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Correlation between Hydrogen Bond Lengths and Reduction Potentials in *Clostridium pasteurianum* Rubredoxin

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Iron−sulfur proteins are found in all forms of life and are involved in numerous important biological processes, including electron transfer.[1−3] Although the reduction potentials of iron−sulfur proteins range widely from −700 to +400 mV,[4] the factors regulating the reduction potentials of various iron−sulfur proteins have not been defined adequately. Rubredoxin, the simplest type of iron−sulfur protein, consists of a single iron ligated by four cysteinyl sulfurs. Despite distinctly similar backbone structures and 50−60% sequence identity, rubredoxins cover a range of reduction potentials between −80 and 40 mV.[5] Because it has been extensively characterized by high-resolution X-ray crystallography, computational modeling, and spectroscopic studies, rubredoxin is an excellent model system to explore the factors that regulate the reduction potential.

Backbone amides from residues Val[8], Cys[9], Tyr[11], Leu[41], Cys[42], and Val[44] participate in hydrogen bonds with the sulfurs of the iron center in *Clostridium pasteurianum* rubredoxin (*Cp*Rd) (Figure 1). These ($H^N\cdots S^\gamma$) hydrogen bonds, which are conserved in rubredoxins as well as in other types of iron−sulfur proteins, are thought to be involved in intermolecular electron transfer.[6,7] In model compounds, deletion or modification of these hydrogen bonds results in changes in reduction potential.[8,9] Of particular interest are recent investigations of the electrochemical and structural features of mutant forms of *Cp*Rd that have focused primarily on mutations of residues that participate in hydrogen bonds with the ligand sulfurs.[10−14] The reduction potentials for these mutants varied over a range of ∼159 mV. Attempts to explain the differences in reduction potential have cited the influences of solvation, ligand field, peptide dipoles, and charged residues.[10−14]

Rubredoxin contains a high-spin iron in both of its accessible oxidation states ($Fe^{3+/2+}$). Interactions between the unpaired electrons of the iron and the surrounding nuclei are manifested in NMR spectra by hyperfine shifts. NMR therefore offers an approach for determining the electronic and geometric structure of the iron center. The ^{15}N hyperfine shift, in the case of rubredoxin, is dominated by Fermi-contact coupling, which arises from the delocalization of unpaired electron spin density through bonds to nearby nuclei.[16] Of the six backbone amides that participate in hydrogen bonds with the Cys S^γ atoms in *Cp*Rd, nitrogen atoms from Val[44] and Tyr[11] exhibit large hyperfine shifts despite being located eight covalent bonds away from the iron center. The conclusion, as corroborated by hybrid density function theory (HDFT) calculations, is that these shifts arise from Fermi-contact interactions mediated by $H^N\cdots S^\gamma$ hydrogen bonds.[15] Such trans-hydrogen-bond electron delocalization may provide pathways for electron transfer. Fermi-contact spin densities at nitrogen nuclei involved in $H^N\cdots S^\gamma$ bonds calculated from structural models of oxidized wild-type *Cp*Rd were found to correlate inverse-linearly with $H^N\cdots S^\gamma$ hydrogen bond lengths derived from these models.[15] Hence the ^{15}N NMR chemical shifts appear to provide a measure of hydrogen bond distance and consequently an approach to determining hydrogen bond strengths. This, in turn, offers a way of investigating whether rubredoxins with different reduction potential have altered $H^N\cdots S^\gamma$ hydrogen bond distances.

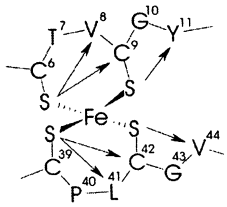

Figure 1. Two-dimensional representation of the network of covalent bonds (lines) and hydrogen bonds (arrows) at the iron−sulfur site in the rubredoxin from *Clostridium pasteurianum*. The S^γ atoms of the four cysteines are denoted by "S"; the other letters are conventional single-letter designators of amino acids with superscripts indicating residue numbers. Reprinted with permission from *J. Am. Chem. Soc.* **1998**, *120*, 4806−4814. Copyright 1998 Am. Chem. Soc.

We have applied this approach to the oxidized forms of four *Cp*Rd variants with different reduction potentials: wild-type *Cp*Rd and mutants V44G, V44A, and V44I (Table 1). 1D ^{15}N NMR spectra of these variants are ordered in Figure 2 according to the reduction potential of the protein (as reported by Wedd and coworkers).[12] For each variant, 12 hyperfine-shifted peaks were detected and then rigorously assigned by selective ^{15}N-labeling (data not shown). The chemical shifts of ^{15}N NMR signals from residues Pro[40], Thr[7], Gly[10], Gly[43], Val[8], Tyr[11], and Leu[41] did not vary significantly among the variants; however, that from the backbone amide at position 44 showed large differences.

Figure 3 shows that the relationship between reduction potential and chemical shift for position 44 is approximately linear. It is clear that as the reduction potential for a mutant increases, the chemical shift of mutated residue 44 shifts progressively to higher frequency.

