Thermodynamic and EPR studies of slowly relaxing ubisemiquinone species in the isolated bovine heart complex I

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Abstract **Previously, we investigated ubisemiquinone (SQ) EPR spectra associated with NADH-ubiquinone oxidoreductase (complex I) in the tightly coupled bovine heart submitochondrial particles (SMP). Based upon their widely differing spin relaxation rate, we distinguished SQ spectra arising from three distinct SQ species, namely SQ_{Nf} (fast), SQ_{Ns} (slow), and SQ_{Nx} (very slow). The SQ_{Nf} signal was observed only in the presence of the proton electrochemical gradient ($\Delta\mu_{H^+}$), while SQ_{Ns} and SQ_{Nx} species did not require the presence of $\Delta\mu_{H^+}$. We have now succeeded in characterizing the redox and EPR properties of SQ species in the isolated bovine heart complex I. The potentiometric redox titration of the $g_{z,y,x} = 2.00$ semiquinone signal gave the redox midpoint potential (E_m) at pH 7.8 for the first electron transfer step [E_{m1}(Q/SQ)] of −45 mV and the second step [E_{m2}(SQ/QH$_2$)] of −63 mV. It can also be expressed as [E_m(Q/QH$_2$)] of −54 mV for the overall two electron transfer with a stability constant (K_{stab}) of the SQ form as 2.0. These characteristics revealed the existence of a thermodynamically stable intermediate redox state, which allows this protein-associated quinone to function as a converter between $n = 1$ and $n = 2$ electron transfer steps. The EPR spectrum of the SQ species in complex I exhibits a Gaussian-type spectrum with the peak-to-peak line width of ~6.1 G at the sample temperature of 173 K. This indicates that the SQ species is in an anionic $Q^{\cdot-}$ state in the physiological pH range. The spin relaxation rate of the SQ species in isolated complex I is much slower than the SQ counterparts in the complex I in situ in SMP. We tentatively assigned slow relaxing anionic SQ species as SQ_{Ns}, based on the monophasic power saturation profile and several fold increase of its spin relaxation rate in the presence of reduced cluster N2. The current study also suggests that the very slowly relaxing SQ_{Nx} species may not be an intrinsic complex I component. The functional role of SQ_{Ns} is further discussed in connection with the SQ_{Nf} species defined in SMP in situ.**

Keywords: NADH:ubiquinone oxidoreductase; Ubisemiquinone; EPR spectrum; E_m

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Abbreviations: Complex I, NADH-ubiquinone oxidoreductase; Complex II, succinate-ubiquinone oxidoreductase; Complex III, ubiquinol-cytochrome *c* oxidoreductase; FMN, flavin mononucleotide; Q, ubiquinone; SQ, ubisemiquinone; EPR, electron paramagnetic resonance; $\Delta\mu_{H^+}$, electrochemical proton gradient; E_m, midpoint redox potential; SMP, submitochondrial particle(s)

1. Introduction

NADH: ubiquinone (Q)[1] oxidoreductase (complex I) is located at the entry point of the aerobic respiratory chain of mitochondria and catalyzes the electron transfer from NADH to ubiquinone 10 (Q_{10}) via a non-covalently bound flavin mononucleotide (FMN), a series of iron–sulfur clusters, and at least 2 protein-associated ubiquinone species [1–3]. Electron transfer in complex I is coupled to transmembrane proton transfer, producing a $\Delta\mu_{H^+}$ across the mitochondrial inner membrane which is utilized for ATP synthesis [4–6]. Although it is still controversial, the consensus value for proton to electron stoichiometry in complex I has been reported to be $H^+/2e^- = 4$ [7–12]. Complex I is composed of two large structural parts; a hydrophilic segment that extends into the mitochondrial matrix, called the promontory domain, and the membrane-spanning domain. The promontory domain contains FMN and 8 iron–sulfur clusters [3,13,14]. The membrane domain contains 7 transmembrane subunits, i.e., ND1 to ND6 and ND4L, encoded by the mitochondrial genome. Perhaps 3 membrane subunits are associated with protein-bound Q species and interact with mobile Q from the Q pool within the phospholipid phase of the mitochondrial inner membrane [15–21]. Iron–sulfur cluster N2, which has the highest E_m in complex I and is also pH dependent [22,23], resides close to the interface between the promontory and membrane domains and, thus, is considered to be the direct electron donor to protein-associated ubiquinone species [3]. Although the H^+ transport mechanism of complex I is still not known, several models implicate the involvement of quinone [24–26]. These models are based in part on the direct involvement of the quinone species in the transmembrane proton transfer reaction. Other energy coupling systems involving quinones are known, for example, the Q cycle mechanism seen in the complex III [27–29], in *E. coli* quinol oxidase [30], and in bacterial photosynthetic reaction centers [31].

