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Selective observation of the Cu(I)-amicyanin metal site by paramagnetic NMR on partially oxidised samples

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Summary

The relaxation enhancement caused by paramagnetic copper(II) is used to observe selectively the metal site of copper(I)-amicyanin by one- and two-dimensional NMR spectroscopy. The paramagnetic effect is communicated to the diamagnetic protein through the electron self-exchange reaction in partially oxidised samples, and can be used for the selective detection of protons around the metal. Relaxation-selective NMR pulse sequences, like super-WEFT and WEFT-NOESY, are used to achieve the desired selection of the signals. The spectra obtained show well-resolved signals corresponding to protons within a radius of ~7 Å from the metal, including almost all protons from the coordinated residues. A significant increase in resolution as well as selection of the most relevant part of the protein (close to the active centre) are the principal advantages of this technique, which can be used to obtain specific information about the metal site in blue copper proteins, to assist in the assignment of their NMR spectra and to determine functional properties like the electron self-exchange rate.

Introduction

The presence of a paramagnetic metal ion in a macromolecule confers on it unique spectroscopic properties that can be used to probe the metal site (Bertini and Luchinat, 1986). Such paramagnetic properties have been extensively exploited by means of so-called 'paramagnetic NMR' spectroscopy, for which special strategies and pulse sequences have been developed in the last few years (Bertini et al., 1993; La Mar and de Ropp, 1993; Banci et al., 1994; La Mar, 1995). For relatively large molecules, like metalloproteins, rotation is slow, and in the presence of a paramagnetic metal centre the correlation time for nuclear relaxation is in most cases dominated by the electron relaxation of the metal ion (Banci et al., 1991, 1994; Bertini et al., 1993; La Mar and de Ropp, 1993; La Mar, 1995). Thus, the actual value of the electron spin relaxation may impose a severe limitation for the detection of paramagnetically affected nuclei by means of NMR (Bertini and Luchinat, 1986; Bertini et al., 1993). In cases where τ_s is in the range of 10^{-11} to 10^{-12} s, like low-spin Fe(III), high-spin Fe(II), high-spin Co(II), Ni(II) and most lanthanides(III), paramagnetic NMR signals will be sharp enough to be detected and studied (Bertini et al., 1993). In contrast to this, the relatively slow electron relaxation of Cu(II) ($1–5\times10^{-9}$ s) (Banci et al., 1991) has normally prevented the observation of paramagnetic signals from Cu(II) proteins. In these cases, substitution of Cu(II) by the faster relaxing Co(II) or Ni(II) in blue copper proteins (Vila, 1994; Piccioli et al., 1995; Salgado et al., 1996), or the increase of the electron relaxation of Cu(II) itself through magnetic coupling with Co(II) or Ni(II) in a bimetallic site, as in the case of superoxide dismutase (Bertini et al., 1994), are successful approaches that have been used to study the metal site by paramagnetic NMR.

Recently, however, we have demonstrated that isotropically shifted ^{1}H NMR resonances can indeed be observed for the paramagnetic forms of small blue copper proteins (cupredoxins), like amicyanin and azurin (Kal-

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Abbreviations: ESE, electron self-exchange; WEFT, water eliminated Fourier transform; 1D, 2D, one-, two-dimensional; NOESY, nuclear Overhauser enhancement spectroscopy.

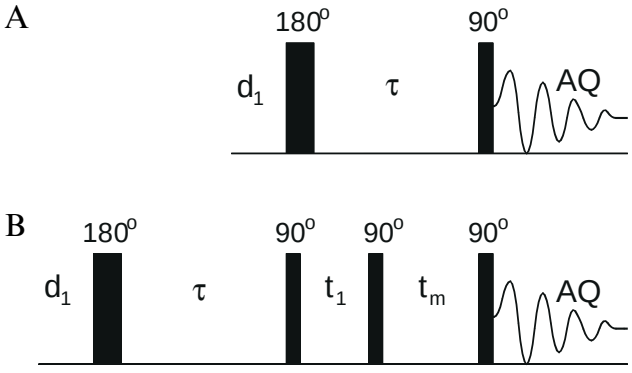

Fig. 1. Schematic representation of the (A) super-WEFT and (B) WEFT-NOESY pulse sequences.

