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Active Site Comparison of Co^{II} Blue and Green Nitrite Reductases

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Abstract: Copper-containing nitrite reductases (NiRs) possess type 1 (T1) and type 2 (T2) copper sites and can be either green or blue in color owing to differences at their T1 centers. The active sites of a green and a blue NiR were studied by utilizing their $T1Cu^{I}$/$T2Co^{II}$ and $T1Co^{II}$/$T2Co^{II}$-substituted forms. The UV/Vis spectra of these derivatives highlight the similarity of the T2 centers in these enzymes and that T1 site differences are also present in the Co^{II} forms. The paramagnetic NMR spectra of $T1Cu^{I}$/$T2Co^{II}$ enzymes allow hyperfine shifted resonances from the three T2 His ligands to be assigned: these exhibit remarkably similar positions in the spectra of both NiRs, emphasizing the homology of the T2 centers. The addition of nitrite results in subtle alterations in the paramagnetic NMR spectra of the $T1Cu^{I}$/$T2Co^{II}$ forms at $pH < 7$, which indicate a geometry change upon the binding of substrate. Shifted resonances from all of the T1 site ligands have been assigned and the Co^{II}–N(His) interactions are alike, whereas the $C^{\beta}H$ proton resonances of the Cys ligand exhibit subtle chemical shift differences in the blue and green NiRs. The strength of the axial Co^{II}–S(Met) interaction is similar in the two NiRs studied, but the altered conformation of the side chain of this ligand results in a dramatically different chemical shift pattern for the $C^{\gamma}H$ protons. This indicates an alteration in the bonding of the axial ligand in these derivatives, which could be influential in the Cu^{II} proteins.

Keywords: copper · metalloproteins · nitrite reductase · NMR spectroscopy

Introduction

The last decade has seen a resurgence in the utilization of NMR spectroscopy to study paramagnetic copper sites in proteins.[1–14] For the mononuclear type 1 (T1) copper centers found in the cupredoxins [small electron transfer (ET) proteins] studies have shown that a number of isotropically shifted ^{1}H NMR resonances can be observed and assigned.[1,8–14] However, most of these signals are extremely broad due to the relatively slow electronic relaxation rate of the metal and a number of active site resonances cannot be observed. Assignment, and in some cases indirect signal observation (using blind saturation-transfer experiments[8–10,12]), has depended on using the electron self-exchange (ESE) reaction to correlate the shifted signals of the Cu^{II} form with their diamagnetic counterparts in the Cu^{I} cupredoxin. It was demonstrated some time ago that hyperfine shifted resonances could be observed for the ET T1 site of copper-containing nitrite reductases (NiRs), which are very large enzymes ($M_r \approx 110$ kDa)[15] but assignment of these signals using the ESE approach was not possible. The NiRs also contain a catalytic type 2 (T2) copper site whose electronic relaxation rate is ~1 order of magnitude slower than that of a T1 copper center,[16] resulting in all paramagnetically shifted resonances from the T2 center being broadened beyond detection.[15] To investigate the active sites of NiRs in more detail by NMR spectroscopy we have prepared derivatives in which the Cu^{II} ions have been replaced with the faster relaxing Co^{II}; an approach which has previously been used to study a number of copper proteins.[17–31]

The copper-containing NiRs function in the dissimilatory denitrification pathway of certain micro-organisms, reducing nitrite to nitric oxide.[32] These proteins are trimeric and in each subunit ($M_r \approx 36$ kDa) the T1 and T2 copper sites (see Figure 1) are in close proximity (~12.5 Å apart) housed in cupredoxin domains.[33,35–38] The T1 copper site has the typical His_2 Cys Met distorted tetrahedral arrangement, whereas at the T2 center the metal is coordinated by three His residues. Two of these His ligands belong to one subunit with the other from the adjacent monomer; the T2 copper site

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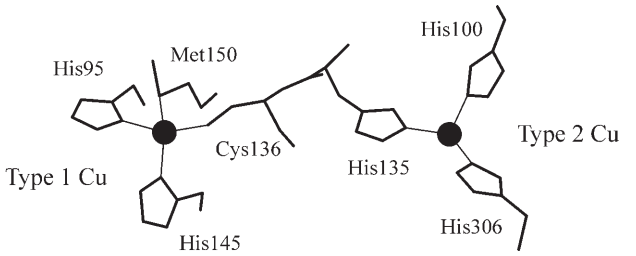

Figure 1. The structure of the copper sites in one of the monomers of *Achromobacter cycloclastes* NiR (PDB accession code 1NIA[33]) drawn with MOLSCRIPT.[34] Black spheres indicate the copper ions and the coordinating residues are labeled. The T2Cu His306 ligand is provided from the adjacent monomer and the water ligand at the T2 site has been omitted. The corresponding ligating residues in the *Alcaligenes xylosoxidans* enzyme are His89, Cys130, His139, and Met144 at the T1 site and His94, His129, and His300 at the T2 center.[35,36]

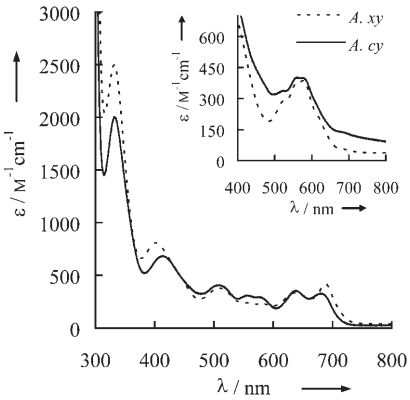

Figure 2. The UV/Vis spectra (25 °C) of the $T1Co^{II}/T2Co^{II}$ NiRs in 10 mM Hepes pH 8.0. The insert shows the spectra (25 °C) of the $T1Cu^{I}/T2Co^{II}$ NiR derivatives. Spectra were acquired in the presence of excess ascorbate.

thus has an inter-subunit location. The T2 site is completed by a water ligand that is displaced by the substrate, which binds through its oxygen atoms in an asymmetric bidentate fashion.[33,35,38]

Two distinct types of copper-containing NiRs that are either green or blue in color are known.[32,33,35–37,39] The UV/Vis and electron paramagnetic resonance (EPR) spectra of the T1 centers in these proteins are very different (see Figures S1 and S2 in the Supporting Information). Similar spectroscopic variations are observed in all cupredoxins, which has led to the classic and distorted (perturbed) T1 copper site classifications.[40–42] Classic T1 sites, such as that in the blue NiRs, have very little absorption at about 450 nm in their UV/Vis spectra and possess axial EPR spectra, whereas distorted centers, as found in the green NiRs, have increased absorbance at about 450 nm and more rhombic EPR signals (see Figures S1 and S2 in the Supporting Information). Previous NMR studies of the Cu^{II} NiRs provided an indication of an altered interaction with the axial Met ligand at the T1 sites of the blue and green enzymes.[15] In this study NMR spectroscopy is used to investigate Co^{II}-substituted forms of a blue and a green NiR to enable a more detailed comparison of both their T1 and T2 sites.

Results

UV/Vis spectra of Co^{II}-substituted NiRs: The UV/Vis spectra of the blue [*Alcaligenes xylosoxidans* (*A. xy*)] and green [*Achromobacter cycloclastes* (*A. cy*)] NiRs, which have Co^{II} only at the T2 site (these samples have copper at the T1 sites, which is maintained in the spectroscopically silent reduced form by the addition of ascorbate, and are thus referred to as the $T1Cu^{I}/T2Co^{II}$ derivatives) are shown in Figure 2. For both proteins three weak ligand-field (LF) bands are observed in the 500 to 600 nm region of the spectrum (see Table 1). Co^{II} has also been incorporated into both the T1 and T2 sites of the *A. cy* and *A. xy* NiRs to give the $T1Co^{II}/T2Co^{II}$ derivatives. The UV/Vis spectra of $T1Co^{II}/T2Co^{II}$ *A. xy* and *A. cy* NiRs (Figure 2) are very similar to those reported previously,[43] and peak positions and intensities are listed in Table 1. The additional bands relative to the spectra of the $T1Cu^{I}/T2Co^{II}$ derivatives must all arise from the T1 Co^{II} centers [the spectra of $T1Co^{II}/T2Co^{II}$ *A. xy* and *A. cy* NiRs show relatively lower intensity for the absorption bands at around 550–600 nm (the T2 LF bands) compared with those reported previously,[43] which can be attributed to either a lower T2 Co^{II} occupancy in our samples or a higher T1 Co^{II} occupancy]. The intense absorption band at 332 nm in the spectrum of $T1Co^{II}/T2Co^{II}$ *A. xy* NiR (333 nm in *A. cy* protein) is attributed to a S(Cys)→Co^{II} ligand-to-metal charge-transfer (LMCT) transition of the T1 site with the band at 402 nm (414 nm in *A. cy* protein) also arising from an LMCT transition involving the same ligand.[43] The absorption band at about 500 nm (which overlaps with a T2 LF transition) and those between 600 and 700 nm have been assigned to LF transitions from the Co^{II}-substituted T1 site.[43] These features are analogous to those found in the UV/Vis spectra of Co^{II} cupredoxins.[25,28,31,44,45]

Table 1. Positions and intensities of the bands observed in the UV/Vis spectra (25 °C) of the Co^{II}-substituted NiRs.

Assignments[a]	*A. xy*		*A. cy*	
	λ [nm]	ε [$M^{-1}cm^{-1}$]	λ [nm]	ε [$M^{-1}cm^{-1}$]
LMCT[b]	332	2500	333	2000
LMCT[b]	402	810	414	680
LF[b]	509	380	509	410
LF[c]	525	290	517	330
LF[c]	560	380	555	400
LF[c]	578	390	576	400
LF[b]	640	340	638	350
LF[b]	688	420	682	330

[a] Taken from reference [43]. [b] From the T1 Co^{II} site of the $T1Co^{II}/T2Co^{II}$ derivatives. [c] From the T2 Co^{II} center of the $T1Cu^{I}/T2Co^{II}$ proteins.

