Supporting Information

Identification of the nitrogen donor hydrogen bonded with the semiquinone at the Q_H site of the cytochrome bo_3 from *Escherichia coli*

Myat T. Lin[1], Rimma I. Samoilova[3], Robert B. Gennis[2*] and Sergei A. Dikanov[4]

[1]Center for Biophysics and Computational Biology, [2]Department of Biochemistry, University of Illinois, 600 South Goodwin Avenue, Urbana, IL 61801 [3]Institure of Chemical Kinetics and Combustion, Russian Academy of Sciences, Novosibirsk 630090, Russia, [4]Department of Veterinary Clinical Medicine, University of Illinois, Urbana, IL 61801

Procedure to construct amino acid auxotrophic C43 (DE3) *E. coli* strains

In order to achieve efficient ^{15}N labeling of cytochrome bo_3 of only the desired amino acid residues, amino acid auxotrophic C43 (DE3) *E. coli* strains are necessary[1]. Each auxotrophic C43 (DE3) strain was constructed by inactivation of an appropriate chromosomal gene, which was achieved by the homologous recombination of a linear double-stranded DNA that replaced the target gene on the chromosome. The linear DNA was generated *in vitro* using a three-step PCR technique resulting in an antibiotic resistance cassette flanked by ~ 500 bp regions homologous to regions upstream and downstream of the target gene (**Figure S1**)[2]. The primers used in the three-step PCR technique are listed in **Table S1**. The linear DNA was then transformed into a C43 (DE3) strain, which already expresses the phage λ Red recombinase from pKD46 plasmid[3]. The new recombinant C43 (DE3) strains were selected by their newly acquired antibiotic resistance, and the deletion of the chromosomal target gene in those strains was verified by sequencing the target chromosomal region. The auxotrophic C43 (DE3) strains used in this study are summarized in **Table S2**. The ^{15}N labeled amino acids were purchased from Cambridge Isotope Laboratories, Inc.

Procedure to prepare selective ^{15}N labeled cytochrome bo_3 samples

Overexpression of cytochrome bo_3 was accomplished using auxotrophic C43 (DE3) *E. coli* strains (**Table S2**) transformed with a pET-17b vector (Novagen) into which was cloned the *cyoABCDE* operon, encoding cytochrome bo_3 with a 6xHis tag at the C terminus of subunit II[4,5]. The cells were grown in M63 minimal medium consisting of 7 g/L K_2HPO_4, 3 g/L KH_2PO_4, 2 g/L NH_4Cl, 2 g/L glucose, 10 mg/L thiamine, 10 μM $CuSO_4$, 30 μM $FeSO_4$, 1 mM $MgSO_4$. When necessary, different combinations of amino

acids were added (**Table S2**). When the density of the culture reached an OD_{600} of 0.5–0.7, the expression of cytochrome bo_3 was induced with 0.5 mM isopropyl 1-thio-β-D-

