Semiquinone intermediates are involved in the energy coupling mechanism of *E. coli* complex I

Madhavan Narayanan, Steven A. Leung 1, Yuta Inaba 1, Mahmoud M. Elguindy, Eiko Nakamaru-Ogiso *

Johnson Research Foundation, Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, United States

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Complex I (NADH:quinone oxidoreductase) is central to cellular aerobic energy metabolism, and its deficiency is involved in many human mitochondrial diseases. Complex I translocates protons across the membrane using electron transfer energy. Semiquinone (SQ) intermediates appearing during catalysis are suggested to be key for the coupling mechanism in complex I. However, the existence of SQ has remained controversial due to the extreme difficulty in detecting unstable and low intensity SQ signals. Here, for the first time with *Escherichia coli* complex I reconstituted in proteoliposomes, we successfully resolved and characterized three distinct SQ species by EPR. These species include: fast-relaxing SQ (SQf) with $P_1/2 = 50$ mW and a wider linewidth (12.8 G); slow-relaxing SQ (SQs) with $P_1/2 = 2–3$ mW and a 10 G linewidth; and very slow-relaxing SQ (Sqv) with $P_1/2 = ~0.1$ mW and a 7.5 G linewidth. The SQf signals completely disappeared in the presence of the uncoupler gramicidin D or squamotacin, a potent *E. coli* complex I inhibitor. The pH dependency of the SQf signals correlated with the proton-pumping activities of complex I. The SQf signals were insensitive to gramicidin D, but sensitive to squamotacin. The SQs signals were insensitive to both gramicidin D and squamotacin. Our deuterium exchange experiments suggested that SQf is neutral, while SQs and Sqv are anion radicals. The SQf signals were lost in the ΔNuoL mutant missing transporter module subunits NuoL, and NuoM. The roles and relationships of the SQ intermediates in the coupling mechanism are discussed.

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1. Introduction

Complex I (NADH:quinone oxidoreductase: EC 1.6.5.3) is an entry point for electrons into the respiratory chains of mitochondria and many aerobic organisms. Complex I transfers two electrons from NADH to ubiquinone, translocates protons across the membrane, and generates a transmembrane electric potential and proton gradient essential for ATP production and cellular maintenance such as the transport of metabolites and nutrients [1–4]. Electron microscopic analyses indicated that complex I has a characteristic L-shaped structure with two distinct domains; a hydrophilic peripheral arm and a transmembrane hydrophobic arm [5–7]. Now, the three-dimensional X-ray crystal structures confirm the L-shaped structure [8,9]. While the hydrophilic peripheral domain comprises electron transfer by flavin mononucleotide (FMN) and a chain of seven iron–sulfur (Fe/S) clusters [2], the hydrophobic membrane domain is responsible for proton translocation and the binding of quinone and/or specific inhibitors [17–22]. The mechanism of how electron transfer is linked to vectorial proton translocation, however, remains largely unknown. Based on experiments with bovine heart submitochondrial particles (SMP), it is believed that semiquinone (SQ) intermediates appearing during the complex I catalysis are key for the coupling mechanism of electron transfer reactions to transmembrane proton translocation in complex I [23,24]. Therefore, the understanding of molecular properties and functions of the individual semiquinone species is a prerequisite for elucidating the energy-coupling mechanism of complex I.

There have been several reports on complex I-associated ubisemiquinone EPR signals [23–27]. Tightly coupled submitochondrial particles showed prominent rotenone-sensitive ubisemiquinone signals upon steady-state oxidation of NADH or succinate. The physicochemical properties of these SQ species differ considerably in their spin relaxation behavior. Because SQ species have their spin densities distributed over several atoms, their spin relaxation rates are strongly determined by the neighboring spin systems via spin–spin interactions [28]. Thus, overlapping SQ signals can be resolved based on their relaxation behaviors. Using tightly coupled bovine submitochondrial particles, at least two types of the complex I-associated SQ species were detected by cryogenic EPR [23]: the fast-relaxing ubisemiquinone (SQf) and the slow-relaxing ubisemiquinone (SQs). The SQf signals were seen better in the presence of oligomycin, which was added to increase the

Abbreviations: DDM, dodecyl-β-D-maltoside; DQ, decylubiquinone; MK, menaquinone; Q, ubiquinone; SMP, submitochondrial particles; SQ, semiquinone; UQ, ubiquinone.