^{1}H NMR spectroscopy of Co^{II}-substituted NiRs: The ^{1}H NMR spectra of the Cu^{II} forms of the *A. cy* and *A. xy* NiRs have been published previously.[15] Only six–seven very

broad shifted signals can be observed, which all arise from protons associated with the T1 site [the spectra of the fully Cu^{II} loaded and T2 copper depleted (T2D) enzymes are identical] and could only be assigned by comparison to data for Cu^{II} cupredoxins (the usual strategy for assignment of paramagnetic signals arising from oxidized T1 copper sites, which involves the use of saturation transfer difference experiments on 1:1 mixtures of Cu^{II} and Cu^{I} protein to correlate shifted resonances to their counterparts in the diamagnetic protein,[8–14] could not be applied to the NiRs owing to the probable absence of ESE and the very broad nature of the Cu^{I} resonances). The spectrum of $T1Cu^{II}/T2Co^{II}$ *A. xy* NiR was investigated to see if the proximity to Co^{II} could influence the relatively slow electronic relaxation of the Cu^{II} T1 site. No significant decrease in the line widths of resonances arising from the oxidized T1 copper ligands was observed. Therefore, unlike in the paramagnetic ^1H NMR studies of Cu_2Co_2 superoxide dismutase,[18–22] in which substitution of the native Zn^{II} with Co^{II} results in enhanced electronic relaxation at the Cu^{II} site, allowing relatively sharp isotropically shifted resonances to be observed, electronic coupling does not occur in this NiR derivative (in superoxide dismutase the metal sites are actually bridged by a His residue, which facilitates coupling). We have therefore studied the ^1H NMR spectra of the $T1Cu^{I}/T2Co^{II}$ and $T1Co^{II}/T2Co^{II}$-substituted forms of the blue and green NiRs to obtain detailed active site information.

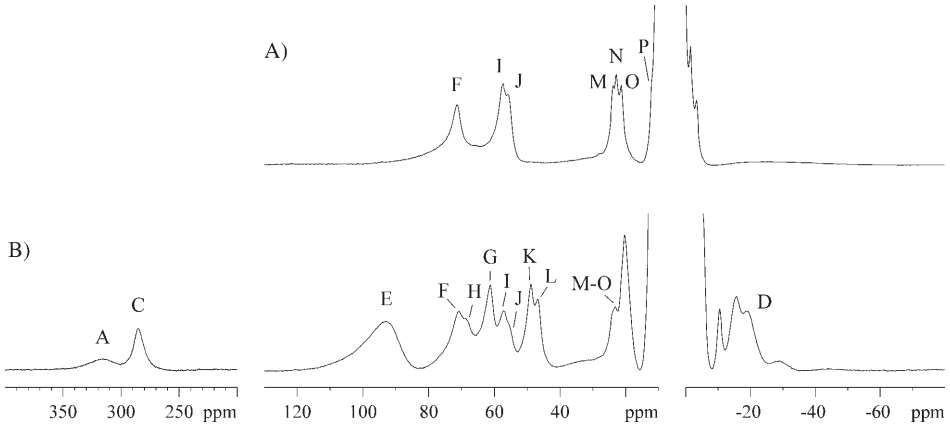

Figure 3. ^1H NMR spectra of (A) $T1Cu^{I}/T2Co^{II}$ and (B) $T1Co^{II}/T2Co^{II}$ *A. xy* NiR in 10 mM phosphate pH 7.8 (90% H_2O/10% D_2O) recorded at 40 °C.

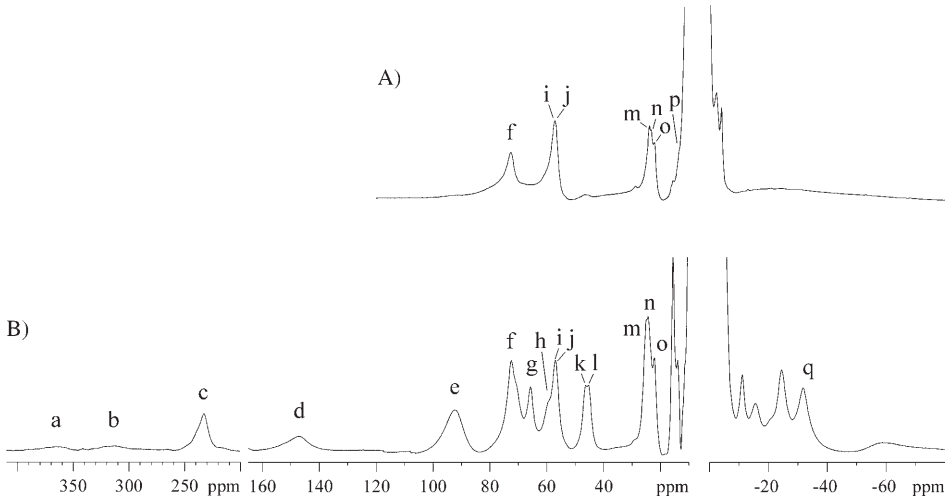

Figure 4. ^1H NMR spectra of (A) $T1Cu^{I}/T2Co^{II}$ and (B) $T1Co^{II}/T2Co^{II}$ *A. cy* NiR in 10 mM phosphate pH 7.7 (90% H_2O/10% D_2O) recorded at 40 °C.

The ^1H NMR spectra of $T1Cu^{I}/T2Co^{II}$ and $T1Co^{II}/T2Co^{II}$ *A. xy* NiR in 10 mM phosphate pH 7.8 (90% H_2O/10% D_2O) at 40 °C are shown in Figures 3A and 3B, respectively, while those of $T1Cu^{I}/T2Co^{II}$ and $T1Co^{II}/T2Co^{II}$ *A. cy* NiR, respectively, in 10 mM phosphate pH 7.7 (90% H_2O/10% D_2O) at 40 °C are shown in Figures 4A and 4B. The hyperfine shifted resonances observed in the spectra of both proteins are listed in Table 2 along with their δ_{obs} and T_1 values. All of these resonances have properties (short T_1 values, large line widths, and hyperfine shifts) that identify them as arising from protons associated with the coordinating amino acids (see Figure 1). Assignments were made by observing dipolar connectivities and utilizing the high-resolution structures of the Cu^{II} proteins [PDB accession codes 2BW4 (0.9 Å) and 1OE1 (1.04 Å) for the green[38] and blue[36] enzymes, respectively] with protons added using Insight II. Assignment has been assisted by comparing the spectra of $T1Cu^{I}/T2Co^{II}$ and $T1Co^{II}/T2Co^{II}$ samples, using the exchangeable behavior of resonances (when appropriate) and in some cases by homology to the assigned spectra of Co^{II}-substituted cupredoxins.[23–31]

The spectra shown in Figures 3A and 4A of the $T1Cu^{I}/T2Co^{II}$ NiRs, which possess paramagnetically shifted resonances from only the T2 site, are extremely similar. In both cases the three signals in the $\delta = 75$–55 ppm region arise from exchangeable protons and one of the overlapping resonances at around $\delta = 55$ ppm (signal I/i in *A. xy*/*A. cy* NiR, respectively) exchanges very slowly (see Figures 5D and 6D). The irradiation of resonance F gives rise to NOEs to peaks M and N (Figure 5B), whilst an NOE is observed to

Table 2. The hyperfine shifted resonances in the paramagnetic ^{1}H NMR spectra of the Co^{II}-substituted NiRs.[a]

A. xy				*A. cy*			
Resonances	δ_{obs} [ppm]	T_1 [ms]	Assignment	Resonances	δ_{obs} [ppm]	T_1 [ms]	Assignment
A	~320	<0.5	Cys130 $C^{\beta}H$	a	~360	n.d.[b]	Cys136 $C^{\beta}H$
C[c]	~290	1.6	Met144 $C^{\gamma}H$	b	~310	n.d.	Cys136 $C^{\beta}H$
E	~94	1.6	Met144 $C^{\varepsilon}H_3$	c	~232	1.9	Met150 $C^{\gamma}H$
F	71.2	2.1	His94 $N^{\delta 1}H$ (T2)	d	~144	1.1	Met150 $C^{\gamma}H$
H	68.7	~1.5	His139 $N^{\varepsilon 2}H$	e	~95	1.5	Met150 $C^{\varepsilon}H_3$
G	61.4	~3.5	His89 $N^{\varepsilon 2}H$	f	72.8	2.3	His100 $N^{\delta 1}H$ (T2)
I	57.2	2.8	His129 $N^{\delta 1}H$ (T2)	g	66.3	n.d.	His95 $N^{\varepsilon 2}H$
J	55.6	2.9	His300 $N^{\delta 1}H$ (T2)	h	59.7	n.d.	His145 $N^{\varepsilon 2}H$
K	49.0	10.6	His89 $C^{\delta 2}H$	i	57.3	2.5	His135 $N^{\delta 1}H$ (T2)
L	47.0	7.5	His139 $C^{\delta 2}H$	j	57.3	~3	His306 $N^{\delta 1}H$ (T2)
M	23.8	11.7	His94 $C^{\beta}H$ (T2)	k	46.7	~8.3	His95 $C^{\delta 2}H$
N	22.9	13.7	His94 $C^{\beta}H$ (T2)	l	~46	~8.3	His145 $C^{\delta 2}H$
O	21.4	6.6	His129 $C^{\beta}H$ (T2)	m	23.8	8.0	His100 $C^{\beta}H$ (T2)
	20.3	6.4		n	23.3	8.4	His100 $C^{\beta}H$ (T2)
P	12.1	n.d.	His300 $C^{\beta}H$ (T2)	o	22.2	3.5	His135 $C^{\beta}H$ (T2)
	−10.1	~11		p	11.3	n.d.	His306 $C^{\beta}H$ (T2)
	−15.0	4.5			−11.2	~8.4	
D	~−19	~2	Met144 $C^{\gamma}H$		−15.7	4.3	
	~−29	n.d.			~−21	~7.5	
	~−20 [d]	n.d.			−24.5	4.3	
				q	−32.0	4.1	Met150 $C^{\beta}H$
					~−59	n.d.	
					~−22 [d]	n.d.	

