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Probing the metal site in *Rhus vernicifera* stellacyanin by Ni(II) substitution and paramagnetic NMR spectroscopy [1]

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Abstract

Stellacyanin from *Rhus vernificera* is a blue copper protein in which the metal is coordinated to a Cys, two His and a Gln residue. We have studied the Ni(II) derivative of stellacyanin by paramagnetic ^{1}H NMR spectroscopy. The NMR spectrum resembles that of Ni(II) Met121Gln azurin, indicating a similar coordination environment. The hyperfine signals were assigned by means of 1D and 2D NOE spectroscopy. Evidence is obtained which indicates that the Gln residue is bound to the metal through its oxygen atom.

Keywords: Paramagnetic NMR; Blue copper proteins; Stellacyanin; Nickel complexes; Metalloprotein complexes

1. Introduction

Copper is often used by proteins for performing electron transfer tasks [1]. This role is accomplished by the mononuclear blue copper centers (or type 1 sites, present in small blue copper proteins and in multicopper enzymes) or by the binuclear Cu_A unit. In both cases, the metals bear a geometric strain imposed by the protein scaffold which allows the reduction step to proceed with a minimal reorganization energy, a fact which enhances electron transfer rates [2,3].

In particular, blue copper proteins are able to accommodate the metal ion in either Cu(II) or Cu(I) oxidation state. They are characterized by an intense absorption at 600 nm, a low $A_{\parallel}$ copper hyperfine coupling constant, and unusually high redox potentials [4,5]. The copper is bound to one cysteine and two histidine residues in a nearly trigonal-planar array, and a weakly coordinated axial methionine is the fourth axial ligand in most of these proteins (Fig. 1(a)) [8]. Stellacyanin (St hereafter), originally isolated from the Japanese lacquer tree *Rhus vernicifera*, has been considered as a chemical puzzle for more than 20 years since all crystallization attempts and spectroscopic studies failed to unveil the structure of the metal site ([9] and Refs. therein). In 1991, Freeman and coworkers proposed that a Gln residue may act as the axial ligand [10]. This prediction has been confirmed recently by NMR spectroscopy [11] and through the X-ray structure of a St from cucumber peelings (Fig. 1(b)) [7]. However, the identity of the coordinating atom is still an unsolved issue, since EXAFS [12] and X-ray studies [7] cannot distinguish between low-Z coordinating atoms. In this way, it is still not known whether the Gln coordinates the metal through the Oε1 or the Nε2 atom.

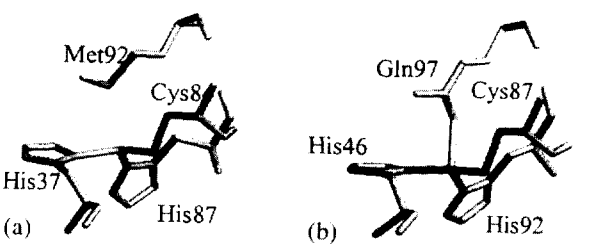

Fig. 1. Schematic representation of the metal site of (a) poplar plastocyanin, from Ref. [6], and (b) cucumber St, from Ref. [7]. The residue numbering corresponds to *Rhus vernicifera* St.

Paramagnetic NMR is a useful spectroscopic tool for the characterization of metal sites in proteins [13]. Until the late 1980s, standard NMR assignment techniques could not be applied to these systems owing to the limitations imposed by the fast nuclear relaxation rates. The contribution of the NMR school in Florence led by Ivano Bertini was fundamental in overcoming this difficulty [13–16]. Nowadays, NMR researchers are more familiar with the feasibility of NOE and

