A POSSIBLE APPROACH TO THE INVESTIGATION OF THE STRUCTURES OF COPPER PROTEINS: ^{1}H N.M.R. SPECTRA OF AZURIN

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SUMMARY. The ^{1}H n.m.r. spectra of apo-, Cu(I) and Cu(II) azurins from Pseudomonas aeruginosa have been measured. Three of the four histidines have been assigned. The effect of the copper(II) ion acting as an intrinsic paramagnetic perturbant leads to the proposal that one of the histidines is far from the metal and another is closer, but not bound, to the copper. The possibility that the remaining two histidines are ligands to the copper is considered. The relationship to the sequence is discussed.

Azurin is a type 1, or 'blue', copper protein (1) with one copper atom per molecule and a molecular weight of approximately 14,000. Though a number of spectroscopic methods have been used in the study of these 'blue' proteins, little is known with certainty about the immediate environment of the copper. We have previously investigated (2) the ^{1}H n.m.r. spectroscopy of a type II copper protein, superoxide dismutase, and concluded that the ligands to the copper were histidines, as has been corroborated by the recently-determined (3) crystal structure. The basis of our approach relies on the fact that whilst copper(I) is dia-

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magnetic, the copper(II) ion is paramagnetic and since it has a relatively long electron spin lattice relaxation time, acts as an intrinsic paramagnetic perturbant, broadening those resonances associated with nearby residues.

Methods. Native azurin was isolated from Pseudomonas aeruginosa NCTC 10332 by the method of Ambler and Brown (4). The protein was chromatographed on a Whatman CM32 column using 0.02 M acetate buffers. Samples were loaded and washed at pH = 4.6 and eluted at pH = 5.3, resulting in the removal of traces of impurities absorbing at around 410 nm. Cu(I) azurin was prepared by reduction with sodium dithionite and the apo-protein by subsequent dialysis against a solution 0.1 M in phosphate, 0.1 M in cyanide and pH = 8 in a Minibeaker fiber dialysis unit obtained from Bio-Rad. Prior to n.m.r. experiments solutions were de-salted, equilibrated in D_2O at room temperature and freeze dried. All n.m.r. samples were between 3-6 mM in protein, 0.1 M in sodium chloride, and 0.02 M in phosphate.

The n.m.r. spectra were recorded in the Fourier Transform mode at 270 MHz using a Bruker spectrometer and an Oxford Instrument Co. 64 K gauss super-conducting magnet. Convolution difference spectra were calculated by the method of Campbell et al. (5). All data were collected at 22°C and chemical shifts are reported relative to TSS (3-trimethylsilylpropane-sulphonate) as internal standard. The symbols pH* and pK* refer to values uncorrected for the deuterium isotope effect.

Results and Discussion: The ^1H n.m.r. spectra of apo-, Cu(I) and Cu(II), azurins are shown in Figure 1A, B and C respectively. The aliphatic residues give rise to resonances upfield

of the HOD (6) whilst the resonances associated with the aromatic residues are apparent between approximately 5.5 and 7.5 p.p.m., and unexchanged NH resonances occur between 6 and 11 p.p.m. The resonances in the vicinity of, and to high field of TSS are due to protons shifted upfield by the ring currents of neighbouring aromatic residues. An immediate comparison of Figures 1B and 1C indicates that the effects of the introduction of the paramagnetic centre are seen in both the aliphatic and aromatic regions of the spectrum.

In the following, assignments of histidine resonances are discussed and comparisons between observations in apo-, Cu(I) and Cu(II) proteins are made. The pH titration of apo-azurin reveals a resonance, (H2 in Figures 1A and 2A), which shifts continuously upfield with increasing pH in a manner typical of a C-2 proton of a freely-titrating histidine residue (7). The rate of proton exchange at imidazole nitrogen is rapid (8) and the observed chemical shift at a particular pH is a weighted average between the C2H chemical shifts of protonated and deprotonated forms of the imidazole ring. The pK* is estimated to be 7.55. The resonance assigned to the C4 proton of this histidine residue, denoted H2' in Figure 1A and 2A, shows a chemical shift difference of about 0.55 p.p.m. between protonated and deprotonated extremes and titrates with a pK* identical to H2. Throughout the pH range, H2' occurs within the region of major aromatic intensity and is most readily observed by use of difference spectroscopy (8,9). Both H2 and H2' are also apparent in the spectra of Cu(I) (Figs. 1B and 2B) and Cu(II) (Figs. 1C and 2C) proteins implying that this residue is a considerable distance from the copper site.

