THE PROTON MAGNETIC RESONANCE SPECTRA OF A COBALT(II) AZURIN

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SUMMARY. The proton magnetic resonance spectrum of a cobalt(II) derivative of Pseudomonas aeruginosa azurin is reported. The temperature dependence of 26 resonances is described together with a study of the pH* titration behaviour over a range 4.7 to 9.3. A few resonances are observed shifted by more than 30 ppm from their diamagnetic positions. Of the remainder most extrapolate to the aliphatic region at $T = \infty$. Two lines are assigned to the C2 and C4 protons of a freely titrating histidine residue far from, and only slightly affected by, the Co(II) centre. A further two lines are assigned to the C2 hydrogen of protonated and deprotonated forms of a histidine residue in slow exchange with bulk aquaeous protons and closer to, but not bound to, the cobalt. The structure of the protein in the vicinity of the paramagnetic centre is found to be essentially insensitive to pH* over the range 4.7 to 9.3.

Azurins are low molecular weight proteins containing a single copper atom per molecule. They are characterized by a deep blue colour, small copper hyperfine coupling constants in the e.p.r. spectra, and high redox potentials, such proteins having been (1,2) termed Type 1 copper proteins. While there is a surfeit of information contained in the ^1H n.m.r. spectra of proteins as small as the azurins, it would be extremely useful to have the resonances of groups of protons displaced outside the largely unresolved main spectral envelope. One method of achieving such an objective is to exploit the paramagnetic shifts due to extrinsic or intrinsic perturbations by metal ions. The intrinsic copper(II) ion perturbs (3) principally the relaxation times of those protons close to it. Since it is known that the high-spin cobalt(II)

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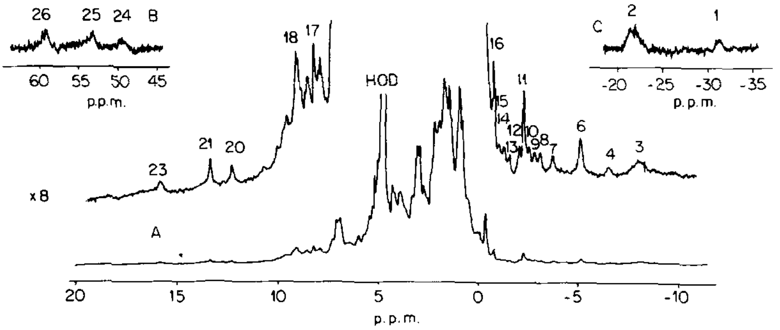
leads to large paramagnetic shifts in simple cobalt(II) complexes (3), or when complexed to proteins such as lysozyme (4), we decided to study the proton magnetic resonance spectra of azurins in which the copper(II) ion is replaced by cobalt(II). Some spectroscopic properties of these derivatives have been recently reported (5,6) by Gray and coworkers.

Methods. The azurin used in this study was isolated from *Pseudomonas aeruginosa* NCTC 10332 by the method of Ambler and Brown (7). Prior to the preparation of the cobalt(II) derivative the protein was chromatographed on Whatman CM 32 carboxymethylcellulose using 0.02M acetate buffers. The protein was loaded and washed at pH 4.6 and eluted at pH 5.3. This procedure removed the last traces of material absorbing at around 410 nm. The Cu(I) protein was prepared by reduction with sodium dithionite and the apo-protein by subsequent dialysis against a solution of 0.1M cyanide, 0.1M phosphate, pH 8.0, in a Minibeaker Fiber dialysis unit obtained from Bio-Rad. This was followed by dialysis against glass distilled water until all salts were removed. The apo-protein was freeze-dried and taken up in D_2O, 0.1M phosphate buffer. Protein concentrations, measured by the absorbance at 280 nm (ε = 8,400 $M^{-1}cm^{-1}$) were usually 2-5 mM and the pH approximately 6. A stoichiometric amount of $CoCl_2.6H_2O$ in D_2O was added. Under these conditions the incorporation of cobalt(II) was markedly accelerated by heating to above 50°C. Finally the cobalt derivative was dialysed against glass distilled water. Prior to pmr investigations, samples were freeze-dried three times from 99.8% D_2O. Unless otherways specified spectra refer to solutions 2-5mM in protein, 0.02M in phosphate buffer and 0.1M in NaCl. Symbols pH* and pK* refer to values uncorrected for the deuterium isotope effect.

