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 $1.3 \pm 0.1$ 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 contributed, 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 ($E_\text{m} \leq -200$ 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 layer 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; $E_\text{m}$, ambient redox potential; $E_\text{p}$, midpoint redox potential; HARR, hexamaminerruthenium (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; $t$, time constant

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2. Materials and methods

2.1. Bacterial growth and purification of Complex I

The E. coli MCW215 (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 (ε = 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 mM ubiquinol oxidase bo3.

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 by the 660 nm protein assay reagent, respectively (both from Thermo Scientific).

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 spectropolarimetric redox titration

UV–vis spectropolarimetric 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...
Complex I turnover time is ~2 ms. The NADH concentration was taken below the apparent $K_m$ (7.2 μM, [14]) to slow down the reaction for better time resolution; (ii) The optical spectra of NADH and Complex I overlap in the UV region, so that the absorbance of NADH at high concentration made the kinetic analysis impossible. In control experiments, we mixed the same solution of NADH with the buffer and obtained an initial NADH spectrum, which was used for the data treatment. Separately, we obtained the spectrum of 8 μM NAD mixed with the buffer. The difference spectrum NADH − NAD$^+$ was subtracted from the data surface. As a background we took the spectrum obtained by the mixing of 3 μM oxidized Complex I with the buffer in a 1:1 ratio. The experimental spectra, taken every millisecond after mixing, are presented in Fig. 1A. The red curve (peak at 340 nm) shows the spectrum of NADH added at zero time. The broad negative peak (minimum at 450 nm) corresponds to the redox changes of FMN and the FeS clusters. The arrow indicates the position of the UQ band (275 nm). The reaction was followed for 50 s. The kinetic curves at 255, 450 and 275 nm are shown in Fig. 1B. Since the UV spectral band for the UQ redox difference spectrum overlaps with the band at 255 nm where spectral redox changes are opposite, the redox changes of UQ during the reaction were measured as the difference:

$$A_{UQ}^{app} = A_{255}^{fl} + (A_{255}^{fl} + A_{297}^{fl})/2$$ (1)

It is clear from the data that the reduction of Complex I occurred mostly in the dead time. Only the tail of this reaction can be observed within the first 70–100 ms. The redox state of the enzyme stayed almost unchanged between 100 and 1000 ms and then re-oxidation proceeded. Global fitting of the kinetic data surface yielded three spectral components (Fig. 2). The first component with a time constant $τ_1$ of 8.3 ms (Fig. 2A) represents the reduction of Complex I. The negative absorbance in the visible area with a broad minimum at 330–340 nm shows NADH oxidation. There could be also some contribution from the reduction of the FeS cluster(s), since the band at 300–360 nm was found in the redox spectra of some of FeS clusters [28]. The positive peak at 255 nm is of unknown origin. The UQ band looks as a shoulder at 273–278 nm between positive and negative peaks. The second component with $τ_2 = 2.1$ s (Fig. 2B) presents a clear spectrum of the redox-active band of NADH with a trough at 340 nm. During this time the redox state of the enzyme does not change and only NADH is consumed. The third, slowest component with $τ_3 = 5.7$ s (Fig. 2C) is practically a mirror reflection of the first fastest component and shows the slow re-oxidation of Complex I after exhaustion of NADH. Fig. 2D shows final and zero time spectra obtained by a global fit. The final spectrum is the sum of all redox changes during the measurements; since the enzyme was reduced and re-oxidated this spectrum derives from NADH oxidation and mainly shows the amount of consumed NADH, although small residual changes at 250–260 nm remain. The zero time spectrum presents the redox events that happen during the dead time. The zero time spectrum is very similar to the first fast components indicating that they reflect the same process of Complex I reduction and NADH oxidation. Fig. 3 shows the sum of these two spectra: it represents the events that happen during ~15 ms after mixing, from which 0.6 of the final spectra (NADH oxidized during dead time and the fast phase) is subtracted. The obtained spectrum of rapidly reduced Complex I consists of the well known redox spectrum of Complex I in the UV–vis region with the major contribution of FeS clusters and possibly FMN (see below), a negative band at 330 nm, also most probably due to FeS Clusters, a positive peak at 255 nm of unknown origin, and a trough at 276–278 nm which can be either due to reduction of FMN or UQ, or both. Both UQ and FMN have