* * Corresponding author at: Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, United States. Tel.: +1 215 898 5153; fax: +1 215 573 2085.
E-mail address: eikoo@mail.med.upenn.edu (E. Nakamaru-Ogiso).

1 These authors contributed equally to this work.
2.2. Isolation of complex I spectral interference from overlapping SQ signals arising from other functional roles in complex I’s electron/proton transfer reaction. We report characteristics of these SQ species and discuss their possible comparison of the biochemical/biophysical program that returned simulated results within minutes and with simulation techniques. We improved an algorithm and made a computer into proteoliposomes by EPR using progressive power saturation and we analyzed SQ signals from puriﬁed complex I reconstituted into proteoliposomes. The generation of a proton gradient was determined by monitoring the ﬂuorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA, Sigma). Proteoliposomes (5–20 μM), 0.2 μM ACMA, and 30 μM decyldiethylenetriamine (DQA) (Sigma) were added to the assay buffer, 5 mM MOPS pH 7.0 containing 50 mM KCl, and incubated at 30 °C for 3–5 min. The fluorescence was detected with a ﬂuoromax-4 spectroﬂuorometer (Horiba) at an excitation wavelength of 430 nm and an emission wavelength of 480 nm. The reaction was started by the addition of 50 μM NADH.

2.3. Preparation of proteoliposomes

Appropriate amount of Avanti-polar lipid (in chloroform, 25 mg/ml stock) was taken in a clean test-tube and dried ﬁrst under N2 and then under vacuum for 4–6 h. The dried lipid was suspended in a 50 mM Bis-Tris at pH 6.0 buffered with 50 mM NaCl to a concentration of 8 mg/ml. After the addition of DDM (to a ﬁnal concentration of 2.5%), the lipid solution was sonicated until all lipids were dissolved, and the solution became clear. Then, the chilled liposome solution was mixed with complex I in a 4:1 ratio and incubated in a shaker for 5 min at 4 °C. Immediately, SM2-biobeads were added (50 μM the weight of DDM), and the sample mixture was shaken for 3 h at 4 °C. At the end of 3 h, the sample was washed 5 times with 3 ml of 50 mM Bis-tris pH 6.0 containing 50 mM NaCl to remove biobeads. The collected supernatant was spun down in a ultra-centrifuge for 30 min at 150,000 × g. The pellet (proteoliposomes) was dissolved in an appropriate pH buffer. The buffers used in suspension of the proteoliposome pellet were: 5 mM MES pH 6.0 containing 50 mM KCl and 2 mM MgCl2, 5 mM MOPS pH 7.0 containing 50 mM KCl, 5 mM HEPES pH 7.5 containing 50 mM KCl, or 5 mM HEPES pH 8.0 containing 50 mM KCl. The protein concentration of proteoliposome suspensions was measured using Bradford assay and found to be ~1.8–2.0 mg/ml. Deuterated proteoliposome was prepared by dissolving the ﬁnal pellets in 4 ml of freshly prepared 5 mM deuterated MOPS pH 7.0 (pD 6.6) buffer containing 50 mM KCl, which was made with 95% D2O (Sigma) and solid KCl, and the solution pH was adjusted with NaOD (Cambridge Isotope Laboratories) [36]. The suspension was incubated for 30 min at 7 °C and was again centrifuged at 150,000 × g for 30 min at 7 °C. The pellet was immediately suspended in the deuterated MOPS buffer mentioned above. The orientation of the proteoliposomes was determined from the ratio of the speciﬁc activities from ferricyanide reductase assay measured in the absence and in the presence of 0.05% DDM [37].

