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Tightly-bound ubiquinone in the *Escherichia coli* respiratory Complex I

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ABSTRACT

NADH:ubiquinone oxidoreductase (Complex I), the electron input enzyme in the respiratory chain of mitochondria and many bacteria, couples electron transport to proton translocation across the membrane. Complex I is a primary proton pump; although its proton translocation mechanism is yet to be known, it is considered radically different from any other mechanism known for redox-driven proton pumps: no redox centers have been found in its membrane domain where the proton translocation takes place. Here we studied the properties and the catalytic role of the enzyme-bound ubiquinone in the solubilized, purified Complex I from *Escherichia coli*. The ubiquinone content in the enzyme preparations was 1.3 ± 0.1 per bound FMN residue. Rapid mixing of Complex I with NADH, traced optically, demonstrated that both reduction and re-oxidation kinetics of ubiquinone coincide with the respective kinetics of the majority of Fe–S clusters, indicating kinetic competence of the detected ubiquinone. Optical spectroelectrochemical redox titration of Complex I followed at 270–280 nm, where the redox changes of ubiquinone contribute, did not reveal any transition within the redox potential range typical for the membrane pool, or loosely bound ubiquinone (ca. +50–+100 mV vs. NHE, pH 6.8). The transition is likely to take place at much lower potentials ($E_m \leq -200$ mV). Such perturbed redox properties of ubiquinone indicate that it is tightly bound to the enzyme's hydrophobic core. The possibility of two ubiquinone-binding sites in Complex I is discussed.

1. Introduction

NADH:ubiquinone oxidoreductase (Complex I) couples the electron transfer from NADH to ubiquinone (UQ) to proton translocation across the membrane. Since no redox centers have been found in the enzyme's membrane domain, the coupling mechanism is considered to be radically distinct from the mechanisms known for other redox-driven proton pumps. Complex I contains a low-affinity UQ binding site involved in the exchange of both UQ and ubiquinol (UQH_2) with the membrane pool. The loosely-bound UQ can be lost during the enzyme isolation as it happens in other UQ-reducing cites e.g. in bacterial photosynthetic reaction centers [1,2], cytochrome bc_1 [3], and cytochrome *bd* [4]. Mutagenesis [5–7] and recent structural studies [8–11] proposed a plausible location for a UQ binding site at the interface between the membrane and the hydrophilic domain of Complex I. The site is located in a cleft of the hydrophilic domain facing the membrane surface but at a significant distance from it. Such a location is quite unique for quinone-binding enzymes: in most of them, quinone headgroup is bound within the membrane dielectric with the quinone ring close to the surface (see e.g. [12,13]). In Complex I, a chain of FeS clusters forms a wire providing rapid electron transport from the FMN bound to the hydrophilic domain to UQ [14]. N2 is the last cluster in the chain, adjacent to UQ, and situated in the hydrophilic domain at a distance of 20–30 Å from the membrane surface [8,11]. Efficient electron transfer between N2 and UQ can only be achieved if the distance between them does not exceed 14–15 Å. To ensure such a distance, the UQ ring should move out from the membrane domain at ~10 Å, leaving at least one of the hydrophobic isoprenoid tail segments exposed to the rather hydrophilic protein milieu between the membrane and hydrophilic domains. If one considered the movement of the same UQ molecule between a membrane-exposed, "exchangeable" site and an N2-proximal, "electron-transfer" site, binding of the hydrophobic tail would present an energetic problem. Brandt and co-authors [5–7] suggested that a local hydrophobic zone in the interface region facilitates binding of the tail at the N2-proximal site. Although the structure of the hydrophilic and hydrophobic domains of Complex I has been resolved at resolutions of 3.1 and 3.0 Å, respectively [9,15], the structure of the interface region formed by the loops of both membrane, Nuo A, H, J, and K, (Nqo 7, 8, 10 and 11) and hydrophilic, Nuo B, and CD (Nqo 4, 5 and 6) subunits remains unclear [11]. However, such hydrophobic segments filling the interface cannot be predicted from the primary amino acid sequences.

Abbreviations: BTP, 1,3-bis(tris(hydroxymethyl)methylamino)propane; DDM, n-dodecyl β-D-maltopyranoside; DQ, decylubiquinone; E_h, ambient redox potential; E_m, midpoint redox potential; HAR, hexaammineruthenium (III) chloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; NHE, normal hydrogen electrode; OTTLE, optically transparent, thin layer electrode; Q1, ubiquinone-1; TMH, transmembrane helix; UQ, ubiquinone; UQH_2, ubiquinol; WE, working electrode; τ, time constant

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The problem of the energetically unfavorable quinone movement between the N2-proximal and exchangeable sites could be overcome if Complex I contained two, rather than one, UQ molecules within the electron-transfer distance between them: one tightly bound at the N2-proximal site and never leaving it, and the other readily exchangeable with the membrane quinone pool. In this case the tail of the exchangeable UQ will always remain in the hydrophobic membrane surrounding. The existence of two UQ-binding sites has been proposed by Ohnishi and co-authors [16–19] based on their EPR study of Complex I in the natural membrane fragments, submitochondrial vesicles. Under turnover, Complex I showed two semiquinone EPR signals originating from two different semiquinone species with different spin-relaxation behavior (Q_{fast} and Q_{slow}) and distances from N2; only one of which being sensitive to transmembrane electric potential. This allowed the authors to conclude that the signals come from UQ located in two different binding sites in Complex I. However, since these signals were not quantified, and the possibility still remains that the two signals originate from two different states of the same UQ molecule, rather than from two different molecules.