[a] Data recorded at 300 MHz in 10 mM phosphate pH 7.8 for *A. xy* NiR and pH 7.7 for *A. cy* NiR (both in 90% H_2O/10% D_2O). Included are the observed chemical shift (δ_{obs}), the spin-lattice (T_1) relaxation times and the assignments that have been made. The δ_{obs} values arise from three contributing factors; δ_{dia} (the shift in an analogous diamagnetic system), δ_{pc} (the pseudo-contact, through space, contribution) and δ_c (the Fermi-contact, through bond, contribution) and $\delta_{pc}+\delta_c = \delta_{iso}$ (the isotropic or hyperfine shift). [b] Not determined due to the very large linewidth and/or overlap. [c] Overlapping with one of the Cys130 $C^{\beta}H$ proton resonances. [d] Observed in the spectrum of $T1Cu^{I}/T2Co^{II}$ sample.

peak O upon irradiation of peak I (Figure 5C) in *A. xy* NiR (it is very difficult to individually irradiate peaks I and J owing to their close proximity in the spectrum but vide infra). An identical pattern of NOEs is seen upon irradiation of signals f and i/j in the spectrum of $T1Cu^{I}/T2Co^{II}$ *A. cy* NiR (see Figure 6). Experiments were also carried out in 10 mM phosphate pH* 7.8 (99.9% D_2O) and the same NOE pattern as obtained when irradiating the overlapping signals I/J and i/j was observed upon irradiation of the slowly exchanging resonances I and i (see Figures 5E and 6E). This demonstrates that the connectivities observed for these signals in the spectra of the samples in 90% H_2O/10% D_2O arise from peaks I/i and not the overlapping signals J/j. The irradiation of peak J in the spectrum of the *A. xy* NiR gives rise to an NOE to signal P (at $\delta = 12.1$ ppm, data not shown) which is not observed when peak I is irradiated in D_2O. An NOE to the peak at $\delta = 11.3$ ppm (peak p) is observed when the overlapping signals i/j in the *A. cy* NiR spectrum are irradiated (data not shown), which is not seen when resonance i alone is irradiated in the experiment in D_2O.

The T2 centers of both NiRs involve coordination of the metal by three His ligands, all through their $N^{\varepsilon 2}$ atoms, and a water ligand (see Figure 1),[33,36,38] and the signals from exchangeable protons in the spectra of the $T1Cu^{I}/T2Co^{II}$ derivatives can only arise from the $N^{\delta 1}H$ protons of the these His ligands (His94, His129, and His300 in *A. xy* NiR and His100, His135, and His306 in the *A. cy* protein). Coordination to Co^{II} by the $N^{\varepsilon 2}$ atom of a His residue results in severe line broadening of the $C^{\varepsilon 1}H$ and $C^{\delta 2}H$ proton resonances[21] (these protons will be ~3 Å away from $T2Co^{II}$) (see Figure 7) and thus are not observed in our spectra. The signals at around $\delta = 20$–10 ppm, which give NOEs to the His ligand $N^{\delta 1}H$ proton resonances in both proteins, must therefore arise from the $C^{\beta}H$ protons of the His ligands. Of the three T2 site His ligands only one (His94 in *A. xy* NiR[36] and His100 in the *A. cy* protein[38]) has a conformation that results in the $N^{\delta 1}H$ proton being close to both of the $C^{\beta}H$ protons (2.65 and 2.81 Å in *A. xy* NiR and 2.71 and 2.86 Å in the *A. cy* protein). Therefore, signal F in *A. xy* NiR (and peak f in the *A. cy* enzyme) can be assigned to the $N^{\delta 1}H$ proton of His94 (His100 in *A. cy* NiR) and peaks M/m and N/n to the $C^{\beta}H$ protons of this His ligand. In the NiRs, the His129/His135 $N^{\delta 1}H$ proton is a hydrogen bond donor to the carbonyl oxygen atom of Cys130/Cys136,[33,35] and thus this proton is expected to exchange slowly. Peak I/i can hence be assigned to the His129/His135 $N^{\delta 1}H$ proton and peak O/o to one of the $C^{\beta}H$ protons of this residue. Interproton distances of 2.58 and 3.78 Å between the His129 $N^{\delta 1}H$ and $C^{\beta}Hs$ in *A. xy* NiR (2.64 and 3.82 Å for His135 in the *A. cy* protein) highlight why an NOE to only one His129 $C^{\beta}H$ (and His135 $C^{\beta}H$) proton resonance is observed. The remaining peak J/j is assigned to the $N^{\delta 1}H$ proton of His300/His306 with signal P/p belonging to one of the $C^{\beta}H$ proton resonances of this His ligand ($N^{\delta 1}H$ to $C^{\beta}H$ interproton distances of 2.85 and 3.85 Å in *A. xy* NiR and 2.83 and 3.86 Å in the *A. cy* enzyme).

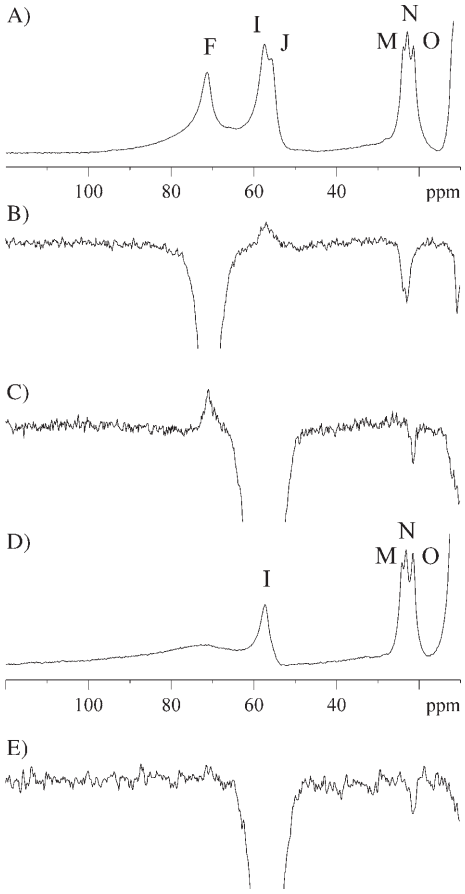

Figure 5. Reference (A and D) and difference (B, C, and E) ^{1}H NMR spectra (300 MHz) of T1Cu^{I}/T2Co^{II} *A. xy* NiR corresponding to 1D NOE experiments performed in 10 mM phosphate (90% H_2O/10% D_2O) at pH 7.4 (A–C) and in 10 mM phosphate (99.9% D_2O) at pH* 7.8 (D and E) at 40 °C. In spectra B and C peaks F and I were irradiated and in spectrum E peak I was irradiated.

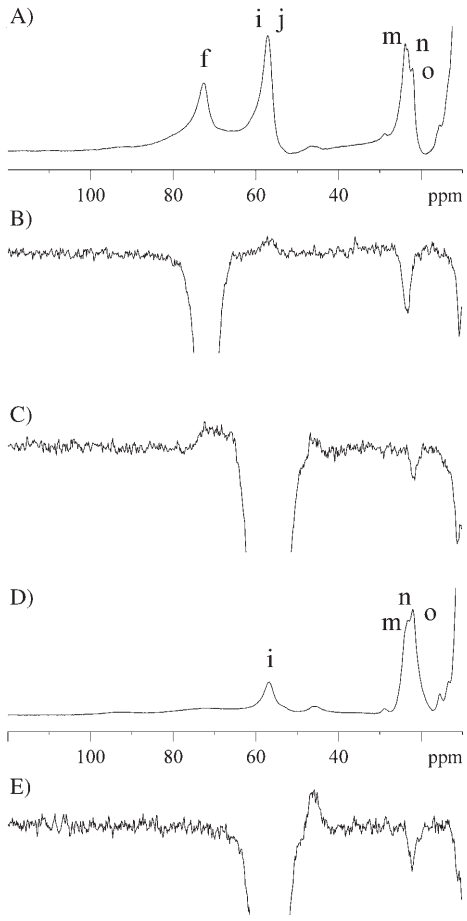

Figure 6. Reference (A and D) and difference (B, C, and E) ^{1}H NMR spectra (300 MHz) of T1Cu^{I}/T2Co^{II} *A. cy* NiR corresponding to 1D NOE experiments performed in 10 mM phosphate (90% H_2O/10% D_2O) at pH 7.7 (A–C) and in 10 mM phosphate (99.9% D_2O) at pH* 7.7 (D and E) at 40 °C. In spectra B and C peaks f and the overlapping peaks i and j were irradiated and in spectrum E peak i was irradiated.

Resonances F, I, J, and M–P as well as f, i, j, and m–p in the ^{1}H NMR spectra of T1Co^{II}/T2Co^{II} *A. xy* (Figure 3B) and *A. cy* (Figure 4B) NiRs, respectively, have already been assigned to T2 His ligand protons (vide supra). The remaining isotropically shifted resonances therefore must belong to the coordinating residues at the T1 center (see Figure 1). Peaks H/h and G/g (see Figures 3B and 4B) are exchangeable (spectra of the T1Co^{II}/T2Co^{II} NiR derivatives in D_2O are shown in Figure S3 in the Supporting Information) and must arise from the N$^{\varepsilon 2}$H protons of the two T1 His ligands (His89/95 and His139/145), which coordinate through their N$^{\delta 1}$ atoms (this results in the C$^{\varepsilon 1}$H proton being about 3 Å from the metal ion and its resonance will be extremely broad, whereas the N$^{\varepsilon 2}$H and C$^{\delta 2}$H protons are much further away and their resonances will be sharper and more readily observed; see Figure 7). The most intense of these two resonances in the spectra of T1Co^{II}/T2Co^{II} *A. xy* and *A. cy* NiRs (peak G/g) is assigned to the N$^{\varepsilon 2}$H proton of the buried His ligand (His89/95).[33,36,38] Resonance H/h can therefore be assigned to the N$^{\varepsilon 2}$H proton of the solvent-exposed His ligand in the NiRs (His139/145). Selective irradiation of peak G

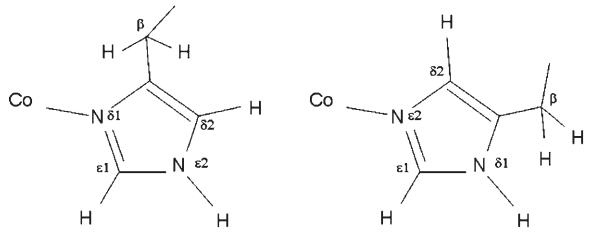

Figure 7. Schematic representation of a His residue coordinating to cobalt through its N$^{\delta 1}$ and N$^{\varepsilon 2}$ atoms.