verda et al., 1996). These are small single copper containing proteins with an electron transfer function and singular spectroscopic properties, like a small $A_{\parallel}$ value in their EPR spectra and an intense absorption band at around 600 nm (Chapman, 1991). The metal ion is tightly bound to one cysteine and two histidine residues and more weakly to a methionine in an axial position (Baker, 1988; Chapman, 1991; Romero et al., 1994). Due to the pseudo-tetrahedral geometry of the copper site and the strong coordination to a (cysteine) sulphur in these proteins, the electron relaxation is faster than in other copper proteins, where the metal coordinates to oxygen and nitrogen donor atoms in a tetragonal geometry (Banci et al., 1991; Kroes et al., 1996). These characteristics make the cupredoxins unique among the copper proteins (Kroes et al., 1996), allowing the observation of isotropically shifted proton signals from the coordinated residues, provided these nuclei are not closer to the metal than ~4.5 Å (Kalverda et al., 1996). Thus, isotropically shifted signals belonging to the four coordinated residues in amicyanin (His^{54}, His^{96}, Cys^{93} and Met^{99}) have been assigned, and from the evaluation of their contact shifts, the amount of unpaired electron spin density delocalised over the different ligand residues was estimated (Kalverda et al., 1996).

In the present paper we describe a different way of profiting from the paramagnetic properties of copper to probe the metal site in the cupredoxins by means of NMR. In this case, protons around the metal centre, belonging to the reduced (diamagnetic) Cu(I) protein, are selectively observed in the NMR spectrum of samples containing small amounts of the oxidised (paramagnetic) Cu(II) protein. As a consequence of the electron self-exchange (ESE) reaction between the reduced and oxidised species (Marcus, 1963), the nuclei close to the copper experience a magnetic perturbation every time the protein becomes oxidised. The result is a sizeable increase in the effective relaxation of the nuclei. Since the paramagnetic relaxation enhancement depends strongly on the nucleus–metal distance (Solomon, 1955; Banci et al., 1991), the effect is felt mainly by protons in the immediate surroundings of the copper, and so it can be used to study selectively the protein metal site.

Materials and Methods

Preparation of samples

Amicyanin was obtained and purified as described previously (Kalverda et al., 1994). Samples for NMR measurements contained 6 mM amicyanin in either 99.95% or 10% D_2O and buffered at pH 7.0 (uncorrected pH meter reading) with 50 mM potassium phosphate. They were prepared by using Amicon ultrafiltration equipment. The reduced Cu(I)-amicyanin was prepared by the addition of sodium dithionite, which was subsequently eliminated from the sample by washing it with oxygen-free buffer in an ultrafiltration unit. Slightly oxidised samples were prepared by the addition of Cu(II)-amicyanin to the reduced protein (both at the same concentration) under oxygen-free conditions.

NMR spectroscopy

^{1}H NMR spectra were recorded on a Bruker DMX 600 MHz spectrometer. For the 1D spectra, the super-WEFT pulse sequence (Inubushi and Becker, 1983) (Fig. 1A) was used. It has been designed for the selective observation of fast-relaxing signals and consists of an inversion recovery sequence which is run under fast pulsing conditions (Inubushi and Becker, 1983). Good signal/noise ratios were achieved by using interpulse delays (τ) which were 70–90% of the total effective relaxation delay (d_1 + AQ in Fig. 1). Thus, typical 1D spectra were recorded using an acquisition time of around 50 ms and a relaxation delay (τ) between the 180° and 90° pulses of 35–45 ms, which gives a repetition rate of around 10 s^{-1}. No presaturation of the residual solvent peak was performed since it is eliminated by the super-WEFT sequence as are the rest of the diamagnetic signals of the spectrum. WEFT-NOESY (Fig. 1B) spectra were performed as described in the literature (Chen et al., 1994), with an acquisition time of 50 ms, an interpulse delay (τ) of 50 ms and a mixing time of 20–50 ms.

1D NMR spectra were processed by using a 60 Hz line broadening (lb) window function for the observation of the paramagnetically shifted signals in completely oxidised samples, or 10 Hz lb in the case of the slightly oxidised samples. 2D spectra were processed with ~60° shifted cosine apodization window functions in both dimensions.

