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HYSCORE Evidence That Endogenous Mena- and Ubisemiquinone Bind at the Same Q Site (Q_D) of *Escherichia coli* Nitrate Reductase A

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In anaerobiosis and in the presence of nitrate, *Escherichia coli* induces the production of two respiratory enzymes: formate dehydrogenase-N and nitrate reductase A. They cooperate in generating a proton motive force across the cytoplasmic membrane through the redox loop mechanism by coupling formate oxidation in the periplasm to nitrate reduction in the cytoplasm thanks to the lipid mobile electron/proton carrier quinol. Nitrate reductase A (NarGHI) is a membrane-bound heterotrimeric enzyme that oxidizes quinols and reduces nitrate to nitrite. It contains eight redox-active metal centers:[1−4] a molybdenum cofactor and a Fe_4S_4 cluster (FS0) in the nitrate-reducing subunit NarG; one Fe_3S_4 cluster (FS4) and three Fe_4S_4 clusters (FS1−3) in the electron-transfer subunit NarH; and two low-spin hemes *b* in the membrane-anchor subunit NarI, which are termed b_D and b_P to indicate their distal and proximal positions with respect to the catalytic site. NarGHI is able to use both menaquinol-8 and ubiquinol-8 as physiological reductants.[5] Interestingly, ubiquinones (UQs) are primarily involved in aerobic respiration, whereas menaquinones (MQs) serve as electron carriers in anaerobic respiration. This is thought to allow facultative anaerobic bacteria to adapt to varying environmental conditions with respect to the redox state. Despite the numerous investigations devoted to studying the interactions of NarGHI with quinones, no general agreement has been obtained concerning the number, nature, and specificity of mena- and/or ubiquinol binding sites in this enzyme.[4,6−9] This illustrates the general difficulty of providing molecular details of the interactions between natural quinones and enzymes.

Importantly, we recently used electron paramagnetic resonance (EPR) techniques to identify an endogenous menasemiquinone (MSQ) intermediate in NarGHI-enriched inner-membrane vesicles (IMVs) of *E. coli*. This species, which is characterized by a midpoint potential $E_{m,pH=7.5}(Q/QH_2) \approx -90$ mV, has been shown to be highly stabilized thermodynamically and located at the Q_D site within NarI in close proximity to heme b_D (Figure 1A).[10,11] In addition, our recent multifrequency HYSCORE study provided direct evidence for nitrogen ligation to the NarGHI-bound MSQ.[12] On the basis of the direct determination of the quadrupolar parameters of the interacting ^{14}N by S-band (∼3 GHz) HYSCORE, we assigned this nucleus to the N_δ imidazole nitrogen of a hydrogen-bonded histidine residue, most likely the heme b_D axial ligand His66.[12] While the properties and binding mode of MSQ to NarGHI could be described in detail,[10−12] information concerning possible ubisemiquinone (USQ) stabilization in this enzyme is still lacking. To investigate whether a NarGHI-bound USQ can be detected without contamination of NarGHI-bound MSQ, NarGHI-enriched IMVs were purified from an MQ-deficient *E. coli* strain, titrated, and studied by EPR spectroscopy. A radical signal with spectroscopic properties ($g \approx 2.004$, peak-to-peak line width ≈ 0.8 mT) and a midpoint potential [$E_{m,pH=7.5}(Q/QH_2) \approx +60$ mV] typical of protein-bound USQ species was detected in this sample,[13,14] with maximal intensity amounting to ∼2% USQ/FS4 (Figure 1B).

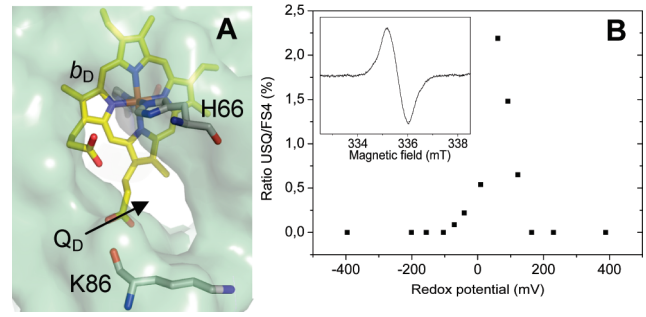

Figure 1. (A) View of the Q_D site in NarI. Residues essential for MSQ stabilization are also indicated. (B) Redox titration of NarGHI-enriched IMVs (pH 7.5) from an MQ-deficient *E. coli* strain. The inset shows the USQ EPR spectrum (microwave frequency 9.407 GHz) of a sample redox-poised at +60 mV.

To determine the origin of this USQ species, control experiments on $NarGHI_{H66Y}$-enriched IMVs were performed. Indeed, the single-point mutation H66Y prevents the insertion of heme b_D as well as binding of quinol analogues and inhibitors to NarGHI, while the overall structure of the enzyme is maintained.[8,15] This mutation leads to complete loss of the NarGHI quinol oxidase activity using menaquinol or ubiquinol analogues as electron donors. Interestingly, a USQ-type radical signal was also detected in these membrane preparations. The protein environments of the USQ species in the two samples were probed by high-resolution pulsed EPR spectroscopy (Figure 2). Comparison of the X-band ^{14}N-HYSCORE spectra allowed two sets of strong peaks to be distinguished. Set 1 (Figure 2B) consists of three narrow lines at $\nu_0 = 1.0$, $\nu_- = 2.3$, and $\nu_+ = 3.3$ MHz along with two broader, intense cross-peaks that correlate $\nu_+ = 3.3$ and the double-quantum transition frequency $\nu_{dq} \approx 5.1-5.2$ MHz. It is worth noting that these frequencies are also resolved in the USQ ESEEM spectrum of NarGHI-deficient IMVs of *E. coli* (see the Supporting Information). This spectrum has been previously described in detail.[16] It arises from the N_ξ of an Arg residue that couples to the USQ formed at the high-affinity Q_H site of cytochrome *bo*.[17] Indeed, this enzyme is present in the membrane fragments when *E. coli* is grown under the semiaerobic conditions used in this work.[18]

