Detection and Classification of Hyperfine-Shifted ^1H, ^2H, and ^{15}N Resonances from the Four Cysteines That Ligate Iron in Oxidized and Reduced *Clostridium pasteurianum* Rubredoxin

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Abstract: Rubredoxins belong to the simplest class of iron−sulfur proteins. They contain a single iron coordinated by four cysteinate sulfurs. The rubredoxin from *Clostridium pasteurianum* was overproduced in *Escherichia coli*, and the metal was incorporated into the apoprotein by *in vitro* reconstitution. Protein samples were prepared at natural isotopic abundance, labeled uniformly with ^{15}N, and labeled specifically with [$^2H^{\alpha}$]cysteine, [$^2H^{\beta 2,\beta 3}$]cysteine, and [^{15}N]cysteine. One-dimensional ^1H, ^2H, and ^{15}N nuclear magnetic spectroscopy was used to study the electron−nuclear interactions. Previously unreported hyperfine-shifted resonance signals were observed in the ^1H and ^2H NMR spectra of rubredoxin samples in both the oxidized and reduced states. Signals from the α- and β-hydrogens of the four cysteines were identified unambiguously from ^1H and ^2H NMR spectra of samples labeled selectively with deuterium. The cysteine hydrogen signals are resolved more clearly by ^2H (lower magnetogyric ratio) than by ^1H (higher magnetogyric ratio) NMR spectroscopy. In the oxidized state, signals from two of the four α-hydrogens are located downfield in the 150−200 ppm range; the other two are found upfield at about −10 ppm. Signals from all eight β-hydrogens were detected downfield in the 300−900 ppm region. Upon reduction, the ^1H NMR signals from all eight β-hydrogens lie downfield between 150 and 240 ppm; signals from two of the four α-hydrogens lie upfield near 0 ppm, and those from the other two are downfield at 16 and 19 ppm. Thirteen hyperfine-shifted signals were resolved in one-dimensional ^{15}N NMR spectra of the sample labeled uniformly with ^{15}N. The two signals located farthest upfield and two signals in the downfield region were assigned to the cysteines that ligate the iron on the basis of selective labeling with [^{15}N]cysteine.

Rubredoxin was the first nonheme iron protein studied by NMR spectroscopy,[1] and it has been the subject of several subsequent NMR investigations.[2−4] The protein also has been studied by a variety of other biophysical techniques.[5] The rubredoxin from *Clostridium pasteurianum* (Rdx) is a small protein with a molecular mass of 6100 Da.[6] Its crystal structure has been solved to a resolution of 1.2 Å,[7,8] and the protein recently was overproduced in *Escherichia coli*.[9] All these factors make it an excellent model protein for detailed NMR study. Here, we report the resolution and characterization of hyperfine-shifted resonances from the four cysteines that ligate the iron of Rdx (the protein has no additional cysteine residues). By combining selective isotopic labeling and one-dimensional ^1H, ^2H, and ^{15}N NMR spectroscopy, we successfully identified resonances from all the H^{α}, H^{β}, and N atoms of the four cysteines of Rdx in both its oxidized and reduced states. Also, some additional hyperfine-shifted ^1H and ^{15}N resonances were discovered.

Experimental Section

The *C. pasteurianum* rubredoxin was overexpressed in *E. coli* by using the bacterial phage T7 RNA polymerase/promoter system. The DNA fragment encoding Rdx was amplified by PCR from the original expression plasmid pCPRD1 and subcolonied into pET3a vector (Novagen) to produce a new plasmid which was called CpRd/pET3a. For general expression, *E. coli* strain BL21(DE3)/pLysS was used as host. The bacteria were grown at 37 °C with vigorous shaking until OD_{600} of the culture reached 1.4, and then 100 mg of IPTG was added to induce the production of Rdx. After additional incubation at 37 °C for 2 h, the cells were harvested by centrifugation and cell pellets were resuspended in 0.5 M Tris·HCl buffer (pH 8.0).[9]

The cells were broken by sonication after a freeze−thaw cycle. After *in vitro* reconstitution of the iron center, the Rdx was further purified