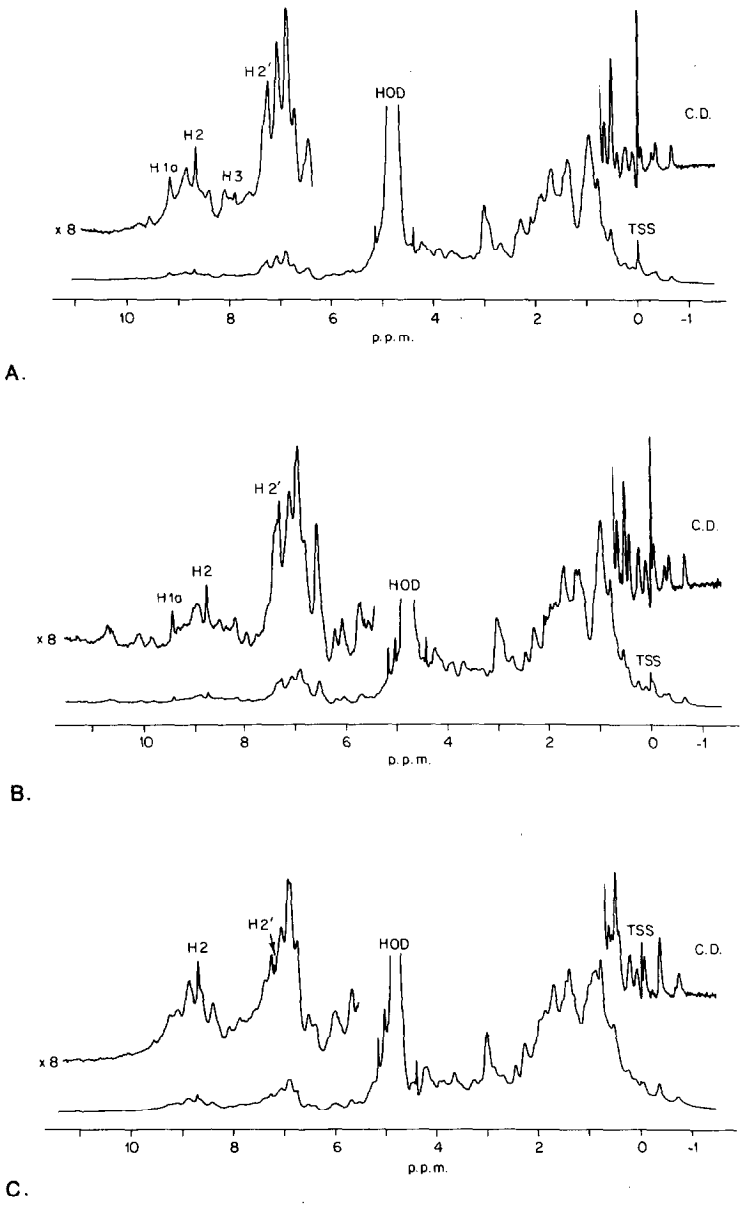

Figure 1.

The 270 MHz ^{1}H n.m.r. spectra of (A) apo-azurin pH* = 4.7 (B) Cu(I) azurin pH* = 4.2 (C) Cu(II) azurin pH* = 4.1. The high field protion of all spectra are also shown as convolution difference (C.D.) spectra (5).

