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An NMR Structural Study of Nickel Substituted Rubredoxin

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Abstract

The Ni(II) and Zn(II) derivatives of *Desulfovibrio vulgaris* rubredoxin (DvRd) have been studied by NMR spectroscopy to probe the structure at the metal centre. The βCH_2 proton pairs from the cysteines that bind the Ni(II) atom have been identified using 1D NOE difference spectra and sequence specifically assigned via NOE correlations to neighbouring protons and by comparison with the published x-ray crystal structure of a Ni(II) derivative of *Clostridium pasteurianum* rubredoxin. The solution structures of DvRd(Zn) and DvRd(Ni) have been determined and the paramagnetic form refined using pseudocontact shifts. The determination of the magnetic susceptibility anisotropy tensor allowed the contact and pseudocontact contributions to the observed chemical shifts to be obtained. Analysis of the pseudocontact and contact chemical shifts of the cysteine H β protons and backbone protons close to the metal center allowed conclusions to be drawn as to the geometry and H-bonding pattern at the metal binding site. The importance of NH-S hydrogen bonds at the metal centre for the delocalization of electron spin density is confirmed for rubredoxins and can be extrapolated to metal centers in Cu proteins: amicyanin, plastocyanin, stellacyanin, azurin and pseudoazurin.

Keywords

NMR; Rubredoxin; [Fe-4S] centre; Paramagnetic protein; nickel

Introduction

Rubredoxin (Rd) belongs to the class of Fe-S proteins containing one Fe atom tetrahedrally coordinated to four cysteinyl sulphur atoms. Rd, isolated from sulphate reducing bacteria, has a molecular weight of ca. 6–7kDa and the metal atom in the native state is high spin Fe³⁺ (S=5/2). The reduced state has high spin Fe²⁺ (S=2). The cysteines that bind the metal have a conserved sequence of the type: -CX₁X₂CG-/-CX₁X₂CG-. More than 20 rubredoxin structures are to be found in the protein data bank (PDB) including 2 very high resolution structures at (0.7Å) from a *P. abyssi* mutant and from *D. gigas* [1,2].

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When applying NMR to paramagnetic metalloproteins a number of problems can be encountered that include large hyperfine shifts and extensive line broadening which can result in loss of NMR signals close to the metal centre. This problem is illustrated for the solution structure of the oxidised and reduced forms of *CpRd*(Fe) where constraints near the metal centre are almost absent resulting in disorder close to the metal [3]. However, compared to tetrahedral Fe(II) or Fe(III) where signal loss is extensive, tetrahedral Ni(II), due to its favourable electronic relaxation properties, allows even the H β resonances of the coordinating cysteines to be observed, albeit at low field [4]. Nowadays the presence of a paramagnetic centre can be used to obtain pseudocontact shifts (PCS) and residual dipolar couplings (RDC) that can be combined with traditional NOESY data for structure determination [5,6]. Even if the system under study does not have an inherent metal centre one can be added to take advantage of these data [7,8]. As Rd is a small accessible protein with easy metal replacement a number of studies have used Rd as a model to attempt new structure determination approaches using PCSs and RDCs [9–15]. Also for *CpRd*(Fe) the relaxation properties and chemical shifts of hyperfine-shifted resonances have given information on the state of hydrogen bonding at and around the metal centre and studies, involving theoretical calculations, have shown a dependence of the redox potential on H-bond strength (essentially distance) [16–21]. The magnetic susceptibility anisotropy tensor (MST) for oxidised and reduced *CpRd*(Fe) has been determined and it was shown that redox dependant chemical shift changes, for protons further than ca. 11 Å from the Fe atom, were due to changes in the MST and not from structural modifications when going from the oxidised to the reduced state [22]. Also, very recently, using selective isotope labelling and novel techniques for detecting fast relaxing resonances, an almost complete assignment of the ^{15}N and ^{13}C signals from oxidized and reduced *CpRd*(Fe) has been carried out [23].

The importance of the H-bonding network in Rds has also been illustrated in a study of the Zn (II) forms of *Cp* and *PfRd* where it was suggested that the thermostability of *PfRd* results from a subtle redistribution of H-bonds in the β -sheet sections of the protein and at the metal centre [11]. A study of diamagnetic derivatives of *Cp* and *PfRd* [24] has further indicated that the symmetry of H-bonds to the metal-coordinated sulfurs is more closely maintained in the hyperthermophile *Pf*.

Ni(II) containing enzymes such as urease and hydrogenase are involved in important biochemical processes and as such they have been extensively studied. NiFe hydrogenase and carbon monoxide dehydrogenase both have a tetrahedral Ni(II) centre bound to 4 sulfurs at the active site, a centre relatively rare in biochemistry [25]. In the past, in order to study the mechanistic reaction of these (or of any metal containing) proteins, theoretical models have been used. As metal substitution is easily carried out for Rd, the Ni(II) derivative is a candidate for a model of the active site of these enzymes. There are very few NMR solution structures of Ni(II) containing proteins (PDB: 1ZRR [26], 2DEF [27], and 2GQK [28]) and the only Ni (II) containing Rd structure determined up until now is the x-ray structure of *CpRd*(Ni). This was resolved at relatively low resolution (2 Å) and therefore no detailed analysis was carried out, however the data indicate that the overall structures of the native Fe and Ni(II) forms are very similar [29].

