Amplification of One-Bond $^1H/^2H$ Isotope Effects on ^{15}N Chemical Shifts in *Clostridium pasteurianum* Rubredoxin by Fermi-Contact Effects through Hydrogen Bonds

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Rubredoxins contain the simplest type of iron−sulfur center found in proteins,[1] a single iron ion coordinated by four cysteine sulfurs. The rubredoxin from *Clostridium pasteurianum* (Rdx)[2,3] is one of the best studied members of this family. Its crystal structure has been solved and refined to high resolution.[4,5] Methods developed for labeling the protein uniformly and selectively with stable isotopes for NMR investigations[6,7] have led to assignments of ^1H, ^2H, ^{13}C, and ^{15}N signals in both the diamagnetic[8] and paramagnetic[7,9] spectral regions of Rdx. Results described herein explain doubling of hyperfine-shifted ^{15}N signals previously observed in spectra of some Rdx samples.[6] Of the 12 backbone amides of Rdx (in either oxidation state) that exhibit hyperfine ^{15}N shifts,[7] four are shown here to have large $^1H/^2H$ one-bond isotope effects on the ^{15}N chemical shifts. The magnitudes of the isotope shifts, −6 to −18 ppm, as compared to −0.6 ppm for a diamagnetic one-bond $^1H/^2H$ isotope effect on ^{15}N,[10] are attributed, on the basis of quantum mechanical calculations, to Fermi-contact interactions between the iron and the nitrogens of backbone amides hydrogen bonded to the iron center.[4] Isotope shifts thus can be used to analyze contributions of Fermi-contact effects transmitted from the iron−sulfur cluster through H-bonds. The resolved splittings have enabled the measurement of previously inaccessible hydrogen exchange (HX) rates for these backbone amides. Isotope effects on hyperfine chemical shifts have not been reported previously for an iron−sulfur protein, but an isotope effect has been observed on the hyperfine ^1H chemical shift of a heme protein.[11]

Recombinant [*U*-^{15}N]-aporubredoxin samples were reconstituted in vitro with iron in ^1H_2O and in ^2H_2O solutions by methods described previously.[6,7] Comparison of 1D ^{15}N NMR spectra of oxidized and reduced forms of these samples revealed an isotope effect on the shifts of peaks 1, 4, 5, and 8 of Fe(III) Rdx and peaks 1′, 2′, 3′, and 8′ of Fe(II) Rdx (Figure 1). The pairing of

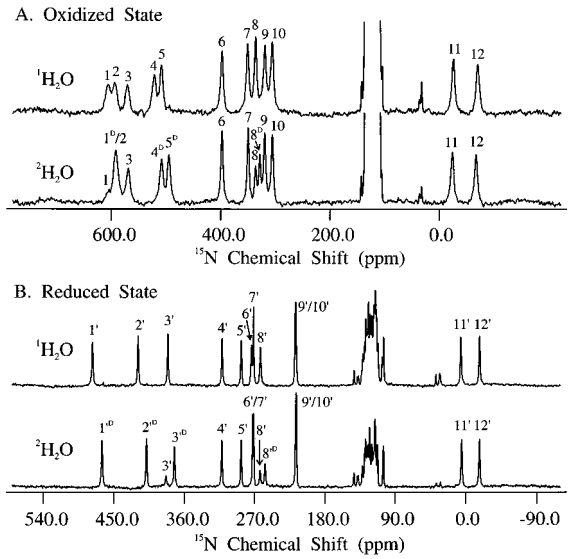

Figure 1. 1D ^{15}N NMR spectra at 50.68 MHz of Rdx samples (at 35 °C, pH 6.0) reconstituted in ^2H_2O and ^1H_2O, respectively: (A) Fe(III) rubredoxin; (B) Fe(II) rubredoxin. The $^{15}N^D$ components of resonances that exhibit large resolved $^1H/^2H$ isotope shifts are denoted by superscript "D". Spectral acquisition and processing were as described previously.[7]