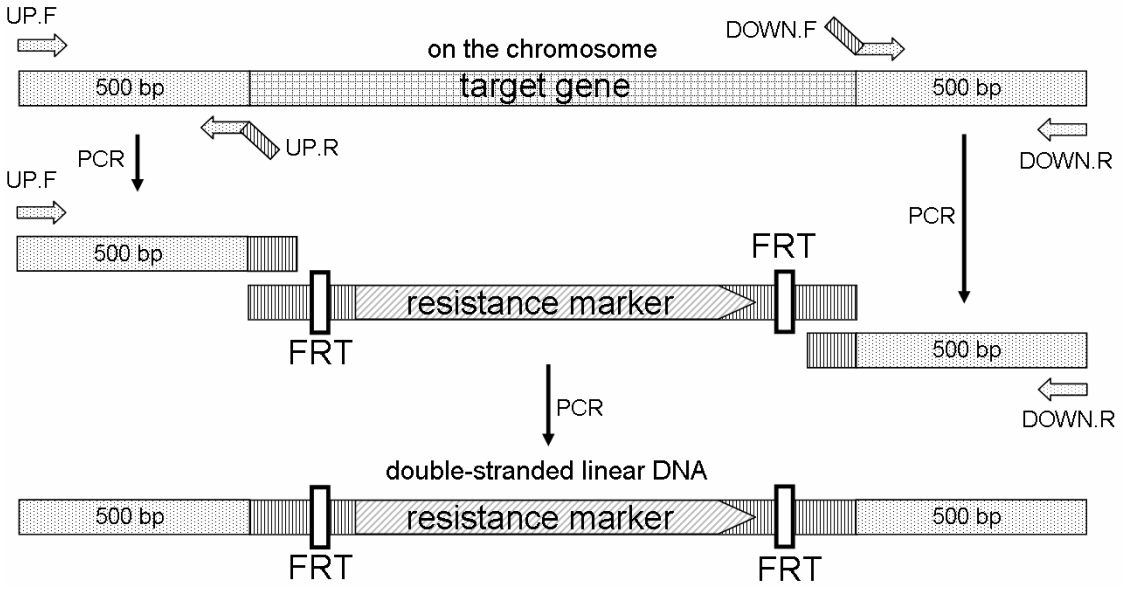


Figure S1. The three-step PCR technique used to generate linear double-stranded DNA with 500 bp homology extensions. In the first step, the upstream 500 bp and the downstream 500 bp of the target chromosomal gene are amplified independently. Each of the UP.R and DOWN.F primers has a short segment homologous to the respective region of the resistance marker. The second step generates the linear DNA from the two homologous 500 bp segments and the one with the resistance marker. The FRT sites flanking the resistance marker allows the simple removal of the marker by pCP20 plasmid, which expresses FLP recombinase[3].

galactopyranoside (IPTG) at 37 °C for 4–5 h. The histidine-tagged cytochrome bo_3 was purified based on the procedure described by Rumbley *et* al[6]. The harvested cells were resuspended in 50 mM KP_i buffer plus 5 mM $MgSO_4$ at pH 8, and broken by three passages through a Microfluidizer® high pressure fluids processor from Microfluidics (Newton, MA) at 10,000 psi. The cell debris was removed by centrifugation at 16,000 x *g* for 20 min. The membranes were then pelleted from the supernatant by centrifugation at 180,000 x *g* for 5 h, and solubilized in 50 mM KP_i buffer at pH 8 with 1 % *n*-dodecyl β-D-maltoside (DDM) at 4 °C for at least 2 h. After the unsolubilized particles were removed by centrifugation at 180,000 x *g* for 1 h, the supernatant was equilibrated with 5–10 mL Ni-NTA resin in the presence of 5 mM imidazole for 1 h. The mixture was then loaded into a column, and the Ni-NTA resin was washed with 5 volumes of 10 mM imidazole, 50 mM KP_i, 0.1 % DDM at pH 8, followed by 5 volumes of 15 mM imidazole, 50 mM KP_i, 0.1 % DDM at pH 8. The histidine-tagged cytochrome bo_3 was eluted with 100 mM imidazole, 50 mM KP_i, 0.1 % DDM at pH 8. Imidazole was removed from the purified cytochrome bo_3 sample, and the buffer was changed to 50 mM KPi, 0.1 % DDM, 10 mM EDTA, 5 % glycerol at pH 8.3, using an Amicon Ultra 15 concentrator with a 100,000 molecular weight cutoff. The concentration of cytochrome

bo$_3$ was determined using an extinction coefficient of 188 $mM^{-1}cm^{-1}$ for the peak of the Soret band (~408 nm). The concentrated protein sample was flash-frozen in liquid nitrogen and stored at − 80 °C. The five cytochrome *bo*$_3$ samples with ^{15}N labels at different sites used in this study are listed in **Table S2**.

Table S1. Primers used in the three-step PCR technique to generate linear double-stranded DNAs.

Target Genes	Primers	Sequences
glnA	UP.F	tcgatgaccagcgtcacttcgttatcg
	UP.R	cttcgaagcagctccagcctacacgtgttttagttgccgtggaaacttttcg
	DOWN.F	ggaataggaactaaggaggatattcatatgactttaactctcctggattggtcatgg
	DOWN.R	tctccaaatcgttggagtccatcacg
hisG	UP.F	gatgtgctccgcttatcagtcctac
	UP.R	cttcgaagcagctccagcctacactttcttattcctctttaaacctgtctgaacc
	DOWN.F	ggaataggaactaaggaggatattcatatgtcgccatgagctttaacacaatcattgac
	DOWN.R	agctgcgccgcataaaggatctc
tyrA	UP.F	ccatgccggttggttttaaaaacggcac
	UP.R	cttcgaagcagctccagcctacacaataaacctcttaagccacgcgagccgtc
	DOWN.F	ggaataggaactaaggaggatattcatatgtaatccagtgccggatgattcacatcatc
	DOWN.R	tgcggcaatggaaaaggttgcacagg
argH	UP.F	ccgtaggtttgtttctcggtgacg
	UP.R	cttcgaagcagctccagcctacactcaatgccggaaagctgcgcctc

	DOWN.F	ggaataggaactaaggaggatattcatatgaggcgattgcttttgcgcaggctc
	DOWN.R	gcttagcgggcaaattcgtaagctg

Table S2. Auxotrophic C43 (DE3) strains used to prepare cytochrome *bo*$_3$ samples with ^{15}N labels at different sites.

