Redox Dependence of Hyperfine-Shifted ^{13}C and ^{15}N Resonances in Putidaredoxin

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Hyperfine-shifted NMR resonances have often been used to characterize the active site of paramagnetic proteins, providing important information concerning structural and electronic properties.[1] Recent results indicate that structural and dynamic differences may exist between the two accessible oxidation states ($Fe^{+3} - Fe^{+3}$ and $Fe^{+3} - Fe^{+2}$) in Fe_2S_2 ferredoxins.[2,3] A detailed knowledge of hyperfine shift patterns in both oxidation states is required if the specific nature of these differences is to be determined. However, hyperfine-shifted ^1H resonances in Fe_2S_2 ferredoxins suffer from severe line-broadening, and have proven difficult to assign sequence-specifically in the absence of crystallographic data.[4] The relatively narrow line widths of ^{15}N and ^{13}C under the same conditions make them more suitable for investigation of hyperfine interactions with the paramagnetic metal center. We now describe the redox dependence of hyperfine-shifted ^{15}N and ^{13}C resonances in putidaredoxin (Pdx), an Fe_2S_2 ferredoxin from the camphor hydroxylase pathway of *Pseudomonas putida*.

The structure of Pdx further than 8 Å from the metal center has been determined by solution NMR methods.[5] But in the absence of a crystal structure and lacking NOE restraints, the structure within 8 Å of the metal center had to be modeled using the crystal structure of a plant ferredoxin.[6] Recently, the paramagnetic domain of oxidized Pdx (Pdx^o) was investigated by ^{15}N NMR spectroscopy.[7] Tentative assignments of some ^{15}N backbone amide resonances were made based on T_1 relaxation times. However, information about the structural and electronic environment around the metal center of Pdx remains sketchy at best. To overcome this problem, we have performed extensive selective ^{15}N and ^{13}C labeling of residues around the metal center in order to identify resonances in both Pdx^o and reduced Pdx (Pdx^r).

Within the paramagnetic region of Pdx, (residues 37−48 and residues 84−87), the selective labeling of Ala43, Ala46, Asp38, Thr47, Leu84, and Gln87 with ^{15}N[9] was accomplished without using auxotrophic strains of *Escherichia coli*. Incorporation of [^{15}N]Gly led to scrambling of labels to Ser and Cys when our standard *E. coli* strain (NCM533) was used for expression. Hence, a [^{15}N]Gly spectrum included paramagnetic resonances from [^{15}N]Ser and [^{15}N]Cys as well. Using auxotrophic strains for Gly (DL39G) and Cys (JM15) for expression,[9] the ^{15}N resonances corresponding to Gly and Cys were identified. Elimination identified the remaining hyperfine resonances in the scrambled sample as serines. Using an approach similar to the one used in assigning a $^{13}C/^{15}N$ pair in the hyperfine-shifted region of a plant-type ferredoxin from *Anabaena*,[10] a selective $^{13}C/^{15}N$ decoupling experiment (Figure 1) was then performed in order to assign the backbone ^{15}N of Ser44 using a Pdx sample doubly labeled at [$^{13}C'$]Ala/[^{15}N]Gly (scrambled to [^{15}N]Ser and [^{15}N]Cys). The difference spectrum (Figure 1b) permitted assignment of the $^{13}C'$ resonance of Ala43 due to decoupling of the ^{15}N resonance of Ser44. In turn, the other hyperfine-shifted serine ^{15}N resonance is assigned to Ser42, and the second hyperfine $^{13}C'$ resonance to Ala46. A similar difference decoupling experiment was performed on Pdx doubly labeled with [$^{13}C'$]Leu/[^{15}N]Cys in order to assign the ^{15}N resonance of the nonligating Cys85 and the $^{13}C'$ resonance of Leu84. ^{15}N resonances of Asp38, Thr47, and Gln87 could be directly assigned since only one residue of each of these amino acids lies in the paramagnetic region. Similarly, the $^{13}C'$ resonances of Cys, Gly, Ser, Ala, and Leu around the metal cluster were identified using selectively $^{13}C'$-labeled samples.[11]