Other metalloproteins containing Ni(II) studied by NMR include the Ni(II) substituted forms of azurin [30–33], amicyanin [34], pseudoazurin [35], stellacyanin [36], and umecyanin [37]. Using the crystal structure of the Cu and Ni(II) forms of azurin it was possible to calculate the axial and rhombic components of the MST. It was found that the Ni(II) form had a lower anisotropy than Co due to the presence of an extra ligand resulting in pentacoordination.

In order to probe the structure in solution of Ni(II) substituted *DvRd*, PCSs were obtained and combined with NOESY data. By obtaining the MST for *DvRd*(Ni) it was possible to predict

the PCS contribution to the chemical shifts of nuclei close to the metal centre and subsequently extract their contact shift contribution. This not only allowed additional assignments to be made but it also allowed the delocalization of unpaired electron spin density onto nuclei near the metal center to be estimated. Conclusions could subsequently be drawn as to the geometry of the H-bonding network at the metal centre in this nickel derivative.

Materials and Methods

Protein purification and metal derivative preparation

Unlabeled DvRd was isolated and purified according to Bruschi *et al* [38]. Isotopic labeling of Rd was carried out using an identical process to that described in Goodfellow *et al.* [39]. The nickel form of Rd was prepared according to Moura *et al.* [4]. The NMR samples were prepared by exchange into phosphate buffer (10mM, pH 7.2) containing 5% D₂O and by repeated concentration/dilution using a centricon YM3 concentrator (Amicon). Final sample concentrations were 1–2 mM.

NMR spectroscopy

For structure determination, backbone ¹H and ¹⁵N resonances (for the Zn(II) and Ni(II) forms of DvRd) were assigned using manual methods with data from the following experiments: [¹H-¹⁵N]-HSQC, 2D-NOESY (mixing time, 150 ms), 2D-TOCSY (mixing time, 70 ms), ¹⁵N-edited NOESY-HSQC (mixing time, 150 ms), and ¹⁵N-edited TOCSY-HSQC (mixing time, 70 ms). A fast recycle 2D NOESY spectrum with a 20ms mixing time, a 300ms recycle delay and a 80ppm sweep width, was also used for assignment in the case of DvRd(Ni). These spectra were run on either Bruker DRX500 or DRX600 (at NMRFAM) spectrometers using TBI and TXI probes respectively. All spectra were processed and analyzed using either NMRPipe [40], Sparky [41] or XEASY [42] software. Chemical shifts were referenced, either directly or indirectly, to DSS at 0 ppm [43].

1D NOE difference spectra were recorded at 400 and 500MHz using the super-WEFT pulse sequence [44] for water suppression (180-t-90-AQ) with t values and recycle times of ca. 150 ms. Selective saturation of the resonances was made during the delay time t. Difference spectra were obtained by subtracting the off-resonance spectra from the on-resonance spectra [45, 46].

Structure determination

Distance constraints for the DvRd(Zn) structure were obtained from 2D-NOESY and 3D ¹⁵N-edited NOESY-HSQC spectra. Structures were generated using the torsion angle dynamics program CYANA [47], followed by manual refinement of the NOE assignments to eliminate consistent violations. The coordinates and experimental constraints have been deposited in the Protein Data Bank (PDB), entry 2QL0, and the chemical shift assignments at BMRB (15374). The structure of paramagnetic DvRd(Ni) was determined using distance constraints from 1D NOE, 2D-NOESY and 3D ¹⁵N-edited NOESY-HSQC spectra. Pseudocontact shift restraints were included using the program PSEUDYANA [48] which is based on DYANA 1.5 [49]. The coordinates and experimental constraints for this form have been deposited in the PDB, entry 2KKD, and the chemical shift assignments at BMRB (15375). The minimization parameters used for the DYANA and PSEUDYANA runs are described in the results section.

The programs FANTASIAN [50] and NUMBAT [51] were used to calculate the MST parameters from PCS data. For the initial PCS tensor calculations only shifts from residues further than 8 covalent bonds from the metal atom were used in order to avoid any possible contact shift contributions (including via H-bonds, *vide infra*). The X-ray structures of the native Fe form of DvRd (8RXN) or the Ni(II) form of CpRd (1R0J) were used in the

calculations. PCSs isosurfaces were calculated using the program NUMBAT. PyMOL [52] was used for all manipulation of structures, for the addition of hydrogen atoms when required and for graphical representations.

Results

DvRd(Zn) solution structure

A total of 90% of the ^1H resonance assignments of *DvRd*(Zn) were obtained through standard procedures. From a total of 47 resonances in an [^1H - ^{15}N]-HSQC spectrum, 44 result from main chain NH groups and 3 from the side chains of N22 (NH_2) and W37 ($\text{NH}\epsilon$). No resonances were observed for residues M1 and K2 due to fast exchange of the amide group with the solvent under these experimental conditions.