Results and Discussion

Influence of the degree of oxidation

When reduced amicyanin (Figs. 2A and B) is mixed with a small amount of the oxidised protein, the 1D NMR spectrum of the sample shows sizeable broadening in some of the resonances, indicating an increase in the

relaxation rate of these protons. This effect, which is due to the ESE reaction between the oxidised and reduced species of the protein in the sample, has been largely used in the past to assign the affected resonances to residues close to the metal centre (Canters et al., 1984), to obtain structural information (Groeneveld and Canters, 1988), as well as to calculate the rate of the ESE reaction (Groeneveld and Canters, 1985,1988; Lommen and Canters, 1990). In all these cases the interpretation of the spectra encountered experimental problems like poor resolution and overlap of the signals.

However, we can take advantage of the paramagnetic effect by recording and processing the spectra in a different way. The increased relaxation rate of some resonances permits their selection from the bulk of the slowly relaxing signals of the protein by using an appropriate pulse sequence, like the super-WEFT (Inubushi and Becker, 1983) (see the Materials and Methods section). The result is shown in Figs. 2C–F, where 1D super-WEFT spectra of amicyanin at different degrees of oxidation are plotted. As can be seen, the increase of relaxation experienced by certain protons of the diamagnetic protein in the presence of only 3% of the oxidised protein is already enough to allow their selection (Fig. 2C). These signals are more prominent when 5% of the Cu(II) protein is present (Fig. 2D), and almost all of them correspond to protons of coordinating residues. If we increase the amount of Cu(II)-amicyanin up to approximately 13%, the initially selected signals become more evident while they are also getting broader (Fig. 2E). At this stage around 30 signals are observed, many of them well resolved. They correspond to protons within a radius of ~7 Å from the copper, in which we basically find the four coordinated residues and a few other ones also close to the metal centre, like Met[29], Tyr[31] and Ile[26] (see Table 1 and Fig. 3).

When increasing the concentration of the oxidised amicyanin up to 25% (Fig. 2F), no further advantage is gained. On the contrary, overlap becomes more severe and resolution decreases because of increased line broadening. Additionally, at this relatively large concentration of Cu(II)-amicyanin, signals arising from the paramagnetic species itself start appearing, which complicates the analysis of the spectra. Eventually, when the concentration of the Cu(II)-amicyanin is high enough, isotropically shifted signals corresponding to the paramagnetic species appear outside the protein envelope (Fig. 4). Some of them are extremely broad and they have been assigned to the coordinated residues of the Cu(II) protein (Kalverda et al., 1996).

2D NMR spectra of partially oxidised amicyanin solutions

The resonances observed in the spectra of Fig. 2 are not shifted during the titration process, indicating that the ESE reaction is slow on the chemical shift time scale (Sandström, 1982). This allows a direct comparison with the spectrum corresponding to the totally reduced amicyanin, whose complete assignment has been reported (Lommen et al., 1991). Such comparative assignment can be tested by looking for the expected dipolar couplings in a 2D NOESY spectrum recorded by using the same relaxation-selective approach. Figure 5B shows a 30 ms mixing time WEFT-NOESY (Chen et al., 1994) spectrum of a sample containing 12% oxidised protein. The result is a 'cleaned-up' version of the NOESY spectrum of the completely diamagnetic protein (Fig. 5A), which specifically shows the dipolar couplings among protons within an ~7