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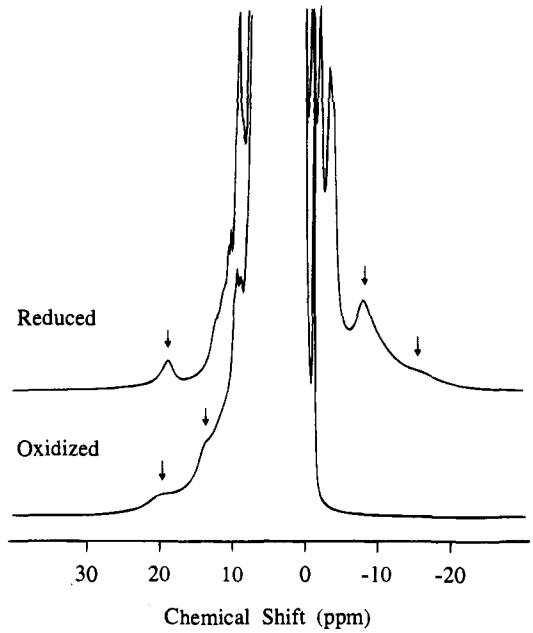

Figure 1. 400 MHz ^1H NMR spectra of oxidized and reduced *C. pasteurianum* rubredoxin (pH 6.0) in ^2H_2O at 35 °C. Peaks indicated by arrows have not been reported previously.

using anion exchange chromatography and gel filtration. Protein fractions with $OD_{490}/OD_{280} > 0.43$ were considered to be pure.[9]

[U-^{15}N]Rdx was prepared by growing the *E. coli* host cell BL21-(DE3)/pLysS with the expression plasmid on M9 medium with $^{15}NH_4Cl$. The cysteines were labeled selectively by using the cysteine auxotrophic *E. coli* strain JM15(DE3)/pLysS as expression host and growing the cells with the expression plasmid in M9 medium plus a mixture of the isotopically labeled cysteine to be incorporated and the unlabeled amino acids. All isotopic labeled compounds were purchased from Cambridge Isotope Laboratories.

Rubredoxin samples were reduced by adding solid sodium dithionite (about 2−3 mg) to the protein solution under anaerobic conditions.

All ^1H and ^2H NMR spectra were collected on a Bruker DMX400 wide-bore NMR spectrometer with a 5 mm ^1H probe or 10 mm broad-band probe, respectively. One-dimensional ^{15}N NMR spectra were recorded on a Bruker DMX600 NMR spectrometer with a 5 mm broad-band probe. A simple one-pulse sequence was used for all one-dimensional ^1H, ^2H, and ^{15}N experiments. Fast pulse repetition rates were used to saturate the diamagnetic resonances.

Results and Discussions

Figure 1 shows the 40 to −30 ppm region of the ^1H NMR spectra[10] of unlabeled oxidized and reduced Rdx at 35 °C. The arrows indicate locations of previously unreported hyperfine peaks for this protein: 19 and 14 ppm (oxidized Rdx); and 19, −8, and −15 ppm (reduced Rdx). The downfield-shifted peaks move toward their diamagnetic positions with increasing temperature ($\Delta\delta/\Delta T < 0$) as do the upfield-shifted resonances (with $\Delta\delta/\Delta T > 0$).

Further vertical expansion of the downfield region (100−250 ppm) of the ^1H NMR spectrum[11] of oxidized Rdx revealed two broad peaks with line widths of about 80 kHz (Figure 2A). These two peaks shift upfield with increasing temperature. The downfield region (270−170 ppm) of the ^1H NMR spectrum of