The torsion angle dynamics program CYANA was used to calculate a family of low target function structures. The Zn(II) atom was included in the calculations by covalently linking it to the C6 S γ atom and introducing 3 constraints to the other cysteine S γ atoms. The tetrahedral geometry of the centre was achieved by constraining, with upper and lower constraints, the distance between the sulfur atoms of the coordinating cysteines (all with a weighting of 1). This resulted in 12 distance constraints for the Zn(II) center. These limits were calculated based on the X-ray structure of oxidised *DvRd*(Fe). A final total of 581 distance constraints were used (intra 122; short 147; med 121; long 203). A CYANA calculation from 200 starting structures gave a final family of 20 with an average target function value of $0.020 \pm 0.003 \text{ \AA}^2$. The global RMSD values for the family were $0.58 \pm 0.18 \text{ \AA}$ and $1.00 \pm 0.19 \text{ \AA}$ for backbone and heavy atoms, respectively. A comparison of the NMR structure closest to the mean structure and an X-ray structure of oxidised *DvRd*(Fe) gave a global backbone RMSD of $1.0 \text{ \AA}$ (excluding the N-terminus and 2 disordered loops; 18–25, 45–47). The RMSD per residue is given as supplementary material.

Assignment of the H β cysteine protons in *DvRd*(Ni)

Figure 1 shows the low field 400–100ppm region of the ^1H NMR spectra of *DvRd*(Ni) where 8 non exchangeable, resonances can be seen (**a–h**). 1D NOE difference spectra were recorded in D_2O indicating that the resonances **a/c**, **b/d**, **e/h** and **f/g** form 4 pairs of neighbouring protons (Table 1). As these peaks are not solvent exchangeable and the cysteine H β protons can be expected to display the largest low field shifts due to their contact contribution, they can be assigned to the coordinating cysteines (C6, C9, C39 and C42 in Rd). Similar observations have been made for reduced *CpRd*(Fe) for samples selectively labelled with [$^2\text{H}\alpha$]Cys or [$^2\text{H}\beta$]Cys [19]. A combination of four 1D NOE difference spectra and a 2D NOESY spectrum (20ms) allowed sequence specific assignment for the cysteine C6 (**e/h**) and C39 (**f/g**) H β protons. As only peaks **g** and **h** show NOEs to the H ζ and H ϵ protons of F49, stereospecific assignment of these peaks to the pro-S protons of C39 and C6 respectively can be made (Table 1). By combining information from a 100ms NOESY spectrum it was also possible to identify another 1D NOE (from the irradiation of peak **h**) as resulting from the CH_3 group of A44. This group is within $3.5 \text{ \AA}$ of the H β of C39. The protons of the **a/c** and **b/d** pairs only show one NOE in the region 30–5ppm. This is consistent with their assignment to either C9 or C42 as there are very few protons within $5 \text{ \AA}$ of these H β pairs. In both cases the closest proton to the H β pair is the H α of the same residue and therefore the most intense NOE observed in both spectra was identified as the H α proton (data not shown). In this case it was impossible to sequentially or stereospecifically assign the H β protons since the intensities of both NOEs were similar.

DvRd(Ni) solution structure

Initial resonance assignment of peaks from residues far from the metallic center was straightforward using previously assigned residues from *DvRd*(Zn). As expected, for residues

close to the metal centre, assignment was more demanding. A combination of a 3D HSQC-NOESY and an HSQC-TOCSY spectrum allowed the assignment of the 2D [^1H - ^{15}N]-HSQC spectrum of *DvRd*(Ni). Of 46 possible HN resonances 33 were observed along with 3 resonances from the side chains of residues N22 (NH_2) and W37 ($\text{NH}\epsilon$). No NH resonances were observed for residues M1, K2, C6-Y11, C39, and V41-A44 in this spectrum (Figure 2). In order to obtain the resonances of nuclei near the metallic centre, 2D NOESY and TOCSY experiments with a mixing times of 20 ms and a recycle time of 300 ms were performed. In this manner a significant number of additional hyperfine shifted resonances were detected and assigned. 1D NOE difference spectra acquired by irradiating the contact shifted cysteine H β protons allowed further assignment. Final assignments were obtained after calculation of the MST and prediction of PCSs via the program FANTASIAN. After this process there were only 2 residues (M1 and G10) for which there were no assigned resonances.

A family of structures was calculated using the program PSEUDYANA [48] which allows for the inclusion of pseudocontact shifts in a torsion angle dynamics protocol. The PCSs of the ^1H and ^{15}N nuclei were determined by subtracting the diamagnetic contribution from the total hyperfine shifts, using the diamagnetic *DvRd*(Zn) analogue. Possible contact shift contributions (via covalent and H-bonds) were avoided by excluding pseudocontact shifts from any nucleus within 8 covalent bonds of the metal, i.e. residues V5-Y11 and V38-A44. A total of 147 ^1H and ^{15}N pseudocontact shifts were initially used as restraints. The initial MST for the structure calculations was determined using the program FANTASIAN. Atomic coordinates from the X-ray structure of the Fe form of *DvRd* (8RXN) and the experimental PCSs served as input for this step. The subsequent calculations in PSEUDYANA use experimental PCSs and calculated NMR structures in the minimisation protocol.

