COSY and NOESY Characterization of Cobalt(II)-Substituted Azurin from *Pseudomonas aeruginosa*

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Azurins are blue copper proteins that probably participate in the denitrification respiratory chains of several bacteria.[1,2] The crystal structure of Cu(II)-azurin from *Pseudomonas aeruginosa* (*Pae*) has recently been determined to 1.93-Å resolution.[3] The copper ion is strongly bound to Sγ of Cys-112 and to Nδ of both His-46 and His-117 and weakly ligated by Sδ of Met-121 and the carbonyl oxygen of Gly-45, resulting in a distorted trigonal-bipyramidal geometry (see the scheme in Figure 1). It has been recently reported that the hydrophobic patch, around the copper-ligand His-117, is involved in the electron-transfer reactions,[4,5] and the residue His-117 has been suggested as a specific pathway for the transit of electrons.[6] On the other hand, *Pae* azurin suffers a pH-induced conformational transition, triggered by the deprotonation of His-35,[3,7-9] whose role in the redox activity of the protein has not been completely clarified.

Paramagnetic metal ions, such as cobalt(II), have been successfully used as spectroscopic probes replacing the copper ion in many blue copper proteins.[2,10-12] Although the study of paramagnetic molecules by using two-dimensional (2D) NMR techniques has been prevented by the fast nuclear relaxation rates,[13] recently NOESY and COSY spectra of paramagnetic metalloproteins have started to appear.[14-16] However, up to now, 2D NMR has been almost exclusively applied to the study of iron proteins and mainly on low-spin iron(III) heme proteins. Here we report the results of the application of 2D ^{1}H NMR spectroscopy as a powerful tool in the assignment of the isotropically shifted proton resonances of Co(II)-azurin, aiming at a better understanding of the structural features in the metal-binding site of this protein in solution.

The ^{1}H NMR spectrum of Co(II)-azurin (Figure 1) shows, at pH 4.4, seven well-resolved signals (a–g) in the far downfield region as well as four other ones (m–p) upfield shifted. These signals have relatively short T_1 values (2.5–8.5 ms) and probably belong to residues directly coordinated to the cobalt ion. Indeed, signals a and b disappear in D_2O, indicating that they are due to the NεH protons of the two histidines bound to the metal ion. A numerous group of other shifted signals is observed close to the diamagnetic region of the spectrum. Most of them are due to proton resonances from residues near cobalt(II) but not bound to it. Their isotropic shifts would be due to through-space dipolar interactions arising when magnetic anisotropy is present, as happens in the case of five-coordinated cobalt(II).[17] However, the signal k at −7.9 ppm integrates three protons and its short T_1 and T_2 values (3.5 and 2.4 ms, respectively) agree with its assignment to the CεH$_3$ group of Met-121.

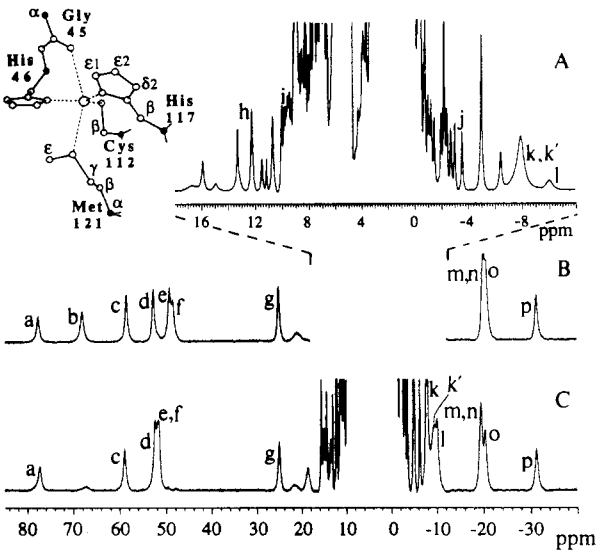

Figure 1. 1D ^{1}H NMR spectrum (400 MHz, 50 mM CH_3COONH_4, 25 °C) of Co(II)-azurin in H_2O solvent at pH 4.4 (B), with expansion close to the diamagnetic region of the same spectrum (A), and spectrum registered at pH 7.0 (C). Only those signals mentioned in the text are labeled. A schematic drawing of the metallic site in *Pae* native azurin based on X-ray crystallographic studies[5] is shown in the upper left corner.