(and signal g) gives rise to an NOE to peak K (and resonance k) (see Figures 8B and 8D). Signals K and k have relatively long T_1 values (see Table 2) and can be assigned to the C$^{\delta 2}$H protons of His89 and His95 in *A. xy* and *A. cy* NiRs, respectively (the reverse NOEs are observed upon irradiation of signals K/k, data not shown). NOE experiments in which signals H and h were selectively irradiated were not possible owing to spectral overlap. However, the relatively long T_1s and δ_{obs} values of resonances L and l indicate

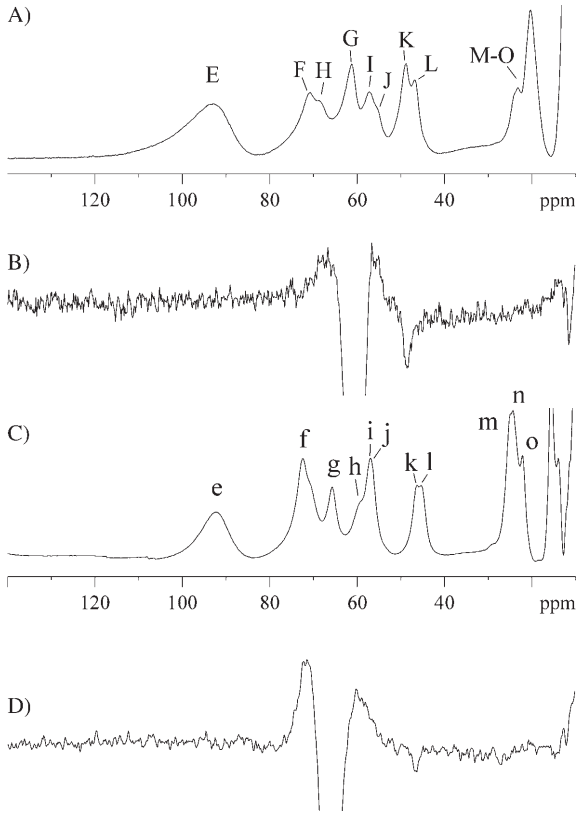

Figure 8. Reference (A and C) and difference (B and D) ^{1}H NMR spectra (300 MHz) of T1Co^{II}/T2Co^{II} *A. xy* (A and B) and *A. cy* (C and D) NiRs corresponding to 1D NOE experiments performed in 10 mM phosphate (90% H_2O/10% D_2O) at pH 7.8 and 40 °C. In spectrum B peak G was irradiated whilst in D resonance g was irradiated.

that they arise from the C$^{\delta 2}$H protons of the His139 and His145 ligands in *A. xy* and *A. cy* NiRs respectively [the C$^{\delta 2}$H proton resonances from the His ligands in Co^{II}-substituted cupredoxins typically have δ_{obs} values between $\delta = 40$ and 60 ppm and are usually found close together (see Table 3)[23–31]].

The most intense isotropically shifted resonances in the ^{1}H NMR spectra of the T1Co^{II}/T2Co^{II} *A. xy* and *A. cy* NiRs are signal E and peak e respectively. These resonances both have intensities equivalent to three protons and therefore can be assigned to the C$^{\varepsilon}$H$_3$ group of the axial Met144 and Met150 T1 ligands in the two enzymes. Irradiation of these signals does not give rise to NOEs to any other hyperfine shifted resonances. Furthermore, the irradiation of the other hyperfine-shifted resonances does not result in any NOEs to peak E and resonance e. This is consistent with observed distances between the Met C$^{\varepsilon}$H$_3$ moiety and other active-site protons, and the T_1 values of these resonances.[31,46]

Signals a–d in the spectrum of the *A. cy* protein (see Figure 4B) all appear to have intensities equivalent to one proton (the width of peaks a and b makes integration difficult). The broad nature of peaks a and b along with their extremely short T_1 values identify them as arising from the C$^{\beta}$H protons of the Cys136 ligand [the corresponding resonances in Co^{II} cupredoxins have similar properties and are usually found close together (see Table 3)[23–31]]. The irradiation of resonances a and b does not result in significant dipolar connectivities to any other hyperfine-shifted resonances (the NOE between a and b cannot be observed owing to the very broad nature of these resonances and their extremely small T_1 values) although very weak NOEs are seen at $\delta = 26.6$, 18.5, and 14.9 ppm upon irradiation of signal a (data not shown). The selective irradiation of peak c results in a strong NOE to peak d (Figure 9B). The reverse NOE is observed when peak d is irradiated (Figure 9C). Such a strong dipolar connectivity identifies these resonances as belonging to a geminal pair of protons which can only arise from the C$^{\gamma}$H$_2$ group of the Met150 ligand. This is confirmed

Table 3. The δ_{obs} values (ppm) of resonances in the ^{1}H NMR spectra of the Co^{II} NiRs compared with those for the corresponding resonances in the spectra of Co^{II}-substituted cupredoxins.

Ligand[a]	Proton	*A. xy* (blue) T1 Co^{II} δ_{obs}[b]	*A. cy* (green) T1 Co^{II} δ_{obs}[c]	Co^{II} AZ[d]			Co^{II} RST[e]			Co^{II} PC[f]	Co^{II} AMI[g]	Co^{II} PAZ[h]
				δ_{obs}	δ_{pc}	δ_c	δ_{obs}	δ_{pc}	δ_c	δ_{obs}	δ_{obs}	δ_{obs}
His	H$^{\delta 2}$	49.0	46.7	50.6	7.8	37.3	59.7	−1.9	53.6	55.8	52.6	53.1
	H$^{\varepsilon 1}$	–	–	97	85.7	4.4	–	–	–	133	118/38	146/57
	H$^{\varepsilon 2}$	61.4	66.3	74.9	16.6	46.9	69.2	10.3	44.2	63.2	62.3	61.9
Cys	H$^{\beta 1}$	~320/290	~360/310	232	5.4	223.2	287/260	69.9/59.7	205/184	299	285	315/267
	H$^{\beta 2}$	~320/290	~360/310	285	−5.3	287.4	287/260	69.9/59.7	205/184	275	285	315/267
His	H$^{\delta 2}$	47.0	46.0	56.4	7.2	42.3	48.7	3.4	38.5	43.8	51.0	43.6
	H$^{\varepsilon 1}$	–	–	75	23.3	45	–	–	–	60	118/38	146/57
	H$^{\varepsilon 2}$	68.7	59.7	65.8	7.5	46.8	80.3	19.9	48.9	75.3	74	71.4
Met	H$^{\beta 1}$	–	–	−18.9	−25.9	5.3	−31.0	−23.2	−9.8	−18.4	−18.6	−31.2
	H$^{\beta 2}$	–	–	−18.5	−26.9	6.1	−24.4	−13.5	−17.4	−27.5	−16.1	–
	H$^{\gamma 1}$	290/−19	232/144	45.3	−19.5	64.4	122.9	−11.5	132.5	254	132.5	105.8
	H$^{\gamma 2}$	290/−19	232/144	−19.1	−20.2	−0.3	285.2	−19.2	301.6	87.8	10.0	271.3
	C$^{\varepsilon}$H$_3$	94	95	−7.3	−31.2	24	103.3	−16.9	130.3	80.4	74.5	90.2
Gly	H$^{\alpha 1}$	–	–	47.8	−10.5	54.2	–	–	–	–	–	–
	H$^{\alpha 2}$	–	–	−29.4	−26.7	−5.9	–	–	–	–	–	–

[a] From top to bottom: His89, Cys130, His139, and Met144 for *A. xy* NiR; His95, Cys136, His145, and Met150 for *A. cy* NiR; His46, Cys112, His117, Met121, and Gly45 for azurin (AZ); His85, Cys138, His143, and Met148 for rusticyanin (RST); His37, Cys84, His87, and Met92 for plastocyanin (PC); His54, Cys93, His96, and Met99 for amicyanin (AMI); His40, Cys78, His81, and Met86 for pseudoazurin (PAZ). [b] This study at 40 °C and pH 7.8. [c] This study at 40 °C and pH 7.7. [d] From *Pseudomonas aeruginosa* at pH 4.5 and 37 °C including the δ_{pc} and δ_c contributions to δ_{obs}.[23,27] [e] From *Thiobacillus ferrooxidans* at pH 6.0 and 20 °C including the δ_{pc} and δ_c contributions to δ_{obs}.[29] [f] From spinach at 30 °C and pH 7.8.[31] [g] From *Paracoccus versutus* at 40 °C and pH 8.0 (the His96 N$^{\varepsilon 2}$H resonance was observed at 22 °C and pH 5.0).[28] [h] From *A. cycloclastes* at 40 °C and pH 8.0.[30]

