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A ^{1}H NMR NOE study on Co(II) stellacyanin: some clues about the structure of the metal site

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Abstract The ^{1}H NMR spectrum of Co(II) stellacyanin is reported, in which four signals not previously observed have been detected. NOE experiments were performed to assign the hyperfine shifted signals corresponding to a Cys and two His residues. Both His residues are solvent-accessible and are shown to bind the metal ion through their Nδ1 atoms. The β-CH_2 Cys proton shifts indicate the presence of a strong axial ligand.

Key words: Stellacyanin; Blue copper protein; NMR; NOE; Co(II); Metal substitution

1. Introduction

Blue copper proteins (BCPs) have attracted the attention of researchers from different fields due to their puzzling spectroscopic and functional properties [1–4]. One of the main questions in the field of BCPs is how the protein framework is able to tune the redox potential of the Cu site, which ranges from 184 mV in stellacyanin to 680 mV in rusticyanin. Stellacyanin (St hereafter) is a glycoprotein isolated from the lacquer tree *Rhus vernicifera* [5,6], which has been considered an outlier in the family of BCPs due to its unusually low redox potential [6], high electron transfer rates [7] and its spectroscopic features. All BCPs contain a so-called type 1 copper center, in which the metal ion lies in a plane defined by three ligands: two histidines and one cysteine [2]. A methionine residue is one axial ligand, and a glycine backbone carbonyl has been found coordinated to the copper in azurin at a longer distance [2]. St lacks a methionine in its sequence [8], and this fact has been considered the main factor responsible for its properties [9]. Three metal ligands have been suggested: residues His-46, Cys-87 and His-92 [10–12]. However, since any attempt to crystallize ST has been unsuccessful, the exact structure of the metal site and the identity of the fourth ligand replacing Met are not known. Sequence alignment [12] and molecular modeling studies [13] led Freeman and co-workers to propose that Gln-97 may be the elusive axial ligand. This is consistent with a recent study on the M121Q mutant of azurin, which exhibits spectroscopic features similar to those of stellacyanin [14]. Two other models for St have been proposed, both suggesting that a sulfur atom from a disulfide bridge may be the fourth ligand [15,16]. One of them includes both histidine ligands bound to the metal through their Nε2 atoms, i.e. in a non-typical coordinating fashion for type 1 copper centers [15].

Metal substitution has proven to be a helpful tool for probing metal sites in metalloproteins [17]. In particular, when Co(II) and Ni(II) replace copper, the NMR signals of nuclei belonging to the metal ligands display considerably larger shifts and linewidths but can still be detected and assigned [18,19]. This strategy has been fruitful for the study of wild-type azurin and its mutants [20–22]. The ^{1}H NMR spectrum of Co(II)St has already been reported [15], but the structural model derived from its assignment conflicts with that coming from molecular modeling [13]. The assignment of paramagnetic NMR signals is no longer problematic since NOE experiments have been successfully performed on paramagnetic proteins [23,24]. We have used this technique to assign the hyperfine shifted signals in Co(II)St. Before identifying the fourth ligand, we have focused our efforts on the first necessary step: the detection and assignment of the proton resonances of the already known three ligands, in an attempt to compare the features of these signals with those of Co(II) azurin.

2. Experimental

Stellacyanin from *Rhus vernicifera* was obtained as previously reported [6]. The apoprotein was prepared by dialysis against thiourea following the procedure of [25], followed by dialysis against 100 mM sodium phosphate, pH 6.0. The Co(II) derivative was prepared by addition of a fourfold excess of cobalt chloride to a buffered solution of apoprotein, followed by dialysis against EDTA to remove excess metal ion. The metal uptake was monitored by optical spectroscopy, and the Co(II) derivative yielded an electronic spectrum similar to the one previously reported [10]. The electronic spectra were recorded in a Gilford Response II spectrometer. The concentrated samples for NMR experiments were obtained using Centricon-10 (Amicon) concentrator units. The D_2O solutions were prepared by dissolving the lyophilized protein in deuterium oxide.

The NMR spectra were recorded at 200 MHz in a Bruker ACE 200 spectrometer using the superWEFT pulse sequence (180°-τ-90°) [26]. Different delays (τ) were used in the superWEFT sequence to optimize the detection of the fastest relaxing signals. Non-selective T_1 values were determined by an inversion-recovery experiment. The difference steady-state NOE spectra were recorded using the methodology reported by Banci et al. [23]. Recycle times of 82 and 41 ms were used in most of the cases, with saturation times ranging from 3 to 30 ms. Protein concentration ranged from 2 to 4 mM, as determined by electronic spectroscopy.