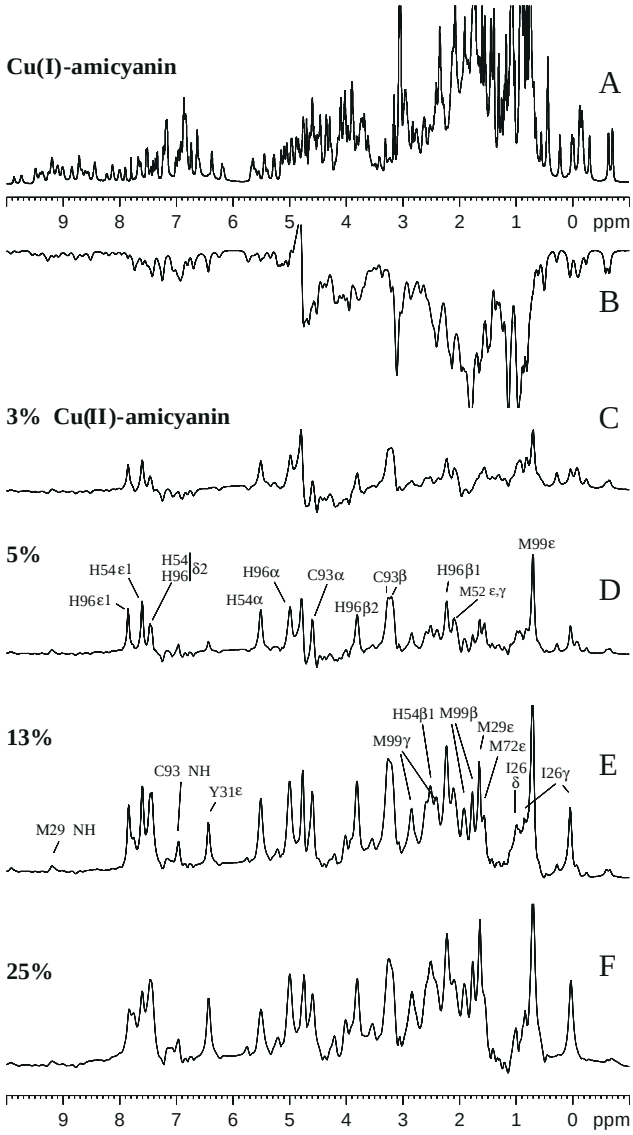

Fig. 2. 1D ^{1}H NMR spectra of amicyanin (pH 7.0, 310 K, D_2O solvent) at different degrees of oxidation. (A) 1D ^{1}H NMR spectrum of the completely reduced sample. The spectrum has been recorded using the d_1-90°-AQ pulse sequence using a low-power presaturation of the water line during the d_1 and a 550 ms recycle time. (C–F) 1D super-WEFT spectra of the same sample as in A but in the presence of increasing amounts of oxidised Cu(II)-amicyanin. When the super-WEFT sequence is applied at the same conditions as in C–F to the reduced amicyanin, no fast-relaxing signals are selected (B).

TABLE 1
^{1}H NMR RESONANCES SELECTED IN SUPER-WEFT SPECTRA OF PARTIALLY OXIDISED AMICYANIN AT pH 7.0 AND 310 K

^{1}H resonance		R[a] (Å)	δ_{red}[b] (ppm)	δ_{ox}[c] (ppm)	^{1}H resonance		R[a] (Å)	δ_{red}[b] (ppm)
His^{54}	H^{α}	2.91	5.50	–[d]	Ile^{26}	$H^{\gamma 1}$	7.35	0.84
	$H^{\beta 2}$	3.36	3.25	–9.2		$H^{\gamma 2}$	5.80[e]	0.04
	$H^{\beta 1}$	4.61	2.48	–2.5		$H^{\delta 1}$	7.04[e]	0.99
	$H^{\delta 2}$	5.23	7.47	43	Met^{29}	H^{N}	6.92	9.19
	$H^{\varepsilon 1}$	3.06	7.58	–[d]		H^{α}	5.09	3.80
	$H^{\varepsilon 2}$	5.00	13.4	27.5		H^{ε}	5.60[e]	1.65
Cys^{93}	H^{N}	5.44	6.97	–[f]	Tyr^{31}	H^{ε}	6.35	6.43
	H^{α}	4.87	4.57	14.1	Met^{52}	H^{ε}	7.25[e]	2.10
	$H^{\beta 2}$	2.99	3.25	–[d]		H^{γ}	4.76	2.10
	$H^{\beta 1}$	3.18	3.15	–[d]	Met^{72}	H^{ε}	5.58[e]	1.57
His^{96}	H^{α}	5.46	5.00	–[f]	Phe^{92}	H^{α}	7.56	5.72
	$H^{\beta 2}$	4.05	2.20	–[d]	Thr^{94}	H^{N}	5.47	9.90
	$H^{\beta 1}$	2.69	3.77	–[d]	Phe^{98}	H^{ζ}	5.96	7.66
	$H^{\delta 2}$	5.34	7.50	50				
	$H^{\varepsilon 1}$	3.44	7.84	–[d]				
Met^{99}	H^{α}	6.85	4.50	–[f]				
	$H^{\beta 2}$	4.92	1.92	–[f]				
	$H^{\beta 1}$	4.21	1.77	–[f]				
	$H^{\gamma 2}$	4.64	2.84	12				
	$H^{\gamma 1}$	5.22	2.41	11				
	H^{ε}	4.20[f]	0.71	–[f]				

[a] Proton–metal distances in Cu(II)-amicyanin (Romero et al., 1994).
[b] Chemical shifts of protons measured in the 1D super-WEFT and 2D WEFT-NOESY spectra of 12% oxidised samples.
[c] Chemical shifts of isotropically shifted protons from the Cu(II)-amicyanin (Kalverda et al., 1996).
[d] Probably broadened beyond detection.
[e] Average value of the distance measured for the three protons of the methyl group.
[f] Not observed, shifted out of the diamagnetic region.