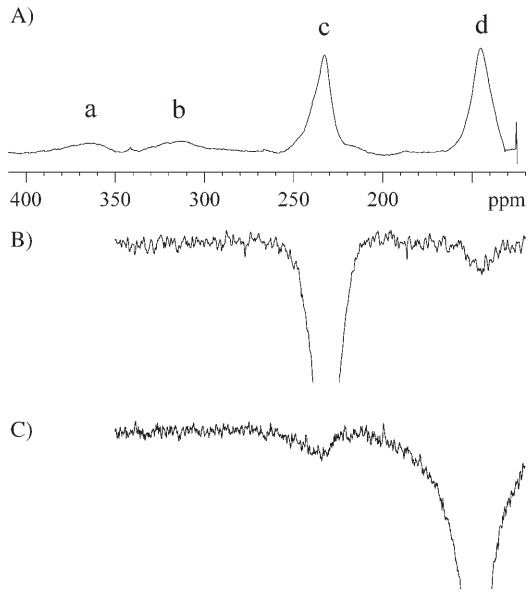

Figure 9. Reference (A) and difference (B and C) ^{1}H NMR spectra (300 MHz) of T1Co^{II}/T2Co^{II} *A. cy* NiR corresponding to 1D NOE experiments performed in 10 mM phosphate pH* 7.7 (99.9% D_2O) at 40 °C. In spectra B and C peaks c and d respectively were irradiated.

by the observation of an NOE to peak q at $\delta = -32$ ppm upon irradiation of resonance c, which can be assigned to one of C$^\beta$H proton resonances of the Met150 ligand [the C$^\beta$H proton resonances of the axial Met ligand of Co^{II} cupredoxins[23–31] are always found in the upfield region owing to a relatively large negative δ_{pc} value and a very small δ_c contribution (see Table 3),[27,29] and usually give NOEs to the C$^\gamma$H proton resonances]. This is consistent with Met150 C$^\gamma$H to Met150 C$^\beta$H interproton distances ranging from 2.39 Å to 3.03 Å in Cu^{II} *A. cy* NiR. The irradiation of signal c also gives a strong NOE to a peak at $\delta = 0.07$ ppm (~700 Hz wide), which could arise from the Met150 C$^\alpha$H proton resonance (the Met150 C$^\gamma$H to C$^\alpha$H interproton distances are 2.11 and 2.92 Å).

The very small T_1 value of peak A (and extremely large linewidth) in the spectrum of T1Co^{II}/T2Co^{II} *A. xy* NiR (Figure 3B) allow assignment of this resonance to one of the C$^\beta$H protons of the Cys130 ligand. Selective irradiation of this signal gives rise to relatively weak NOEs to resonances at $\delta = 20.3$ and 12.4 ppm (data not shown), which is similar to the NOE pattern observed upon irradiation of peak a in *A. cy* NiR (vide supra). Given that the C$^\beta$H proton resonances of the Cys ligand are always found close together and at around $\delta = 300$ ppm in the NMR spectra of Co^{II} cupredoxins with an axial Met ligand (see Table 3), and that resonance C has an intensity equivalent to two protons, we assume that the second Cys C$^\beta$H proton resonance overlaps with peak C. A strong NOE to the broad signal D ($\Delta\nu_{1/2}$ ~3000 Hz) at $\delta = -19$ ppm and a resonance at $\delta = 0.1$ ppm ($\Delta\nu_{1/2}$ ~550 Hz) are observed upon irradiation of peak C (data not shown). The strong dipolar connectivity between peaks C and D, indicates that these resonances belong to a geminal pair of ligand protons and can be assigned to the C$^\gamma$H$_2$ moiety of the Met144 ligand. This assignment is supported by the strong NOE to the signal at $\delta = 0.1$ ppm, which can be tentatively assigned, as for the *A. cy* protein, to the C$^\alpha$H proton of the axial Met ligand (the Met144 C$^\gamma$H to C$^\alpha$H interproton distances are 2.31 and 3.39 Å). The Cys130 C$^\beta$H proton resonance that overlaps with the Met144 C$^\gamma$H signal resulting in peak C is not expected to give any strong NOEs to other isotropically shifted resonances (as seen in the experiment in which peak b in the spectrum of the *A. cy* NiR was irradiated).

Only one of the Met C$^\beta$H$_2$ proton resonances has been assigned (signal q) in the spectrum of T1Co^{II}/T2Co^{II} *A. cy* NiR, and as already mentioned these signals are usually shifted upfield (δ_{obs} ~ −20 to −40 ppm) in the ^{1}H NMR spectra of Co^{II} cupredoxins (with T_1 values of ca. 5–10 ms).[23–31] One of the other resonances in the δ ~ −10 to −30 ppm region of the T1Co^{II}/T2Co^{II} *A. cy* NiR spectrum therefore probably arises from the second C$^\beta$H proton of the Met150 ligand. The resonances at $\delta = -10.1$ and −15.0 ppm in the spectrum of T1Co^{II}/T2Co^{II} *A. xy* NiR are tentatively assigned to the C$^\beta$H$_2$ protons of Met144. A number of other resonances in the spectra of T1Co^{II}/T2Co^{II} *A. xy* and *A. cy* NiRs that only experience relatively small isotropic shifts have not been assigned and could arise from the C$^\alpha$H protons of the coordinated Cys residues or the C$^\beta$H protons of the T1 His ligands.

Temperature dependence of the δ_{obs} values of resonances in the ^{1}H NMR spectra of the Co^{II}-substituted NiRs: The temperature dependence of the δ_{obs} values of the hyperfine-shifted resonances in the spectra of Co^{II}-substituted *A. xy* NiR was studied between 15 and 50 °C and between 25 and 50 °C for the *A. cy* enzyme. All of the T2 Co^{II} resonances in the spectra of T1Cu^{I}/T2Co^{II} *A. xy* and *A. cy* NiRs show Curie-type behavior (see Figure 10). The temperature dependencies of the T1 Co^{II} resonances in the spectra of the T1Co^{II}/T2Co^{II} NiRs are shown in Figure 11. For the *A. xy* enzyme all of the T1 hyperfine-shifted resonances exhibit Curie-type behavior (Figure 11A). The only unusual feature is the δ_{obs} value of about −150 ppm at infinite temperature for peak C (which arises from two overlapping signals). For T1Co^{II}/T2Co^{II} *A. cy* NiR Curie-type behavior is seen for all of the shifted resonances except peak d (see Figure 11B), and signal c exhibits a δ_{obs} value at infinite temperature of almost −400 ppm (the unusual behavior of peaks c and d, possibly due to a temperature-induced change in conformation of the axial Met150 ligand,[36,37] further supports their assignment to the C$^\gamma$H protons of this residue).

The influence of nitrite on the paramagnetic ^{1}H NMR spectra of the T1 Cu^{I}/T2 Co^{II} NiRs: The paramagnetic ^{1}H NMR spectrum of T1Cu^{I}/T2Co^{II} *A. xy* NiR was monitored in the presence of increasing nitrite concentrations at pH 7.8 and 6.7 (data not shown). At pH 7.8 very little effect was observed, whereas at pH 6.7 the δ_{obs} values of resonances F, I, J, and M–O change by about 1 ppm upon addition of a 100-

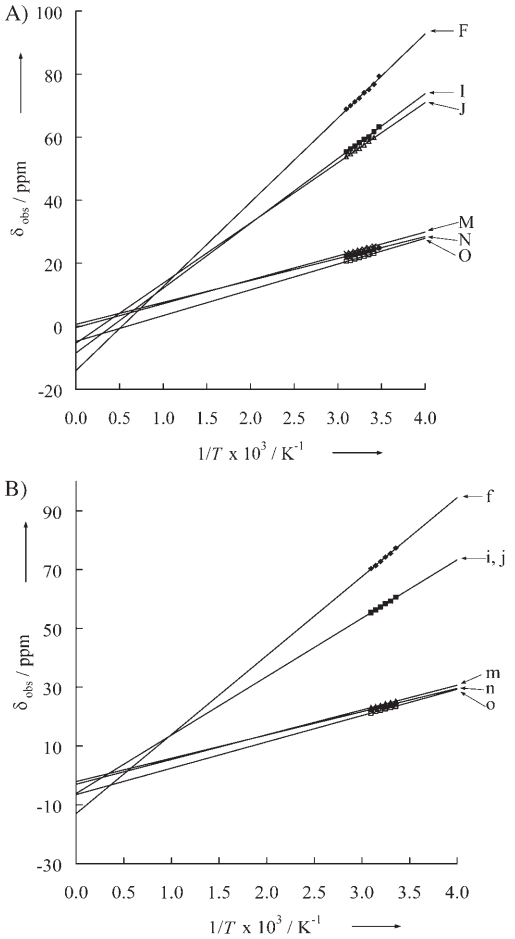

Figure 10. The temperature dependence of the δ_{obs} values of the hyperfine-shifted resonances in the ^{1}H NMR spectra of the T1Cu^I/T2Co^{II} NiRs; (A) from *A. xy* in 10 mM phosphate pH 7.8 (90% H_2O/10% D_2O) and (B) from *A. cy* in 10 mM phosphate pH 7.7 (90% H_2O/10% D_2O).