3. Results

Fig. 1a shows the spectrum of Co(II)ST recorded at pH 8.2, which displays a considerable number of hyperfine shifted signals and resembles the one previously reported [15]. Table 1 reports the measured parameters for the detected signals experiencing the larger hyperfine shifts. Spectra obtained at lower

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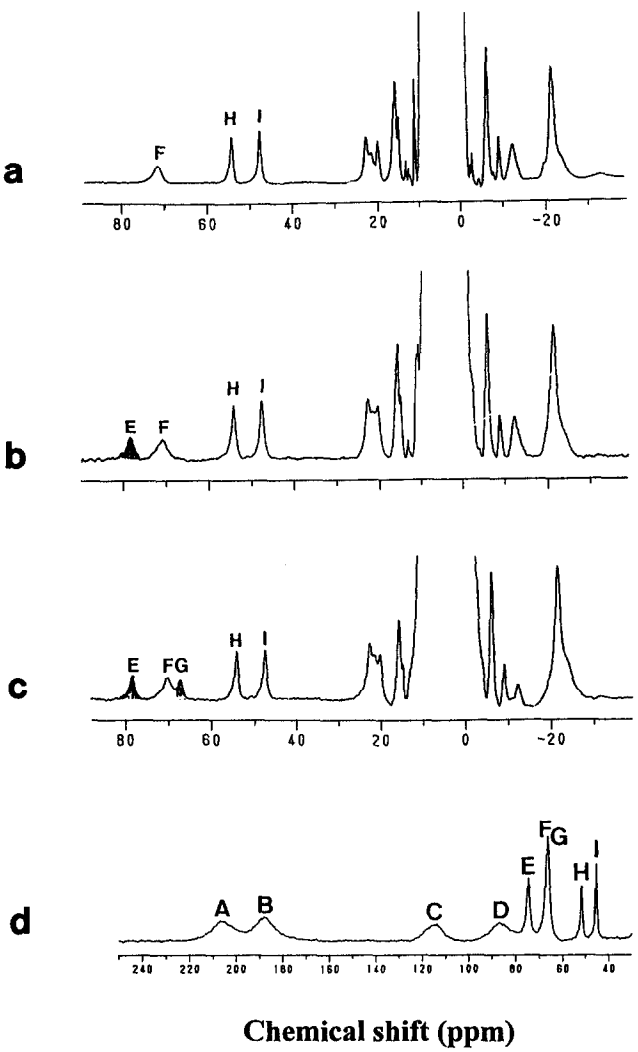


Fig. 1. ^{1}H NMR 200 MHz spectra of Co(II)ST recorded at 298 K and (a) pH 8.2, (b) pH 5.0, and (c) pH 4.0. (d) Spectrum recorded at pH 4.0 with a faster repetition rate. Shaded signals are absent when the spectra are recorded in D_2O solution.

pH values allowed us to detect two further signals, E and G (Fig. 1b and c). Signals E and G are clearly broadened beyond detection at pH values above 6.0 and 5.0, respectively. However, at higher temperatures they are broadened further, thus preventing their detection even at pH 4.0. Both correspond to exchangeable protons since they are absent when the spectra are recorded in a D_2O solution.

Spectra performed with a faster repetition rate (25–70 s^{-1}) and larger spectral widths (125 kHz) allowed us to detect two additional broad well-shifted downfield signals (A and B in Fig. 1d). Also signals C and D can be better observed under these acquisition conditions. All of these four signals are also present in the spectrum recorded in a D_2O solution.

A series of NOE experiments were performed in order to assign the paramagnetic proton signals. Since the hyperfine signals are broader in Co(II)St with respect to Co(II) azurin, due to the higher molecular weight of the title protein, these experiments are more difficult to perform. In spite of the large linewidths of these signals, we have been able to detect some dipolar connectivities arising from them. Signals E,I and G,H were shown to be dipolarly connected pairwise (Fig. 2). Furthermore, irradiation of the very broad signals A–D was also attempted. In the case of signals C and D, no NOEs could be detected. On the contrary, when signal A was irradiated, a small NOE on signal B was observed (data not shown). Since a strong decoupling rf had to be used, the off-resonance irradiation frequency was located symmetrically with respect to signal B so that the spillover operative when irradiating A was compensated for during the off-resonance irradiation and its effects were cancelled in the difference spectrum. This allowed us to rely on the small NOE detected between signals A and B.