2.4. Proton translocation activity

The generation of a proton gradient was determined by monitoring the ﬂuorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA, Sigma). Proteoliposomes (5–20 μM), 0.2 μM ACMA, and 30 μM decyldiethylenetriamine (DQA) (Sigma) were added to the assay buffer, 5 mM MOPS pH 7.0 containing 50 mM KCl, and incubated at 30 °C for 3–5 min. The fluorescence was detected with a ﬂuoromax-4 spectroﬂuorometer (Horiba) at an excitation wavelength of 430 nm and an emission wavelength of 480 nm. The reaction was started by the addition of 50 μM NADH.

2.5. EPR spectroscopy

EPR samples were prepared under strict anaerobic conditions. Puriﬁed complex I samples were reduced with 6 mM NADH or 20 mM neutralized sodium dithionite solution. Reconstituted complex I proteoliposome samples were transferred into EPR tubes, incubated with 400 μM DQ for 15 min. The NADH-DQ oxidoreductase reaction was initiated by the addition of 2 mM NADH, and the mixture was immediately frozen at 10 s except for the time course experiment. We used a special mixer for mixing samples quickly in EPR tubes, which was previously described [38]. EPR spectra were recorded by a Bruker Elexys E500 spectrometer at X-band (9.4 GHz) using an Oxford Instrument ESR900 helium ﬂow cryostat. EPR spectra of the semiquinone signals were simulated by Easyspin (http://www.easyspin.org). Simulation of the power saturation curves was performed using MATLAB software (MathWorks Inc.), utilizing the trust region reﬁnement algorithm and simplex for non-linear least-square ﬁtting. Power saturation data were analyzed by a ﬁtting method as described previously [24,39].
2.6. Other analytical procedures

NADH:DQ and NADH:ferricyanide activities in proteoliposomes were spectrophotometrically measured at 30 °C using a Cary 60 UV-visible spectrophotometer (Agilent, Santa Clara, CA). The buffers used were: 5 mM MOPS buffer (pH 7.0) containing 50 mM KCl. Reaction mixtures contained 150 μM NADH and either 30 μM DQ or 1 mM potassium ferricyanide. Extinction coefficients of ε340 = 6.22 mM⁻¹ cm⁻¹ for NADH and ε420 = 1.00 mM⁻¹ cm⁻¹ for ferricyanide were used for activity calculations. Reported values are the average of three measurements. SDS-PAGE and two dimensional SDS-PAGE were performed according to Laemmli [40], Schägger [41], and ref. [42]. The existence of bound quinones in purified complex I was immunochemically determined using antibodies specific to NuoL [14] and NuoM [10]. The quantification of bound quinones in purified complex I was performed as described in ref. [35].

3. Results

3.1. Purified complex I from the wild-type and the ΔNuoL variant

To investigate how SQ intermediates are linked to the catalytic reactions, we newly constructed a ΔNuoL mutant strain derived from the (His)9-nuoE MC4100 strain for purification. The ΔNuoL complex I has previously been shown to have reduced electron transfer and proton pumping activities [14]. We purified complex I from this strain and from the wild-type. The SDS-PAGE pattern of complex I isolated from the ΔNuoL strain demonstrated the presence of all the subunits NuoA-N except NuoL and NuoM (Fig. 1A). Western blot analysis confirmed that these subunits NuoL and NuoM were below the detection limit in complex I purified from the ΔNuoL strain (Fig. 1B). Because the presence of NuoN in the ΔNuoL complex I was not very clear in the 1D-SDS page (Fig. 1A), we performed two dimensional SDS-PAGE which has been shown to be very effective in separating highly hydrophobic membrane proteins from water soluble proteins such as mitochondrial respiratory chain complexes [42] or synaptic vesicles [43]. By comparing to known patterns of bovine heart complex I [20], we were able to assign the hydrophobic spots as NuoL, NuoM, and NuoN in the wild-type complex I (Fig. 1C). We found that our preparation of the ΔNuoL complex I contained NuoN, but not NuoL and NuoM. Interestingly, the purified ΔNuoL complex I contained 1.11 ± 0.06 moles of ubiquinone per one mole of complex I in contrast to the wild-type which always contained ~2 moles of ubiquinone per one mole of complex I [35]. The representative patterns for proton translocation by the wild-type and ΔNuoL variant after reconstitution in proteoliposomes are shown in Fig. 1D. The proton gradient dissipated after the addition of the uncoupler gramicidin D. The NADH:DQ activity and initial proton pumping rate of the reconstituted ΔNuoL complex I were greatly reduced to ~40% and ~10% of the control, respectively.