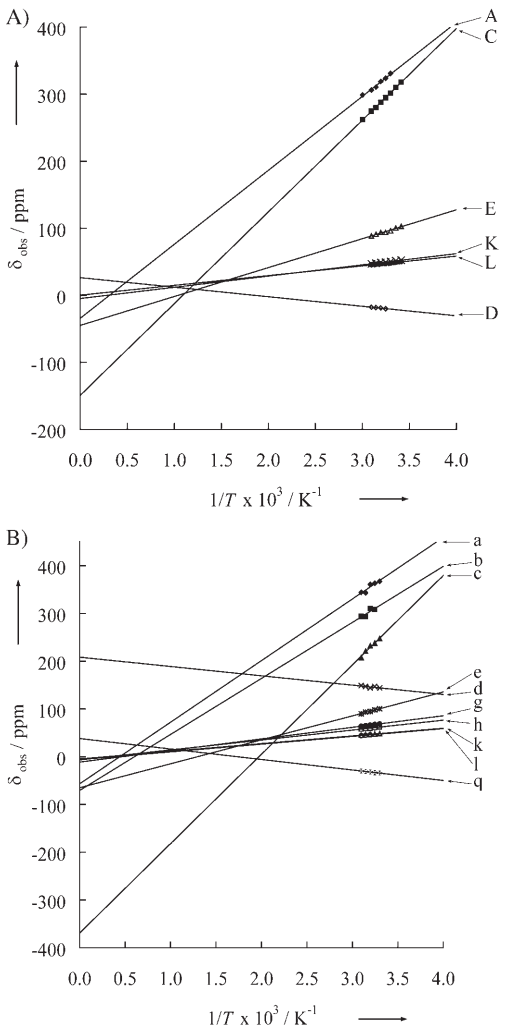

Figure 11. The temperature dependence of the δ_{obs} values of the hyperfine-shifted resonances in the ^{1}H NMR spectra of the T1Co^{II}/T2Co^{II} NiRs; (A) from *A. xy* in 10 mM phosphate pH* 7.8 (99.9% D_2O) and (B) from *A. cy* NiR in 10 mM phosphate pH 7.7 (90% H_2O/10% D_2O).

fold excess of nitrite (200 mM). Nitrite has a limited effect (data not shown) on the NMR spectrum of T1Cu^I/T2Co^{II} *A. cy* NiR at pH 7.7 with peak f shifting upfield by about 0.6 ppm and the overlapping signals i and j shifting upfield by about 0.3 ppm in the presence of a 100-fold excess (200 mM). When the experiment was performed at pH 6.7 peak f experienced a slightly larger shift whilst the overlapping peaks i and j broadened (data not shown). These effects were not sufficiently large to allow an association constant for nitrite at the T2 Co^{II} site to be determined. Titrations at pH ~8 monitored by UV/Vis spectroscopy confirmed the limited effect of the addition of nitrite. Attempts to repeat these experiments at pH < 7 were hampered by the formation of precipitate upon the addition of nitrite.

Discussion

UV/Vis spectra of Co^{II}-substituted NiRs: The preparation of the T1Cu^I/T2Co^{II} derivatives of the NiRs has allowed the UV/Vis spectra of the Co^{II} T2 sites to be observed. The spectra of these derivatives are remarkably alike for the *A. xy* and *A. cy* enzymes and both exhibit three LF transitions in the 500 to 600 nm region, analogous to what has been seen for Co^{II}-substituted T3 copper sites.[47–49] The intensities and positions of these bands are consistent with a regular tetrahedral geometry at the T2 Co^{II} sites, which probably involves a water ligand (as in the Cu^{II} proteins).[33,36,38] The main S(Cys)→Co^{II} T1 LMCT band in the UV/Vis spectra of the Co^{II} NiRs is in very similar positions in the *A. xy* and *A. cy* enzymes. The second LMCT transition occurs at slightly longer wavelength in *A. cy* NiR and the intensities of both bands are lower in this derivative. The T1 LF feature at highest wavelength is sharper and more intense in the *A. xy* derivative than in the green NiR. This results in this region of the UV/Vis spectrum of the *A. xy* NiR being similar in appearance to that of Co^{II} amicyanin (AMI),[28] whilst the corresponding region of the *A. cy* NiR spectrum is not directly comparable to any published for a Co^{II} cupredoxin.

^{1}H NMR spectra of the T2 Co^{II} site of the blue and green NiRs: The assignment of the isotropically shifted resonances from the T2 Co^{II} site of the *A. xy* and *A. cy* NiRs demonstrate that this center is comparable in these two enzymes. The δ_{iso} values are alike for the N$^{\delta 1}$H and C$^{\beta}$H proton resonances of the three His ligands (it is assumed that their δ_{dia} values are similar). This indicates that not only is the spin density distribution onto the His ligands similar for the T2 Co^{II} sites in the blue and green NiRs, but also the conformations of these three ligands (and the orientation of the magnetic susceptibility tensors) are alike (the δ_c and δ_{pc} values are probably comparable, which is supported by almost identical temperature dependencies of δ_{obs} for the T2 Co^{II} site resonances giving similar chemical shift values at infinite temperature). This is in agreement with the reported crystal structures of the native Cu^{II} enzymes in which almost identical bond lengths and angles are observed for the T2 sites in the *A. xy* and *A. cy* NiRs.[33,36,38] Theoretical studies have investigated structural differences, which occur at the T2 copper sites of some NiRs,[50] and alterations, which are mainly found in the green NiR from *Alcaligenes faecalis*,[51] have been associated with movements of the solvent ligand.

The addition of nitrite has very little effect on the ^{1}H NMR spectra of the T1Cu^{I}/T2Co^{II} derivatives at slightly alkaline pH. At pH <7 more significant changes are observed with slightly different effects seen in the blue and green NiRs, and more substantial differences for the former. The gradual changes in the δ_{obs} values of resonances upon the addition of nitrite indicates that the substrate binds at the Co^{II} site and that exchange between free and bound nitrite is fast on the NMR time scale. The subtle changes (all <1 ppm) show that the Co^{II}–N(His) interactions are not significantly altered by the binding of nitrite (up to a 100-fold excess) or that the binding of nitrite is not particularly strong. In both proteins, the isotropically shifted signals broaden in the presence of nitrite. This could be the result of exchange broadening or may be due to a decreased electronic relaxation rate for the substrate-bound form. The limited effect of nitrite on the NMR spectra at alkaline pH indicates that substrate binding is much weaker under these conditions, which is consistent with competition from hydroxide binding to the T2 Co^{II} site.[52] More sizable changes upon the addition of nitrite could be anticipated at even lower pH values, however the T1Cu^{I}/T2Co^{II} NiR derivatives were not stable under these conditions.

^{1}H NMR spectra of the T1 Co^{II} site of the blue and green NiRs: The assignment of hyperfine-shifted ^{1}H NMR resonances arising from all of the ligands at the T1 Co^{II} sites of the *A. xy* and *A. cy* NiRs allows us to make a detailed comparison of these centers in the blue and green enzymes. When interpreting these data it should be remembered that the δ_{obs} values for isotropically shifted resonances at T1 Co^{II} sites can have sizable pseudocontact (δ_{pc}, dipolar) contributions. These have been determined for Co^{II} azurin (AZ),[27] which has a classic T1 copper site, and Co^{II} rusticyanin (RST),[29] which possesses a distorted center (see Table 3). The δ_{pc} values are similar and are not that large for the His ligand resonances in both Co^{II} AZ and Co^{II} RST (except for the C$^{\varepsilon 1}$H proton signals, which are not observed in this study). In the case of the Cys C$^{\beta}$H proton resonances the δ_{pc} contributions are minimal for the classic T1 site of AZ but are much more significant for the distorted T1 site of RST. For the axial Met ligand the discrepancy in the δ_{pc} values for classic and distorted T1 sites is not so large. Another feature that should be considered when comparing the NMR spectra is the influence of ligand conformation on δ_c values. At a T1 Co^{II} site the δ_c of the Cys C$^{\beta}$H protons is not only influenced by the spin density on the thiolate sulfur but is also dependent on the Co^{II}-S$^{\gamma}$-C$^{\beta}$-H$^{\beta}$ dihedral angles (a $\cos^2\theta$ dependence on the dihedral angle θ has been suggested for the δ_c values of the C$^{\beta}$H proton resonances in Co^{II} AZ[27]). The δ_c values of the axial Met C$^{\gamma}$H proton resonances are dependent on the Co^{II}-S$^{\delta}$-C$^{\gamma}$-H$^{\gamma}$ dihedral angles (again a $\cos^2\theta$ dependence has been suggested for Co^{II} AZ[27]), but the C$^{\varepsilon}$H$_3$ proton resonance of this ligand will not display any angular dependence.

Resonances from the two coordinated His residues at the T1 site of the blue and green NiRs display remarkably similar δ_{obs} values, which indicates that the spin density distribution on these two ligands is almost identical in these enzymes. This is also the case if NMR data for Co^{II} (see Table 3)[23–31] and Cu^{II}[1,8–14] cupredoxins are considered, and thus these metal–ligand bonds do not vary significantly and are not dramatically influenced by the features that give rise to classical or distorted spectroscopic features (vide infra).

The Cys C$^{\beta}$H proton resonances display larger δ_{obs} values in the NMR spectrum of the green NiR than in the spectrum of the blue enzyme. The conformation of the Cys ligand is comparable in the *A. xy*[36] and *A. cy*[38] NiRs (Cu^{II}-S$^{\gamma}$-C$^{\beta}$-H$^{\beta}$ dihedral angles of $-48°$ and 72° in the former and $-48°$ and 64° in the latter) consistent with an analogous shift pattern for the C$^{\beta}$H proton resonances (the absence of stereospecific assignments for the Co^{II} derivatives of the blue and green NiRs prevents any detailed discussion of this point). The different δ_{obs} values of the Cys C$^{\beta}$H proton resonances could be partly due to more sizable δ_{pc} contributions to their δ_{obs} values in the green NiR (the *A. cy* NiR possesses a more distorted T1 site than RST as judged by the $A_{\sim 450}$ to $A_{\sim 600}$ UV/Vis ratio). It would therefore appear that the Co^{II}-S(Cys) interactions are similar in the Co^{II} enzymes. The interaction of the native metal with this ligand is also comparable in the blue and green NiRs with reported Cu^{II}–S(Cys) bond lengths of 2.20 Å[36] and 2.23 Å[38] respectively. It is interesting to note that electron nuclear double-resonance studies of the green NiR from *Rhodobacter sphaeroides* and its T1 Met to Thr variant, which is blue, have found almost identical hyperfine couplings for the Cys C$^{\beta}$H protons.[53] However, in this case the blue site possesses a weak axial oxygen ligand (vide infra).