The Ni(II) atom was included in the calculations using a series of linker residues placed at the C-terminus with additional constraints between the metal and the cysteine S_γ and C_β atoms and between all cysteine S_γ atoms. In order to keep the center in a tetrahedral environment these constraints were given a weighting of 20. In addition, experimental constraints from the 2D and 3D NOESY spectra, from the fast recycle 2D NOESY and from the 1D NOE difference experiments were included to give a total of 529 constraints (161 intra, 95 short, 96 medium, 177 long). The experimental PCS were included with a weighting of 5 compared to the NOE constraints. This was due to lower weightings giving poorer definition of the metal atom. From an initial total of 800 conformers 15 gave a lowest target function of $3.65 \text{ \AA}^2$. This value is rather high, target function values of less than $1.5 \text{ \AA}^2$ are normally considered as acceptable. However, inspecting the constraint violations indicated that the PCSs for a number of side chain resonances and many ^{15}N resonances were being violated. It has been noted previously that the use of ^{15}N PCSs can be problematic. A study of the use of lanthanide based PCS for structure assignment [53] found that for 2 diamagnetic reference compounds, although backbone ^1H chemical shifts did not change between apo- $\epsilon 186$ and $\epsilon 186(\text{La}^{3+})$, the ^{15}N shifts varied considerably. Also due to the small size of Rd, a large percentage of the residues are surface exposed with the possibility of motional averaging. Therefore the calculation was repeated using only backbone PCSs and no ^{15}N PCSs giving a total of 66 restraints. PSEUDYANA does not minimize the magnitude of the MST during the structure calculation only its position and orientation. Therefore, after calculating an initial family of structures a new MST was calculated and this tensor was used in a new round of structure calculations [54]. This process was repeated 5 times resulting in an average MST with magnitude of $\chi_{\text{ax}} -3.85 \pm 0.02 \times 10^{-32} \text{ m}^3$ and $\chi_{\text{rh}} -0.62 \pm 0.02 \times 10^{-32} \text{ m}^3$. The family of structures with a MST closest to this value was subsequently used.

This family of 15 structures with a maximum TF of $1.34 \text{ \AA}^2$ was obtained from an initial total of 400 conformations. Here there were 3 NOE constraints greater than $0.43 \text{ \AA}$. The global

RMSD for the family was 0.96 ± 0.22 Å for the backbone and 1.52 ± 0.22 Å for the heavy atoms. The constraints and RMSD per residue are given in supplementary material.

Determination of the magnetic susceptibility tensor in *DvRd*(Ni)

The program NUMBAT was used to calculate the MST parameters for 3 different rubredoxin structures: *DvRd*(Fe) (8RXN); *CpRd*(Ni) (1R0J); *DvRd*(Ni) (2KKD). Experimental PCS data from *DvRd*(Ni) was used in conjunction with the above atomic coordinates. For these tensor calculations only the reduced set of backbone PCSs, excluding residues further than 8 covalent bonds from the metal atom, were used to avoid any possible contact shift contribution. The ^{15}N resonances were also excluded. The origin of the MST was constrained to the coordinates of the metal atom in the structures used for the calculations. The resulting tensors and their orientations are shown in Table 2. By plotting experimental PCSs and PCS values calculated from the fitted MST for all the PCS used in the calculation and the PCS shifts from nuclei within 8 covalent bonds of the Ni(II) atom, Figure 3, the presence of contact shift contributions to the observed chemical shifts in *DvRd*(Ni) can be seen (*vide infra*). A chemical shift pseudocontact shift isosurface at ± 1 ppm for *DvRd*(Ni) using the MST parameters obtained using the *DvRd*(Ni) NMR structure is shown in Figure 4a.

Estimation of Contact shifts in *DvRd*(Ni)

In order to estimate the contact shift contributions to the observed chemical shifts the diamagnetic and PCS contributions must be factored out ($\delta_{fc} = \delta_{\text{exp}} - \delta_{\text{psc}} - \delta_{\text{dia}}$). Using the calculated MSTs from the NMR data from *DvRd*(Ni) and the coordinates from the *DvRd*(Ni) NMR structure and the *DvRd*(Fe) and *CpRd*(Ni) x-ray structures the PCS contribution to the chemical shift of nuclei close to the metal center could be calculated. These PCS values (see supplementary material) and the chemical shifts from the diamagnetic zinc form of *DvRd* were subtracted from the observed chemical shifts to estimate the contact shift contribution in *DvRd*(Ni).

Table 3 shows the average (from the 3 structures) estimated contact shifts (and the standard deviation) for the ^1H nuclei within 7 bonds (covalent and/or H-bond) of the Ni(II) atom. The nuclei included in the table and the numbers in parentheses, which indicate the number of bonds removed from the metal centre, assume that the H-bonding pattern at the metal center, Figure 5, is of the standard Rd type [16].