3.2. SQ signals in the wild-type and the ΔNuoL complex I

In order to resolve the spectra of multiple SQ signals, we examined the power saturation profiles of the SQ signals at g = 2.004 at 150 K using a computer simulation program. Power saturation curves were analyzed by fitting the curves to the equation: \( A = \Sigma \frac{P_{1/2}}{1 + P / P_{1/2,1}} 0.5h \), where \( A \) is the amplitude of the total signal observed, \( C_i \) is a coefficient for the actual amplitude of the i-th type free radical in the sample, \( P_{1/2}(i) \) is the half-saturation power, and \( h_i \) is the “inhomogeneity parameter” [39,44]. A power saturation curve of the signal amplitude was plotted as log signal amplitude divided by square root microwave power versus square root microwave power.

Fig. 1. SDS-PAGE analyses of purified complex I from the wild-type and the ΔNuoL variant, and their proton translocation activities after reconstitution into proteoliposomes. (A) 1D Tricine SDS-PAGE stained with Coomassie Brilliant Blue (left) and silver (right). (B) Immunoblotting with anti-NuoL and anti-NuoM antibodies. (C) Two dimensional SDS analysis. (D) Generation of a proton gradient monitored by the quench of the ACMA fluorescence. The NADH:DQ and NADH:ferricyanide activities were 12.23 and 58.58, 5.15 and 137.63 μmol/min/mg for the WT and ΔNuoL preparations, respectively. The data were normalized based on the complex I concentrations of the wild-type (MW 537 kDa) and the ΔNuoL variant (MW 417 kDa, lacking NuoL and NuoM) and the complex I orientation factor in the proteoliposomes (79% for the wild-type; 62% for the ΔNuoL variant).
As shown in Fig. 2A1, A2, A3 and A4, broad isotropic SQ species were present in both the wild-type and ΔNuoL complex I in their as-isolated form, respectively. As microwave power was increased, the peak-to-peak linewidth (ΔHpp) also increased without changing the center g values. However, after purified complex I was reconstituted into proteoliposomes, three distinct SQ species were detected in the wild-type (Fig. 2B1 and B2): fast-relaxing SQ signals with P1/2 (half-saturation power level) = ~50 mW, which is equivalent to SQNs reported in bovine heart complex I; slow-relaxing SQ signals with P1/2 = ~2 mW, which is equivalent to SQns in bovine heart complex I; and the reconstituted complex I at 150 K (A), the reconstituted complex I at 150 K (B) and 40 K (C). Panels A1, B1, and C1, or A2, B2, and C2 are EPR data for the wild-type or the ΔNuoL variant, respectively. (A) The purified complex I (the wild-type, 8.28 mg/ml; the ΔNuoL variant, 5.0 mg/ml) were anaerobically reduced with 6 mM NADH. The EPR data was analyzed as a three component system. "Data" and "Reconstituted" represent "actual EPR data" and "combined data of three resolved components after the fitting analysis", respectively. The parameters obtained for the wild-type complex I are SQN1 (SQ species 1), C = 0.335; P1/2 = 0.017; b = 1.452. The parameters for the ΔNuoL variant are SQN1: C = 0.662; P1/2 = 5.609; b = 1.211; SQN2: C = 0.261; P1/2 = 0.292; b = 1; SQN3: C = 0.667; P1/2 = 0.006; b = 1 (B). The reconstituted proteoliposomes were incubated