4. Discussion

Signals A–I are well-shifted downfield and display very short T_1 values. These features suggest that they correspond to residues directly coordinated to the Co(II) ion, experiencing a contact contribution to the shift [18,19]. Signals E and G are the only exchangeable signals experiencing considerable hyperfine shifts, and they are therefore assigned to the NH protons of the Co(II)-bound His. Both protons are solvent-accessible, as seen by the absence of these signals in spectra recorded at pH values above 6.0. This behavior has been previously observed for only one of the NH resonances of Co(II)-substituted azurin [21]. This indicates a larger accessibility of ST in the solvent with respect to azurin, thus resembling the metal site of the cucumber basic protein [12] which has been used as a starting point for constructing a three-dimensional model for ST [13]. This finding could explain the faster electron transfer rate found for ST with respect to azurin [7].

When signal E is irradiated, an NOE is observed on signal I (and vice versa), and the same is found for the pair of signals G and H. Signals I and H may be safely assigned to CH protons of the bound histidines vicinal to the NHs. We should note that signals I and H are sharper than signals E and G. If the His residues were bound to the metal ion via their Nε2 atoms (see Scheme 1), as previously suggested, signals E and I would correspond to the Hδ1 nuclei of His-46 and His-92, and signals I and H should be assigned as the Hε1 nuclei of these residues. However, the latter signals should be sizeably broader, as would be expected for protons adjacent to the purported coordinating Nε2 atoms. Since this is not the case this possibility must be excluded. Furthermore, the four His resonances are

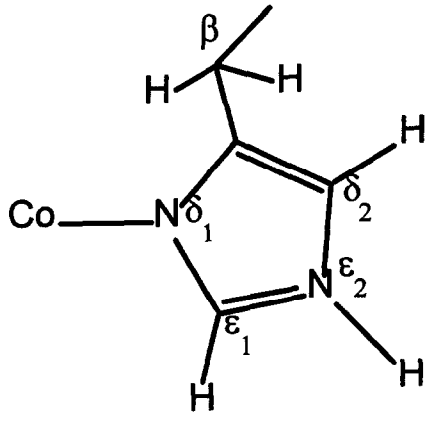
Table 1
^{1}H NMR paramagnetically shifted signals at 200 MHz and 298 K (pH 6.0 except when indicated)

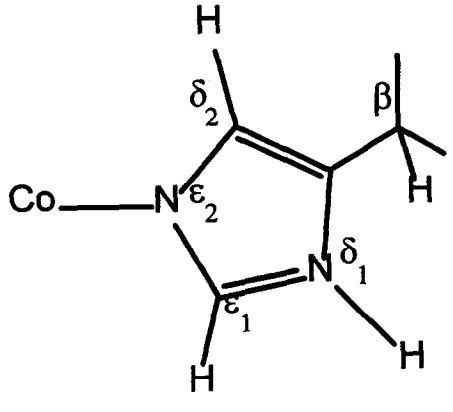
Signal	δ (ppm)	T_1 (ms)	Linewidth (Hz)	Assignment
A	220	[a]	2600	Hβ1 Cys-87
B	190	[a]	2000	Hβ2 Cys-87
C	127	[a]	1800	Hε1 His-46(92)
D	90	[a]	1850	Hε1 His-92(46)
E	78.1[b]	1.9	440	Hε2 His-46
F	70.7	1.2	640	
G	68.2[b]	1.2	550	Hε2 His-92
H	53.9	5.0	250	Hδ2 His-92
I	47.8	5.9	260	Hδ2 His-46

[a] T_1 value under 0.1 ms.
[b] Measured at pH 4.0.

not so broad, and therefore they should correspond to histidine ring protons which are three bonds away from the coordinating nitrogen atom. This indicates that both His are bound to the metal via their Nδ1 atoms (see Scheme 1). Even if both Nϵ2 protons are solvent-accessible, one of them exchanges faster. In all BCPs (including the cucumber basic protein), the more exposed histidine is the one nearest the C-terminus. On this basis, it may be proposed that signals E and I correspond to His-46 and G and H to His-92.