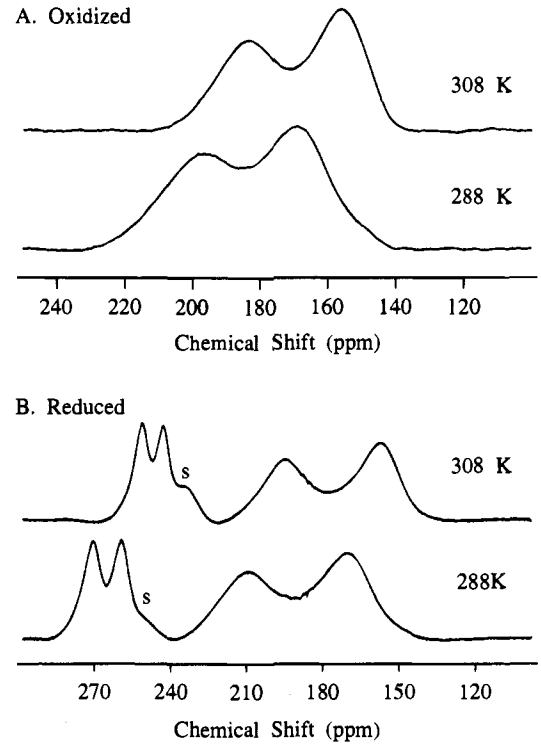

Figure 2. ^1H NMR spectra of (A) oxidized and (B) reduced rubredoxin (pH* 6.0) in ^2H_2O, at the temperatures indicated. Only parts of the downfield region are shown. The shoulder "s" in the spectrum of reduced Rdx becomes a fully resolved peak at higher temperature.

reduced Rdx contains five resonances (Figure 2B), two with sharper line widths than the other three. Similar peaks in this region were reported for reduced *Desulfovibro gigas* rubredoxin and assigned to β-protons of cysteines.[12] In Rdx, five peaks are resolved in this region instead of four as reported previously;[12] the additional peak appears as a broad shoulder to the high field of one of the sharper peaks. Our results, confirmed and extended below by use of ^2H NMR spectroscopy, clearly indicate that the 2-fold degeneracy initially proposed for the eight β-protons of *D. gigas* rubredoxin[12] does not occur for the reduced *C. pasteurianum* rubredoxin. It is worth noting that the pattern of peaks in Figure 2B compares with those detected for rubredoxin model compounds involving bidentate peptide ligands.[13] Moreover, the similarity of the chemical shift range observed here with Rdx in water and with model compounds in the less polar acetonitrile[13] confirms the highly hydrophobic and solvent-shielded environment of the rubredoxin active site.[7,8]

In order to detect and classify all H^α and H^β resonances from the cysteines, we prepared Rdx samples selectively labeled with [$^2H^\alpha$]cysteine or [$^2H^\beta$]cysteine and studied these samples by both ^2H and ^1H NMR spectroscopy.[14] Figure 3 shows ^2H NMR spectra of the [$^2H^\alpha$]Cys- and [$^2H^\beta$]Cys-labeled Rdx samples at 35 °C. In oxidized Rdx (Figure 3A), two of the four H^α signals appear downfield (180 and 150 ppm), while the other two appear upfield (−10 ppm, overlapped). Upon reduction, they all shift

(10) The chemical shifts of the ^1H NMR spectra were referenced to external DSS. Fifty thousand scans were collected for a spectral width of 100 ppm by using 4096 data points. The repetition time was ~62 ms. The protein sample was in ^2H_2O (pH* 6.0) at a concentration of about 8 mM.

(11) Two hundred thousand transients were collected over a bandwidth of 500 ppm by using 4096 data points. The center of the spectrum was 4.7 ppm for oxidized Rdx and 50 ppm for reduced Rdx. The repetition rate was about 25 ms. The protein sample was the same one described in ref 10.

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(14) For [$^2H^\alpha$]Cys Rdx, 150 000 scans were collected with a spectral width of 600 ppm (oxidized state) or 1500 ppm (reduced state) and a repetition period of ~56 or ~12 ms, respectively. For oxidized [$^2H^{\beta2,\beta3}$]-Cys Rdx, 300 000 scans were averaged with a spectral width of 1196 ppm (center at 700 ppm) and repetition period of about 10 ms. For reduced [$^2H^{\beta2,\beta3}$]Cys Rdx, 100 000 scans were recorded with a 400 ppm spectral width. The labeled protein samples were dissolved in ^2H-depleted ^1H_2O at pH* 6.0.

