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Orientation-Selected ^{15}N-HYSCORE Detection of Weakly Coupled Nitrogens around the Archaeal Rieske [2Fe−2S] Center

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Proteins containing Rieske-type [2Fe−2S] clusters with two histidyl and two cysteinyl 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] The distinct biological function of this protein family is in part associated with the cluster redox potential, for which its rough correlation with the number of hydrogen bonds to the cluster has been proposed.[5−7]

In the cytochrome bc_1/b_6f family, a high-potential Rieske protein constitutes a high-potential electron-transfer fragment of the bifurcated mechanism at the quinol-oxidizing Q_o site.[4,8] The energy released from the electron-transfer reaction is captured in the form of a transmembrane electrochemical proton gradient, which is then used for energy-requiring processes such as ATP synthesis, ion transport, and flagellar movement in the cells.

In the catalytic quinol oxidation at the Q_o site, extraction of a proton, as well as an electron, from bound quinol to the Rieske protein is a crucial step, and the pK_a values of the N_ϵ of the terminal histidyl ligands of the high-potential Rieske proteins may be of particular mechanistic importance in the coupled electron−proton transfer in the cytochrome bc_1/b_6f family.[4,8] However, since spectroscopic techniques for the specific characterization of the N_ϵ and peptide nitrogens are very limited,[7,9] it has not directly been addressed experimentally how electron transfer is coupled to proton transfer at the Rieske center to initiate the Q_o site catalysis.

Toward deeper mechanistic understanding of the coupled electron−proton transfer event of the biological iron−sulfur cluster system, we have employed X-band one- and two-dimensional ^{14}N electron spin−echo envelope modulation (ESEEM) techniques to characterize bacterial cytochrome bc_1 complex (with natural abundance of nitrogen, 99.63% of ^{14}N) in complex with bound quinol and various inhibitory Q_o site occupants.[10] This study was based on the analysis of the ESEEM from two coordinated $^{14}N_\delta$ of the histidyl ligands that dominantly contribute to the spectra. Other weakly coupled nitrogens around cluster, i.e., N_ϵ and peptide nitrogens, did not show readily recognizable lines in the spectra, due to the influence of nuclear quadrupole interaction requiring special relations between nuclear Zeeman frequency and hyperfine coupling.[11] Consequently, no quantitative information about these weakly coupled nitrogens has previously been reported for the Rieske protein system with natural abundance of nitrogen.[10,12−15]

Belonging to the high-potential class of the Rieske protein family, a new tractable model protein of interest is the hyperthermostable archaeal sulredoxin (SDX) from *Sulfolobus tokodaii* strain 7 ($E_{m,acid\ pH} \approx +190$ mV), which is weakly homologous to the extrinsic cluster-binding domain of cytochrome bc_1-associated, high-potential Rieske proteins (DDBJ accession number, AB023295).[9,16−18] Recombinant SDX has been overproduced in *Escherichia coli* and can be obtained in appropriate forms for isotope labeling, site-directed mutagenesis, crystallization, and various spectroscopic analyses.[9,15,18,19] Here, we report the orientation-selected hyperfine sublevel correlation spectroscopic (HYSCORE) analysis of the uniformly ^{15}N-labeled, archaeal SDX (prepared as reported previously),[18] with the specific aim to detect and characterize weakly coupled $^{15}N_\epsilon$ and peptide $^{15}N_p$ in the immediate Rieske cluster environment without any interference from other redox centers nearby. This sample is particularly suitable for the ESEEM analysis of the weakly coupled nitrogens because ^{15}N does not possess the quadrupole moment.

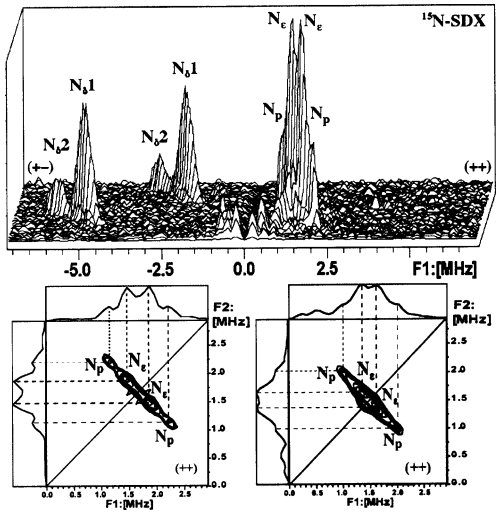

Figure 1. Typical ^{15}N-HYSCORE spectrum of the uniformly ^{15}N-labeled SDX recorded near g_x (top). Magnetic field, 386 mT ($g = 1.796$); $\tau = 136$ ns; microwave frequency, 9.7015 GHz. The contour presentations of the weakly coupled ^{15}N cross-peaks in the (++) quadrant near g_x (magnetic field 386 mT, $g = 1.796$; $\tau = 136$ ns; left) and g_z (magnetic field 344.5 mT, $g = 2.012$; $\tau = 136$ ns; right) are also shown (bottom).