The 270 MHz Fourier transform pmr spectra were obtained using a modified Brucker HFX-90 console and 64 K gauss super-conducting magnet (Oxford Instruments Ltd.) of the Oxford Enzyme Group. All chemical shifts are reported relative to sodium, 2,2-dimehtyl-2-silapentane-5-sulphonate



The 270 MHz ^{1}H nmr spectrum of Co(II) azurin. pH* = 4.7; 1A 10 KHz spectral width, 21°C; 1B lowest field lines, 23.8°C; 1C highest field lines, 26.5°C resonances are numbered 1 to 26 from high field to low field . The same numbering scheme is used in all diagrams and tables.

as internal standard. Positive chemical shifts imply low field shifts.

Results and Discussion. In diamagnetic proteins, the majority of resonances are observed between about 10 and -1 p.p.m. and indeed the major spectral intensity in cobalt(II) azurin also lies within this range. However a number of resonances are observed lying outside the main spectral envelope (Fig.1). In total the spectral range investigated spanned 126 p.p.m., and the lowest and highest field peaks are shown in Figs. 1B and 1C respectively. Such large shifts undoubtedly derive from the influence of the high-spin cobalt(II) ion and should in consequence be temperature-dependent as is indeed the case (Fig.2) in which representative data are given. Before proceeding with a discussion of the temperature dependence it is necessary to consider the problem of assignment. The aromatic residues most readily assigned are (8) histidines, since the associated resonances are usually pH-dependent. Resonances 17 and 18 (termed H_2' and H_2 respectively) occur to lower field than is usually found for histidine resonances in diamagnetic proteins. However, the chemical shifts of these lines (at 17.3°C) vary from 9.12 to 8.02 p.p.m. (H_2) and 7.93 to 7.36 p.p.m. (H_2') with increasing pH, in a manner character-

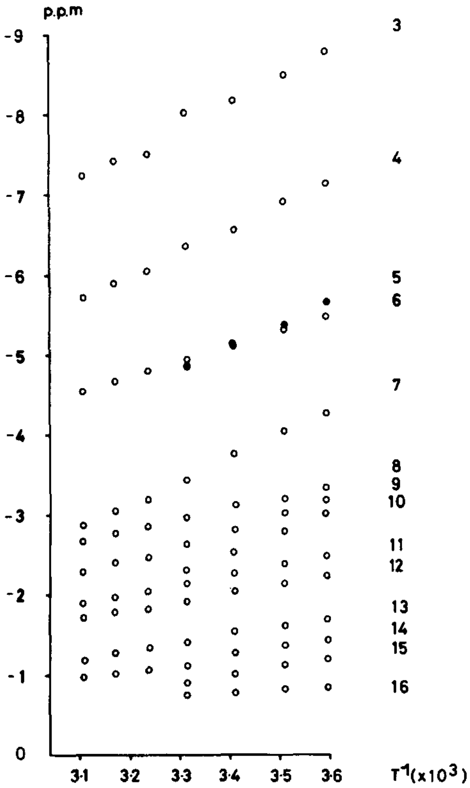


Figure 2.

The temperature dependence of the chemical shifts of representative resonances: O pH* = 4.7; ● pH* = 6.7. The choice of abscissa (T^{-1}) is for presentation purposes only. Only those resonances which were not clearly resolved at pH* = 4.7 are included in the data which refer to measurements obtained at pH* = 6.7.

istic of the C-2 (line 18) and C-4 (line 17) protons of a freely titrating histidine residue (8). The pK_a^* of 7.45 is very similar to that of the analogous H_2 and H_2' resonances observed (9) in the spectra of the apo-, copper(I) and copper(II) azurins. The small paramagnetic shift in the spectrum of cobalt(II) azurin and the relatively long relaxation times of the resonances in the spectrum of the copper(II) azurin imply that this residue is far from both metal ions.

Of those lines occurring outside the main spectral envelope the most marked dependence on pH is shown by resonances 19 and 21. With

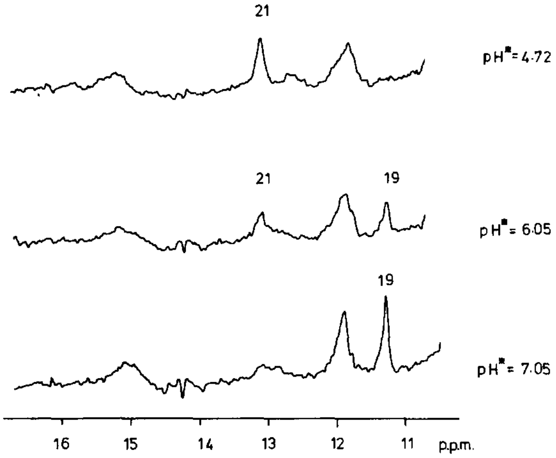


Figure 3.

