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^{15}N HYSCORE Characterization of the Fully Deprotonated, Reduced Form of the Archaeal Rieske [2Fe−2S] Center

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Proteins containing Rieske-type [2Fe−2S] clusters with two histidine N_δ and two cysteine S_γ ligands play important roles in many biological electron-transfer reactions, such as aerobic respiration, photosynthesis, and biodegradation of various alkene and aromatic compounds.[1−4] In the cytochrome bc_1/b_6f family, which is composed of redox-linked proton pumps, interaction of some Q_o-site occupants with the Rieske center occurs via formation of a hydrogen bond with one of two imidazole ring N_ϵ of the histidyl ligands to the reduced cluster.[4−7] A coupled proton and electron transfer have been proposed to occur along this hydrogen bond during the Q_o-site catalysis.[7]

The reduced Rieske-type cluster exhibits strong antiferromagnetic exchange interaction between the electron spins of Fe^{3+} ($S = {}^5/_2$) and Fe^{2+} ($S = 2$) and produces a paramagnetic $S = {}^1/_2$ ground state with a relatively low g_{av} value of 1.90−1.92 for a biological [2Fe−2S] cluster around physiological pH[1,3] [e.g., $g_z = 2.01$, $g_y = 1.91$, $g_x = 1.79$ (g_{av}=1.90) for sulredoxin (SDX), a high-potential Rieske protein from the hyperthermophilic archaeon *Sulfolobus tokodaii* strain 7 (DDBJ accession code, AB023295; $E_{m,acid\ pH}$ of ∼+190 mV)[8−12]]. Electrothermodynamic[13,14] and density functional theory (DFT)[15] analyses of the Rieske-type [2Fe−2S] cluster system suggest that N_ϵ of the two histidyl ligands of reduced proteins are fully protonated around physiological pH, while they become deprotonated almost concomitantly near and above pH ∼13. In agreement with these results, Tiago de Oliveira et al.[16] have reported a novel EPR spectrum for the fully deprotonated, reduced Rieske protein from *Thermus thermophilus* at pH 14, which is characterized by the principal values of $g_z = 2.14$, $g_y = 1.94$, $g_x = 1.81$, an unusually high $g_{av} \sim 1.97$, and very broad spectral components. The observed changes have been interpreted as a consequence of the antisymmetric exchange in the cluster in combination with the decrease of the exchange interaction from $J \sim 150$ cm^{-1} (pH 7) to ∼40 cm^{-1} (pH 14). Further spectroscopic analysis of this species is expected to provide a useful fingerprint for studying the Q_o-site catalysis in the cytochrome bc_1/b_6f family in greater detail.

We have previously shown that the two-dimensional hyperfine sublevel correlation (HYSCORE) spectra uniquely resolved the cross-peaks from several types of ^{15}N nuclei around the fully protonated, reduced Rieske [2Fe−2S] cluster in the uniformly ^{15}N-labeled SDX (^{15}N-SDX) at pH 7.[17a] The CW EPR spectrum of the dithionite-reduced SDX undergoes the same type of changes near and above pH 13 as reported for the *T. thermophilus* Rieske protein at pH 14,[16] that is, a marked increase of the g_z principal value ($g_z = 2.13$, $g_y = 1.92$, $g_x = 1.78$; $g_{av} = 1.95$) and significant broadening of spectral components (data not shown). Both of the thermophilic Rieske proteins are weakly homologous to the cluster-binding domain of cytochrome bc_1-associated, high-potential Rieske proteins and have a conserved disulfide linkage that covalently connects the two cluster-binding loops around a [2Fe−2S] cluster.[10,18,19] This peculiar structural feature seems to contribute to the striking tolerance of their cluster-binding subdomains against very alkaline pH (at least for the short time required for the EPR sample preparations), which is unusual for protein-bound systems and could be useful for future design of metalloprotein-based, hyperstable nanodevices.

Because of the mechanistic importance of a redox-linked imidazolate−imidazole transition of a metal−histidine ligand in the biological redox system, in general, we herein report the orientation-selected HYSCORE characterization of the ^{15}N-SDX reduced at pH 13.3 to quantify the deprotonation effects through the hyperfine couplings with ^{15}N nuclei in the cluster environment.