At low pH the apo protein shows a distinctively sharp resonance at 9.2 p.p.m. denoted H1A, which exhibits a marked pH dependence. Increasing pH results in a decrease

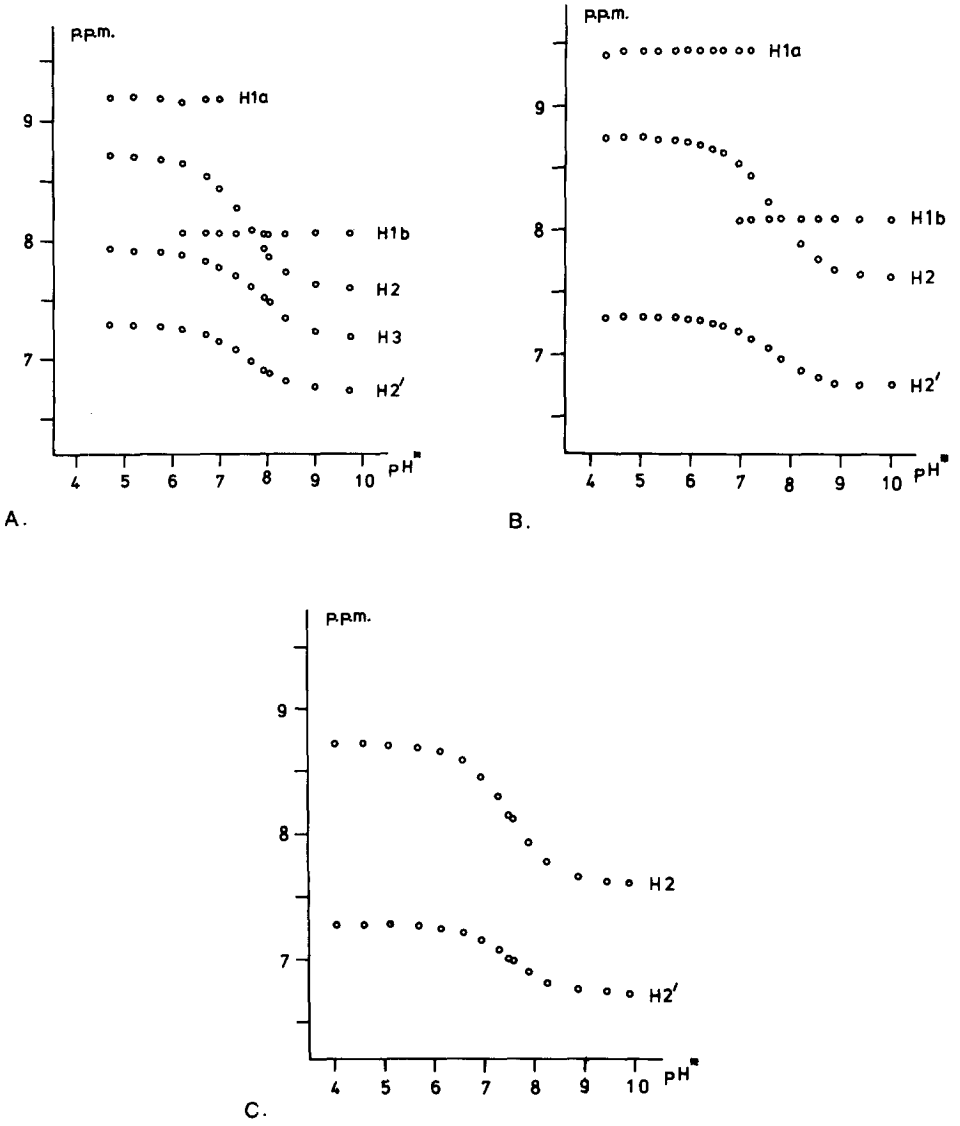

Figure 2.