Discussion

The solution structures of *DvRd*(Zn) and *DvRd*(Ni)

The backbone conformation of the Zn form of *DvRd* is very similar to the backbone fold of the x-ray structure of *DvRd*(Fe), as expected. In fact the global fold of rubredoxins is conserved in almost all organisms: the maximum RMSD for the backbone alignment of the high resolution (0.5–1.5 Å) x-ray structures for *Pyrococcus furiosus*, *Clostridium pasteurianum*, *Desulfovibrio gigas*, *Desulfovibrio vulgaris*, *Pyrococcus abyssi* and *Desulfovibrio desulfuricans* is 1.5 Å².

The solution structure of the Ni(II) form has a relatively good global backbone RMSD with poorer definition near the paramagnetic Ni(II) centre, especially the C6–C9 region, due to a lack of experimental NOE constraints. The backbone RMSD, excluding the N-terminus, to the x-ray structure of the Fe form is 1.09 Å². The backbone RMSD of the solution structures of the Zn(II) and Ni(II) forms is 1.6 Å². A comparison with the published x-ray structure of *CpRd*(Ni) indicates that the backbone conformation is very similar (bb RMSD, residues 1–52, 1.1 Å², Figure 4b). The RMSD per residue for these comparisons is shown as supplementary material. Comparison of the geometry at the metal centre is more problematic due to the poorer definition in the NMR structure, however as the sequences of *CpRd* and *DvRd* differ only

slightly near the binding cysteines: *DvRd* -CTVC-/-CPVCGA- and *CpRd* -CTVC-/-CPLCGV-; the backbone geometries would be expected to be similar.

The H-bonding network at the metal center in Rubredoxins is well known and includes a number of NH-S γ H-bonds (Figure 5). In order to probe the H-bonding in *DvRd*(Ni), NH to S γ distances in the family of solution structures were measured. It was found that of the 2 of the 6 NH-S γ H-bonds present in most Rds, Y11-C9 and V41-C39, had longer distances than normally found: 4.50(0.54) and 3.01(0.29) Å. In order to confirm the presence of these H-bonds an analysis of the contact shift contribution to the observed chemical shifts in *DvRd*(Ni) was carried out.

Contact shifts in *DvRd*(Ni)

The estimation of contact shifts requires that the PCS and diamagnetic contributions be known. As Ni(II) has no accessible diamagnetic oxidation state another diamagnetic metal must be used. Zn(II) is a good candidate as it adopts tetrahedral coordination and Zn-S bonds lengths are comparable to Ni-S bond lengths. Figure 3 shows how the calculated PCSs for a number of backbone chemical shifts deviate from their experimental values. The error bars shown in Figure 3 are the standard deviations for the calculated PCSs using the solution structure and the 2 X-ray structures for *DvRd*(Fe) and *CpRd*(Ni). The largest standard deviations are seen for HA from residue 44 and the HN for residues 8 and 41. The variations for HN 8 and HN 41 result from structural differences between the solution and x-ray structures. This is most probably due to the poor definition, due to the lack of experimental constraints, in the solution structure for the backbone near the Ni(II) center. In fact the standard deviation for calculated PCSs between the *DvRd*(Fe) and *CpRd*(Ni) X-ray structures for these atoms is 0.24 and 0.29 respectively compared to 2.8 and 6.0 for all 3 structures (Table 3). For HA 44 the standard deviation does not change significantly by considering the X-ray structures alone (2.0 compared to 1.7). However, even assuming that the solution structure may not be well defined near the Ni(II) center structural differences alone do not explain the deviations of the calculated PCS from their experimental values. Contact shift contributions to the observed chemical shifts however can be used to explain these deviations.

A number of studies, both experimental and theoretical, have shown the importance of H-bonds (NH-O and NH-S) in Rd and changes in H-bond strength have been found to modulate the redox potential of the active site [16]. The presence of these H-bonds, especially NH-S, at the metal center can be confirmed for *DvRd*(Ni) in solution by considering the pattern of contact shifts shown in Table 3. Contact shifts result from the presence of unpaired electron spin density at a nucleus and it is assumed that the larger the contact shift the more unpaired electron spin density resides at a nucleus. This proportionality is valid for a single electron in an orbital which is well separated from any other excited level. This spin density can arrive via covalent bonds or via H-bonds. For *DvRd*(Ni), most of the larger contact shifts (> 2 ppm) in Table 3 can be explained by invoking an H-bonding network near the Ni(II) center that facilitates unpaired electron spin delocalization. Appreciable contact contributions to observed chemical shifts are seen here for protons up to 5 covalent bonds removed from the Ni(II) atom and importantly for atoms further than 5 covalent bonds from Ni(II). For instance, the ¹H NH resonances for V8 and V41 are both 8 covalent bonds removed from the Ni(II) atom but have contact shifts of 12.43 and 13.64 ppm respectively. These large contact shifts can be explained by the fact that they are also involved in NH-S H-bonds to Ni-ligating S γ atoms. Also, for the NH protons of C9 and C42 the unpaired electron spin density from the Ni(II) atom arrives via 5 covalent bonds and via 2 bonds involving an NH-S H-bond (Table 3) resulting in these resonances being undetectable under our experimental conditions. Conversely, the NHs of C6 and C39 are not involved in NH-S H-bonds and only receive unpaired electron spin density via 5 covalent bonds and are therefore detectable.