In the case of the T1 axial Met ligand, almost identical δ_{obs} values are found for the resonances of the C$^{\varepsilon}$H$_3$ moiety. A slightly larger negative δ_{pc} contribution to the δ_{obs} of this signal is found for a classic T1 Co^{II} site such as that found in

the blue NiR (see Table 3). The spin density on the axial Met therefore appears to be similar in the blue and green NiRs, which is consistent with Cu^{II}–S(Met) bond lengths of 2.45 [36] and 2.49 Å,[38] respectively. A dramatic difference is found for the shift pattern of the $C^{\gamma}H$ proton resonances of the axial Met ligand in the spectra of the Co^{II} NiRs. In the case of the *A. xy* NiR the Met144 $C^{\gamma}H$ proton signals are found at $\delta = 290$ and -19 ppm, whereas in the green enzyme these resonances are at $\delta = 232$ and 144 ppm. This different shift pattern is consistent with an altered conformation of the axial Met ligand as seen in the structures of the Cu^{II} proteins (see Figure 12).[36,38] Similar differences in the conformation of the axial Met ligand are also present at other T1 copper sites;[55] this influences the shifts of the $C^{\gamma}H$ proton resonances in the paramagnetic NMR spectra of their Co^{II} derivatives (see Table 3). In the proteins whose axial Met adopts a gauche conformation[55] (*A. xy* NiR, see Figure 12,[36] AZ[56] and AMI[57]) only one of the $C^{\gamma}H$ proton

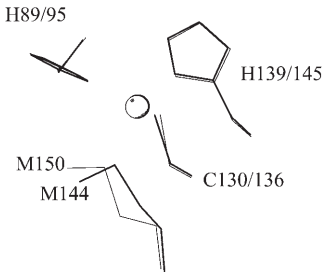

Figure 12. Overlay of the T1 Cu^{II} sites of the *A. cy* (PDB code 2BW4, thin lines and a white copper ion)[38] and *A. xy* (PDB code 1OE1, thicker lines and a black copper ion)[36] NiRs. The overlay was obtained using Swiss PDB Viewer[54] and the picture was drawn using MOLSCRIPT.[34] The Cu^{II}–S(Cys130) and Cu^{II}–S(Met144) bond lengths are 2.20 and 2.45 Å respectively in the *A. xy* protein whilst the Cu^{II}–S(Cys136) and Cu^{II}–S(Met150) bond lengths are 2.23 and 2.49 Å respectively in *A. cy* NiR.

resonances exhibits a sizable δ_c value. In the proteins in which the Met side chain is in a trans arrangement[55] [*A. cy* NiR (see Figure 12),[38] plastocyanin (PC),[58] pseudoazurin (PAZ)[59] and RST[60]] both $C^{\gamma}H$ proton resonances have large δ_c values. Comparisons of the strength of the M^{II}-S-(Met) interactions as indicated by the shifts of the $C^{\gamma}H$ proton resonances therefore should only be made within these two groups. In the proteins with a gauche Met the δ_{obs} values for the most shifted $C^{\gamma}H$ proton resonance of $\delta =$ 45.3, 132.5, and 290 ppm are consistent with Cu^{II}–S(Met) bond lengths of 3.15, 2.88, and 2.45 Å for AZ,[56] AMI,[57] and *A. xy* NiR,[36] respectively (the same trend is found for the δ_{obs} values of the resonances from the Met $C^{\varepsilon}H_3$ moiety). For the proteins with a trans Met the $C^{\gamma}H$ proton resonances exhibit similar δ_{obs} values consistent with comparable Cu^{II}–S(Met) distances,[38,58–60] except for the *A. cy* NiR, in which this bond is ~0.4 Å shorter. It has been suggested that owing to the weak bond between copper and the axial Met, the influence of the conformation of this ligand on the active site's properties is more likely to be steric in nature than electronic.[55] However, at the T1 copper sites of the NiRs the axial bond is significantly shorter and thus alterations in the conformation of the Met may influence the electronic structure of the site.

The different conformation of the axial Met ligand does not significantly alter the Cu^{II}-S^{δ}-C^{γ}-H^{γ} dihedrals (values of $-171°$ and $-59°$ are found for the Met150 $H^{\gamma 1}$ and $H^{\gamma 2}$ protons, respectively, in *A. cy* NiR, whilst these dihedrals are 61° and 180°, respectively, for the corresponding Met144 protons in the *A. xy* enzyme). The observation that one of the $C^{\gamma}H$ proton resonances in the *A. xy* enzyme probably has almost no δ_c contribution (the small negative δ_{obs} value most likely being made up of mainly a negative δ_{pc} shift) suggests a $\sin^2\theta$ dependence on the Co^{II}–S^{δ}–C^{γ}–H^{γ} dihedrals for the δ_c of the Met144 $C^{\gamma}H$ protons in this NiR (providing the dihedral angles for the Cu^{II} site are maintained in the Co^{II} derivative), indicative of a dominant π-type spin delocalization mechanism.[61] For Co^{II} AZ, which also has a gauche axial Met ligand, spin transfer by σ bonds to the Met $C^{\gamma}H$ protons has been suggested.[27] Small angular changes in the Co^{II} derivatives could alter this interpretation but what is clear is that in the Co^{II} green NiR both of the Met $C^{\gamma}H$ proton resonances exhibit sizable δ_{obs} (and presumably also δ_c) values and thus a different dependence, compared with the Co^{II} blue NiR, (most likely $\cos^2\theta$) on the dihedral angles must prevail. This suggests that a σ-type spin delocalization mechanism dominates in this case.[61] It therefore appears that the different conformation of the axial Met in the blue and green NiRs results in alterations in the bonding interactions of this ligand with the metal.

The spectroscopic properties of blue (classic) and green (distorted/perturbed) T1 copper sites such as those found in the NiRs studied herein have been discussed previously.[35,37,40–42,62–69] The distorted UV/Vis features (see Figure S1 in the Supporting Information) of the green NiR have been assigned to a rotation of the $d_{x^2-y^2}$ orbital, which gives rise to an enhanced interaction with the S(Cys) pseudo-σ orbital (responsible for the LMCT transition at 460 nm) and a decreased overlap with the S(Cys) π orbital (which gives rise to the 600 nm LMCT band) compared to a classic T1 copper site.[40] It is interesting to note that detailed spectroscopic and theoretical studies, mainly of PC, have assigned the weak band at ~450 nm for a classic T1 site to charge-transfer transitions involving the Met and His ligands,[70] with the S(Cys) pseudo-σ→Cu^{II} $d_{x^2-y^2}$ transition occurring at lower energy and overlapping with the 600-nm band. A number of geometric features have been considered to be responsible for the classic/distorted spectroscopic differences. It has been suggested that an increased axial Cu–S(Met) interaction, which pulls the copper out of the plane of the equatorial ligands, is a major contributing factor.[62–64] However, the Cu–S(Met) bond lengths and the Cu to His_2Cys plane distances are almost identical in the blue and green NiRs [there is very little correlation between the ratio of the absorption bands at about 460 nm and about 600 nm and the axial Cu–S(Met) bond length (and the Cu to His_2Cys plane

distances) when a range of structurally characterized T1 copper sites are considered].

Another suggested geometric cause of these spectroscopic differences at T1 copper sites is alterations in the angle (ϕ) between the $N_{His}CuN_{His}$ and $S_{Cys}CuS_{Met}$ planes (see Figure S4, Supporting Information).[40–42] The coupled distortion model associates a decrease in this angle with an increased axial Cu–S(Met) interaction which results in a weaker Cu–S(Cys) bond and these together produce a more tetragonal site (for an ideal trigonal site ϕ is 90°).[40–42] The ϕ values are 66° and 74° for the green and blue NiRs, respectively. The value for the blue NiR is quite small for a protein with a classic T1 copper site (the archetypal classic T1 site of PC has a ϕ value of 82°, whilst for the distorted site of PAZ ϕ is 75°) and the difference relative to that for the green enzyme is not that large. However, it appears that the ϕ values for the NiRs occur in a range in which small alterations lead to large spectroscopic variations (see Figure S4).[40,67,68] The removal of the axial Met ligand, in the Met182Thr *R. sphaeroides* NiR variant results in the green site of the WT protein becoming blue (the crystal structure of this variant is available with the PDB code 1MZZ and demonstrates that the O^{γ} of Thr182 is about 3.5 Å from the copper center).[66,69] The EPR spectrum is hardly altered by this mutation (is still rhombic[66]), which has been explained by similar LF strengths in the Met182Thr and WT proteins.[69] The Cu^{II}–S(Cys) interaction is increased in the Met182Thr variant and the site is less tetragonal than the WT protein, which is consistent with the coupled distortion model.[69] However, this model does not seem to be able to rationalize the presence of classic UV/Vis properties at the T1 site of the blue *A. xy* NiR, which has a strong axial Met ligand. The conformation differences of the Met in the blue and the green enzymes,[36,38] being gauche in the former and trans in the latter, seems to alter the bonding interaction of this residue with Co^{II}. A similar adjustment in the conformation of the axial Met ligand has been found at the T1 copper site of the green NiR from *R. sphaeroides* upon reduction (the Met is trans in the Cu^{II} structure and gauche in the Cu^{I} form), and it has been noted that this will influence the Cu–S(Met) orbital interactions.[37] It could be that changes in the conformation of the axial Met ligand, as well as differing Cu^{II}–S(Met) bond strengths, affect the degree of tetragonal distortion at a T1 copper site.

Conclusion

In this study we show that NMR spectroscopy can be used to obtain detailed active-site information about large, complex, paramagnetic metalloproteins. Derivatives of the blue and green NiRs have been prepared, which possess Co^{II} at just their T2 and also their T1 and T2 sites, and thus the spectral properties of these two centers can be distinguished and investigated. The UV/Vis spectra demonstrate that the T2 Co^{II} sites of the blue and green NiRs are alike, whereas differences are seen for the T1 Co^{II} centers. Detailed T2 and T1 center information is obtained from NMR spectra, which show the close homology of the T2 sites in the blue and green enzymes. The binding of substrate, which may not be particularly strong, results in subtle alterations in the geometry of the Co^{II} T2 site and is inhibited by hydroxide. A comparison of the NMR spectra for the T1 Co^{II} centers highlights that the strength of the interactions with the Cys and Met ligands are similar for the blue and green NiRs. Dramatic alterations in the shift pattern of the $C^{\gamma}H$ protons of the axial Met ligand are observed in the spectra of these proteins, indicating that a conformational change of this residue alters the bonding interaction with the metal. The conformation of the axial Met ligand could contribute to the different spectral properties of the T1 centers in the Cu^{II} proteins.