The HYSCORE spectrum of ^{15}N-SDX at pH 7 shows (i) two pairs of cross-peaks with narrow contours in the (+ −) quadrant from the two histidine N_δ ligands of SDX (N_δ1,2) (located close to the dashed lines $|\nu_1 + \nu_2| = 2\nu_I$ parallel to the diagonal of the (+ −) quadrant) (Figure 1, top left), and (ii) two cross-features around the diagonal point with ^{15}N Zeeman frequency in the (++) quadrant for weakly coupled N_ϵ of the histidine imidazole groups (a smaller coupling of ∼0.3−0.4 MHz) and peptide nitrogen N_p (a larger coupling of ∼1.0 MHz)[17a] (Figure 1, top right). The isotropic constant and axial anisotropic hyperfine tensor of N_δ1,2 were determined to be $a = 6$ MHz, $\mathbf{T} = (2.4, -1.2, -1.2)$ MHz and $a = 7.8$ MHz, $\mathbf{T} = (2.6, -1.3, -1.3)$ MHz, respectively, based on the contour line shape analysis of the spectra recorded at several field positions along the EPR line shape (Figure S-1).[21]

The fully deprotonated, reduced Rieske [2Fe−2S] cluster of ^{15}N-SDX at pH 13.3 shows the same number of cross-features from ^{15}N nuclei as those detected at pH 7, but with striking changes in the HYSCORE spectra (Figure 1). In the (+ −) quadrant, the two cross-features from the strongly coupled N_δ1′,2′ are shifted from the $|\nu_1 + \nu_2| = 2\nu_I$ line, from which the orientation of the contours of one pair is significantly deviated, clearly indicating a substantial change of the anisotropic hyperfine couplings[20] (Figure 1, bottom left). The correlation of the cross-features for N_δ1′,2′ from the spectra (pH 13.3) recorded at different field positions showed that the frequencies recalculated to the same field[21] do not fit on linear regression corresponding to the axial approximation, unlike the spectra at pH 7. These results indicate a significant nonaxiality of the N_δ1′,2′ hyperfine tensors, which are estimated to be $a = 7$ MHz, $\mathbf{T} = (-4, -0.5, 4.5)$ MHz and $a = 5$ MHz, $\mathbf{T} = (-5, 1, 4)$ MHz on the basis of the spread of the nuclear frequencies in the ν_1^2 versus ν_2^2 presentation (Figure S-2).[20] In the (++) quadrant, the cross-features for the weakly coupled N_ϵ of the histidine imidazole groups

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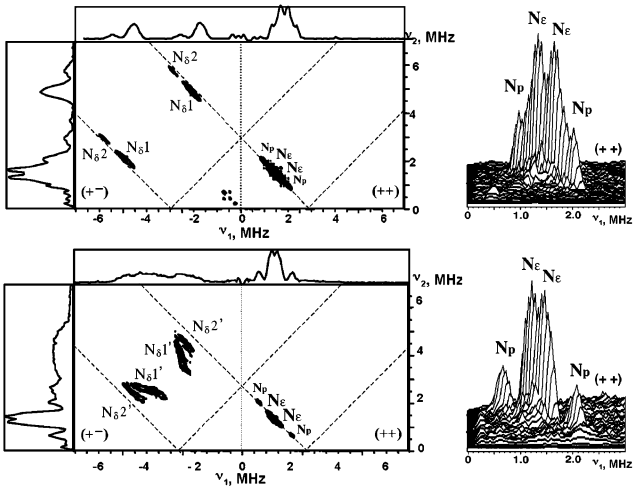

Figure 1. The contour plots (left) and the 3D presentations (for the weakly coupled ^{15}N in the (++) quadrant only; right) of ^{15}N HYSCORE spectra of the fully protonated, reduced ^{15}N-SDX at pH 7 (recorded near $g_x = 2.01$; magnetic field, 343.5 mT) (top) and the fully deprotonated, reduced ^{15}N-SDX at pH 13.3 (near $g_x = 2.13$; magnetic field, 310 mT) (bottom); $\tau =$ 136 ns; microwave frequency $\sim$ 9.70 GHz, $T = 10$ K. The dashed lines in the contour plots (left), $|\nu_1 + \nu_2| = 2\nu_I$ lines with $\nu_1 = 1.43$ MHz (pH 7) and 1.34 MHz (pH 13.3). The cross-peaks from the strongly and weakly coupled ^{15}N nuclei are located in (+ −) and (++) quadrants, respectively, within the strip defined by these lines at any values of isotropic and anisotropic hyperfine couplings[20] (left). The uniformly ^{15}N-labeled SDX was prepared as described previously.[10,17a]

(0.2 MHz) and peptide nitrogen N_p (1.4 MHz) are better resolved at pH 13.3 than those at pH 7[17] (Figure 1, right). These couplings do not show any field dependence, indicating that their predominantly isotropic characteristics are similar to those at pH 7.