Our previous work showed that ^{15}N hyperfine shifts can be used to determine hydrogen bond lengths and that, for oxidized *Cp*Rd, shortening the $H^N\cdots S^\gamma$ length by 0.1 Å results in a shift to higher frequency of 88.7 ppm.[15] On the basis of the chemical shift data, mutation of residue Val[44] of *Cp*Rd to Ile, Ala, or Gly decreases the length of the hydrogen bond between the backbone amide hydrogen of residue 44 and the S^γ of Cys[42] (Table 1). Given that the precision of atomic positions in crystal structures is approximately $^1/_5$ to $^1/_{10}$ of the reported resolution,[19] the hydrogen bond distances determined here by NMR agree within experimental error with those from single-crystal diffraction (Table 1, last column).

Our results confirm that a small change in the length of a single hydrogen bond is capable of tuning the reduction potential of *Cp*Rd by nearly 80 mV. This indicates that hydrogen bonding is likely to

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Table 1. Hydrogen Bond Distances between the Backbone Amide Hydrogen of Residue 44 and the Side Chain Sulfur of Cysteine-42 in Variants of the Rubredoxin from *Clostridium pasteurianum* (*Cp*Rd)

		$H^N\cdots S^\gamma$ hydrogen bond distance (Å)	
*Cp*Rd variant	reduction potential (mV)[a]	from ^{15}N NMR hyperfine shifts[b]	from X-ray structures[c]
wt	−77	2.87	3.01[d]
V44I	−53	2.75	2.84[e]
V44A	−24	2.55	2.60[f]
V44G	0	2.36	unavailable

[a] From Xiao et al.[12] [b] From the present study as calculated by the method of Wilkens et al.[15] [c] MacroModel software (Schrödinger, Portland, OR) was used to calculate the positions of the hydrogens from the heavy atom coordinates of each X-ray structure. [d] From PDB 1IRO (1.1 Å resolution).[18] [e] From coordinates supplied by A. G. Wedd (1.6 Å resolution).[12] [f] From PDB 1C09 (1.6 Å resolution).[11]

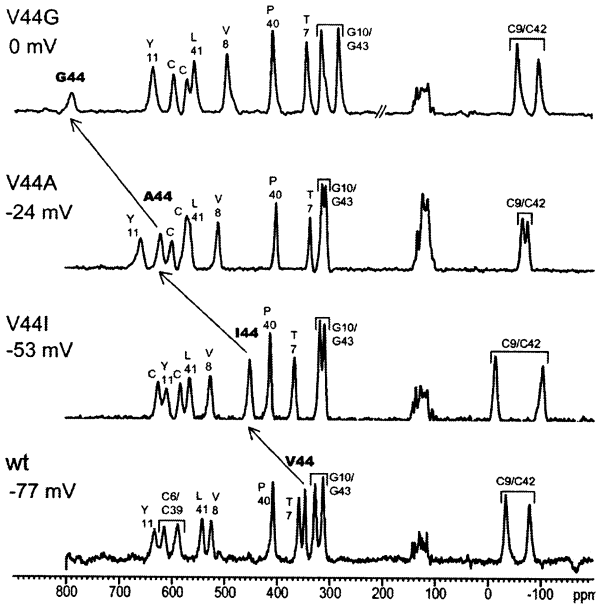

Figure 2. 1D ^{15}N NMR spectra of variants of the rubredoxin from *Clostridium pasteurianum* all in the oxidized (Fe^{3+}) state (proteins labeled uniformly with ^{15}N, dissolved in 90% H_2O/10% D_2O, 20 °C, pH 6.0, 50 mM potassium phosphate buffer). Spectra were recorded on a Bruker DMX500 NMR spectrometer with a 5 mm broad-band probe under the following acquisition parameters: spectral width, 1000 ppm; data points, 2048; repetition time, ∼20 ms; number of scans, ∼300 000. A Superweft pulse sequence was used to suppress diamagnetic signals.[17] The strategy used in assigning the signals was residue-specific labeling either by addition of a specific [^{15}N]-amino acid or by [U-^{15}N] labeling plus addition of the specific [^{14}N]-amino acid. The reduction potentials indicated at the left of the spectra are those determined for these proteins by square wave voltammetry by Wedd and co-workers; they are reported relative to the standard hydrogen electrode.[12]

be an important determinant of reduction potential in rubredoxins and other iron−sulfur proteins. The mechanism by which a shorter hydrogen bond stabilizes the reduced state in *Cp*Rd could involve either an increased capacity for electron delocalization or an increased proximity of partial positive charge to the iron center.

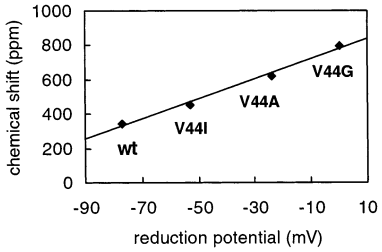

Figure 3. Plot of the ^{15}N NMR chemical shifts of the backbone amide nitrogen of residue 44 of variants of the rubredoxin from *Clostridium pasteurianum* [wild-type with Val44 (wt), Ile44 (V44I), Ala44 (V44A), and Gly44 (V44G)] versus the reduction potential of the protein.

Further work is needed to elucidate the relative importance of these two mechanisms. Nonetheless, this study demonstrates that paramagnetic NMR provides an impressively sensitive tool that allows us to explore contributions to reduction potential in a novel manner.

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