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2D experiments in paramagnetic proteins. Moreover, it is now possible to solve the structure of a paramagnetic metalloprotein by NMR [17]. In cases where metals are not magnetically active (such as Zn(II)) or display fast electron relaxation rates (such as Cu(II)) [15], metal substitution into metal sites has been an advantageous strategy, largely exploited by Bertini's team. When Co(II) and Ni(II) replace Cu(II), the NMR signals of nuclei belonging to the metal ligands display narrower lines than in the Cu(II) proteins and can be better detected and assigned [13]. This strategy has been successfully applied to blue copper proteins [11,18–25]. In particular, paramagnetic NMR techniques have contributed to the elucidation of the identity of the axial ligand in Co(II)St [11]. An NMR analysis of the Ni(II) derivative of Met121Gln azurin, which successfully mimics the St metal site, was able to show that the Gln residue binds the metal ion via the amide oxygen atom [20]. We therefore undertook the study of Ni(II)St by means of paramagnetic NMR techniques in an attempt to clarify this issue.

2. Experimental

St from *Rhus vernicifera* was obtained as previously reported [26]. The apoprotein was prepared by dialysis against thiourea, followed by dialysis against sodium phosphate 100 mM at pH 6.0 [27]. The Ni(II) derivative was prepared by addition of a five-fold excess of nickel sulfate (Titrisol, Merck) to a buffered solution of apoprotein, followed by dialysis against EDTA to remove the excess metal ion. When Ni(II) was added at pH 6.0, the metal uptake was very slow (1–2 weeks). The same operation performed at pH 8.5 rendered the desired metal derivative in ca. 2 days with a 70–80% yield. The metal uptake was monitored by optical spectroscopy, and the Ni(II) derivative yielded an electronic spectrum similar to that previously reported [28]. The electronic and ^{1}H NMR spectra of Ni(II)St obtained at both pH values were identical.

The electronic spectra were recorded on Gilford Response II and LKB Ultraspec 2 spectrophotometers. The concentrated samples for NMR experiments were obtained using Centricon-10 (Amicon) concentrator units. The D_2O solutions were prepared by dissolving in deuterium oxide the lyophilized protein.

The NMR spectra were recorded on Bruker ACE 200 and MSL 300 spectrometers operating at proton frequencies of 200.13 and 300.13 MHz, respectively. All chemical shifts were referenced to the chemical shift of water at the appropriate temperature, which in turn was calibrated against internal DSS. 1D experiments were performed using the superWEFT pulse sequence (180°–τ–90°) [29] or by presaturating the water resonance. Different delays (τ) were used in the superWEFT sequence to optimize the detection of the fastest relaxing signals and to eliminate the solvent H_2O or HDO signal. Nonselective longitudinal relaxation times were determined using the standard inversion recovery pulse sequence. The T_1 values were obtained from the initial slope of the semilogarithmic plots [30]. NOE experiments were performed by using a modified superWEFT sequence, irradiating the resonance of interest during the intermediate delay [31]. WEFT–NOESY spectra [32] were recorded in the TPPI mode [33]. Recycle times ranging from 30 to 80 ms were used, with mixing times between 6 and 15 ms. The delay τ between the initial 180° and the 90° pulses was optimized according to the total recycle time in order to minimize the absorption in the diamagnetic region. The NMR data sets were processed using the software WinNMR(Bruker). The 2D NMR data sets were Fourier transformed using square sine-bell weighting functions shifted by 45, 60 or 90°. The spectra were phase-corrected and base-line leveled in both dimensions.

3. Results and discussion

3.1. ^{1}H spectrum of Ni(II)St

The ^{1}H NMR spectra of Ni(II)St recorded under different conditions are illustrated in Fig. 2. Nine well-resolved isotropically shifted resonances (A–I) are found in the downfield region, whereas two additional signals (y,z) fall upfield from the diamagnetic envelope. Their shifts span from 200 to −25 ppm. Two of them (A,B) are well shifted downfield