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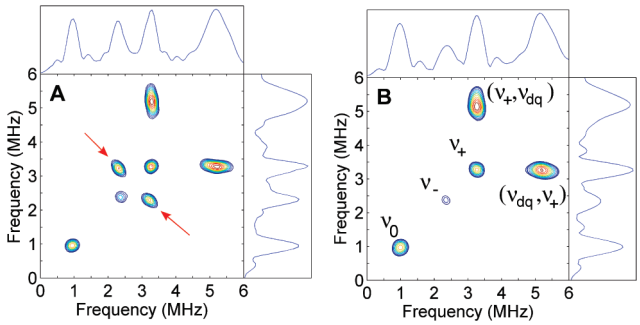

Figure 2. HYSCORE spectrum of the USQ stabilized in (A) NarGHI-enriched IMVs from an MQ-deficient *E. coli* strain and (B) $NarGHI_{H66Y}$-enriched IMVs from an LCB3063 strain. Magnetic field, time τ, and microwave frequency: (A) 345.2 mT, 136 ns, 9.695 GHz; (B) 344.4 mT, 136 ns, 9.666 GHz.

Intriguingly, an additional pair of off-diagonal crosspeaks (set 2) was resolved in the spectrum of membrane fractions issued from an MQ-deficient *E. coli* strain. They are indicated by arrows in Figure 2A. These correlation peaks are lost upon substitution of NarGHI His66 by a Tyr (Figure 2B), thus showing clearly that they arise from a NarGHI-bound USQ species. These cross-peaks correlate nuclear transition frequencies at 2.3 ± 0.1 and 3.2 ± 0.1 MHz. Therefore, these peaks could be assigned to the double-quantum transition frequencies $\nu_{dq+} = 3.2$ MHz and $\nu_{dq-} = 2.3$ MHz from a ^{14}N nucleus. The calculated isotropic hyperfine coupling constant is $A_{iso} = 0.6 \pm 0.1$ MHz, with a quadrupole coupling constant in the range $0.39 \leq \kappa \leq 0.51$ (see the Supporting Information). The latter values are within the range measured for histidines that are H-bonded to other protein-bound semiquinones [e.g., $0.35 < \kappa < 0.41$ (see refs listed in ref 12)]. Moreover, they are very similar to those found for the ^{14}N interacting with the MSQ radical at the Q_D site of NarGHI ($A_{iso} \approx 0.8$ MHz, $\kappa = 0.49$, $\eta = 0.50$), which was previously assigned as the His66 N_δ.[12] Additionally, this interaction was also detected in the HYSCORE spectra of the USQ species stabilized in NarGHI-enriched IMVs from the *E. coli* LCB3063 strain (see the Supporting Information).

In conclusion, our EPR experiments reported herein show for the first time that an endogenous USQ intermediate can be formed in NarGHI. Detailed analysis using ESEEM/HYSCORE spectroscopy has demonstrated that it binds to the protein via a H-bond to a His ^{14}N. This interaction is specifically lost upon substitution of His66 at the Q_D site by a Tyr residue, which preserves a native-like conformation of NarI. The only change occurs in a localized region that forms part of the Q_D site (see the Supporting Information). Altogether, these results demonstrate unambiguously that MSQ and USQ bind to the same His66 residue, the only His residue present in the Q_D site of NarGHI. Moreover, these results invalidate previous models suggesting that ubi- and menaquinol analogues deliver their electrons at two different sites of NarGHI[6,7] and that a second Q site could be located in an elongated hydrophobic cavity exposing both hemes to the lipid bilayer.[8,9]

Many respiratory enzymes can use different types of quinones as substrates. However, the direct binding of the two kinds of endogenous quinones at the same Q site has been only partially demonstrated.[19,20] The remarkable stability of the two kinds of semiquinone herein thus offers the unique possibility of further investigating the details of the catalytic mechanism with the two substrates. Interestingly, the amount of NarGHI-bound USQ is less than that measured for MSQ. This could arise from a lower affinity of NarGHI for USQ or from a lower stability constant for USQ. Knowledge of similarities and differences in hydrogen bonding is essential for understanding how the accommodation of the two substrates proceeds.[19] Advanced EPR studies to address this point are in progress. Furthermore, selective ^{15}N amino acid labeling will be used to probe the potential role of the Lys residue located at the entrance of the Q_D binding pocket.

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Supporting Information Available: Experimental procedures, analysis of ^{14}N-ESEEM/HYSCORE spectra, model of the Q_D site in NarGHI and $NarGHI_{H66Y}$, HYSCORE spectra of MSQ and USQ prepared from the LCB3063 strain, and ESEEM spectrum of NarGHI-deficient IMVs. This material is available free of charge via the Internet at http://pubs.acs.org.

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