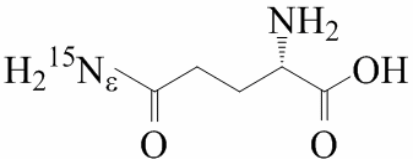
Selective ^{15}N labels in the cytochrome *bo*$_3$ samples	Genes deleted in C43 (DE) strains	Amino acids added to M63 minimal medium
$^{15}N_{\varepsilon}$ Gln	*glnA*	730 mg/L $^{15}N_{\varepsilon}$ Gln
U–$^{15}N_3$ His	*hisG*, *tyrA*	15 mg/L $^{15}N_3$ His, 20 mg/L Tyr
$^{15}N_{\eta}$s Arg	*argH*	100 mg/L $^{15}N_{\eta}$s Arg
U–$^{15}N_4$ Arg	*argH*	80 mg/L $^{15}N_4$ Arg
$^{15}N_P$ Arg	*argH*	80 mg/L $^{15}N_P$ Arg

Procedure to generate the semiquinone radical at the Q_H site of cytochrome *bo*$_3$

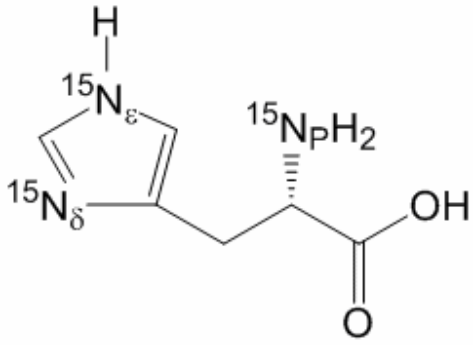
The cytochrome *bo*$_3$ sample was dialyzed against 0.5 L of 50 mM KP_i buffer, 0.1 % DDM, 10 mM EDTA, 5 % glycerol at pH 8.3 for 10 h. The sample was then concentrated with Microcon YM-100 to about 200-400 μM. After the concentrated sample was made anaerobic by equilibration with argon gas using a vacuum line, about 500-fold excess sodium ascorbate in an anaerobic solution of the same buffer was added to the sample. After the mixture was incubated at 4 °C for 3 h, it is rapidly transferred to an argon-flushed EPR tube, and immediately frozen with liquid nitrogen[5,7].

Scheme.

^{15}N labeling of Q-101 and H98



$^{15}N_{\varepsilon}$ –Q101



Uniformly ^{15}N labeled H98

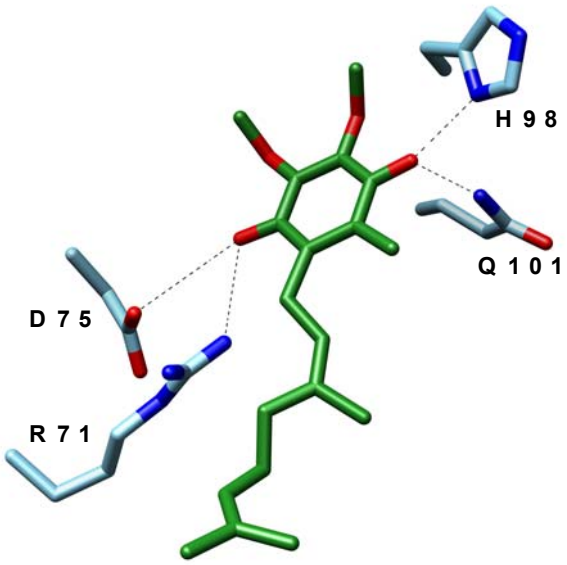


Figure S2. Model of the quinone binding at the Q_H site of the *cyt* bo_3. The figure was generated according to the model based on the X-ray crystal structure by Abramson *et al.* (8). Taken from ref. (5).