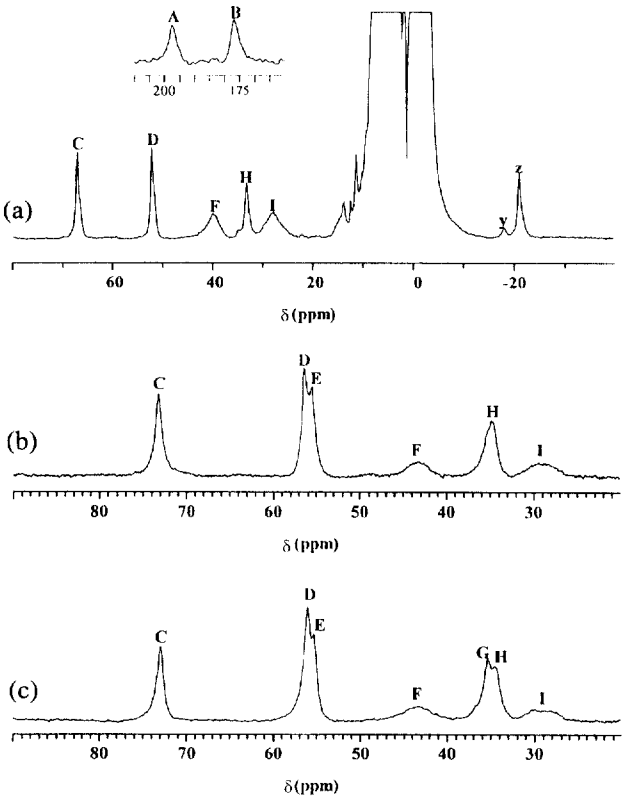

Fig. 2. ^{1}H NMR 300 MHz spectra of *Rhus vernicifera* Ni(II)St recorded at (a) 313 K in D_2O, pH 7.0 (The inset shows the most downfield shifted region); (b) 290 K in H_2O, pH 5.0; (c) 290 K in H_2O, pH 4.0.

and exhibit the largest line widths. Table 1 summarizes the spectral features of the observed resonances.

Signals E and G are absent when the spectrum of Ni(II)St is recorded in D_2O, whereas the intensity of resonance y falls to ca. 15% its value (Fig. 2(a)). The two downfield resonances E and G can be assigned to the exchangeable Hε2 protons of the two bound histidines (His46 and His92) by analogy with the shifts found in metal-bound His in Ni(II)-substituted proteins [13,20,24]. Both are present in spectra recorded at pH 4.0 in H_2O solution (Fig. 2(c)), but as the pH is raised, they broaden beyond detection, entering into a fast exchange regime with the bulk solvent. The same effect is observed at higher temperatures. A subtle difference may be traced: signal G is no longer observed at pH 5.0 (Fig. 2(b)), whereas signal E becomes undetectable only above pH 6.0. This behavior clearly differentiates the exchange rates of the two protons. Considering that His92 is more solvent exposed [11,7,23], we assign the more labile signal G to the Hε2 of this residue and signal E is ascribed to the His46 Hε2.

Variable temperature ^{1}H NMR spectra of Ni(II)St were recorded between 288 and 323 K. The hyperfine shifts showed a marked temperature dependence (not shown), most of them exhibiting a Curie behavior, i.e. intercept values at infinite temperature within or close to the diamagnetic region. Signals A and B are the only exception, indicating that these resonances bear a sizable dipolar contribution to the isotropic shifts.

Table 1
Spectral features and assignments of the proton hyperfine shifted signals of *Rhus vernicifera* Ni(II)St recorded at 300 MHz, pH 4.0 and 313 K

Signal	Assignment	δ (ppm)	T_1 (ms)
A	Hβ2 Cys87	197	1.5
B	Hβ1 Cys87	177	1.0
C	Hδ2 His92	67.1	20.5
D	Hδ2 His46	52.1	18.4
E	Hε2 His46	50.8	20.0
F	Hε1 His92 (46)	39.8	2.0
G	Hε2 His92	33.9	15.2
H	Hγ1 Gln97	33.2	14.4
I	Hε1 His46 (92)	28.0	1.6
x	Hβ Gln97	0.5	
y	Hε21 Gln97	−18.0	12.8
z	Hγ2 Gln97	−21.0	20.3