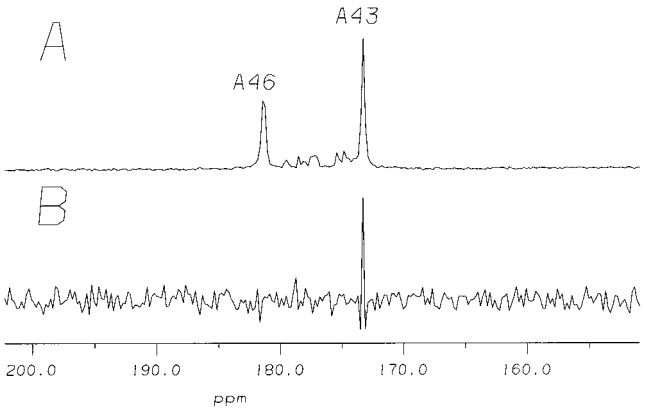

Figure 1. (A) ^{13}C NMR spectrum of a Pdx^o sample (pH 7.4, 10% D_2O, 290 K) selectively labeled with [$^{13}C'$]Ala/[^{15}N]Gly (scrambled to [^{15}N]-Ser and [^{15}N]Cys). (B) Difference spectrum obtained by subtraction of a spectrum with selective decoupling at the ^{15}N frequency of neighboring Ser44 from a reference spectrum where the decoupler frequency was set off-resonance (~90 ppm).

Many of the newly identified ^{15}N and ^{13}C resonances show considerable differences in their hyperfine shifts as a function of metal cluster oxidation state (Table 1). The ^{13}C chemical shifts of Cys (Figure 2) and Ala residues show considerable changes upon going from the oxidized to the reduced state. The origin of these changes is not yet clear, although pseudocontact contributions (which are mainly due to dipolar interactions) cannot be ruled out, because of close proximity of these residues to the metal center. A more detailed understanding of these $^{13}C'$ hyperfine

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(9) ^{15}N and ^{13}C selectively labeled samples were grown and purified from cell extracts by using methods as previously described[8] Auxotrophic strains, DL39G and JM15, used for preparing selectively labeled samples were obtained from *E. coli* Genetic Stock Center, Yale University.

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(11) All NMR ^{15}N and ^{13}C direct detection experiments were recorded at 290 K on a Bruker AMX-500 spectrometer, operating at 50.68 and 125.76 MHz for ^{15}N and ^{13}C respectively, and equipped with a 5-mm broadband probe for heteronuclear detection. A typical ^{15}N or ^{13}C rapid acquisition experiment (recycling time <50ms) consisted of 4 K data points with a spectral width of 50 000 Hz. Broadband ^1H decoupling was used during all ^{15}N acquisitions. The rapid acquisition of signals allowed for the efficient detection of paramagnetic resonances due to suppression of the diamagnetic resonances. For the selective decoupling experiments, a modified Bruker triple resonance probe (doubly tuned to ^{15}N and ^{13}C in the inner coil) was used, allowing increased sensitivity for ^{13}C and ^{15}N. NMR data were processed and analyzed on Silicon Graphics workstations using Felix version 95.0 (Biosym, San Deigo, CA) software.

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Table 1. Comparison of ^{15}N, ^{13}C Chemical Shifts in Pdx^o and Pdx$^{r a,b}$

	Gly	Ser	Cys	Ala	Leu
			^{15}N δ, ppm		
Pdx^o	109.5	132.1 (S44)	107.2	131.0	120.6 (L84)
	117.6	139.5 (S42)	126.7	137.5	
	156.2		135.4 (C85)		
			136.2		
			151.0		
Pdx^r	105.6	87.9 (S44)	139.8 (C85)	120.7	122.8 (L84)
	124.2	176.6 (S42)	140.8	121.0	
	124.8		182.5		
			260.5		
			272.7		
			^{13}C δ, ppm		
Pdx^o	174.8	174.2	171.0	173.5 (A43)	178.0 (L84)
	175.0	175.2	173.4	181.6 (A46)	
	175.4		173.7		
			175.7		
			177.3		
Pdx^r	169.0	173.2	169.5	166.6 (A43)	177.9 (L84)
	172.8	177.1	173.4	176.1 (A46)	
	178.9		174.8		
			180.9		
			186.2		

[a] The residue designators in parentheses indicate residues for which assignments are available. [b] All ^{15}N chemical shifts are reported relative to external liquid ammonia, and ^{13}C chemical shifts are reported relative to the methyl resonance of external DSS. [c] Other sequence-specific ^{15}N backbone assignments, listed as (Pdx^o/Pdx^r), include D38 (121.7/120.8), T47 (146.6/85.6), and Q87 (129.8/160.2).