on ice with 400 μM SQ transferred into EPR tubes, and brought into an anaerobic chamber. The samples were anaerobically frozen at 10 s after the addition of NADH to a concentration of 2 mM. The parameters obtained for the wild-type are SQN1: C = 0.0426; P1/2 = 50; b = 2; SQN2; C = 0.2118; P1/2 = 1.624; b = 1.918; SQN3; C = 0.8782; P1/2 = 0.1; b = 2. The parameters for the ΔNuoL variant are SQN1: C = 0.0010; P1/2 = 50; b = 2; SQN2; C = 0.0000; P1/2 = 2.0; b = 1.431; SQN3; C = 1.0163; P1/2 = 0.1; b = 1. (C) The same proteoliposome samples described in (B) were used. The parameters for the wild-type are SQN1: C = 0.2213; P1/2 = 2.307; b = 2; SQN2; C = 1.242; P1/2 = 0.078; b = 2; SQN3; C = 2.0012; P1/2 = 0.006; b = 2. The parameters for the ΔNuoL variant are SQN1: C = 0.2135; P1/2 = 1.405; b = 2; SQN2; C = 0.001; P1/2 = 0.229; b = 1.594; SQN3; C = 2.4572; P1/2 = 0.017; b = 1.452. The g signal of cluster N1a signal, which partially overlaps with the SQ signals at high microwave powers, was subtracted using the corresponding data obtained from their counter samples that were anaerobically reduced with 20 mM dithionite. Other EPR conditions were: microwave frequency, 9.45 GHz; modulation frequency, 100 kHz; modulation amplitude, 6 G; time constant, 82 ms. The concentrations and the specific activities of the proteoliposome samples are, wild-type (WT)-proteoliposomes (PL): [1.8 mg/ml], NADH-DQ activity = 7.96 μmol/min/mg, NADH:ferricyanide activities = 78 μmol/min/mg; ΔNuoL-PL: [1.0 mg/ml], NADH-DQ activity = 2.38 μmol/min/mg, NADH:ferricyanide activities = 80.4 μmol/min/mg. The data shown in the figure are the representative data from three different samples for WT, WT-PL, and ΔNuoL-PL and two samples for ΔNuoL.
very slow-relaxing SQ signals with $P_{1/2} = -0.1$ mW, which is possibly equivalent to $SQ_{\text{N}2}$ that was originally reported, but later dismissed as a non-intrinsic complex I component [45]. Therefore, to avoid any possible confusion, in this study, the very slow-relaxing SQ signals were newly designated as $SQ_{\text{N}2}$ signals. To our surprise, only two SQ species $SQ_{\text{f}}$ and $SQ_{\text{N}2}$ were detected in the $\Delta$NuoL variant (Fig. 2B$_2$ and B$_3$). While the signal amplitudes of the $SQ_{\text{f}}$ species in $\Delta$NuoL decreased to 3.7% of the total signal at 51 mW compared to that in the wild-type (51.9%), the $SQ_{\text{N}2}$ signal was virtually absent in $\Delta$NuoL (only 0.000003% of the total signal at 1 mW), as shown in Fig. 2B$_4$. The data clearly suggest that protein-bound SQ signals are extremely sensitive to protein conformation and that reconstitution into proteoliposomes, which are likely to provide an environment closer to physiological membrane states, is necessary to characterize SQ signals involved in the coupling mechanism. To confirm these power saturation profiles of SQ signals at 150 K, we also analyzed the EPR data obtained at 40 K (Fig. 2C$_1$ and C$_2$). We expected that lower temperatures would slow down relaxation rates of SQ signals, and indeed, the $P_{1/2}$ values for all three SQ signals were drastically decreased (Fig. 2C$_3$ and C$_4$).