Three-pulse ESEEM spectra

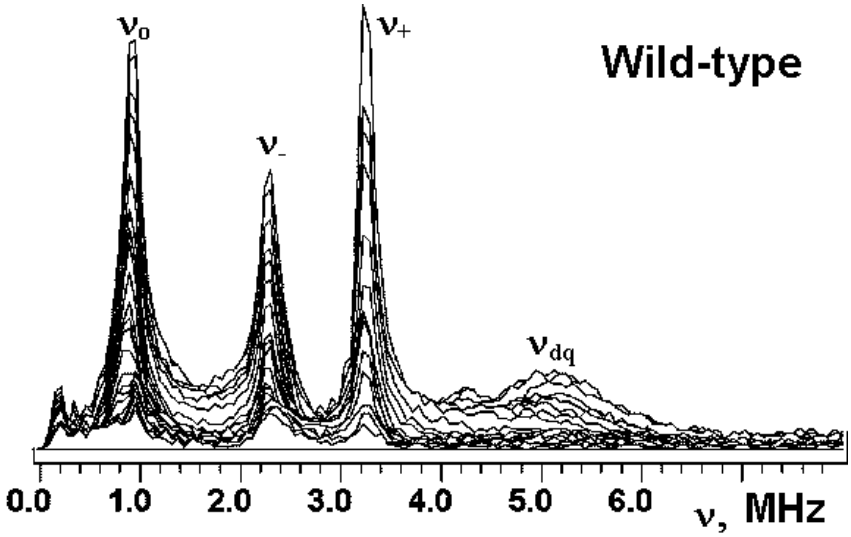
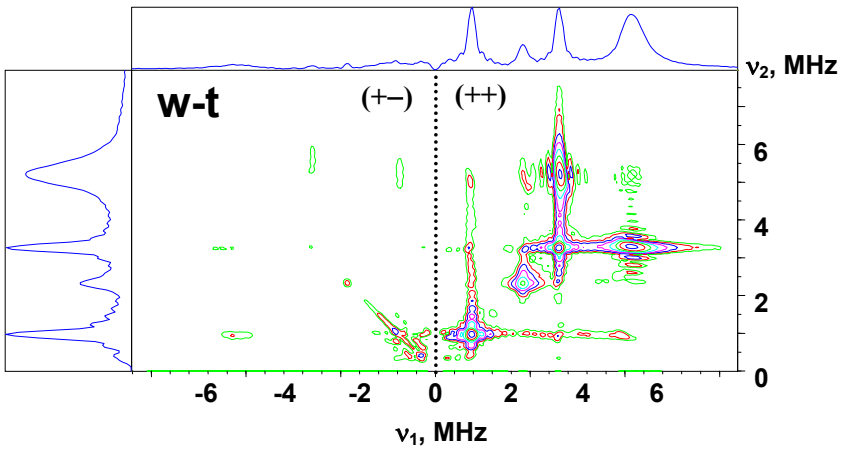


Figure S3. Stacked plots of three-pulse ESEEM spectra of the SQ at the Q_H site of wild type cyt bo_3. The spectra show modulus Fourier transforms along the time T (between second and third microwave pulses) axis (512 points with a 16 ns step) at different times τ. The initial time τ (between first and second pulses) is 88 ns in the farthest trace and was increased by 16 ns in successive traces. The microwave frequency was 9.7 GHz, and the magnetic field was 346.0 mT.