Signals C and D are very broad, indicating that they belong to protons near to the metal center. Two candidates could be the *ortho*-like protons of the bound histidines, as suggested for Co(II) azurin [22]. Signals A and B, the signals shifted the most downfield, both exhibit T_1 values under 0.1 ms and are dipolarly connected. Since dipolar connectivities are hard to detect in extremely broad signals, it is expected that they correspond to geminal protons. The best candidates therefore are the β-CH_2 protons of the bound cysteine residue, namely Cys-87. This assignment is in agreement with the recent finding of similarly shifted signals for Co(II) azurin [22]. It is relevant to point out that in this case they are considerably less shifted (220 and 190 ppm) than the signals corresponding to Cys-112 in Co(II) azurin (280 and 230 ppm, according to [22]). The shifts of cysteine residues bound to paramagnetic metal ions are mainly due to a contact contribution [18], so that it is reasonable to conclude that the electron delocalization onto the cysteine ligand is smaller in Co(II)St. One possible explanation is that the presence of a stronger axial ligand displaces the Co(II) ion somewhat from the plane defined by the equatorial ligand triad reducing the Co(II)St orbital overlap.





Scheme 1.

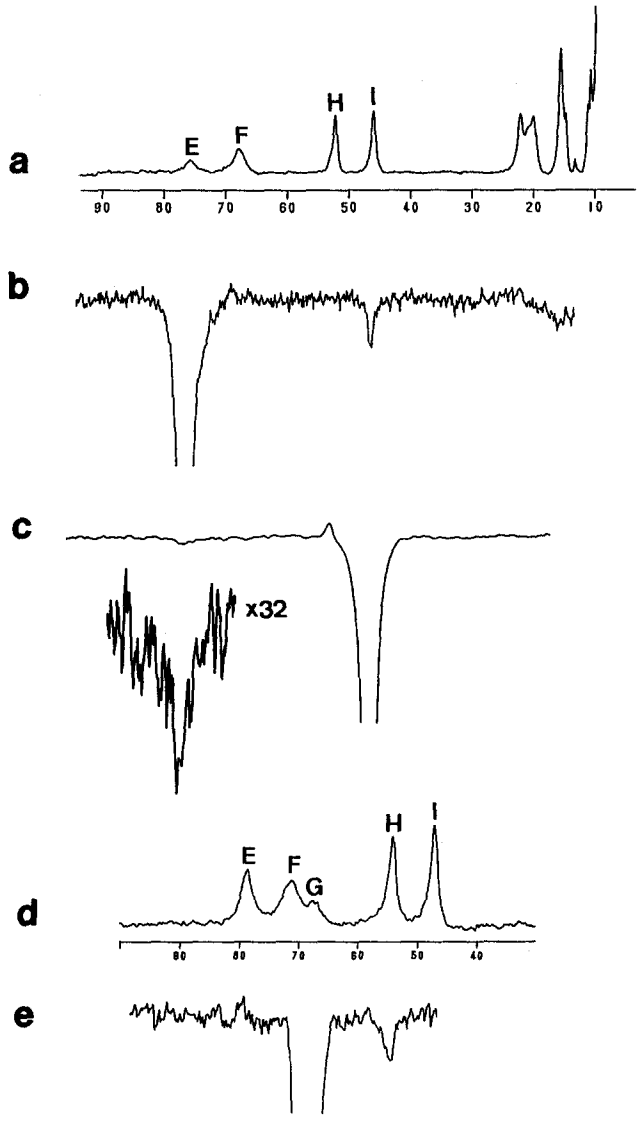


Fig. 2. ^{1}H NMR 200 MHz spectrum at 303 K (a) of Co(II)ST, and NOE difference spectra under the same experimental conditions obtained by saturation of (b) signal E and (c) signal I. In (e) and (d) the NOE difference spectrum obtained at 279 K by saturation of signal G and the corresponding reference spectrum are reported, respectively.

In the present study, the paramagnetic ^{1}H NMR signals corresponding to the two bound His and to the Cys residues in Co(II)St have been identified and assigned. Four proton resonances not previously reported have been detected and we have demonstrated that they belong to metal-bound residues. It has also been shown that both His ligands are solvent-accessible, resembling the site of the cucumber basic protein. These

two His residues are shown to be bound to the metal ion by their Nδ1 atoms, as in all the hitherto characterized type 1 copper centers. The hyperfine shifts found for the cysteine proteins are indicative of the existence of an axial ligand stronger than a methionine. This strategy has proven to be a useful tool for probing the metal site structure in copper proteins the structures of which are not known, and gives strong spectroscopic support in favour of one of the proposed models for St [13]. Further experiments could be helpful in assessing the identity of the hitherto unknown fourth ligand.

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