signals to $^{15}N^H$ and $^{15}N^D$ from a given residue was determined by selective ^{15}N labeling.[7,12] These signals have been assigned to the amide nitrogens of Val[8], Tyr[11], Leu[41], and Val[44] (Table 1).[7,12] Full ^2H occupancy was not observed for peaks 1 and 8 (oxidized) and 3′ and 8′ (reduced) for the sample reconstituted in ^2H_2O; this indicates that back exchange at residues Tyr[11] and Val[44] occurred during purification of Fe(III) Rdx in ^1H_2O solutions. The X-ray structures of oxidized[4] and reduced[5] clostridial rubredoxin show that the amide hydrogen of each of these residues donates an H-bond to the sulfur atom of a cysteine ligated to the iron. This suggests that the unusual isotope effect originates from the hyperfine shift transmitted through the H-bond and that this transmission is greater for ^{15}N-$^1H\cdots S$ than for ^{15}N−$^2H\cdots S$. The isotope shifts are temperature dependent in both oxidation states, with larger isotope shifts at lower temperatures. At 5 °C, the largest isotope shift was −22.4 ppm. The other 8 hyperfine-shifted ^{15}N peaks exhibited $^1H/^2H$ isotope effects smaller than the line widths.

For data from each oxidation state at 35 °C, a linear correlation (slope = −0.0335; $R^2 = 0.99$) was found between the magnitude of the large $^1H/^2H$ isotope shift and the paramagnetic component of the ^{15}N chemical shift (Figure 2). The reduced data fit the same line as the oxidized, which indicates that the dipolar contribution to these four shifts in each oxidation state is minimal as proposed before.[9] The amide of Cys[9] and Cys[42] each donates an H-bond to the sulfur of another cysteine. Their nitrogens exhibit hyperfine shifts to low frequency and small isotope effects (Figure 2), in agreement with calculations indicating that the cysteine side chain provides an efficient pathway for unpaired electron delocalization, which does not involve the H-bond.[9] The slope of the chemical shift dependence of the isotope shifts of these two cysteines shows an interesting dependence on the oxidation state not seen for the other four residues.

Chemical shift changes of the magnitude and direction observed were replicated in high-level, all-electron, density functional

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Table 1. Backbone Amides Hydrogen Bonded to Cysteine Sulfurs in the Iron Center of Oxidized and Reduced *Clostridium pasteurianum* Rubredoxin: One-Bond $^1H/^2H$ Isotope Effects on ^{15}N Chemical Shifts and $^1H \rightarrow {}^2H$ Hydrogen Exchange Rates[a]

oxidation state	assignments[7,9,12]	Val[8]	Tyr[11]	Leu[41]	Val[44]
	^{15}N peak number	5	1	4	8
Fe(III)	$^{15}N^H$ chemical shift (/ppm)	508.9	606.0	521.9	336.6
	^{15}N ($^1H/^2H$ isotope shift) (/ppm)[b]	−14.4	−17.7	−14.4	−8.2
	calculated isotope shift (/ppm)	−8.3	−20	−10.2	−9.4
	HX rate k_{ex} ($/hr^{-1}$) [c]	$3.5_2 \times 10^{-1}$	$3.6_6 \times 10^{-3}$	$5.1_2 \times 10^{-2}$	$<1.0 \times 10^{-3}$ [d]
	^{15}N peak number	1′	3′	2′	8′
Fe(II)	$^{15}N^H$ chemical shift (/ppm)	477.7	381.6	419.6	262.8
	^{15}N ($^1H/^2H$ isotope shift) (/ppm)[e]	−12.5	−8.8	−11.0	−6.4
	HX rate k_{ex} ($/h^{-1}$)[c]	$3.67_6 \times 10^{-3}$	$1.04_4 \times 10^{-3}$	$2.71_3 \times 10^{-3}$	$1.06_4 \times 10^{-3}$
	k_{ex} (ox)/k_{ex} (red)	96	3.5	19	<1

[a] Chemical shifts[7] and isotope shifts reported here are from spectra collected on a Bruker DMX500 spectrometer (50.68 MHz ^{15}N) at 35 °C. [b] Estimated error is ±1.5 ppm. [c] The subscripts represent errors for the last digit. [d] Limitations in protein stability prevented more accurate measurement of this exchange rate. [e] Estimated error is ±0.5 ppm.