The pH* titration curves of histidine resonances of (A) apo- (B) Cu(I) and (C) (Cu(II) azurins.

in the intensity of H1A concomitant with an increase in intensity of a line H1B 1.12 p.p.m. to higher field. These lines are attributed to the C2 proton of a histidine residue in protonated and deprotonated forms respectively with the pH dependence resulting from a rate of proton exchange

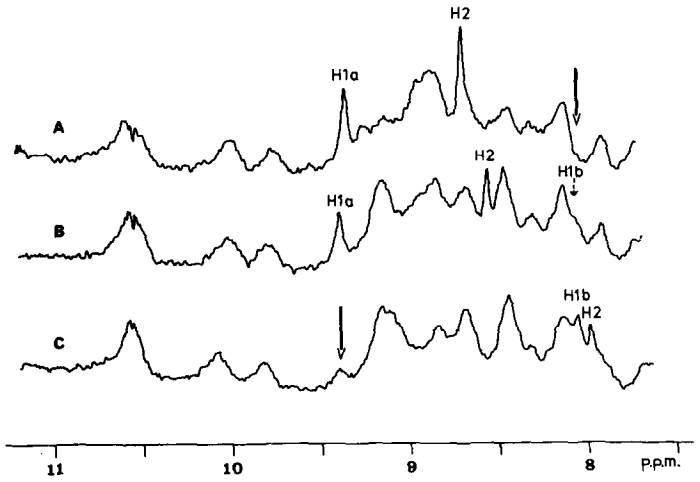

Figure 3.

The pH* dependence of H1 in the spectra of Cu(I) azurin. (A) pH* = 4.2 (B) pH* = 6.8 (C) pH* = 7.9.

slow with respect to the chemical shift difference between H1A and H1B. A similar effect is observed in the Cu(I) protein and is shown in Figure 3. The pK* of the histidine giving rise to H1 is estimated to lie within the range 6.4 ± 0.6. The pH behaviour of H1 is qualitatively similar in the apo-, and Cu(I) proteins though the pK* of H1 in the latter is the higher. While this suggests that this histidine is not a ligand to the copper, it is sufficiently close for its resonance to be broadened beyond detection in the spectrum of the Cu(II) azurin.

A third histidine residue is observed in the spectrum of the apo azurin, (H3 in Figures 1A and 2A) and titrates with a pK* of 7.8. The chemical shift difference between the protonated and deprotonated forms is smaller than usual. This resonance is not observed in either the diamagnetic Cu(I) or the paramagnetic Cu(II) proteins which is consistent

with, but not proof of, binding to the copper. Throughout the pH titrations, the high field ring-current-shifted resonances shown in Figures 1A-C change only slightly in position, implying that the conformations of the relevant portions of the proteins are not particularly pH sensitive.

Evidence from different sources is accumulating (10-12) which indicates that a sulphur atom is bound to copper in the 'blue' proteins. A cysteine residue is favoured though the possibility of a methionine cannot be ruled out. ^{1}H n.m.r. spectra studies of the related proteins, the plastocyanins, implicate two histidines as ligands to the copper (13) and indeed there are two strongly conserved histidines. All four histidine residues in P. aeruginosa azurin are conserved in the sequence of nine azurins. A limited comparison of the sequences of the plastocyanins and azurins is given below.* If the only cysteine available

Azurin -- His 35 --- His 46 --- His 83 --- Cys 112 -- His 117 --- Met 121

Plastocyanin ------- His 38 ---------- Cys 85 -- His 88 --- Met 93

in the azurin sequences (cys 112) is a ligand to copper then His 117 also appears as a possible ligand and indeed the similarities between azurin and plastocyanin sequences in this region are quite striking. Further, copper(II) strongly perturbs the aromatic region of the ^{1}H n.m.r spectrum and we note that four of the ten aromatic residues (excluding histidines) lie between positions 108 and 117. Another possible histidine ligand may be His 46, analogous to His 38 in the plastocyanins, and proximate to Trp 48 which fluorescence studies have suggested (10) may be close to

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* Met 93 is absent in Dock plastocyanin (14). The sequence numbers are from P. aeruginosa azurin (4) and French Bean plastocyanin (15).

the metal centre. The non-conserved histidine in Anabena variabilis plastocyanin, His 61, titrates freely (13). Further assignments and a more quantitative use of copper(II) as an intrinsic relaxation probe should allow a more reliable assessment of this plausible model.

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