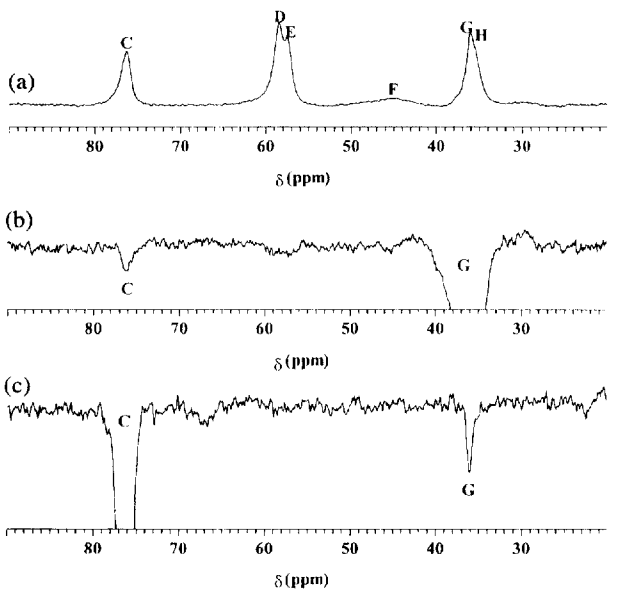

Fig. 3. ^{1}H NMR 300 MHz NOE experiments of *Rhus vernicifera* Ni(II)St in H_2O at 278 K and pH 4.0: (a) reference spectrum; (b) NOE difference spectrum performed by irradiation of signal G; (c) NOE difference spectrum performed by irradiation of signal C.

3.2. Signal assignments

The isotropically shifted resonances have been assigned on the basis of 1D and 2D NOE experiments. Several primary NOEs, establishing the nearest neighbor connectivities, are apparent in Figs. 3 and 4. Resonances E and G are the starting point to assign the signals corresponding to the His residues.