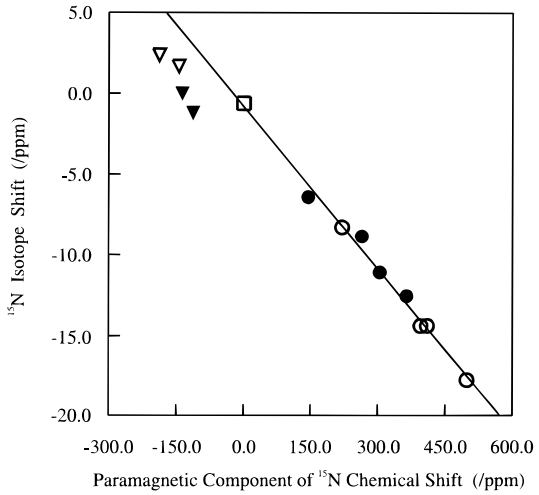

Figure 2. Plot of one-bond $^1H/^2H$ isotope effects on the ^{15}N resonances of *C. pasteurianum* rubredoxin as a function of the corresponding paramagnetic component of ^{15}N chemical shift in ^1H_2O, both determined at 35 °C: residues Val[8], Tyr[11], Leu[41], and Val[44] in the oxidized (○) and reduced (●) states; residues Cys[9] and Cys[42] in the oxidized (▽) and reduced (▼) states. The paramagnetic component of the ^{15}N chemical shift was obtained by subtracting (diamagnetic) random coil ^{15}N chemical shifts for each residue from the experimental shift. A point (□) was added at 0 ppm paramagnetic shift and −0.6 ppm isotope shift to simulate the result for a diamagnetic protein. The line indicates the best fit to a linear function that includes this point plus the eight points for Val[8], Tyr[11], Leu[41], and Val[44] in oxidized and reduced rubredoxin.

calculations (B3LYP/6-311G** level)[9] of Fermi-contact spin densities on a model for the iron center derived from one of the crystal structures of oxidized Rdx (Protein Data Bank[13] file 5RXN)[3] by moving H-bonded hydrogens 0.01 Å closer to the nitrogen to mimic the distance change expected from the asymmetry of the H-bond potential well for an ^1H to ^2H substitution.[14] The change in bond lengths resulted in different calculated Fermi-contact spin densities ($\rho_{\alpha\beta}$) for the backbone nitrogens, which were used to compute the isotope shift.[15] The calculations yielded large, negative, one-bond isotope shifts for Val[8], Tyr[11], Leu[41], and Val[44] (Table 1) but very small isotope shifts for the other hyperfine-shifted ^{15}N signals, in agreement with experiment. The calculations support the idea that the $^1H^N\cdots S-{}^{113}Cd$ and $^1H^N\cdots S-{}^{199}Hg$ couplings reported for two homologous residues (Ile[6] and Ile[40]) of metal-substituted *Pyrococcus furiosus* rubredoxins are scalar (through-hydrogen-bond) in origin:[16] the magnitudes of these couplings were reported to be equivalent within experimental error,[16] and the hyperfine ^{15}N shifts of the homologous residues of Fe(II) Rdx (Val[8] and Leu[41]) are similar (Table 1).

HX rates for H-bonded groups in rubredoxin have not been reported previously. It would not be possible to derive them from exchange effects on ^1H or ^2H signals from the amide, because such signals have never been resolved (presumably because of severe paramagnetic broadening). The separate signals from $^{15}N^H$ and $^{15}N^D$, however, serve as reporters for HX. One-dimensional ^{15}N spectra of oxidized and reduced samples of Rdx reconstituted in ^1H_2O were taken at different intervals after the samples were dissolved in ^2H_2O. These data yielded HX rates for Val[8], Tyr[11], Leu[41], and Leu[44] (Table 1).

Counterintuitively, in each oxidation state, the amide with the smallest hyperfine shift (and presumably the weakest H-bond to the iron center) has the slowest HX rate (Table 1). The rank order of HX rates by residue assignment was similar in the two oxidation states, but the range of HX rates was much larger for Fe(III) Rdx than for Fe(II) Rdx. Except for Val[44], HX rates were slower in reduced than in oxidized Rdx. Qualitative data available on hydrogen exchange at the analogous hydrogen-bonded amides of *P. furiosus* zinc-substituted rubredoxin[17] are, as expected, in better agreement with those of Fe(II) Rdx than of Fe(III) Rdx.[18]

Supporting Information Available: Two figures showing HX data and determinations of HX rates (3 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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