Paramagnetic NMR studies of copper-containing nitrite reductases

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Paramagnetic signals have been observed in the ^{1}H NMR spectra of copper-containing nitrite reductases ($M_r \approx 110$ kDa), which provide a detailed fingerprint of the type 1 centres in these proteins.

Copper-containing nitrite reductases (NiRs) function in the dissimilatory denitrification pathway of certain micro-organisms, reducing nitrite to nitric oxide.[1] Structural studies have shown that these proteins are trimeric[2–4] with each subunit ($M_r \approx 36$ kDa) possessing a type 1 copper site. In addition, the protein possesses three inter-subunit type 2 copper centres (sites of nitrite binding) in close proximity to the type 1 centres. The arrangement of the two copper centres in one of the monomers of nitrite reductase is shown in Fig. 1. The presence of type 1 centres in NiRs results in them having unique spectroscopic features, including intense LMCT bands in their visible spectra. NiRs can be either green or blue, with the distinction due to differences at the type 1 centre.[4–7] Herein, we investigate the differences at the type 1 centres of the green NiR from *Achromobacter cycloclastes* and the blue NiR from *Alcaligenes xylosoxidans* using paramagnetic ^{1}H NMR spectroscopy.

Recent studies have demonstrated that isotropically shifted ^{1}H NMR resonances can be observed and assigned for simple, low molecular weight ($M_r \approx 12$ kDa), type 1 copper proteins (cupredoxins).[8,9] In all cases, a number of broad resonances can be observed and assigned. The assigned spectra provide a detailed fingerprint of the active sites of the various cupredoxins. We now show that similar resonances can be observed for the type 1 centre in the more complex copper-containing nitrite reductases. This is possibly the largest copper protein that has been studied to date using paramagnetic NMR spectroscopy. Interpretation of the isotropic shifts experienced by these resonances provides details of the structure of the type 1 copper centres in these proteins and highlights differences between blue and green NiRs.

The paramagnetic ^{1}H NMR spectrum[10] of the type 2 depleted (T2D, *i.e.* with copper removed from the type 2 centres) green NiR from *A. cycloclastes*[12] is presented in Fig. 2A. Also shown (Fig. 2B) is the spectrum of the protein which possesses > 80% Cu(II) at its type 2 centre.[14] The spectrum of the T2D blue NiR from *A. xylosoxidans*[15] is shown in Fig. 2C. The chemical shifts of the observed resonances are listed in Table 1 along with spin–lattice (T_1) relaxation times.

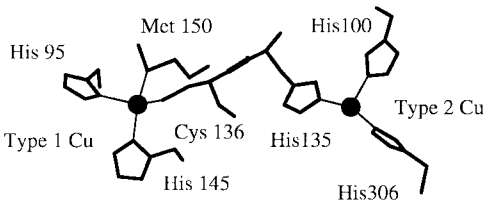

Fig. 1 Structure of the Cu(II) sites in one of the monomers of NiR (the His306 ligand of the type 2 copper centre originates from the adjacent monomer). Black spheres indicate the copper ions. This picture was produced using the coordinates of the crystal structure of the protein from *A. cycloclastes*.[3]

The spectra of the green NiR and the corresponding T2D enzyme show no significant differences in chemical shift values and relaxation times (see Table 1). This clearly demonstrates that all of the observed resonances are due to protons associated with the type 1 centre, and that the binding of Cu(II) to the type 2 centre has no effect on the structure, or electronic properties, of the type 1 site.[16,17] The temperature dependence of the chemical shifts of the resonances observed in Fig. 2A has been investigated in the range 292–328 K, and in all cases they exhibit Curie-type behaviour (data not shown).

In previous paramagnetic ^{1}H NMR studies on the oxidised forms of the cupredoxins amicyanin[8] and plastocyanin,[9] the assignment of paramagnetic resonances has relied upon the ability to correlate these peaks to their counterparts in the spectrum of the reduced (diamagnetic) protein, utilising the electron self-exchange reaction. This is clearly not possible in the case of NiRs. However, a comparison to the assigned cupredoxin spectra provides a very reliable initial analysis of the NiR spectra.