Å sphere around the metal. Furthermore, in a spectrum of this type, optimisation of both the mixing time and the delay (τ) between the 180° and 90° pulses (see Fig. 1B) can be combined to achieve the desired selection of the signals (Chen et al., 1994). It should also be realised that, due to the increased line broadening, a larger number of scans are normally needed to record the spectra under paramagnetic conditions. However, also as a consequence of the faster relaxation of the nuclei, these spectra can be acquired much faster, resulting in a partial compensation of the time needed to complete the spectrum.

Paramagnetic NMR on blue copper proteins: Fully versus partially oxidised samples

In a previous investigation we have demonstrated that Cu(II) can actually be used as an internal probe to inves-

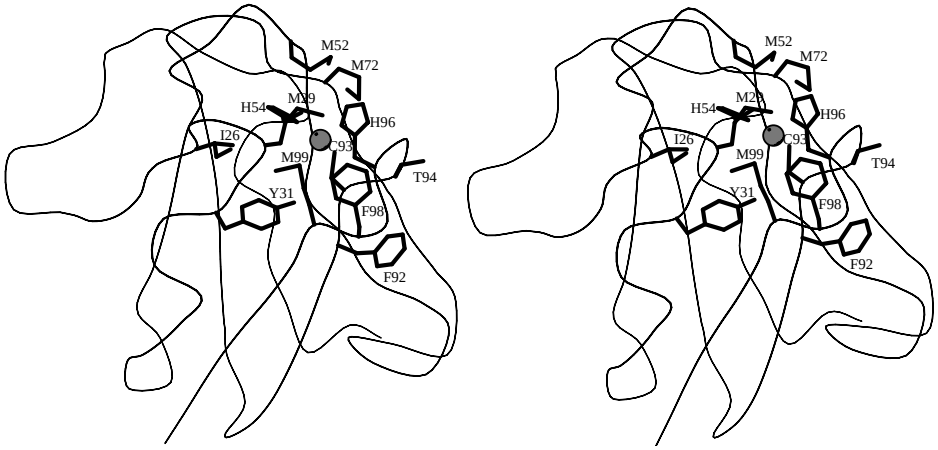

Fig. 3. Schematic stereo representation of the Cu(II)-amicyanin structure (Romero et al., 1994). The copper ion is shown as a grey sphere. The side chains of the amino acid residues which are totally or partially observed in the spectra of Figs. 2 and 5B are drawn with thick lines. The figure has been generated by using the program MOLSCRIPT (Kraulis, 1991).

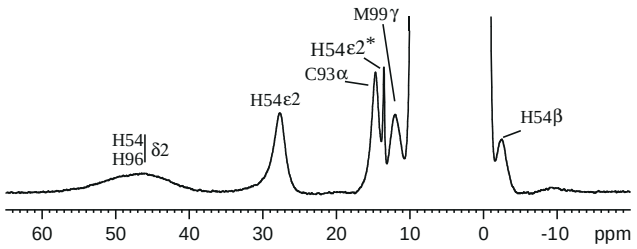

Fig. 4. Super-WEFT spectrum of an ~80% oxidised sample of copper-amicyanin in H_2O solvent at 310 K and pH 7.0 (120 kHz spectral window, 17 s^{-1} repetition rate). The labels indicate the assignment of the signals as taken from Kalverda et al. (1996). All the labelled signals correspond to isotropically shifted protons belonging to the Cu(II)-amicyanin, except the signal at 13.4 ppm, marked with an asterisk, which comes from the Cu(I)-amicyanin. This later signal displays an exchange cross peak with the signal at 27.5 ppm in an EXSY spectrum (Kalverda et al., 1996). The spectrum has been baseline corrected using the conventional Bruker software.