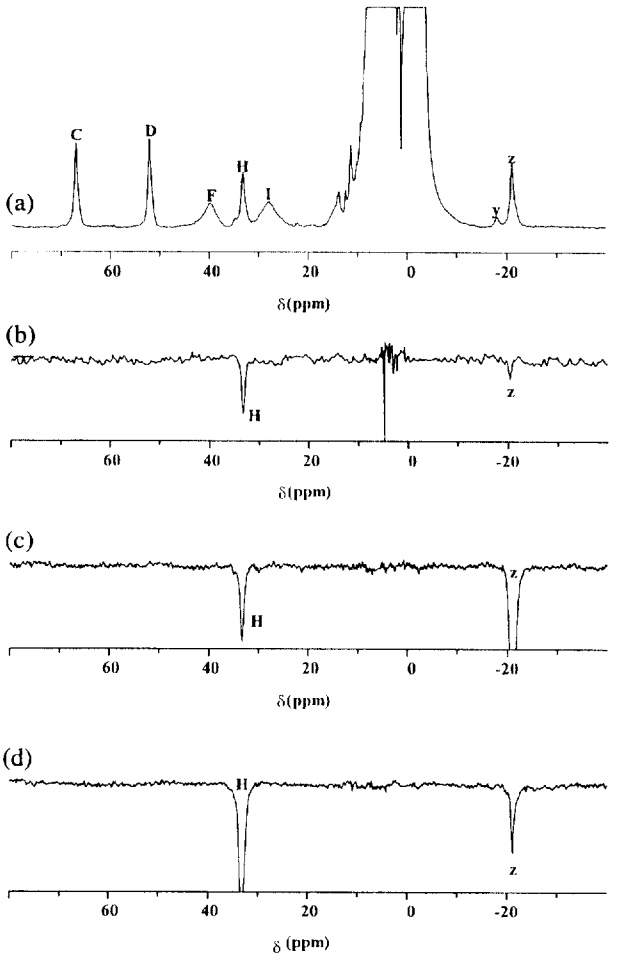

Fig. 4. ^{1}H NMR 300 MHz NOE experiments of *Rhus vernicifera* Ni(II)St in D_2O at 313 K and pH 7.0: (a) reference spectrum; (b) NOE difference spectrum performed by irradiation of signal B (located outside the spectral window); (c) NOE difference spectrum performed by irradiation of signal z; (d) NOE difference spectrum performed by irradiation of signal H.

Fig. 3(a) illustrates the downfield region of the ^{1}H NMR spectrum of Ni(II)St in H_2O at pH 4.0 and 278 K. The temperature and pH conditions were selected so as to provide the optimal conditions for NOE detection, according to the exchangeable nature of these protons. Saturation of the exchangeable signal G yields a detectable NOE with resonance C (Fig. 3(b)). Irradiation of signal C yielded the expected reciprocal NOE to signal G (Fig. 3(c)), but no NOEs to other nonlabile protons could be detected. Since the His ligands in blue copper proteins bind the metal through their Nδ1 atom, the Hε2 is equidistant to the Hδ2 and Hε1 nuclei. The Hε1 nuclei, being closer to the metal, give rise to broader signals and it is difficult to find NOEs on them [23]. This allows us to conclude that signal C corresponds to the Hδ2 of His92. By comparison with previous assignments in other Ni(II)-substituted blue copper proteins [20,24], resonance D at 57 ppm is the most likely candidate for the Hδ2 of His46. Unfortunately, selective irradiation of signal D was difficult to achieve, owing to its partial overlap with resonance E.

Signals A and B are a peculiar feature of the ^{1}H NMR spectrum of Ni(II)-substituted blue copper proteins (Fig. 2(a)). The large hyperfine shifts and fast nuclear relaxation rates indicate that they correspond to a β-CH_2 moiety of a Cys residue strongly bound to the metal ion [20,24]. Additional evidence for the identity of A and B came from the following experiments. Irradiation of signal A gives very weak NOEs with signals H and z (not shown), whereas resonance B exhibits a clear NOE with resonance H and a smaller NOE with resonance z (Fig. 4(b)). According to the structure of the metal site in St, the Cys 112 β-CH_2 moiety is found facing the Gln γ-CH_2 [11,7]. These data are consistent with signals H and z corresponding to protons from the axial Gln 97. To confirm the proposed origin of resonances H and z, as well as their assignments to individual protons, additional NOE experiments were performed. Thus, saturation of peak z yielded a strong NOE (ca. 50%) with H (Fig. 4(c)). The reciprocal NOE is also observable when signal H is irradiated (Fig. 4(d)). The observed intensities for these NOEs allow us to establish that signals H and z correspond to a pair of geminal protons. This pattern of connectivities is compatible with signals H and z being Gln Hγ1 and Hγ2, respectively. A WEFT–NOESY experiment allowed us to detect an intense cross-peak between signals H and z. Both resonances also exhibit a common dipole–dipole connectivity with a signal located at 0.5 ppm, most likely a Gln Hβ (Fig. 5). Signal z exhibited another cross-peak with a signal at 3.0 ppm, attributable also to a proton belonging to the Gln ligand.

No dipolar connectivities could be detected for the two broad signals F and I. Their shifts and relaxation times are typical of the Hε1 of histidines coordinated to Ni(II) through their Nδ1 atoms [20,24]. We therefore tentatively assign them as the Hε1 of His46 and 92.