The presence of an equimolar amount of UQ in detergent-solubilized, purified Complex I can itself indicate its tight binding. In the literature, the ratio between the UQ and FMN content (UQ:FMN) in isolated, purified Complex I varies between the substoichiometric value of 0.2–0.4 [20] and 1.0 [21]. Previously, we also found one molecule of UQ per protein in the purified Complex I from *Escherichia coli* [22]. Here we have further studied the physical–chemical properties of this enzyme-bound UQ, and elucidated its role in enzyme turnover.

2. Materials and methods

2.1. Bacterial growth and purification of Complex I

The *E. coli* MWC215 (Sm^R *ndh*::Cm^R) strain was grown in LB medium at 37 °C in a 25 L fermentor and harvested at the late exponential growth phase. The membranes for Complex I purification were prepared by passing the cells through the APV Gaulin homogenizer, as described in [23]. Complex I was purified by the two consecutive chromatography steps using anion exchanger DEAE-Trisacryl M (Bio-Sepra) columns and gel filtration on Superdex 200 prep grade (GE Healthcare), respectively [24].

2.2. Measurements of catalytic activity

Hexaammineruthenium (HAR) and decylubiquinone (DQ) reductase activities of membrane-bound or purified Complex I were measured by following NADH oxidation at 340 nm ($\varepsilon = 6.2\ mM^{-1}cm^{-1}$) in Buffer A containing 25 mM HEPES-BTP, pH 7.5, and 3 mM KCl at +30 °C. Substrates (100 μM DQ, 360 μM HAR and 200 μM NADH) were added, as indicated. For the measurements of the ubiquinone reductase activity of purified, solubilized Complex I, Buffer A was supplemented with 0.005% DDM and 20 nM ubiquinol oxidase bo_3.

2.3. Analytical procedures

Protein concentration was determined in the membrane-bound and the purified enzyme by the BCA protein assay reagent kit and Pierce 660 nm protein assay reagent, respectively (both from Thermo Scientific). Bovine serum albumin was used as a standard in both cases. FMN was extracted by acid [25] and its content was determined fluorimetrically on a Hitachi F-7000 spectrofluorimeter. UQ was extracted with methanol and cyclohexane as described [21] and its content was determined by the difference UV spectra between borohydride-reduced and air-oxidized states ($\varepsilon_{272} = 12.3\ mM^{-1}\ cm^{-1}$).

2.4. Stopped-flow experiments

Complex I (3 μM) in Buffer A (200 mM MOPS/KOH, pH 6.75) was rapidly mixed (dead time 3 ms) with an equal volume of Buffer a containing 8 μM NADH. Rapid mixing experiments were carried out using a stopped-flow spectrophotometer equipped with a diode array detector capable of recording spectra at a rate up to one per millisecond (Unisoku Instruments). All the experiments were conducted at room temperature.

2.5. UV–vis spectroelectrochemical redox titration

UV–vis spectroelectrochemical redox titration of Complex I was performed at 21 °C using the OTTLE cell (0.3 mm optical path length) as described in [26] with minor modifications. The working electrode (WE) potentials within the range from −450 mV to +100 mV vs. NHE were applied using a PAR263A potentiostat (Princeton Applied Research). Absolute optical absorption spectra were recorded in the spectral range of 240–650 nm using a Cary 300 spectrophotometer. At each potential step, the onset of equilibrium on WE was determined as the point where changes in the cell current were no longer significant. To accelerate the redox equilibrium between WE and the enzyme, the following redox mediators were added: hexaammineruthenium ($E_m = +50$ mV), 200 μM; pentaaminechlororuthenium ($E_m = -130$ mV), 200 μM; cobalt (III) sepulchrate ($E_m = -350$ mV), 200 μM. No optical contribution from hexaammineruthenium was detected in the studied spectral range. Small contribution of cobalt sepulchrate and pentaaminechlororuthenium was registered and subtracted from the data. Both oxidative and reductive titrations were performed without significant hysteresis. For the redox titrations, 45 μl of Complex I (40 mg/ml), solubilized in Buffer B, containing 100 mM MOPS/KOH (pH 6.75), 100 mM KCl, and mediators was degassed and saturated with 99.99% Ar gas (AGA) using a locally-built vacuum/gas line and anaerobically loaded into the OTTLE cell. The cell was also washed with anaerobic Buffer B before loading with the sample. Since the concentrated enzyme stocks before dilution contained large amount of detergent (DDM), it was not necessary to add it to Buffer B.

2.6. Data analysis

Data analysis was carried out using the MATLAB software (the Mathworks, Inc.). Decomposition of the kinetic data surfaces was achieved by global fitting run under a MATLAB interface using the Rakowsky algorithm as described (Eq. 9 and Supporting Material: http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)00696-1 in [27]).

3. Results

3.1. Properties of purified Complex I

The enzyme activity measured as the rate of NADH oxidation upon the reduction of an artificial donor (HAR) and the quinone reductase activity measured as the rate of NADH oxidation upon reduction of DQ were 120–140 and 20–25 μmol min^{-1} mg^{-1}, respectively. Quantification of FMN and quinone in the Complex I preparations provided a UQ:FMN (mol/mol) ratio slightly higher than unity (1.3 ± 0.1).

3.2. Kinetics of the reduction and re-oxidation of Complex I

A stopped-flow setup was used to study the rapid kinetics of the reduction initiated by NADH and subsequent re-oxidation of Complex I. The enzyme (~3 μM) was mixed with NADH (8 μM) at a 1:1 volume ratio. Such a low NADH concentration was used for two reasons: (*i*) The dead time of the stopped-flow setup was 3 ms, while the