In the reduced Rieske-type [2Fe−2S] cluster system, two histidine N_δ ligands bind the Fe^{2+} site. According to the vector-coupling model, the antiferromagnetic exchange effectively scales the hyperfine tensors of individual ^{15}N at pH 7, so that the coupling seen at the Fe^{2+} is (−4/3)A, while that seen at the Fe^{3+} is (7/3)A.[22] We have found an approximate doubling of the maximum component of anisotropic hyperfine tensors **T** for $N_\delta1',2'$ at pH 13.3. This cannot be explained by the influence of a smaller antiferromagnetic exchange interaction[23] or by the influence of an antisymmetric exchange on scaling coefficients. In this connection, we note that a recent DFT analysis of the mitochondrial Rieske [2Fe−2S] cluster by Ullmann et al.[15] suggests the negligible difference of reduction energy between two iron sites in the totally deprotonated form at very alkaline pH (i.e., reduction of either iron site or valence delocalization between both sites are possible); the reduction of the cysteine-coordinated iron would then increase the scaling coefficient for histidine $N_\delta1',2'$ by $\sim|^7/_4|$ times. This could reasonably explain the observed changes of anisotropic hyperfine tensors, where the major contribution results from direct dipole interaction with the spin of the nearest iron.

The isotropic constants of ^{15}N nuclei observed in HYSCORE spectra, besides responding to the scaling coefficient, also depend on the spin density on the 2s orbitals. On going from pH 7 to 13.3, none of the changes of these isotropic constants follow the alterations of the anisotropic tensors (i.e., no proportional double increase of constants). This indicates an additional influence of the redistribution of unpaired electron spin density on the hyperfine couplings.

In conclusion, the present HYSCORE data firmly establish that (i) ^{15}N-SDX treated at very alkaline pH, which exhibits a significantly modified EPR line shape for the fully deprotonated, reduced protein, partially retains essential geometric features for a Rieske-type [2Fe−2S] cluster (including the coordination of two histidyl ligands to the iron site ($N_\delta1',2'$) and the modified hydrogen bond network involving the peptide nitrogen N_p[17b]), and (ii) there is marked redistribution of the unpaired spin density at pH 13.3, which is indicative of a possible difference in the mixed-valence state of the reduced cluster. The hyperfine couplings for ^{15}N nuclei at pH 13.3 determined in this study contribute to the experimental characterization of the protein-bound, "native-like" [2Fe−2S] cluster environment under extreme alkaline conditions and can be used in the theoretical analysis for selection of an appropriate model of the mixed-valence state of the fully deprotonated, reduced Rieske center.

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Supporting Information Available: The plots of $(\nu_1)^2$ versus $(\nu_2)^2$ for recalculated frequencies, from which the anisotropic N_δ hyperfine tensors of ^{15}N-SDX at pH 7 (Figure S-1) and 13.3 (Figure S-2) were obtained, and additional discussion for the accuracy in determination of hyperfine tensors (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) (a) Iwasaki, T.; Kounosu, A.; Uzawa, T.; Samoilova, R. I.; Dikanov, S. A. *J. Am. Chem.* Soc. **2004**, *126*, 13902−13903. (b) The coupling for N_p has been assigned primarily to the peptide nitrogen involved in the NH−S-type hydrogen bond(s) with the bridging sulfide atom(s) of the reduced cluster. Its substantial increase from ~1.0 MHz at pH 7 to 1.4 MHz at pH 13.3 may reflect a proportional increase of unpaired electron spin density onto the bridging sulfide atoms in the fully deprotonated, reduced Rieske center at pH 13.3, providing additional support for redistribution of the unpaired spin density over the reduced cluster. Additional contribution to this phenomenon is local conformational adjustment of the immediate cluster environment, which is expected for a protein-bound system at pH 13.3, as reported for the oxidized protein near and above pH 12 by resonance Raman spectroscopy (ref 11).
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