As occurs in the native protein,[3,7-9] as well as in the Ni(II) metalloderivative,[12] cobalt(II)-azurin undergoes a pH-induced conformational change, and consequently, most of the isotropically shifted signals change their position in slow-exchange regime on the NMR time scale. Furthermore, signal b disappears (Figure 1) when the pH is increased suggesting that the corresponding NεH proton enters fast-exchange conditions with bulk water, being due to either a slight pH-induced structural modification or general base catalysis. The analysis of the pH dependence of the spectra (data not shown) allows us to obtain a pK_a = 5.7 completely consistent with that associated to the ionization equilibrium of His-35 in *Pae* Cu(II)-azurin.[8]

2D NMR experiments have led us to the assignment of the main hyperfine-shifted resonances. Figure 2 represents the 400-MHz COSY spectrum of Co(II)-azurin in D_2O solvent. Five scalar connectivities numbered 1–5 allow us to distinguish three sets of signals: e,p (giving cross peak 1), f,n,o,j (2, 3), and g,h,i

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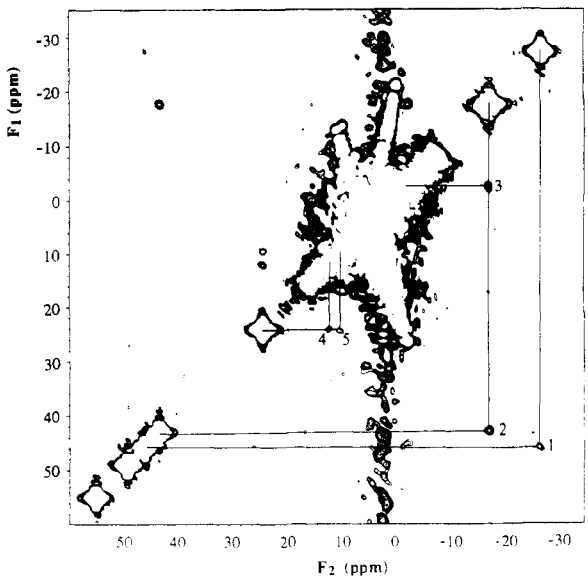

Figure 2. 400-MHz magnitude COSY map of 8 mM Co(II)-azurin in D_2O (pH 4.5, 50 °C) with 256 experiments (8192 scans each) using a 70-kHz bandwidth in the *F*1 dimension over 1 K data points in the F2 dimension. Data were processed in each dimension using a 0°-shifted sine-bell-squared function.

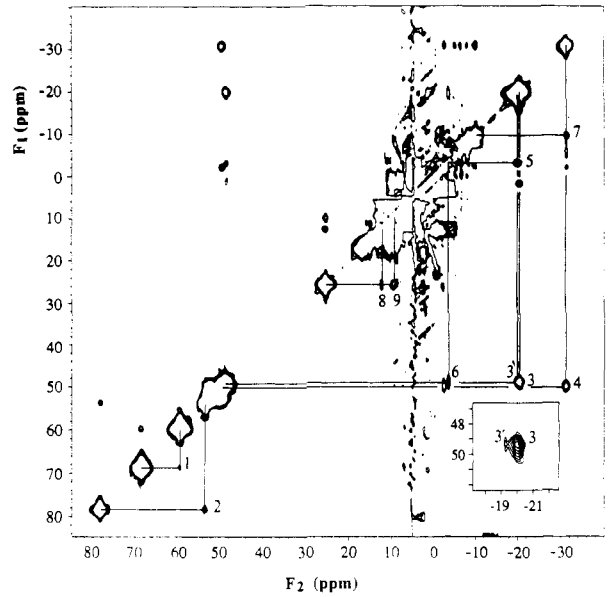

Figure 3. Phase-sensitive (TPPI) ^{1}H NOESY spectrum obtained at 400 MHz on a 4 mM sample of Co(II)-azurin in water (pH 4.5, 25 °C). This map was collected with a 6-ms mixing time, 256 *t*1 values (4096 scans each) over a 70-kHz bandwidth using 1 K data points in the *F*2 dimension. A 80°-shifted sine-bell-squared weighting function was applied in both dimensions The insert shows cross peaks 3 and 3′ of the same spectra processed to find more resolution.