Experimental Section

Cloning of the *A. xy* NiR gene: The *A. xy* NiR gene was cloned using a procedure described previously[71] with slight modifications. Genomic DNA was isolated from *A. xy* NCIMB11015 and used as a template for polymerase chain reaction (PCR). The forward primer, CATATGCAGGACGCCGACAAGCTGCCCCATACCAAGGTCACGCTGG, introduces an *NdeI* restriction site in front of the mature NiR sequence with the ATG from the *NdeI* restriction site used as a start codon. The reverse primer,
CTCGAGTCAGCGCGGAATCGGCGCGGGCGCCTTGATCTGCTTCATCAGG, incorporates an *XhoI* restriction site after the stop codon of the NiR sequence. Both strands of the PCR product were sequenced and found to be the same as that of mature *A. xy* NiR[72] and the gene was cloned into *NdeI*/*XhoI* digested pET22b vector (Novagene) creating pET22b-bNiR.

Overexpression and purification of recombinant *A. xy* NiR: Overexpression of recombinant *A. xy* NiR was carried out using the same procedure as Ho et al.[71] except that BL21 cells harboring pET22b-bNiR were grown for 6 h at 30 °C after induction. The cell pellet from 2 L of culture was resuspended in 20 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes, Sigma) pH 6.0, disrupted by sonication, and centrifuged. $CuSO_4$ was added to the supernatant (final concentration 1 mM) and after incubation at 4 °C for 2 h, 0.5 % (w/v) of streptomycin sulfate was added and incubated at 4 °C for 1.5 h. The solution was centrifuged and the supernatant loaded onto an SP Sepharose (Amersham Biosciences) column equilibrated in 20 mM Mes pH 6.0. Bound proteins were eluted with a 0–0.3 M NaCl gradient in 20 mM phosphate pH 6.0. The NiR-containing fractions were combined and exchanged into 20 mM tris(hydroxymethyl)aminomethane (Tris, Aldrich) pH 7.5 containing 200 mM NaCl by ultrafiltration (Amicon stirred cell, PM30 membrane). The final purification step involved a Superdex 200 (Amersham Biosciences) gel filtration column. The purified protein gave a single band on a 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and had a A_{280}/A_{594} ratio of <11 for the fully oxidized enzyme. Typically 60 mg of pure protein was obtained from 1 L of culture. Protein concentrations were determined using the molar absorption coefficient of $5200\,M^{-1}cm^{-1}$ at 594 nm, which we have determined (vide infra).

Isolation and purification of *A. cy* NiR: *A. cy* was grown and protein isolation was performed as described previously.[11] The crude cell-free extract of *A. cy* was passed down a carboxymethyl Sepharose (Amersham Biosciences) column equilibrated with 20 mM phosphate pH 6.0 to separate NiR (which does not bind) from PAZ and the eluted solution was loaded onto a diethylaminoethyl (DEAE) Sepharose (Amersham Biosciences) column equilibrated with the same buffer. The bound proteins were eluted with a 0–0.4 M NaCl gradient in 20 mM phosphate pH 6.0. The green fractions were pooled and concentrated by ultrafiltration and

loaded onto a Sephadex G100–120 (Sigma) gel filtration column equilibrated with 20 mM Tris at pH 7.0 containing 150 mM NaCl. The NiR-containing fractions were combined and exchanged into 20 mM phosphate pH 6.0 using ultrafiltration. The protein solution was loaded onto a DEAE Sepharose column equilibrated in the same buffer. Pure NiR was eluted from this column with a 0–0.3 M NaCl gradient in 20 mM phosphate pH 6.0. The purity of the protein was confirmed by SDS-PAGE (12.5% gel) and had a A_{280}/A_{459} ratio of <16 for the fully oxidized enzyme.[39] Protein concentrations were determined using a value of $2400\,M^{-1}cm^{-1}$ for the molar absorption coefficient at 459 nm.[39]

Inductively coupled plasma atomic emission spectroscopy: Inductively coupled plasma atomic emission (ICP-AE) studies were carried out on a Unicam 701 spectrometer. The samples were fully oxidized and washed with 1 mM EDTA and then exchanged into 10 mM phosphate pH 8.0. The UV/Vis spectra of the samples were measured at 25 °C on a λ35 (Perkin Elmer) spectrophotometer. The molar extinction coefficient of WT *A. xy* NiR was determined to be $5200\,M^{-1}cm^{-1}$ at 594 nm using a T2D sample.

T2 copper depletion: T2 copper-depleted *A. xy* and *A. cy* NiRs were prepared using the conditions reported by Suzuki et al.[39] except that incubation was carried out for only two days. The reductant and chelating agents were removed by dialysis (4 °C) in 0.1 M Tris pH 7.0 overnight under nitrogen. The protein sample was further dialyzed against 0.1 M Tris pH 7.0 for 3 h followed by 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) at pH 8.0 (4 °C). Depletion of the T2 copper was confirmed by EPR spectroscopy.

Preparation of apo *A. xy* and *A. cy* NiRs: The removal of both T1 and T2 copper was achieved by dialyzing T2D NiR (~300 μM) against 0.1 M Tris pH 8.0 containing 20 mM KCN and 5 mM ascorbate at 4 °C. After four days the cyanide-containing buffer was refreshed and dialysis was carried out for a further four days. Cyanide was removed by dialyzing the protein against 0.1 M Tris pH 8.0 and finally against 10 mM Hepes pH 8.0 (4 °C).

Cobalt substitution: For cobalt substitution, either T2D or apo NiR were diluted to 200–500 μM in 10 mM Hepes pH 8.0. The protein solution was dialyzed against 10 mM Hepes pH 8.0 containing a 2-fold excess of Co^{II} (against free metal sites), and 0.2 M NaCl in the case of *A. xy* NiR, under nitrogen. After five days the excess Co^{II} was removed by dialysis against 10 mM Hepes pH 8.0 (also under nitrogen). The protein was filtered and exchanged into 10 mM phosphate buffer pH 8.0 using ultrafiltration. Cobalt concentrations were determined by ICP-AE spectroscopy. The $T1Cu^{I}/T2Co^{II}$ and the $T1Co^{II}/T2Co^{II}$ *A. xy* and *A. cy* NiRs are all inactive.

EPR spectroscopy: X-band EPR spectra of $T1Cu^{II}/T2Cu^{II}$ and T2D NiRs were acquired on a Bruker EMX EPR spectrometer at 77 K. The samples were fully oxidized by the addition of a sufficient amount of a solution of 20 mM $[Fe(CN)_6]^{3-}$; the excess oxidant was removed by ultrafiltration and the protein was exchanged into 40 mM Tris pH 7.6. Glycerol was added to the EPR sample to give a final concentration of 40%.

Samples for NMR spectroscopy: The Co^{II}-substituted NiR samples for NMR spectroscopy (typically 2–6 mM) were prepared in 10 mM phosphate buffer pH 7.8 for *A. xy* NiR and pH 7.7 for the *A. cy* protein in 90% H_2O/10% D_2O or 99.9% D_2O using ultrafiltration. Ascorbate was always present (~5–10 mM) and the samples were kept under argon in the NMR tube. For nitrite binding experiments, samples were prepared in either 10 mM phosphate pH 7.7 or 6.5 (both in 90% H_2O/10% D_2O, containing 10 mM ascorbate) for *A. cy* NiR and 10 mM phosphate pH 7.8 or 6.7 (both in 90% H_2O/10% D_2O, containing 10 mM ascorbate) for *A. xy* NiR. Aliquots of a 2 M stock solution of KNO_2 dissolved in 10 mM phosphate pH 7.7 or 6.5 for *A. cy* NiR and in 10 mM phosphate pH 7.8 or 6.7 for *A. xy* NiR were added to the proteins. The sample was flushed with argon and sealed in an NMR tube. The pH values quoted for deuterated solutions are uncorrected for the deuterium isotope effect and thus are indicated by pH*.

NMR spectroscopy: All ^1H NMR spectra were acquired at 300 MHz on a Bruker Avance 300 spectrometer usually at 40 °C. The water-suppressed equilibrium Fourier transform (WEFT) sequence (d1–180°-τ-90°-acq, in which d1 is the relaxation delay and acq the acquisition time) was typically used with spectral widths of approximately 100 kHz, and spectra were processed using 50–200 Hz of exponential line broadening as apodization. Steady-state 1D NOE difference spectra were acquired using the WEFT sequence with τ values and a total relaxation delay (d1 plus acq) ranging from 20 to 50 ms. The resonance of interest was irradiated during τ with a selective decoupler pulse. Spectra were acquired in blocks of eight scans alternating between on-resonance, off-resonance in an upfield direction, on-resonance, and off-resonance in a downfield direction (the offset was equal to that utilized in the upfield direction). The off-resonance spectra were subtracted from their on-resonance counterparts to produce the NOE difference spectrum. The spin-lattice (T_1) relaxation times of the hyperfine-shifted resonances were determined using the WEFT sequence. Peak widths were measured using spectra that had not undergone any baseline correction. The temperature dependence of the observed shifts (δ_{obs} values) of resonances was studied between 25 and 50 °C for *A. cy* NiR and between 15 and 50 °C for the *A. xy* NiR samples (resonances became too broad to be observed below 15 °C in the spectra of the *A. xy* NiR derivatives and at $<$ 25 °C in the substituted forms of the *A. cy* protein).

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