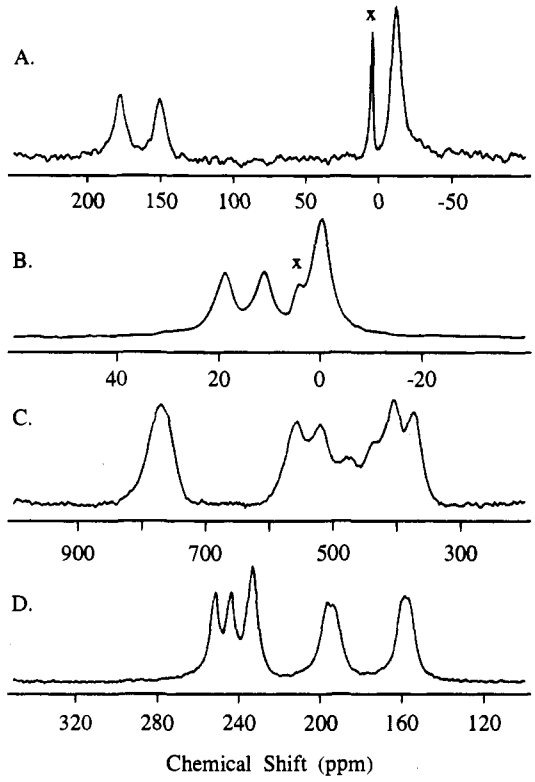

Figure 3. ^2H NMR spectra of ^2H-cysteine-labeled rubredoxin at 35 °C, pH 6.0: (A) oxidized [$^2H^{\alpha}$]Cys-labeled rubredoxin; (B) reduced [$^2H^{\alpha}$]Cys-labeled rubredoxin; (C) oxidized [$^2H^{\beta 2,\beta 3}$]Cys-labeled rubredoxin; (D) reduced [$2H^{\beta 2,\beta 3}$]Cys-labeled rubredoxin. The peak labeled "x" arises from residual $^1H^2HO$.

toward the diamagnetic region: at 35 °C, the downfield peaks of unit intensity are at 19 and 16 ppm, and one upfield peak of two-unit intensity is at 0 ppm (Figure 3B). Spectra of oxidized [$^2H^{\beta}$]Cys Rdx reveal signals from all eight H^{β} with one overlapped doublet in the extreme downfield region between 900 and 300 ppm (Figure 3C). In the reduced state, the eight H^{β} signals all appear in the 280−150 ppm range as two resolved single peaks, one overlapped doublet, and two partially resolved doublets (Figure 3D).

Thirteen hyperfine-shifted resonances were observed in the ^{15}N NMR spectrum[15] of [U-^{15}N]Rdx in both oxidation states (Figure 4A,B, top spectra). Two of them appear in the upfield region and shift downfield with increasing temperature, whereas the remaining 11 peaks are located in the downfield region and shift upfield with increasing temperature. In order to identify the ^{15}N resonances from the cysteines, an Rdx sample was labeled selectively with [^{15}N]cysteine and studied by ^{15}N NMR at 35 °C (Figure 4). Two hyperfine-shifted peaks from [^{15}N]-cysteine appeared in the upfield region (−27 and −71 ppm in oxidized Rdx; 3.5 and −21 ppm in reduced Rdx), and the other two appeared downfield (593 and 570 ppm in oxidized Rdx; 310 and 285 ppm in reduced Rdx).

Some generalizations can be made concerning the pattern of hyperfine-shifted resonances from the cysteinyl ligands. The isotropic shifts of the H^{β} nuclei are larger than those of the H^{α} nuclei. This is consistent with a predominant contact mechanism with σ spin delocalization.[16] All eight H^{β} nuclei yield

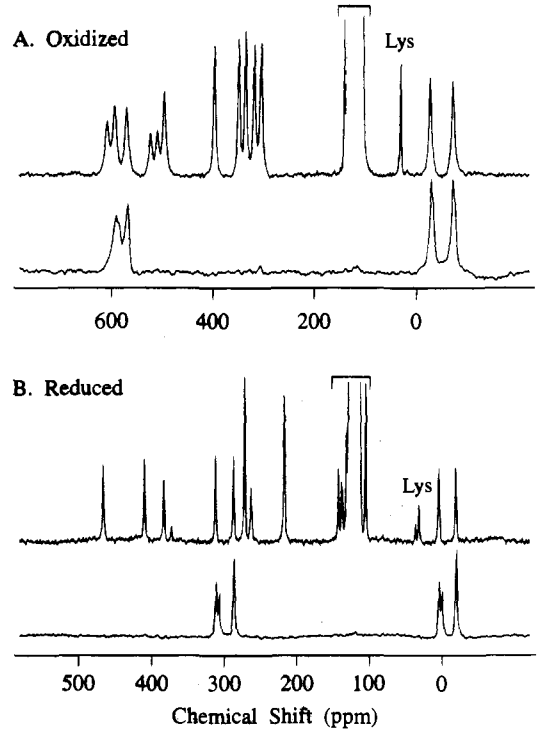

Figure 4. ^{15}N NMR spectra at 35 °C of [U-^{15}N]-labeled rubredoxin (top) and [^{15}N]Cys-labeled rubredoxin (bottom): (A) in the oxidized state; (B) in the reduced state. The brackets enclose the diamagnetic resonances. Signals from the side-chain nitrogens of the four lysine residues are indicated. Two of the ^{15}N signals of the selectively labeled sample show the presence of a minor component (upfield shoulder) whose chemical shifts do not match those of the uniformly labeled samples. The identity of this minor species is unknown at present.