Dithionite-reduced Rieske [2Fe−2S] center in ^{15}N-SDX shows a rhombic EPR spectrum ($g_{z,y,x} = 2.008, 1.91, 1.79$).[15,16] HYSCORE spectra measured at different points of the EPR line contain the cross-features produced by different types of nitrogens (Figure 1). In the (+−) quadrant, two pairs of cross-peaks with a contour parallel to the diagonal are detected, which are attributed to the two coordinated $^{15}N_\delta$1,2 with the hyperfine splittings of the order 6 and 8 MHz. The contour line shape analysis of these features recorded at different field positions[20] gave the hyperfine tensors in

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the axial approximation of $a = 6.0$ and $T = 1.2$ MHz for $N_\delta 1$, and $a = 7.8$ MHz and $T = 1.3$ MHz for $N_\delta 2$ in ^{15}N-SDX. These tensors are very similar to those reported for other Rieske-type proteins by the orientation-selected ^{15}N Q-band electron−nuclear double resonance (ENDOR).[21]

Of particular interest in the HYSCORE spectra of ^{15}N-SDX is the (++) quadrant, in which two superimposed but well-resolved pairs of the cross-features are clearly detected at (2.2, 1.1) MHz (N_p) and (1.9, 1.5) MHz (N_ϵ) near g_x, with the splittings of 1.1 and 0.4 MHz, respectively, and at (2.04, 0.97) MHz (N_p) and (1.63, 1.38) MHz (N_ϵ) near g_z, with the splittings of 1.07 and 0.25 MHz, respectively (Figure 1). These features are centered symmetrically around the diagonal point with ^{15}N Zeeman frequency and are attributed to weakly coupled ^{15}N in the immediate cluster environment. They were also observed in the HYSCORE spectra recorded at some intermediate field positions as well as near g_x and g_z (Figure 1, bottom), indicating their predominantly isotropic character as a result of the transfer of the unpaired spin density onto the corresponding nuclei.

The intense pair of the cross-features with the smaller splitting of 0.25−0.4 MHz can be assigned to the N_ϵ of the histidyl ligands of ^{15}N-SDX (N_ϵ in Figure 1). The available values of isotropic hyperfine couplings for coordinated N_δ and remote N_ϵ of the imidazole rings ligated to Cu(II) and VO(II) in various model complexes and proteins have an almost constant ratio of about 20 between the N_δ and N_ϵ couplings (although both couplings in the Cu(II) complexes are much larger than those in the VO(II) complexes).[22,23] The couplings of ∼6−8 MHz for two N_δ coordinated to the Rieske-type [2Fe−2S] clusters are very close to those of the N_δ equatorially coordinated to the VO(II) complexes,[23] and the N_ϵ nuclei in the latter compounds have the coupling 0.3−0.4 MHz, as observed in the present HYSCORE spectra of ^{15}N-SDX (Figure 1). This probably reflects analogous spin density distribution over the imidazole ring in the reduced Rieske cluster system, which is controlled by the protonation state of the N_ϵ atoms.

In light of these arguments, the coupling of ∼1.1 MHz for the less intense cross-features in the HYSCORE spectra does not fit the typical ratio between the N_δ and N_ϵ couplings, thus requiring different assignment. ESEEM spectra of the plant and vertebrate ferredoxin-type [2Fe−2S] clusters with four cysteinyl ligands exhibit lines from the peptide backbone nitrogens (N_p), which hold some unpaired spin density transferred from the cluster via the hydrogen bonding network.[11] A typical hyperfine coupling for $^{14}N_p$ is ∼1 MHz (or ∼1.5 MHz for $^{15}N_p$), which is very close to the coupling of ∼1.1 MHz observed for ^{15}N-SDX (Figure 1). We therefore assigned this coupling to the peptide backbone $^{15}N_p$ involved in the hydrogen bonding network around the reduced Rieske cluster.

In conclusion, the weakly coupled $^{15}N_\epsilon$ and $^{15}N_p$ nuclei around the archaeal Rieske center in the uniformly ^{15}N-labeled SDX produce readily observable signatures in the HYSCORE spectra. Two well-pronounced spectral contributions assigned to the $^{15}N_\epsilon$ and peptide backbone $^{15}N_p$ with the couplings of 0.3−0.4 and 1.1 MHz, respectively, are resolved for the hyperthermostable tractable protein. These nitrogens do not show unambiguous patterns in the ^{14}N ESEEM spectra of the cognate natural abundance protein[15] as well as many other biological metal centers and clusters. Thus, the HYSCORE experiment with ^{15}N-labeled proteins can offer a new practical tool, applicable for the detailed structure-mechanism studies of a wide range of the biological redox protein system involving these nitrogens. Among the particular instances are the quantitative and mechanistic investigation of histidine N_ϵ of ^{15}N-labeled Rieske proteins in context of the Q_o-site catalysis of the cytochrome bc_1/b_6f family,[8,10] and probing the peptide backbone $^{15}N_p$ involved in hydrogen bonding network around Rieske-type proteins with different redox potentials.[6,7]

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