HYSCORE spectra



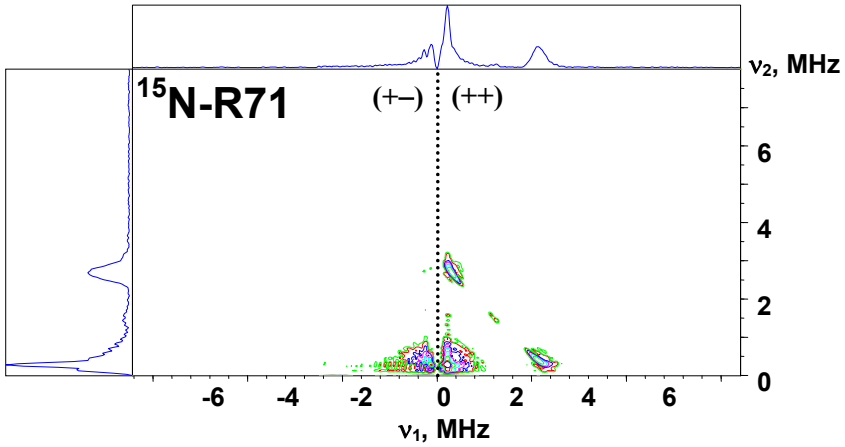
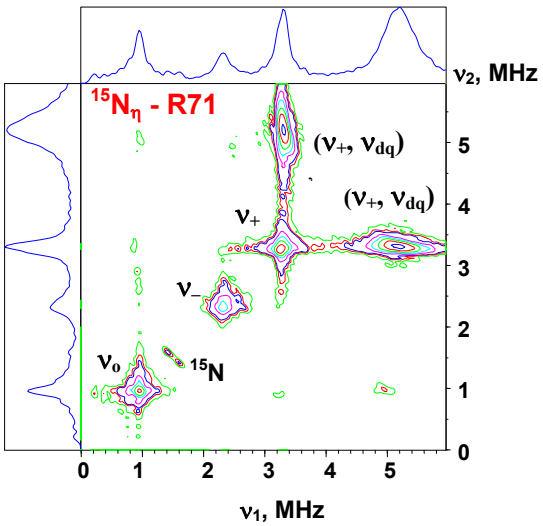
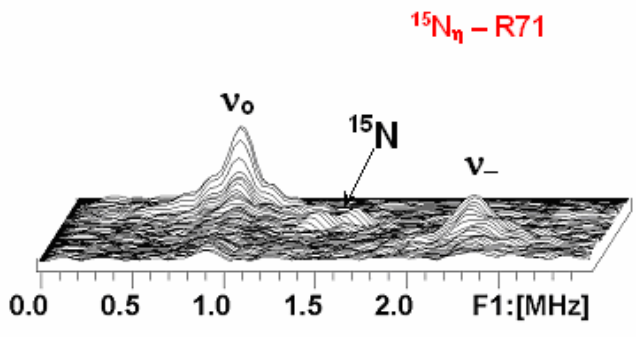


Figure S4. The contour HYSCORE spectra of the SQ at the Q_H site of the *cyt* bo_3. In addition to the spectra in Figures 1a and 1d these spectra show both (++) and (+–) quadrants of the spectra.