The exchangeable resonance d arises from one of the N$^{\varepsilon 2}$H of the two His ligands. Spectra were acquired of the proteins in H_2O–D_2O (9 : 1, v/v) at lower pH values, but there was no evidence for a second such resonance.[18] These observations

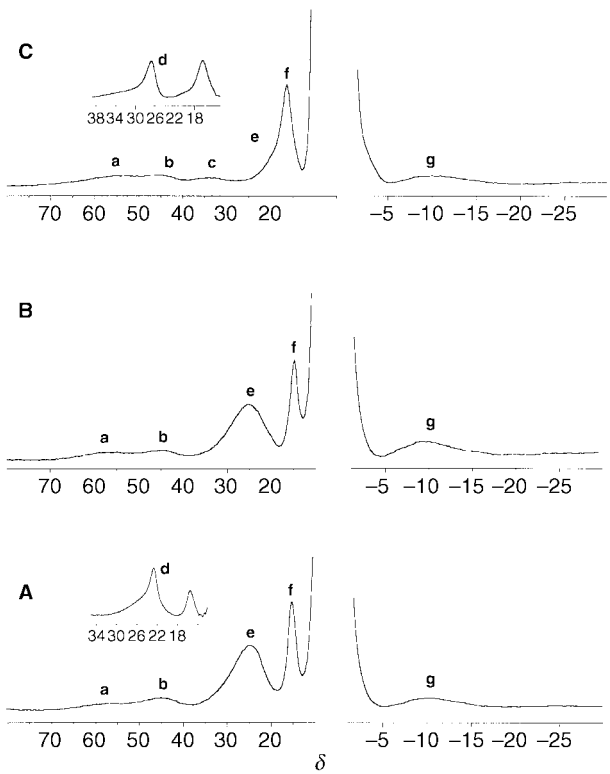

Fig. 2 ^{1}H NMR spectra of Cu(II) NiRs (500 MHz, 25 °C): A, T2D green NiR in D_2O at pH 7.5 with the inset showing part of the spectrum in H_2O at pH 7.5; B, green NiR in D_2O at pH 7.0; **C**, T2D blue NiR in D_2O at pH 7.5 with the inset showing part of the spectrum in H_2O at pH 7.5.

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Table 1 Hyperfine shifted resonances in the 500 MHz ^{1}H NMR spectrum of Cu(II) NiRs at 25 °C

	T2D green[a] NiR		Green[a] NiR		T2D Blue[b] NiR		
Resonance	δ_{obs}[c]	T_1[d]/ms	δ_{obs}[c]	T_1[d]/ms	δ_{obs}[c]	T_1[d]/ms	Assignment[e]
a	~58	n.d.[f]	~58	n.d.	~54	n.d.	His C$^{\delta 2}$H
b	~45	n.d.	~45	n.d.	~44	n.d.	His C$^{\delta 2}$H
c					~33	n.d.	His C$^{\varepsilon 1}$H
d	22.7	2.9			26.6	2.6	His95 N$^{\varepsilon 2}$H
e	25.1	0.46	25.3	0.40	< 18	n.d.	Met150 C$^{\gamma}$H
f	15.3	6.3	15.1	6.0	16.3	4.8	Asn96 C$^{\alpha}$H
g	−10.8	n.d.	−10.5	n.d.	−10.5	n.d.	Cys136 C$^{\alpha}$H

[a] *A. cycloclastes.* [b] *A. xylosoxidans.* [c] Estimated error ±0.1 for sharp resonances and ±0.3–0.5 for broader peaks. [d] Estimated error is ±5%; this is larger for the very fast relaxing signals. [e] The numbering of the residues is as found in NiR from *A. cycloclastes*. [f] n.d. = not determined.

point to peak d belonging to the N$^{\varepsilon 2}$H of the more buried His95 ligand in both proteins.[19] The corresponding resonance of the exposed His145 ligand is broadened beyond detection owing to fast exchange with the bulk solvent.

The two very broad resonances a and b have counterparts in the spectra of amicyanin[8] and plastocyanin,[9] and can be assigned to the two C$^{\delta 2}$ protons of the histidine ligands. Resonance e has an apparent T_1 value of 0.46 ms in the T2D green NiR and is most likely due to one or both of the C$^{\gamma}$H signals of the axial Met150 ligand. In the paramagnetic ^{1}H NMR spectra of other cupredoxins a very broad peak, comprised of the two C$^{\varepsilon 1}$H resonances of the two histidine ligands, has been found at δ *ca.* 30–35, which could also be present in the spectrum in Fig. 2A overlapping with one, or both, of the Met C$^{\gamma}$H signals. The histidines C$^{\varepsilon 1}$H peak is present in the spectrum of the blue NiR (peak c), whilst peak e (Met150 C$^{\gamma}$H) now overlaps with peak f (*vide infra*).

Resonance f is the least paramagnetic signal in the spectra of the two T2D NiRs (see Table 1). A corresponding resonance has been found in the region δ *ca.* 12–18 in all such studies on proteins possessing type 1 centres. In the published study on Cu(II) amicyanin[8] this resonance was assigned to the C$^{\alpha}$H of the coordinated Cys ligand. However, in the Cu(II) plastocyanin work[9] the corresponding resonance has been assigned to the C$^{\alpha}$H of Asn38, the backbone amide group of which makes a strong hydrogen bond to the thiolate sulfur of the coordinated Cys ligand (the Cys C$^{\alpha}$H is upfield shifted and has a T_1 value of < 1 ms in plastocyanin, *vide infra*). The long spin–lattice relaxation time and relatively small linewidth of peak f clearly point to it belonging to the C$^{\alpha}$H of Asn96 in the NiRs, whose backbone amide makes a hydrogen bond with the S$^{\tau}$ of the ligand Cys136.