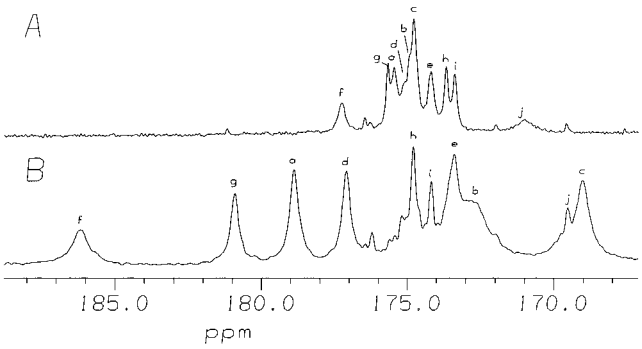

Figure 2. ^{13}C NMR spectra[9] of Pdx (pH 7.4, 10% D_2O, 290 K) selectively labeled with [$^{13}C'$]Gly (scrambled to [$^{13}C'$]Ser and [$^{13}C'$]Cys: (A) Pdx^o and (B) Pdx^r. Peaks a−c correspond to [$^{13}C'$]Gly, d−e to [$^{13}C'$]-Ser and f−j to [$^{13}C'$]Cys resonances.

shifts is needed before interpretation of their chemical shift change as a function of oxidation state can be attempted. The ^{15}N resonances for two of the metal-cluster bonded cysteines show a downfield shift >100 ppm in Pdx^r with a strong Curie-type temperature dependence,[12] indicating an interaction of these two cysteines with the Fe^{3+} center and the other two with the Fe^{2+} center. ^{15}N resonances of Thr47, Gln87, and Ser44 also show a significant chemical shift change as a function of oxidation state. Thr47 is especially significant, since this residue is conserved in several Fe_2S_2 ferredoxins. Recently, the crystal structure of a related ferredoxin,[13] bovine adrenodoxin (Adx), revealed the presence of a H-bond between the backbone NH of Thr54 and the γ-sulfur of Cys52 (corresponding to Thr47 and Cys45 in Pdx). Cys52 ligates the iron thought to accept the electron upon reduction (Fe1 in both the Pdx and Adx structures). The high degree of structural, functional and sequence homology between Pdx and Adx suggests that a similar H-bond may also exist between the backbone nitrogen of Thr47 and Cys45. The strong anti-Curie temperature dependence of the ^{15}N resonance of Thr47 in Pdx^r supports the assumption that this amide group is H-bonded to one of the cysteine γ-sulfurs ligating the Fe^{2+} center, possibly Cys45. A similar argument can be made for the ^{15}N resonance of Ser44, which also shows a strong anti-Curie type behavior in the reduced protein. The presence of these H-bonds is also consistent with the predictions that the Fe atom closer to the protein surface is the one which likely is reduced.[4]

Recently, the presence of such H-bonding patterns near the metal cluster has been inferred on the basis of observation of $^1H/^2H$ isotope effects on ^{15}N chemical shifts in *Clostridium pasteurianum* rubredoxin.[14] These effects are attributed to the Fermi contact interactions between the iron and the nitrogens of backbone amides transmitted through H-bonds. The characterization of H-bonding patterns is important in that it is likely a major determinant of redox potential in Fe_2S_2 ferredoxins.[15] Also, H-bonds tend to delocalize unpaired electron density away from the metal cluster and provide potential pathways for electron transfer. In addition, H-bonds provide structural restraints for modeling the metal center in NMR-based structural calculations.

Finally, the ^{15}N resonance of Asp38 which has been implicated in the binding interactions of Pdx and its redox partner, cytochrome $P450_{cam}$,[16] is fairly narrow and not very hyperfine-shifted in Pdx^o or Pdx^r. It will be of interest to see if any change occurs for this resonance upon complexation with cytochrome $P450_{cam}$.

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Supporting Information Available: ^{15}N and ^{13}C NMR spectra of Pdx^o and Pdx^r selectively labeled with [^{15}N]Gly (scrambled to [^{15}N]Ser and [^{15}N]Cys), [^{15}N]Ala, [^{15}N]Asp, [^{15}N]Thr, [^{15}N]Gln, [^{15}N]Cys, [15N]-Gly, [$^{13}C'$]Cys, [$^{13}C'$] Gly and [$^{13}C'$]Leu/ [^{15}N]Cys difference decoupling experiment (10 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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