Using tightly coupled bovine heart submitochondrial particles (SMP), we previously resolved EPR spectra from three distinct SQ species in complex I in situ by quickly freezing the steady state NADH-Q_1 reductase reaction or the succinate-NAD^+ reverse electron transfer reaction [32–35]. These SQ radicals were distinguished from each other as SQ_{Nf} (fast), SQ_{Ns} (slow), and SQ_{Nx} (very slow) species because their spin-relaxation properties differ considerably. The SQ_{Nf} species was found to be highly sensitive to the $\Delta\mu_{H^+}$ poised

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across the mitochondrial inner membrane, while the slowly relaxing SQ species were insensitive to $\Delta\mu_{H^+}$. We further analyzed the EPR spectrum of the SQ_{Nf} species in SMP and the detailed temperature profile of difference spectra (coupled minus uncoupled), revealing a direct spin–spin interaction [1] between SQ_{Nf} and cluster N2, which is predominantly exchange coupled with a center to center distance of 12 Å. Other physicochemical properties of the SQ_{Nf} species remain to be determined, such as its E_m value, stability constant (K_{stab}), and the Q binding residues, along with the topographical location within the membrane in order to understand the proton/electron transfer mechanism of complex I.

Here, as a first step to understand the role of these semiquinone species, we have focused on the slowly relaxing SQ species examined using bovine heart complex I isolated with detergents. The slowly relaxing SQ signals can be detected in the absence of $\Delta\mu_{H^+}$ and, thus, in the isolated complex I system, there are no EPR spectral interference from overlapping SQ_{Nf} signals. Furthermore, there is much less interference from SQ signals arising from other respiratory enzyme complexes, such as SQ_S in complex II or SQ_i in complex III [29,36,37] than in an SMP system in situ. The SQ species detected in isolated complex I was found to be a single species with slow spin relaxation. In the current study, both potentiometrically poised and substrate (NADH)-reduced complex I systems were used in combination with complex I specific inhibitors. Presently, the SQ signals have been characterized only in the bovine heart complex I, but not yet in complex I isolated from bacterial [38,39] or fungal membranes.

2. Materials and methods

2.1. Isolation of complex I from bovine heart mitochondria

Bovine heart complex I was isolated according to the original methods of Hatefi [40] and Ragan [41] with some modifications as described in [42]. Complexes I–III were solubilized away from complex IV (cytochrome *c* oxidase) by selective solubilization using deoxycholate and KCl and selectively precipitated by removal of salts and detergent by dialysis. Complexes I–III were further purified by a series of ammonium acetate fractionations in the presence of deoxycholate. Complexes I and III were split by ammonium sulfate fractionation in the presence of sodium cholate. This preparation of complex I contains only complexes III and IV as contaminants. We determined the content of these three complexes by measuring FMN, cytochromes b_L+b_H and a + a_3 contents. Then, the molar ratios of these three complexes were estimated based on the [FMN], [b_L+b_H]/2, [a + a_3]/2 concentrations. Usually, complex IV contamination is very low. Repeated ammonium sulfate fractionation in the presence of sodium cholate will remove complex III. However, the repeated fractionation will also remove phospholipids, SQ signals and inhibitor sensitive ubiquinone reductase activity. We chose not to completely remove complex III. In order to exclude the interfering effects of SQ_i signal from complex III, we pretreated our complex I preparation with antimycin A. There is no contamination by complex II in our preparations, as determined by the absence of the EPR signals from [3Fe–4S] cluster S3. Our cholate-extracted complex I preparation used in the current work contained 0.93 nmol FMN per mg protein and had a molar ratio of [C.I.]/[C.III.]/[C.IV.] as 1.0/0.24/0.09. The quinone content was 3.3 bound Q molecules per complex I, 306 nmol phospholipids per mg protein, and its NADH: decyl-ubiquinone oxidoreductase activity with a turnover number of 1500 per FMN while retaining 99% sensitivity to the complex I inhibitors, such as piericidin A and rotenone.