(4, 5). The pair of signals e–p can be clearly assigned as geminal protons of a CH_2 group. On the other hand, since cross peak 4 is clearly stronger than cross peak 5, signals g and h must correspond to another CH_2 group, and so signal i would be a vicinal proton in this group. In the case of the signals f, n, o, and j the situation is apparently more confusing because at this temperature signals n and o are in the same position. However, a COSY spectrum registered at 25 °C permitted us to distinguish these signals and we were able to associate cross peak 2 with signals f and o and cross peak 3 with signals j and n. Additionally, the comparison of the relaxation times of signals j (T_1 = 30 ms) and n (4 ms) indicates that these values are too different for two geminal protons.[18]

The NOESY spectrum of Co(II)-azurin in water solvent (Figure 3) gave us valuable information to achieve a more complete assignment since we can now observe the two exchangeable NϵH protons of the coordinated histidines (signals a and b). So, signals c and d, connected through NOESY cross peaks with signals b and a, respectively (Figure 3, cross peaks 1 and 2), must correspond to the CδH protons of the two coordinated histidines. A specific assignment of these histidine protons can be easily deduced from the inspection of the X-ray structure of *Pae* azurin. Of the two coordinated histidines only His-117 is accessible to the solvent, and its NϵH proton is hydrogen-bonded to a water molecule.[7] So, considering the greater lability of signal b, it is assigned to the NϵH proton of His-117, and then signal c is assigned to the CδH proton of the same histidine. It follows that the signals a and d can be obviously assigned as NϵH and CδH protons of the other coordinated histidine, His-46.

In agreement with the COSY experiment, the two pairs of protons, f–o and e–p, exhibit strong NOESY cross peaks as expected for geminal protons (Figure 3, cross signals 3 and 4). We can also observe dipolar connectivities between signals n–j (5), f–j (6), and f–n (3′). This pattern suggests to us that signals f, j, n, and o belong to the same residue. According to the crystallographic data,[3] the proton–metal distances corresponding to the CβH$_2$ protons of the coordinated residues Cys-112, His-117, and His-46 range from 3.0 to 3.5 Å, which are typically associated with very large relaxation rates and so resulting in very broad resonances. So the aforementioned set of signals must correspond to the Met-121 residue. Consequently, considering the COSY data, signal j can be assigned as the α proton of Met-121 and signal n as one of its β protons. It follows that the pair of signals f–o corresponds to the CγH$_2$ protons of the same residue. The other β proton could correspond to signal m, but signals n and m are not resolved enough to allow the detection of any connectivity.

On the other hand, signal p gives a NOE connectivity with signal l (Figure 3, cross peak 7). This later signal disappears in D_2O after several weeks, showing the characteristic behavior of an internal labile amide proton. This pattern fits well with the assumption of signal l being the backbone amide proton of Gly-45 and so the pair of signals e–p being the two α protons of this coordinated residue.

Finally, as mentioned above two additional scalar couplings involving g, h, and i have been detected, and the same pattern is observed in the NOESY map (Figure 3, cross peaks 8 and 9). Additionally, signal i shows strong COSY and NOESY cross peaks with a signal at 5.50 ppm (data not shown). This set of signals probably corresponds to Met-13, a residue close to the metal ion but not bound to it. However, their definitive assignment requires a deeper analysis of the crowded region of the spectra, which is out of the aim of this paper.

2D NMR experiments have allowed us to assign the most significant signals of the ^{1}H NMR spectrum of Co(II)-azurin. The spectroscopic features of this paramagnetic metallo derivative have proved valuable for its structural study in solution by using modern high-resolution NMR techniques. A more complete assignment, which is actually in progress, will open new possibilities of connecting the observed changes in the spectrum with structural modifications affecting specific residues in the protein.

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(18) The T_1 values can be interpreted in terms of ^{1}H-paramagnetic center distances according to the Solomon equation (Solomon, I. *Phys. Rev.* **1955**, *99*, 559).