The pH titration of resonances 19 and 21 at 40^{0}C.

increasing pH, resonance 21 gradually disappears with the concomitant appearance of resonance 19 (Figure 3). A similar phenomenon is observed in the pH-titration of Cu(I) and apo-azurin. The resonances can be assigned to the C-2 proton of a histidine in the protonated (H1A, resonance 21) and depronotaed (H1B resonance 19) forms respectively. The rate of proton exchange between the imidazole nitrogen and bulk water is slow compared to the chemical shift difference between resonances 19 and 21. An approximate pK_a^* of 6 is estimated as the pH* at which resonances H1A and H1B are of equal intensity. Whilst the paramagnetic shifts of H1A and H1B are much larger than those of H_2 and H_2' they are much smaller than those of 1,2,24,25 and 26 and smaller than might be expected for protons of an imidazole bound directly to the cobalt(II). This is consistent with the results derived from studies of the copper(II), copper(I) and apo forms which indicate that of the four histidines, one is distant from the metal and had ready access to solvent, and another is close to, but not bound to, the metal and may be relatively inaccessible to solvent. The resonances of the two remaining histidines have not been assigned. The possibility remains that they are coordinated.

Paramagnetic shifts may derive from both contact and dipolar contributions (3). The residues associated with resonances 1,2,24,25 and 26, which show the largest observed shifts, are probably very close to the cobalt centre. As expected these resonances are also the most broadened. In the following we treat the temperature dependence of lines 3 to 23 on the basis that the majority of observed shifts are predominantly due to a dipolar mechanism. While this is not proven it would seem a reasonable initial assumption which might be amended pending further assignments. For a dipolar mechanism the temperature dependence is expected (10) to be of the form:

$$\Delta_i = A_i(T^{-1} + BT^{-2}) + C_i$$

where Δ_i is the observed chemical shift, C_i is the diamagnetic chemical shift, A_i is a constant for a particular resonance and B is a constant for all dipolar shifted resonances. Using the values of the diamagnetic chemical shifts observed in the spectra of apo- and Cu(I)azurin for H1A (9.31 p.p.m.) and H1B (8.07 p.p.m.) allows B in the above equation to be esgimated. This permits the computation of the diamagnetic shifts (C_i) of the remaining resonances by extrapolation of the observed temperature variation to $T = \infty$. The calculated diamagnetic shifts for lines 3-23 are given in the table, and are expected to be indicative rather than accurate. It is obvious that most resonances are associated with aliphatic residues, as indeed might be expected from the amino-acid composition, and this is consistent with observations on the copper(I) and copper(II) proteins. The extrapolated values for other assigned resonances 17,18,19 and 21 are in reasonable agreement with the shifts observed in apo- and copper(I) proteins (see that Table). Most of the remainder of the resonances studied are not significantly pH-dependent and this suggests that the structure of the protein in the vicinity of the cobalt is not particularly sensitive to pH. Other work has demonstrated (11,12) that

Table

Resonance	Calculated Diamagnetic Shift (p.p.m.)		Resonance	Calculated Diamagnetic Shift† (p.p.m.)	
	pH*4.7	pH*6.7		pH*4.7	pH*6.7
3	0.7		13	1.1	
4	0.8	0.6	14	1.6	1.3
5		2.1	15	1.7	1.5
6	0.2	0.1	15	0.3	
7	3.7	4.5	17	7.1(7.3)	
8	0.4	1.0	18	8.8(8.7)	
9	1.8	2.0	19		8.0(8.1)
10	3.6		20	5.8	5.5
11	0.8	1.0	21	9.4(9.3)	
12	0.6	0.8	22		7.3
			23	6.6	5.2

† Observed values in parentheses.

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this part of the protein is inaccessible to solvent. These preliminary results indicate that the use of intrinsic paramagnetic perturbants of the chemical shifts of protons is of value. Full exploitation awaits further assignments of the paramagnetically-shifted resonances.

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