tigate the metal site in cupredoxins by paramagnetic NMR of the completely oxidised protein (Kalverda et al., 1996). The analysis of the spectra allows the extraction of important information, like the spin density distribution over the coordinated ligands, but the observation of protons in the surroundings of the metal is restricted to those that belong to the coordinated residues and so exhibit a significant contact contribution to the isotropic shifts. Additionally, signals from protons which are located at a distance < ~4.6 Å from the Cu(II) are broadened beyond detection. Due to these limitations, in the case of amicyanin, out of the 21 protons belonging to the coordinated residues, only eight are resolved in the paramagnetic region: the $C^{\delta 2}H$, $N^{\varepsilon 2}H$ and $C^{\beta}H_2$ of His^{54}, the $C^{\delta 2}H$ of His^{96}, the $C^{\alpha}H$ of Cys^{93} and the $C^{\gamma}H_2$ of Met^{99} (Kalverda et al., 1996) (Table 1). Protons like the $C^{\beta}H_2$ of Cys^{93}, or the $C^{\varepsilon 1}H$ of both His^{54} and His^{96}, which are ~3 Å from the copper, relax too fast to be detected in the spectrum of Cu(II)-amicyanin, and the $C^{\beta}H_2$, $C^{\alpha}H$ and $C^{\varepsilon}H_3$ protons of Met^{121}, despite being at a distance of 4.2–6.8 Å from the paramagnetic metal ion, do not shift outside the protein envelope (Kalverda et al., 1996). An additional drawback of the spectra of Cu(II)-amicyanin is the large width of some of the observed resonances, which in practice limits the possibility of obtaining dipolar or contact couplings between these nuclei.

The experimental approach presented here permits the selective observation of the resonances from almost all the protons in the immediate surroundings of the copper. This selection is based on the increased relaxation in the reduced Cu(I) protein in the presence of small amounts of the oxidised one. Since paramagnetic relaxation rapidly

