. The conformational change of the His^{95} in amicyanin was reflected in the broadening of its Cε1H resonance in the proton NMR spectrum at low pH [21].

Inspection of the apo-azurin one-dimensional proton NMR spectra at a pH below that of the crystallisation (pH 5.5) did not reveal the presence of two forms in solution. No broadening of the His^{117} Cε1H is observed. Moreover, this resonance has normal intensity corresponding to approximately one proton. This means that at low pH under NMR conditions the equilibrium between forms 1 and 2 is shifted quantitatively to one side or the interconversion between them is fast on the NMR timescale. This latter interpretation is supported by the fact that at high pH (i.e. above the His^{117} pK_a) the broadening of several resonances indicates an exchange process involving His^{35}, His^{117} and Met^{121} and probably also His^{46} which we tentatively interpret as the interconversion between form 1 and 2 of apo-azurin.

A surprising result of this study is the apparent flexibility of the binding site region in apo-azurin. At first sight, this seems to be in contradiction to the widely held view that the irregular coordination geometry of the metal in the blue copper proteins is imposed on the copper by the polypeptide structure [22,23]. It is conceivable, however, that the different apo-azurin forms seen in this study represent the two rigid conformational states characteristic for the protein structures before (form 2) and after (form 1) metal incorporation.

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