Finally, resonance g is the only upfield shifted signal observed in the spectra of the NiRs. In studies on amicyanin and plastocyanin two upfield shifted resonances have been observed. For the former, these have been assigned as the two C$^{\beta}$ protons of one of the His ligands.[8] For plastocyanin, the most upfield shifted resonance has been assigned to the C$^{\alpha}$H of the Cys ligand with the other upfield shifted resonance due to a His C$^{\beta}$H.[9] Due to the assignment of peak f as the C$^{\alpha}$H of Asn96 we conclude that peak g must belong to the C$^{\alpha}$H of Cys136.

The hyperfine shifts of the ^{1}H NMR signals associated with type 1 copper centres are made up mainly of a Fermi-contact contribution, and therefore directly provide information about the spin density distribution of the unpaired electron. The shifts observed for the two NiRs are, on the whole, very similar and are also reminiscent to those observed in the published studies on type 1 copper centres.[8,9] This is especially so for the signals derived from the two histidines, the Cys C$^{\alpha}$H and the Asn C$^{\alpha}$H resonances, consistent with very similar type 1 architectures in the various proteins. The major difference between the spectra of the blue and green NiRs lies in the shifts experienced by the Met C$^{\gamma}$H resonances (peak e). This signal in the green NiR lies at δ = 25.1, *cf.* δ < 18 in the blue enzyme. This indicates *ca.* 1.5 times more spin density on this proton in the green protein, which cannot be due to differences in the Cu–S(Met) distances as structural studies have shown this bond length (precision *ca.* 0.1 Å) to be very similar in the green (2.56 Å)[3] and blue (2.64 Å)[4] NiRs. These variations are therefore probably due to differences in the Cu–S$^{\delta}$–C$^{\gamma}$–H$^{\gamma}$ dihedral angles in the two proteins.[20] Further paramagnetic NMR studies on Co(II)-substituted NiRs are currently underway to clarify this issue.

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12 The green NiR from *A. cycloclastes* was isolated and purified as described previously.[13] The isolated enzyme possesses *ca.* 10% copper at its T2 centre and this sample was used for the T2D spectra.
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14 The incorporation of copper at the T2 centre of *A. cycloclastes* NiR was achieved by dialysing (Spectra/Por CE DispoDialyzer, MWCO 10000) the protein in 50 mM Tris buffer at pH 7.0 against a similar buffer solution containing 1 mM $Cu(NO_3)_2$. Dialysis was carried out over 4–7 days immediately prior to the acquisition of NMR spectra. The excess copper was dialysed out of the protein solution against 50 mM Tris at pH 7.0.
15 The blue NiR from *A. xylosoxidans* was isolated and purified according to a literature method.[13] The isolated enzyme possesses *ca.* 70% copper at its T2 centre. The T2D protein was obtained as described in the literature[13] and contained < 10% copper at the type 2 centre.
16 Spectra of green NiR in the presence of nitrite were also obtained, and exhibited very little difference to those of either NiR or of the T2D enzyme. This shows that binding of the substrate at the type 2 centre has very little effect on the structure and electronic properties of the type 1 site.
17 Spectra were obtained of the blue NiR with and without Cu(II) at its type 2 centre. No significant differences were observed between these spectra. For example the δ_{obs} and T_1 values of peak d in blue NiR are 26.5 and 2.9 ms, respectively.
18 In the spectrum of the T2D blue NiR in H_2O–D_2O (9:1, v/v) there is an indication of a second exchangeable resonance at δ *ca.* 46. However, this peak appears to be quite broad and overlaps with resonances a and b making any definite conclusions impossible.
19 The type 1 ligands are numbered as in *A. cycloclastes* NiR. The corresponding residues in *A. xylosoxidans* NiR are His89, Cys130, His139 and Met144.
20 Analysis of the crystal structures of the green and blue NiRs reveals that one of the Cu–S$^{\delta}$–C$^{\gamma}$–H$^{\gamma}$ dihedral angles is in the region 50°–60°. If the contact shift of the Met C$^{\gamma}$H$_2$ protons follow a $\cos^2\theta$ Karplus law, where θ is the Cu–S$^{\delta}$–C$^{\gamma}$–H$^{\gamma}$ dihedral angle, then small changes in this angle could result in quite large differences in the observed shift. It should be noted that in the crystal structures of *A. cycloclastes* NiR small differences (greater than the estimated precision of 3°) are observed in these dihedral angles for the type 1 sites in the different monomers, and also in the proteins crystallised under different conditions (see ref. 3).