Lowering temperatures from 150 K to 40 K did not change the microwave dependence of each SQ species except shifting to lower microwave powers by about one tenth, as seen in Fig. 2B$_3$ and C$_3$ for the microwave dependence of each SQ species except shifting to lower $P_{1/2}$ values for all three SQ signals were drastically decreased (Fig. 2C$_3$ and C$_4$).

3.3. Effect of gramicidin D and squamotacin on SQ species

To characterize SQ species, first, the effects of the uncoupler gramicidin D and the potent $E. coli$ complex I inhibitor squamotacin on these three SQ signals were investigated. The fast-relaxing $SQ_{\text{f}}$ signals completely disappeared in the presence of gramicidin D and in the presence of squamotacin (Fig. 3A). The $SQ_{\text{N}2}$ signals were almost insensitive to gramicidin D, but they were sensitive to squamotacin and the $SQ_{\text{N}2}$ signal intensity decreased to less than 30% of the control intensity (Fig. 3A). The $SQ_{\text{N}2}$ signals were insensitive to both gramicidin D and squamotacin (Fig. 3A). These results strongly suggest that the three SQ species distinguished by their relaxation rates indeed have
different biochemical properties and are probably localized in different sites in complex I. When substrate NADPH was used, almost no SQNf signals (less than 1% of the control intensity with NADH) were observed. However, the signal amplitude of SQNs and SQNvs increased by 68% and 16%, respectively. Compared to experiments using NADH, SQNs became less sensitive to squamotacin, while SQNvs increased. The SQNf signals in ΔNuol were also not observed in the presence of gramicidin D, squamotacin, or with NADPH (Fig. 3B). Interestingly, the SQNvs signals in ΔNuol were partially reduced by gramicidin and squamotacin.

3.4. Temperature-dependence of SQ species

In order to investigate the interaction of SQ signals in the wild-type complex I with neighboring paramagnetic species [23], we plotted SQ signal amplitudes as a reciprocal function of temperature in the range from 4 K to 100 K (data not shown). SQ signals detected at 0.01 mW, which contain mostly SQNs and SQNvs, were inversely proportional to temperature (following the Curie law), indicating that SQNs and SQNvs components are magnetically isolated from the environment and that their interaction with paramagnetic centers (cluster N2 in this case) is very weak. In contrast, SQ signals detected at 10 mW, which contain all three semiquinone signals, showed weak but some deviation from temperature dependence at very low temperatures (<10 K). Once SQNf signals were abolished by the addition of the uncoupler gramicidin D, this feature almost disappeared in the remaining signals. This suggests a weak magnetic interaction between SQNf and cluster N2. However, we did not detect splitting signals due to the magnetic (exchange and dipolar) interactions between SQNf and cluster N2, which have previously been reported in tightly coupled bovine SMP [27].

3.5. Simulated EPR spectra of three isolated SQ species

We isolated and simulated individual EPR spectra for these three distinct SQ species observed in the wild-type (Fig. 4). Because at low microwave powers, the slowest SQNvs can predominantly be detected, we first chose the EPR data measured at 0.08 mW from the samples treated with squamotacin containing almost no SQNf as a representative EPR spectrum for the SQNf species (Fig. 4C). The principal g values were \( g_x = 2.0061, g_y = 2.0061, \) and \( g_z = 2.0051 \). Then, using these parameters, we obtained a representative EPR spectrum for the SQNv species (Fig. 4B) by subtracting the contribution of the SQNf signals based on 2D-power saturation analysis from the EPR data taken from the samples treated with gramicidin D, which contain no SQNf at 3 mW. The principal g values for SQNv were \( g_x = 2.0065, g_y = 2.0065, \) and \( g_z = 2.0049 \). Then, the EPR spectrum of the fast-relaxing SQNf species was isolated (Fig. 4A) by subtracting the contribution of both SQNs and SQNvs, from the EPR data measured at 20 mW. The peak-to-peak linewidths (\( \Delta H_{pp} \)) for SQNf, SQNs, and SQNvs were 12.8 G, 10 G, and 7.5 G, respectively. Our data are consistent with the characteristics of bovine counterparts for which SQNf has a wider linewidth (8.4 G) [27] than SQNs (7.0 G) [45]. Interestingly, in addition to the difference in their linewidths, the spectral line shape was also very different among those SQ species. The Gaussian and Lorentzian broadening ratios for SQNf, SQNs, and SQNvs were 1 to 0, 3 to 1, and 0 to 1, respectively. The linewidths are shown in gauss.