downfield-shifted resonances in both oxidation states. The cysteinyl H^{α} resonances can be divided into two groups of two according to the sign of the isotropic shift. This is also true for the cysteinyl ^{15}N resonances of Rdx. In both cases, the isotropic shifts downfield are much larger than those upfield.

An upfield isotropic shift can arise from one or more of the following three factors: a negative hyperfine coupling constant (A), antiparallel electron spin due to antiferromagnetic coupling, or deshielding pseudocontact interaction with the iron. In reduced [2Fe−2S] ferredoxins, upfield-shifted hyperfine hydrogen peaks are assigned to cysteines that are ligated to the Fe(II) whose electron spin is antiparallel to the external field as the result of antiferromagnetic coupling to Fe(III), which has the larger magnetic moment.[17] Because only one iron is present in Rdx, such a mechanism cannot account for the upfield isotropic shifts observed here. A likely explanation is that significant dipolar interaction contributes to the hyperfine shift. The pseudocontact shift depends upon, among other parameters, the anisotropy of the g-tensor and the orientation of the observed nuclei toward the paramagnetic center.[18] EPR spectroscopy has indeed revealed a highly anisotropic g-tensor for rubredoxin.[19] Furthermore, the four cysteines of Rdx[7,8] can be classified into two groups according to the pattern of their hydrogen bonding and the conformation of their side chains in the X-ray crystal structure (Table 1): Cys6 and Cys39 in one category and Cys9 and Cys42 in the other. The amide protons of cysteines 9 and 42 hydrogen-bond to the sulfur atoms of cysteines 6 and 39, respectively. The sulfur atoms of Cys6 and Cys39 each accept

(15) ^{15}N NMR spectra of both [U-^{15}N]Rdx and [^{15}N]Cys Rdx were collected with a spectral width of 1405 ppm in the oxidized state, or 1005 ppm in the reduced state, by using 2048 data points. The repetition rate was about 13 ms for oxidized samples and 17 ms for reduced samples.

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Table 1. Dihedral Angles (deg) of the Cysteines That Ligate Fe in Oxidized *C. pasteurianum* Rubredoxin[a]

cysteines	Fe−S−C^{β}−C^{α}	S−C^{β}−C^{α}−H^{α}	S−C^{β}−C^{α}−N	Fe−S−C^{β}−$H^{\beta 2}$	Fe−S−C^{β}−$H^{\beta 3}$
Cys6	−167.34	−62.33	176.52	71.83	47.02
Cys9	−90.67	−170.67	74.74	147.89	29.79
Cys39	−172.36	−62.50	177.55	68.09	52.26
Cys42	−86.97	−170.67	72.28	150.02	35.56

[a] From the X-ray crystal structure.[8]

two hydrogen bonds, whereas the sulfurs of Cys9 and Cys42 accept only one each.[20] For these reasons, the relative contributions of contact and pseudocontact shifts may vary significantly for various atoms surrounding the iron ion. In addition, the signs of the electron−nuclear hyperfine coupling constants may be opposite for the two groups of cysteines.

Although rubredoxin has a simple iron ligation pattern, its NMR hyperfine-shifted resonances have remained poorly characterized. Because the iron is high-spin in both oxidation states, the paramagnetism of the iron center has a strong effect on nuclei in its vicinity, leading to extreme line broadening and large isotropic shifts. Selective labeling with low magnetogyric ratio nuclei (^{2}H and ^{15}N)[21] has proved to be an effective way to overcome this problem. Recent advances have been made in the theoretical description of NMR spectral properties of iron−sulfur proteins with [2Fe−2S] and [4Fe−4S] clusters.[22] By contrast, no theory is available for proteins with mononuclear iron coordinated by four cysteines. It is hoped that the present results will stimulate progress in this area.

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