At this point, only signal y remains unassigned. This resonance corresponds to an exchangeable proton, since it is absent in spectra recorded in D_2O solution. A similar signal in Ni(II)Met121Gln azurin has been assigned to the Hε21 of the axial Gln ligand [20]. With the rest of the spectral assignments being similar, we feel confident in assigning this resonance to the equivalent amide proton of Gln 97 in Ni(II)St. We tried unsuccessfully to find a dipolar connectivity between this resonance and signal z. If the Gln binds the metal via the nitrogen atom, the amide protons will be too close to the metal ion to be observable. On the other hand, assuming an oxygen-mediated binding, the Gln Hε21 and Hε22 are expected to be at 4.5 and 3.1 Å from the metal ion, respectively [7]. This confirms that only the former proton can give rise to a detectable NMR signal. This reasoning implies that the possibility that Gln 97 is binding the metal through the nitrogen atom may be discarded, as in the Met121Gln azurin mutant. These data, together with a recent study on the alkaline transition in Cu(II) and Co(II) St, clearly demonstrate that the nitrogen atom binds the metal in the whole pH range in which the protein integrity is preserved.

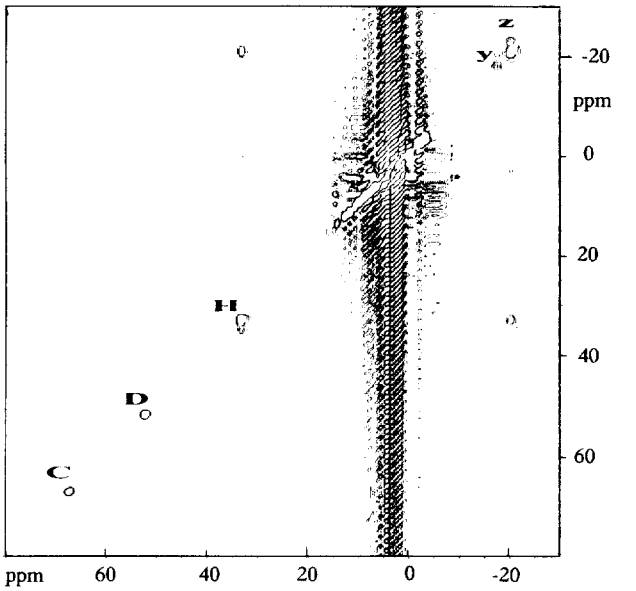

Fig. 5. ^{1}H NMR 200 MHz WEFT-NOESY spectrum of *Rhus vernicifera* Ni(II)St in D_2O at pH 7.0 and 313 K. A mixing time of 10 ms, a recycle time of 60 ms and an intermediate delay $\tau = 40$ ms were used.

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References

[1] H.B. Gray and W.R. Ellis, Jr., in I. Bertini, H.B. Gray, S.J. Lippard and J.S. Valentine (eds.), Bioinorganic Chemistry, University Science Books, Mill Valley, CA, 1994, p. 315.

[2] J.A. Guckert, M.D. Lowery and E.I. Solomon, J. Am. Chem. Soc., 117 (1995) 2817.

[3] B.E. Ramirez, B.G. Malmstrom, J.R. Winkler and H.B. Gray, Proc. Natl. Acad. Sci. USA, 92 (1995) 11 949.

[4] A.G. Sykes, in A.G. Sykes (ed.) Advances in Inorganic Chemistry, Academic Press, New York, 1991, p. 377.

[5] E.I. Solomon, M.J. Baldwin and M.D. Lowery, Chem. Rev., 92 (1992) 521.

[6] J.M. Guss, P.R. Harrowell, M. Murata, V.A. Norris and H.C. Freeman, J. Mol. Biol., 192 (1986) 361.

[7] P.J. Hart, A.M. Nersissian, R.G. Herrman, R.M. Nalbandyan, J.S. Valentine and D. Eisenberg, Protein Sci., 5 (1996) 2175.

[8] E.T. Adman, Adv. Protein Chem., 42 (1991) 144.