These contact shift results confirm the V8-C6, C9-C6, C42-C39 and A44-C42 NH-S H-bonds seen in the *DvRd*(Ni) solution structure. They also confirm that the V41-C39 NH-S H-bond is present as well, something that could not be confirmed using the solution structure alone. The final Y11-C9 NH-S H-bond was not detected in the solution structures, and the Y11 ^1H NH resonance could not be detected under our experimental conditions. However this fact in combination with the results of Wilkens et al [19] where DFT calculations indicated the nitrogen of Tyr11 as having the largest calculated contact electron density and the shortest NH to S H-bond in a *CpRd*(Fe) structure and of Lin et al [23] where the ^{15}N resonance of Y11 in oxidised *CpRd*(Fe) is the most low field shifted of all the ^{15}N hyperfine signals, suggests that the absence of this NH resonance may be due to relaxation broadening or a large hyperfine shift due to unpaired electron spin density arriving via a NH to S H-bond.

The use of NMR, more specifically a combination of experimental chemical shifts from paramagnetic and diamagnetic forms of *DvRd* in combination with available structures, allows the distribution of unpaired electron spin density to be determined from contact shifts and H-bonding networks to be inferred even in regions close to the metal where there is a lack of experimental constraints.

Analysis of the H β shifts from the binding cysteines in *DvRd*(Ni)

In order to determine the amount of electron spin density present at the H β nuclei the average chemical shift of a H β proton pair ($\delta_{1/2}$) is often used [55] as it is less sensitive to conformational changes in the H β -C β -S γ -M dihedral angle [56]. The $\delta_{1/2}$ values for the H β proton pairs in *DvRd*(Ni) and *CpRd*(Fe) show (Table 1) that C9 (321/320 ppm) and C42 (320/321 ppm) have much higher electron spin density present at the nucleus than C6 (183 ppm) and C39 (178 ppm). A comparison of experimental NMR data with density functional calculations for the hyperfine shifts in oxidised and reduced *CpRd*(Fe) showed that NH-S H-bonds are very effective in transferring electron spin density from the metal to other nuclei and that small changes in NH-S H-bond distances create large changes in spin density at the resonating nuclei [18]. Intuitively, as the S γ atoms for C6 and C39 have two NH atoms H-bonded, it may be expected to have less electron spin density due to these “extra” outlets for spin delocalisation when compared to the S γ atoms (and attached nuclei) for C9 and C42 which only have one NH-S H-bond each. The experimental $\delta_{1/2}$ values, seen for *DvRd*(Ni), confirm that the H β protons of C9 and C42 have higher unpaired electron spin density compared to C6 and C39 and that the same electron spin delocalization (H-bond) pathway may be active here. This type of pattern is also seen for oxidised and reduced *CpRd*(Fe) where the ^2H hyperfine shifts for Cys9 and Cys42 were seen further downfield (Table 1) than those for C6 and C39 [19].

In general it appears that, for a paramagnetic metal bound to cysteine in Rd, the spin density on the cysteine H β nuclei depends, not only on the H β -C β -S γ -M dihedral angle, but also on the number of NH-S H-bonds to the S γ atom : 2 NH-S H-bonds compared to 1 allow more electron spin density to be siphoned off resulting in lower $\delta_{1/2}$ values for the corresponding H β protons. NH-S H-bonds are also present in blue copper proteins and their derivatives where one ligating cysteine is present with approximately the same dihedral angle. NMR chemical shift data is available for a number of Ni(II) derivatives along with structural data from the native Cu forms. Table 1 presents the $\delta_{1/2}$ values for the cysteine H β protons for Ni(II) forms of azurin [33,34], pseudoazurin [35], stellacyanin [36], umecyanin [37] and amicyanin [34] where it can be seen that the presence of NH-S H-bonds may be correlated to a decrease in $\delta_{1/2}$ values. Those cysteine H β protons whose S γ atom has 2 H-bonds appear at higher field. It must be remembered however that other factors such as differences in M-S bond strength/length and the presence of axial ligands will also affect chemical shifts and may also play a role in these cases [34,37].

Conclusions

The large number of NMR studies using diamagnetic and paramagnetic forms of the small Fe-S protein Rubredoxin in order to validate theoretical calculations, test new pulse sequences and probe unfolding pathways confirms Rd as an important metalloprotein model system. In this work the Ni(II) form of this protein was studied not only because it acts as a model for Ni(II) containing enzymes but also because Ni(II), due to its relaxation properties, allows more of the protein to be seen by NMR compared to the native Fe form. Initially solution structures of the Zn(II) and Ni(II) forms of *Dv*Rd were determined by NMR. The assignment of the spectra of the paramagnetic Ni(II) form required the use of tailored NMR experiments in conjunction with the MST obtained via PCSs. The structure of the Ni(II) form was subsequently determined using constraints from standard 2D/3D spectra, 1D NOE difference spectra and from PCS data. The structures were found to be very similar to the numerous published Rd structures obtained using NMR and x-ray.