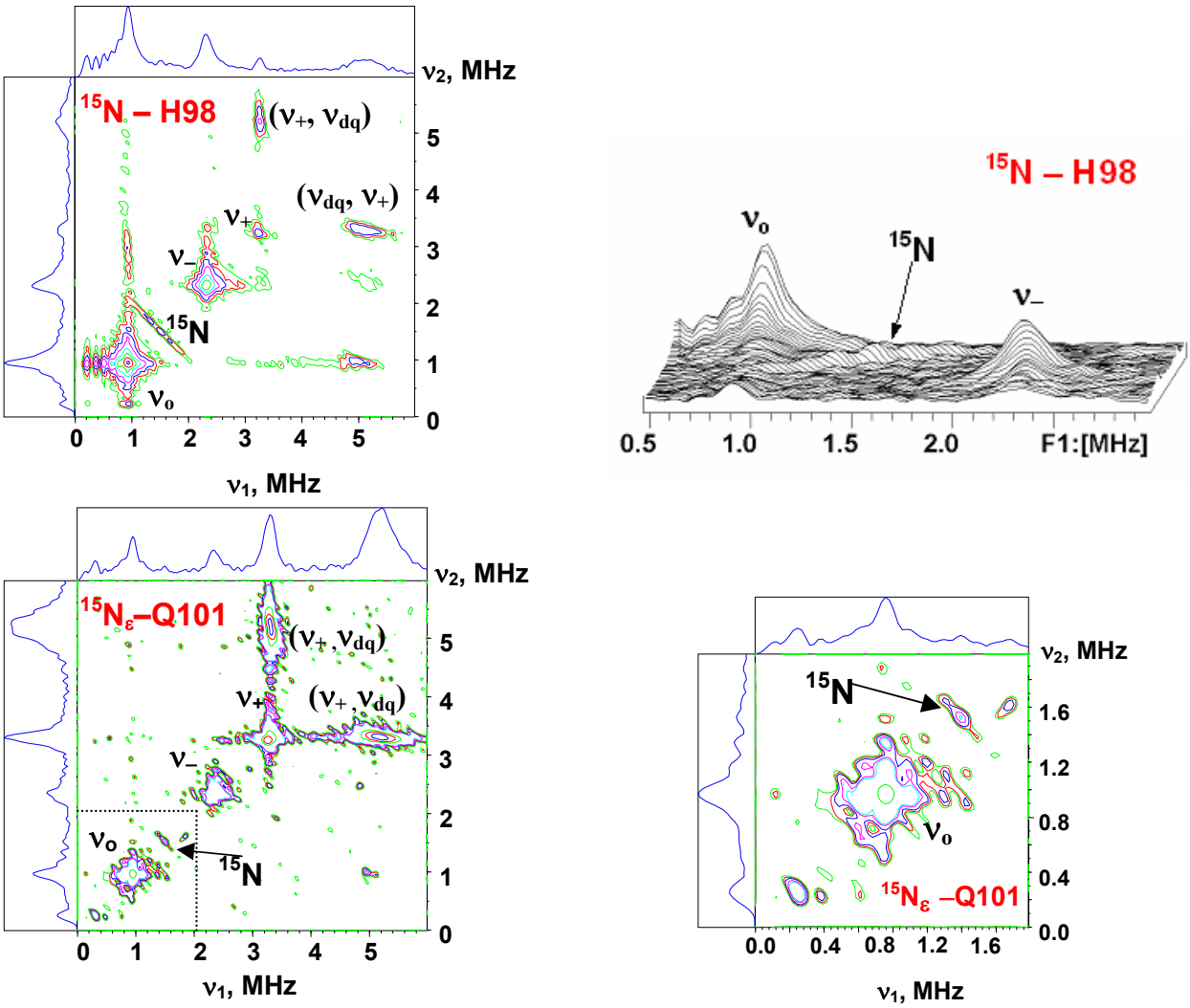


Figure S5. The contour and 3D stacked presentations of the HYSCORE spectra of the SQ at the Q_H site of the *cyt* bo_3 with $^{15}N_\eta$-R71 (346.3 mT, τ=136 ns, 9.712 GHz); uniformly ^{15}N labeled H98 (346.3 mT, τ==200 ns, 9.708 GHz); $^{15}N_\varepsilon$-Q101 (346.4 mT, τ=136 ns, 9.711 GHz).

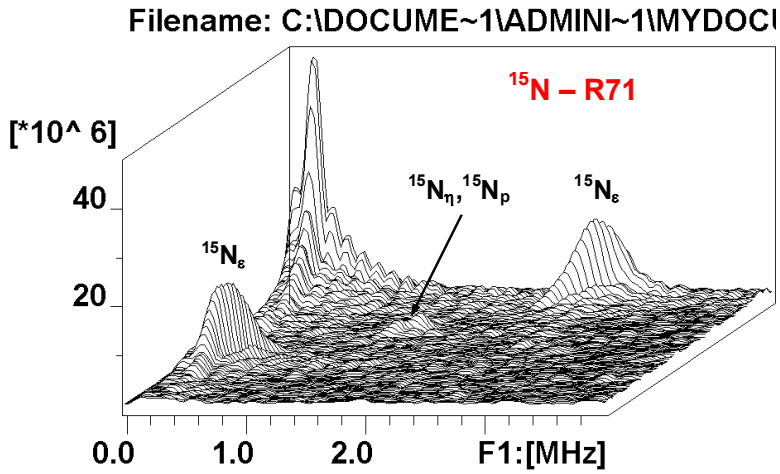


Figure S6. 3D stacked HYSCORE spectrum of the SQ at the Q_H site of the *cyt* bo_3 with uniformly ^{15}N labeled R71.

Transferred spin density. The existence of a nonzero isotropic constant, *a*, for the interacting N_ε of R71 indicates that unpaired electron spin density is transferred from the SQ onto this atom. The isotropic constant is determined mainly by the unpaired spin density in its 2*s* orbital. It is proportional to the value of 1811 MHz for ^{14}N, which is computed for unit spin density in this orbital (9). Thus the isotropic hyperfine coupling ~2.0-2.4 MHz determined from HYSCORE spectra corresponds to the transfer of only a small fraction of this computed spin density, with $\rho_s \sim (1.2 \pm 0.1) \cdot 10^{-3}$ on the 2s orbital of nitrogen. An additional unpaired spin density will be located on the $2p^3$-orbitals of the nitrogen depending on the exact configuration of the sp-hybridization.

References.

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