Fig. 4A shows the pH dependency of these three SQ signals in the wild-type. As pH was raised above 7, the SQNf signal intensity decreased.

**Fig. 4.** Three distinct SQ species in the wild-type complex I resolved by power saturation and simulation analyses. The SQNf spectrum was obtained by subtracting 35% of SQNf, and 15% of SQNvs, from the control EPR data (reduced with NADH in the presence of DQ) at 20 mW. The SQNv spectrum was obtained by subtracting 70% of SQNvs, from the EPR data reduced with NADH in the presence of DQ and gramicidin D at 3 mW. The SQNf spectrum was obtained from the EPR data reduced with NADH in the presence of DQ and squamotacin at 0.08 mW. The EPR conditions were the same as described in Fig. 2 except the data were accumulated 10 times. Simulated spectra are shown as dotted lines. The g-tensor principal values are: SQNf, \( g_x = 2.0046, g_y = 2.0046, \) and \( g_z = 2.0049 \); SQNs, \( g_x = 2.0049, g_y = 2.0065, \) and \( g_z = 2.0065 \); SQNvs, \( g_x = 2.0051, g_y = 2.0061, \) and \( g_z = 2.0061 \). The ratios of Gaussian to Lorentzian broadenings are 1 to 0, 3 to 1, and 0 to 1 for SQNf, SQNs, and SQNvs, respectively. The linewidths are shown in gauss.
respective. Proton pumping activities were mean ± SD (μmol/min/mg, NADH:ferricyanide = 49.01, 40.87, 39.73, and 27.67 μmol/min/mg, NADH:DQ = 13.94, 9.16, 8.12, and 0.64 μmol/min/mg and NADH:ferricyanide = 78 μmol/min/mg. We measured three different sets of samples at 0 and 10 s, two sets of samples at 0, 5, and 10 s, and two sets of samples at 0, 5, 10, and 60 s.

The SQNf signal intensity at pH 8, for example, decreased to one tenth of the amplitude at pH 7. In stark contrast, the SQns and SQNvs signal intensities significantly increased as pH increased. The pH dependency of the SQns signals correlated with the proton-pumping activities at pH 7 and above (Fig. 6B) and with NADH:DQ activities (data not shown). We observed much lower proton pumping activities at pH 6, although the SQNf signal intensity was nearly as high as that observed at pH 7. This pH dependence profile of SQNf was similar to that of SQNf observed in bovine SMP [27].

3.8. Deuterium effect on SQ species

It is known that protonation increases spectral linewidth because of an asymmetric perturbation of the spin density on the quinone ring [31, 48]. According to previous literatures, the linewidths of neutral ubisemiquinones are always much larger (~12 G) than those of the corresponding anion radicals (7–9 G). Therefore, the wider linewidth (12.8 G) of the SQNf signal suggests that the SQNf species could be in a neutral form (QH•). To investigate this possibility, we prepared proteoliposomes in a deuterated buffer. As expected based on previous studies [49–51], the peak-to-peak linewidth decreased to 10.2 G (Fig. 7A), indicating that SQNf could be a neutral semiquinone radical. No difference was observed in the linewidths of SQNs and SQNvs (data not shown), relaxation profiles of three SQ species (Fig. 7B), or the gramicidin D responsiveness of three SQ species (data not shown). However, the NADH:DQ activity and initial proton pumping rate in the deuterated complex I proteoliposomes were greatly reduced to ~50% and ~40% of the control, respectively (data not shown).