In order to probe the geometry and H-bonding network at the metal center the contact shifts for the observable resonances near the Ni(II) center were determined by subtracting the experimental diamagnetic and calculated dipolar (PCS) shifts from the observed chemical shifts. The subsequent pattern of contact shifts for *Dv*Rd(Ni) observed in solution can be explained by invoking a H-bond network similar to that seen in a published low resolution x-ray structure from *Cp*Rd(Ni). The results also confirm the importance of the NH-S H-bonds in the distribution of electron spin density in Rd and show how structural information can be obtained from the distribution of contact shifts even when there is a lack of experimental NOE constraints.

Analysis of the contact shift dominated $\delta_{1/2}$ values from the cysteine H β protons shows how NH-S H-bonds as well as H β -C β -S γ -M dihedral angles are important in unpaired electron spin density delocalization on these atoms. A S γ atom involved in 2 NH-S H-bonds results in electron spin density being siphoned off and an observed shift for the H β protons of C6 and C39 of approximately 150–200 ppm. The presence of 1 NH-S H-bond to a S γ atom results in more electron spin density at the H β protons as is the case for C9 and C42 (shifts of approximately 300–250 ppm). This type of analysis can also be applied to the H β protons from the single ligating cysteine ligand found in the Ni(II) forms of azurin and azurin type Cu proteins. Here a similar correlation between the $\delta_{1/2}$ values of the H β protons and the number of NH-S H-bonds (for the same H β -C β -S γ -M dihedral angles) was found suggesting that these of type H-bonds may also play a role in the distribution/delocalization of electron spin density in these native Cu proteins as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used

NOESY	nuclear Overhauser enhancement spectroscopy
Rd	rubredoxin
<i>Dv</i>	<i>Desulfovibrio vulgaris</i>
<i>Cp</i>	<i>Clostridium pasteurianum</i>
<i>Pf</i>	<i>Pyrococcus furiosus</i>
MST	magnetic susceptibility anisotropy tensor

HSQC	heteronuclear single quantum coherence
TOCSY	total correlation spectroscopy
NOE	nuclear Overhauser enhancement
RDC	residual dipolar coupling
PCS	pseudocontact shift
AZ	Azurin
UMC	umecyanin
PA	pseudoazurin
STC	stellacyanin
AMI	amicyanin

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FIGURE 1.

The 1D ¹H NMR spectrum of *DvRd(Ni)* at 302K. The low field contact shifted H β protons from the 4 binding cysteines can be observed between 350 and 150 ppm (**a–h**). Other contact and pseudocontact shifted peaks can be seen outside of the diamagnetic envelope up to +30 and down to –30 ppm. The difference in peak intensity seen for the low field shifted peaks is due to an uneven excitation profile.

FIGURE 2.

The $[^1\text{H}-^{15}\text{N}]$ -HSQC spectra of *DvRd*(Zn) (blue) and *DvRd*(Ni) (red) at 296K in phosphate buffer at pH 7.2. Assignments are indicated along with selected hyperfine shifts.

FIGURE 3.

A plot of the calculated versus experimental PCSs for DvRd(Ni). The data were obtained using the MST calculated from the mean solution structure and backbone chemical shifts excluding those within 8 covalent bonds of the metal center (black circles). Also shown are the experimental and average calculated PCSs (using the DvRd(Fe), CpRd(Ni) and mean NMR structures) from backbone nuclei within 8 covalent bonds (grey circles). The error bars are the standard deviations for the results from the 3 structures. The equation of the line is $y=0.984x-0.005$ with an R^2 of 0.98

FIGURE 4.

a) A pseudocontact chemical shift isosurface at -1 (dark grey) and +1 (light grey) ppm using the MST parameters obtained from the *DvRd(Ni)* NMR structure; b) A stereo overlay plot of the backbone and metal centre for the x-ray structures of *DvRd(Fe)* (dark grey) and *CpRd(Ni)* (light grey) and the mean NMR structure for *DvRd(Ni)* (black).

FIGURE 5.

Representation of the NH to S γ H-bonding network found at the metal center in rubredoxins. Arrows indicate the S γ to NH direction. Amino acid numbering is for DvRd. Figure adapted from reference [19].

¹H NMR chemical shifts for the ligating cysteine H β protons from the Ni(II) and Fe(II) forms of *Dv* and *Cp* rubredoxin respectively and published chemical shifts for a number of Ni(II) containing azurin-like proteins. Dihedral angles for the C α carbon of the cysteines ligating the metal atom in *Dv*Rd(Fe), *Cp*Rd(Ni) and *Cp*Rd(Fe) are taken from the x-ray structures 8RXN, 1R0J and 5RXN respectively. The average NMR chemical shifts for the cysteine H β protons in *Dv*Rd(Ni) and *Cp*Rd(Fe) are included along with the number of HN-S hydrogen bonds each cysteinyl sulfur is involved in. The structural data for the azurin-like proteins are for the native Cu forms and the NMR data for the Ni(II) derivatives.