[1] Yano, T., Dunham, W.R., and Ohnishi, T., Biochemistry, in press.

2.2. Generation of ubisemiquinone radicals in complex I

Potentiometric redox titration of complex I was conducted anaerobically as described by Dutton [43]. Detailed experimental method is provided in the legend of Fig. 1. Potentiometric titration of the intrinsic SQ free-radical signals in the presence of added redox mediator dyes (including Q-analogues) requires careful discrimination of the EPR signals from the intrinsic complex I-associated semiquinone species from that of the added redox dyes. Initially, we selected $n = 2$ redox dyes of appropriate E_m values [44] for the titrations. This combination of redox dyes was monitored for SQ signal contribution by titrating with varying concentrations of all dyes in the absence of complex I (with added bovine serum albumin at 1 mg/ml) or relative to fixed concentrations of complex I. We concluded that the dye contribution to the SQ signal was negligible. However, further precautions were taken. Experiments were designed in such a way that the intensity of target SQ signals was significantly higher than signals arising from the added mediator dyes. Combining all of these control titrations allowed us to determine the thermodynamic parameters of protein-associated forms of the intrinsic semiquinone species as reported here with confidence. We observed that the spin relaxation of the intrinsic free radical signals was enhanced by the neighboring fast relaxing redox components in SMP. This strongly suggested that the SQ signals arose from intrinsic SQ in complex I. On the other hand, the spin relaxation of the redox dye signals is usually not enhanced and, furthermore, these signals diminish by power saturation with a proper use of high microwave power, as reported in the case of FMN [45], SQ_S [36], and SQ_i [37]. We had to carefully work out experimental conditions for the potentiometric titration of free radicals which do not have some specific characters, such as hyperfine structure or enhanced spin-relaxations.

We have also examined the properties of SQ free radical signals which were generated by the treatment of complex I with an appropriate concentration of NADH (0.15 mM) under aerobic condition at room temperature. Complex I (19.4 μM FMN; the same batch of complex I used for potentiometric titration presented in Fig. 1) in 0.35 ml aliquots (with 50 mM Bis-tris-propane buffer and 10% glycerol) was placed in small Eppendorf tubes and incubated at room temperature for 8 min. This amount of NADH is equivalent to about 70% of all intrinsic components of complex I or at an NADH concentration not quite sufficient to fully reduce all endogenous redox centers in complex I in the absence of added redox mediator dyes [46,47]. The rise in the SQ signal was slow and required 7–10 min to reach the maximum signal state. This SQ EPR signal was stable for over 30 min at room temperature.

2.3. EPR measurements

EPR spectra for iron–sulfur clusters were recorded on a Bruker ESP 300E spectrometer, using an Oxford Instrument ESR-9 helium flow cryostat, except at temperature 173 K, where a Bruker E580 EPR spectrometer was used for SQ analyses, using a nitrogen flow system. Bruker standard TE102 rectangular cavities were used with both spectrometers.

2.4. EPR characterization of ubisemiquinone in cholate-isolated complex I

Complex I as isolated in the presence of detergents cannot generate a proton electrochemical potential ($\Delta\mu_{H^+}$), because no vesicles are present to support the proton gradient. Taking advantage of the fact that the SQ_{Nf} species requires a $\Delta\mu_{H^+}$, we used the isolated complex I system for thermodynamic and EPR characterization of uncoupler-insensitive, slowly relaxing SQ species. Spin relaxation properties of SQ species are extremely sensitive to their molecular environment and the spin relaxation rate of the SQ spins detected in the isolated complex I is much slower than what we typically observe in SMP in situ. The SQ species in isolated complex I are grossly power-saturated at 40 K, and as such, we used liquid nitrogen flow system at temperatures between −120 and −100 °C (153–173 K) for detection and measurement. We have temporarily designated this SQ species as SQ_{slow}, because individual SQ_{Ns} or SQ_{Nx} are defined with $P_{1/2}$ values of 1–10 mW and ~0.1 mW at 40 K, in situ, respectively [34,35]. We cannot assign SQ species detected in isolated complex I directly to either SQ_{Ns} or SQ_{Nx}, based on the $P_{1/2}$ values defined at 40 K, because SQ species observed in the isolated complex I shows spin-relaxation properties