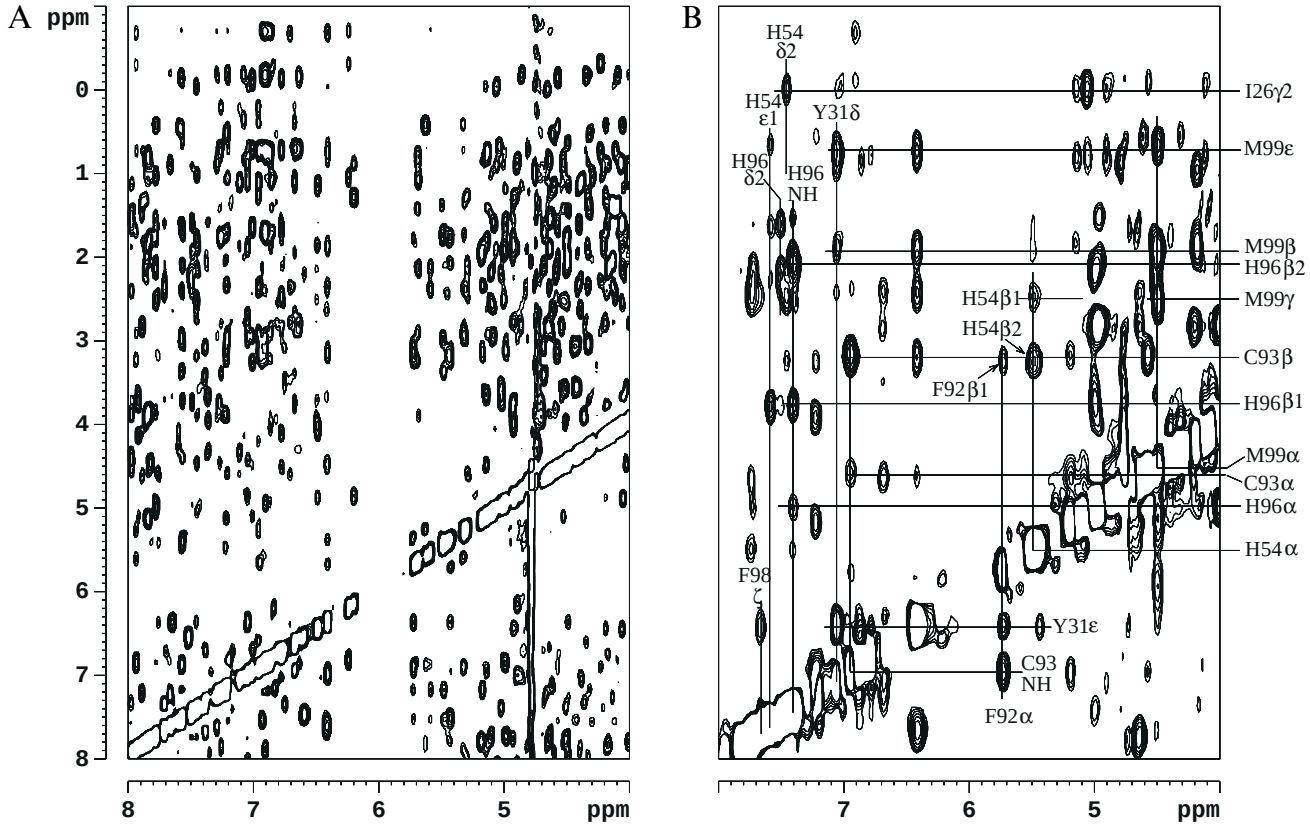

Fig. 5. Parts of the NOESY spectra of amicyanin recorded at 310 K and pH 7.0. (A) Conventional phase-sensitive NOESY spectrum recorded on a completely reduced amicyanin sample using a 120 ms mixing time, a 1 s relaxation delay and a 68 ms acquisition time. (B) Spectrum recorded by using the WEFT-NOESY pulse sequence with an interpulse delay of 50 ms, a mixing time of 30 ms and an acquisition time of 50 ms.

decays with distance, the selection principally extends up to a radius of ~5.5 Å from the metal. Protons placed as close as 2.6 Å from the copper (like the His^{96} $C^{\beta 1}H$, see Table 1) are observed in the super-WEFT spectra as relatively sharp signals. As a result, the spectra of slightly oxidised samples (less than 10%) basically contain the signals of protons from the coordinated residues. The radius of selection of signals can be extended up to 7–7.5 Å by increasing the oxidation of the sample up to 12–15%, so that signals from other, noncoordinated residues can also be resolved. The region finally covered is shown in Fig. 3. It is gratifying to see that this zone corresponds to the most significant part of the amicyanin molecule, as far as the function is concerned, since not only does it contain the residues of the metal site but also residues of the hydrophobic patch located in the surface close to the metal, like Met^{29}, Met^{52}, Ile^{26}, Met^{72}, Thr^{94} and Tyr^{31}. This has been shown to be the docking patch on amicyanin for its redox partner methylamine dehydrogenase (Chen et al., 1992).

Conditions of applicability

The successful application of the technique illustrated above hinges on the distinction that can be made with the help of the WEFT sequence between protons with normal and protons with shortened longitudinal relaxation times, the shortening being due to the occurrence of the ESE reaction. Further, as shown in the present case, the peaks in the WEFT spectra of partially oxidised samples appear to occur at positions which are identical to their positions on the diamagnetic species. This characteristic is vital when the technique is used as an assignment aid. In discussing the applicability of the method presented here, two points should therefore be briefly addressed: how do the T_1 values of the protons under study depend on the rate of the ESE reaction, and under what conditions do the peaks in the WEFT spectrum occur at their diamagnetic positions.

(1) *Longitudinal relaxation:* The way in which the relaxation of protons is affected by the exchange reaction depends on the relative values of the (first-order) exchange rate (k) and the difference between the nuclear spin relaxation rates in the diamagnetic and paramagnetic species ($T_{i,ox}^{-1} - T_{i,red}^{-1}$, i = 1,2) (McLaughlin and Leigh, 1973). In the general case of exchange between two species, the nuclear spin lattice relaxation becomes biexponential (Leigh, 1971). Two limiting cases, in which the magnetisation decay becomes monoexponential, can be distinguished depending on the magnitude of the exchange rate k, which is given by:

$$k = (1/\tau_{ox} + 1/\tau_{red}) = k_{ese}[red] + k_{ese}[ox] = k_{ese}[prot] \quad (1)$$