[9] A.M. Nersissian, Z. Mehrabian, R.M. Nalbandyan, P.J. Hart, G. Fraczkiewicz, R.S. Czernuszewicz, C.J. Bender, J. Peisach, R.G. Herrman and J.S. Valentine, Protein Sci., 5 (1996) 2184.

[10] B.A. Fields, J.M. Guss and H.C. Freeman, J. Mol. Biol., 222 (1991) 1053.

[11] A.J. Vila and C.O. Fernandez, J. Am. Chem. Soc., 118 (1996) 7291.

[12] R.W. Strange, B. Reinhammar, L. Murphy and S.S. Hasnain, Biochemistry, 34 (1995) 220.

[13] I. Bertini, P. Turano and A.J. Vila, Chem. Rev., 93 (1993) 2833.

[14] I. Bertini and C. Luchinat, NMR of Paramagnetic Molecules in Biological Systems, Benjamin/Cummings, Menlo Park, CA, 1986.

[15] L. Banci, I. Bertini and C. Luchinat, Nuclear and Electron Relaxation. The Magnetic Nucleus — Unpaired Electron Coupling in Solution, VCH, Weinheim, 1991.

[16] I. Bertini and C. Luchinat, NMR of Paramagnetic Substances, Elsevier, Amsterdam, 1996.

[17] L. Banci, I. Bertini, L.D. Eltis, I. Felli, D.H.W. Kastrau, C. Luchinat, M. Piccioli, R. Pierattelli and M. Smith, Eur. J. Biochem., 225 (1994) 715.

[18] M. Piccioli, C. Luchinat, T.J. Mizoguchi, B.E. Ramirez, H.B. Gray and J.H. Richards, Inorg. Chem., 34 (1995) 737.

[19] J. Salgado, H.R. Jimenez, A. Donaire and J.M. Moratal Mascarell, Eur. J. Biochem., 231 (1995) 358.

[20] J. Salgado, H.R. Jimenez, J.M. Moratal Mascarell, S. Kroes, G.C.M. Warmerdam and G.W. Canters, Biochemistry, 35 (1996) 1810.

[21] J.M. Moratal Mascarell, J. Salgado, A. Donaire, H.R. Jimenez and J. Castells, Inorg. Chem., 32 (1993) 3587.

[22] J.M. Moratal Mascarell, J. Salgado, A. Donaire, H.R. Jimenez and J. Castells, J. Chem. Soc., Chem. Commun., (1993) 110.

[23] A.J. Vila, FEBS Lett., 355 (1994) 15.

[24] J.M. Moratal Mascarell, J. Salgado, A. Donaire, H.R. Jimenez, J. Castells and M.-J. Martinez Ferrer, Magn. Reson. Chem., 31 (1993) S41.

[25] C.O. Fernández, A.I. Sannazzaro and A.J. Vila, Biochemistry, 36 (1997) 10 566.

[26] B. Reinhammar, Biochim. Biophys. Acta, 205 (1970) 35.

[27] J.A. Blaszak, D.R. McMillin, A.T. Thornton and D.L. Tennent, J. Biol. Chem., 258 (1983) 9886.

[28] V. Lum and H.B. Gray, Isr. J. Chem., 21 (1981) 23.

[29] T. Inubushi and E.D. Becker, J. Magn. Reson., 51 (1983) 128.

[30] G.N. La Mar and J.S de Ropp, in L.J. Berliner and J. Reuben (eds.) Biological Magnetic Resonance, Vol. 12: NMR of Paramagnetic Molecules, Plenum, New York, 1993, p. 1.

[31] L. Banci, I. Bertini, C. Luchinat, M. Piccioli, A. Scozzafava and P. Turano, Inorg. Chem., 28 (1989) 4650.

[32] Z. Chen, J.S de Ropp, G. Hernandez and G.N. La Mar, J. Am. Chem. Soc., 116 (1994) 8772.

[33] D. Marion and K.J. Wüthrich, Biochem. Biophys. Res. Commun., 113 (1982) 967.