4. Discussion

In this study, for the first time, we successfully detected and characterized the distinct molecular properties of three SQ signals resolved by their different spin-relaxation behaviors in purified E. coli complex I reconstituted in proteoliposomes. Two of them, SQNs and SQNvs, are equivalent to the SQ species that have been observed in bovine heart SMP. We confirmed previously described, important features like the presence of SQNf is dependent on the membrane potential and its complete disappearance with the addition of uncouplers, while SQNs is insensitive to uncouplers [23,27]. The differences in their sensitivities to the potent E. coli complex I inhibitor squamotacin is also very similar to that observed with rotenone in bovine SMP [52]. In this study, we further revealed new details regarding SQNs and SQNvs.

One of our most important findings is that there was no SQNf signal in the ΔNuoL mutant, while the SQNf signal was still detectable. This ΔNuoL mutant showed only ~10% of the control’s proton pumping activities, although the electron transfer activity (NADH-DQ) was ~40% of the wild-type. This suggests a lower proton pumping ratio in this variant. Although SQNs was previously suggested to be remotely located from cluster N2 (estimated > 30 Å) [23], the location of SQNs is unknown. Plus, it is not clear why the ΔNuoL variant, which contains up to NuoN that extends 100 Å away from the primary catalytic site, contains only one bound quinone, while the wild-type complex I contains two bound quinones per complex I. Further studies are required to elucidate how the loss of the secondary bound Q is related to the disappearance of SQNs in ΔNuoL. At least, it is reasonable to conclude that SQNs plays a critical role for the proton pumping mechanism of complex I.

Our other important finding is that the SQNf species in E. coli complex I is seemingly protonated, in contrast to the SQNf species in bovine heart complex I, which was found to be anionic [27]. The highly conserved Tyr84 in the NuoD subunit of complex I is only ~7 Å away from cluster N2, and it faces the quinone binding site based on the crystal structures of Thermus thermophilus complex I [9,53]. Mutational analyses of the corresponding residue Tyr144 in Yarrowia lipolytica revealed that this residue is essential for complex I activities in both electron and proton transfer [54]. Therefore, it is likely that this Tyr84 residue (and/or possibly Glu83) could be a proton donor for the enzyme’s SQNf.

SQNf is the direct electron acceptor from cluster N2 with an estimated distance of 12 Å [27], which was calculated based on a strong magnetic interaction between cluster N2 and SQNf observed in bovine SMP. Our results including the extremely high sensitivities to uncouplers and inhibitors, and the pH dependency of the SQNf signals
correlating with the proton-pumping activities of complex I, support the possibility that the SQNf is directly involved in proton pumping activities. According to the recent crystal structures of the entire T. thermophila complex I with the quinone analogues piericidin A (a complex I inhibitor) and DQ, "the quinone-reaction chamber" (equivalent to the SQNf binding site) is unusually long, narrow and enclosed [9]. The 100% Gaussian broadening feature (Fig. 4A) also supports the conclusion that SQNf is located deeply in a dense environment interacting with amino acid side chains such that it could trigger conformational changes in proton transfer subunits.

Based on several features of SQNs such as the 75% Gaussian broadening features (Fig. 4C) which suggests that SQNs is largely surrounded by protein environment, complex I inhibitor sensitiveness, the generation of SQNvs by NADPH, and the absence of SQNs signals in ΔNuoL, it is likely that SQNs, could be linked to the catalytic site through an unknown mechanism.

Regarding SQNvs, its insensitivity to squamotacin and the 100% Lorentzian broadening features (Fig. 4C) suggest that it is in a free environment likely very close to the Q pool. But SQNvs is still a legitimate complex I-associated SQ species, since it appears after the addition of NADH and disappears faster than SQNs as NADH is consumed. The role of SQNvs in the complex I catalytic mechanism is totally unknown at this moment, however, it was reported in a study of steady state kinetics of SQ binding sites are probably involved in this complex I during turnover. Our present results strongly suggest that both SQNf and SQNs are involved in the energy coupling mechanism of complex I.

Conflict of interest

The authors declare no conflict of interest.

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