Here k_{ese} is the second-order exchange rate constant, τ_{red} and τ_{ox} are the lifetimes of the reduced and oxidised species, [red] and [ox] are their corresponding concentrations, and [prot] is the total protein concentration. In the fast-exchange limit, the difference in relaxation is very small compared to the exchange rate ($k \gg T_{i,ox}^{-1} - T_{i,red}^{-1}$), and the spins in the reduced and oxidised species relax with a common relaxation rate. In the slow-exchange limit, the difference between the two spin relaxation rates is so large compared to the rate of exchange between the paramagnetic and diamagnetic environments ($k \ll T_{i,ox}^{-1} - T_{i,red}^{-1}$) that the two spins relax independently and with different rates. Simplified expressions for the proton relaxation rates of the diamagnetic species have been derived corresponding to the above two conditions (McLaughlin and Leigh, 1973). These are, for the longitudinal relaxation rates of importance here,

$$T_1^{-1} = T_{1,red}^{-1} + f_{ox}\, k \text{ (slow-exchange limit)} \quad (2)$$

$$T_1^{-1} = (1 - f_{ox})T_{1,red}^{-1} + f_{ox}\, T_{1,ox}^{-1} \text{ (fast-exchange limit)} \quad (3)$$

where f_{ox} is the molar fraction of the paramagnetic (oxidised) species. These equations clearly show that the increase of the relaxation of protons in the diamagnetic species depends on the exchange rate of the equilibrium, for the slow-exchange conditions, and on the intrinsic relaxation of the proton in the paramagnetic species, for the fast-exchange conditions. It is important to realise at this point that different exchange limits may apply for different protons of the same molecule, since, although they are under the same ESE equilibrium, the paramagnetic relaxation will vary strongly depending on the distance of these protons to the paramagnetic metal ion. Thus, protons very close to the copper, essentially those that belong to the coordinated residues, are most likely to experience slow-exchange conditions, while fast-exchange conditions apply for protons placed farther away, typically arising from noncoordinated residues. As stated above, in the intermediate case the nuclear relaxation becomes biexponential and a detailed numerical analysis is needed to see if the WEFT parameters can be chosen such that a discrimination is possible between the signals of the protons under consideration and the main body of the diamagnetic signals of the protein.

Having the possibility of selection of nuclei in the NMR spectra depending on their relaxation rates, it is now clear how the number of protons selected is limited by the condition imposed by Eq. 3, since, when we move far from the paramagnetic centre, $T_{1,ox}^{-1}$ decreases rapidly and the corresponding protons do not exhibit enough relaxation to be significantly selected by the super-WEFT sequence. Additionally, according to Eq. 2 the exchange rate should be large enough to significantly increase the relaxation of the protons under slow-exchange conditions. The blue copper proteins exhibit normally fast ESE rates and so they are suitable systems for the application of the methodology reported here.

(2) *Peak positions:* A different criterion applies when we take into consideration the positions at which the peaks occur in the WEFT spectra. The critical parameter in this case is the difference in the chemical shifts of the protons from the reduced and oxidised species, $\Delta\nu$ (McLaughlin and Leigh, 1973; Sandström, 1982). If $\Delta\nu$ is large as compared to the first-order exchange rate, different positions are observed for the resonances of the oxidised and reduced species ('well-resolved' case), provided that $\Delta\nu$ is also larger than the width of each resonance (T_2^{-1}). On the other hand, when the exchange is larger than $\Delta\nu$ ('exchange narrowed' case), both resonances average to one line, the position of which depends on the concentration of the two exchanging species (McLaughlin and Leigh, 1973; Sandström, 1982). For the protons of the coordinated residues in amicyanin, well-resolved conditions apply, since $\Delta\nu$ is large due to relatively large contact shifts in the Cu(II) protein. If the exchange rate is larger, as in the case of azurin, this may come close to the intermediate case, and so lead to considerable line broadening (Kalverda et al., 1996). With regard to the protons of the noncoordinated residues, their resonance positions in the spectra of the oxidised and reduced species are similar since the pseudocontact contribution to the isotropic shifts in the Cu(II) species is small (Kalverda et al., 1996). So the chemical shifts of the later resonances do not change appreciably in the presence of different (small) concentrations of Cu(II)-amicyanin and we can assume them to be identical to those in the completely reduced sample.

Conclusions

In conclusion, the paramagnetic properties of Cu(II) can be used to selectively observe the signals from the metal-site residues in the reduced Cu(I)-amicyanin. This selection is based on the increase of relaxation that some protons from the diamagnetic species experience in the presence of the paramagnetic protein due to the ESE reaction, and is limited to nuclei within a radius of ~7 Å around the metal ion. The combination of this approach with the study of the isotropic shifts of the completely oxidised Cu(II) protein (Kalverda et al., 1996) is a powerful method for the investigation of blue copper proteins that can be of great help for the characterisation of their metal sites by means of NMR.

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