TABLE 1

	H β	δ_{obs}	$\delta_{1/2}$	M-S γ -C β -C α	HN-S H-bonds ^g
<i>Dv</i> Rd(Ni)	C9/C42	a(c) 362 (279)	321/320	-94.35/-89.65 ^{8RXN}	1
	C42/C9	b(d) 360 (269)	321/320	-89.65/-94.35	1
	C6 proR	e(h) 198 (161)	183	-172.69	2
	C39 proR	f(g) 188 (167)	178	-175.33	2
<i>Cp</i> Rd(Fe)	C42 proS	a(c) 251 (233)	242 ^a	-83.8 ^{5RXN}	1
	C9 proS	b(d) 244 (233)	239 ^a	-90.5	1
	C6 proR	e(h) 196 (157)	178 ^a	-177.7	2
	C39 proR	f(g) 193 (159)	176 ^a	-178.2	2
<i>Cp</i> Rd(Ni)	C42			-84.7 ^{1R0J}	1 ^h
	C9			-95.2	1
	C6			-167.7	2
	C39			-178.8	2
UMC	C85 proS	224(167)	196 ^b	-169.6 ^{1X9U}	2
AZ	C112 proS	238 (197)	218 ^c	-161.4 ^{2AZA}	2
AZ	C112 proS	233 (187)	210 ^d	-171.0 ^{4AZU}	2
AZ ^{N12IQ}	C112 proS	237(178)	208 ^c	169.1 ^{1URI}	2
AZ	C112 proS	238(194)	216 ^j	-172.7 ^{2CCW}	2
STC	C87 proS	197(177)	187 ^e	-176.2 ^{1JER - CαSTC}	2
PA	C78 proS	297 (274)	285 ^f	-169.2 ^{1BOK}	1
AMI	C93 proS	254 (296)	275 ⁱ	-171.6 ^{1ID2}	1

^a²H shifts from the reduced form, data taken from [19].

b taken from ref [37]
 c taken from ref [31]
 d taken from ref [33]
 e taken from ref [36]
 f taken from ref [35],
 g within 2.8 Å,
 h 3.1 Å,
 i taken from [34],
 j taken from ref [32]

TABLE 2

The MSTs calculated using experimental PCSs from *Dv*Rd(Ni) and the coordinates from the *Dv*Rd(Ni) NMR structure and the *Dv*Rd(Fe) and *Cp*Rd(Ni) x-ray structures. The errors presented in the table are standard deviations calculated from 100 sets of randomized (0.1 Å standard deviation) atom coordinates via NUMBAT.

	ΔY_{ax}^a	ΔY_{in}^a	α	β	γ
<i>Dv</i> Rd(Fe) – PCS: 8RXN	–3.61(0.10)	–0.88(0.05)	148(0.3)	69(0.6)	167(1.8)
<i>Cp</i> Rd(Ni) – PCS: 1R0J	–3.41(0.08)	–0.92(0.05)	57(0.5)	96(0.4)	59(1.8)
<i>Dv</i> Rd(Ni) – PCS: 2KKD	–3.86(0.09)	–0.62(0.07)	174(0.6)	121(0.9)	81(2.9)

^a ×10^{–32} m³

TABLE 3

The estimated contact shifts for all ¹H nuclei within 7 bonds (covalent and/or H-bonds and assuming a standard Rd H-bonding pattern at the metal center [16]) of the Ni(II) atom. The number of bonds that separate each nucleus from the Ni(II) atom is presented in parentheses. ND = not detected. The contact shifts presented (with standard deviations) are average values calculated using the mean NMR solution structure and x-ray structure from CpRd(Ni) and DvRd(Fe).

	covalent	NH-O	NH-S	δ _{con}	SD	covalent	NH-O	NH-S	δ _{con}	SD
V5	Hα (7)			-0.03	0.1	V38	Hα (7)		0.39	0.1
C6	HN (5)			-3.94	1.2	C39	HN (5)	HN (7)*	-2.11	0.3
	Hα (4)			-18.89	1.2		Hα (4)		ND	
	Hβ (3)			168.5	5.7		Hβ (3)		164.0	4.9
				124.0	6.3				139.09	4.3
T7	HN (6)		HN (7)	-1.48	0.8	P40				
	Hα (7)		Hα (6)	0.78	0.2		Hα (7)	Hα (6)	1.65	0.7
V8			HN (2+7)	12.43	2.8	V41		HN (2+7)	13.64	5.7
	Hα (7)		Hα (5+6)	4.61	0.3		Hα (7)	Hα (5+6)	4.29	0.3
C9	HN (5)		HN (2+7)	ND		C42	HN (5)	HN (2+7)	ND	
	Hα (4)		Hα (5)	-3.10	0.3		Hα (4)	Hα (5)	-3.03	0.6
	Hβ (3)		Hβ (6)	325.8	4.8		Hβ (3)	Hβ (6)	326.1	4.5
				264.5	1.4				257.4	1.2
G10	HN (6)	HN (6)	HN (7+7)	ND		G43	HN (6)	HN (7+7)	ND	
	Hα (7)		Hα (6)	ND			Hα (7)	Hα (6)	1.64	0.6
									0.34	0.4
Y11			HN (2)	ND		A44				
			Hα (5)	1.01	0.2			Hα (5)	1.28	2.0
E12			HN (7)	-0.03	0.2	E50		HN (7)	0.39	0.1

* via NH(39)-O(44)