![Fig. 1. Reaction of Complex I with NADH. (A) A set of spectra taken every 1 ms after mixing Complex I with NADH. The red curve is the spectrum of the redox-active band of 4 μM NADH present in the solution after mixing at zero time. (B) The kinetic transients after mixing, taken at 450 nm (FMN and FeS, blue line) and 255 nm (an unknown compound, green line) and at the 275 nm band ($ΔA_{297} = (ΔA_{297} + ΔA_{275})/2$, FMN and UQ, red line). The kinetics shows that the main reduction events happen during the dead time of the setup, followed by a quasi-equilibrium state and then by re-oxidation. In (A), arrow shows the position of the UQ redox difference absorption band. For conditions, see: Materials and methods.](Image)

![Fig. 2. Spectral components obtained by global fitting of the experimental kinetic data. (A) The fastest component ($τ_1 = 8.3$ ms) representing the very beginning of the reaction. (B) The $τ_2 = 2.1$ s component showing mostly the NADH consumption. (C) The slowest component ($τ_3 = 5.7$ s) representing re-oxidation of the enzyme. (D) The final spectrum (the sum of all spectral changes during the reaction) comprising mostly the consumed NADH, but also showing a contribution of the unknown 255 nm band. Zero time spectrum (dashed line in panel D) represents the spectral changes taking place in the dead-time of the setup; it is similar to the fastest component (panel A). Data are taken from Fig. 1.](Image)

![Fig. 3. Fast redox changes in Complex I. The sum of zero time spectrum and the fastest components corrected for the redox active NADH band (solid line) presents the redox events that happen in approximately 15 ms upon reduction of Complex I (1.5 μM). For comparison, the redox difference spectra of 1 μM ubiquinone-1 (Q1, dashed line) and 1 μM FMN (dotted line) are shown. Conditions: as for Fig. 1.](Image)
redox-active bands at 270–280 nm as shown in Fig. 3, and could contribute to the 275 nm trough. However, Complex I was not fully reduced upon the reaction; only 2.4 μM NADH was oxidized during the first 15 ms after mixing. This amount is not enough to reduce all redox centers of 1.5 μM Complex I, which can hypothetically accept 11 or 12 electrons per molecule (1 FMN, 8 FeS clusters and 1 UQ). FMN has the lowest midpoint redox potential among all redox centers of Complex I (−350 mV); therefore, it seems highly improbable that it significantly contributes to the redox spectrum of the fast events. Indeed, comparison of this spectrum with the redox titration data obtained in our group previously also indicates that Complex I was less than 50% reduced during the fast events and that FMN was in the oxidized state (Fig. 4, see also [26]).

The stopped-flow experiment was also performed at the same conditions but in the presence of 6 μM rolliniastatin, a specific inhibitor of Complex I, which was added to Complex I before mixing with NADH (final rolliniastatin concentration after mixing was 3 μM). We note that neither amplitude nor kinetics of the rise phase at 275 nm (Eq. (1)) is affected by the inhibitor. This result may indicate that (a) the 275 nm kinetics does not reflect the UQ reduction, which is unlikely; (b) the 275 nm kinetics shows the UQ reduction and the latter is not affected by the inhibitor (Fig. 5). Re-oxidation of FeS clusters was slightly slower in the presence of rolliniastatin and the most prominent effect of the inhibitor was a decrease of the rates of both reduction and re-oxidation of the unassigned band at 255 nm (Fig. 1B). Under the experimental conditions, the oxidation phase is slow because it is limited by the lack of added electron acceptor; the autooxidation of Complex I by oxygen is a relatively slow process, which does not necessarily directly involve UQ. In separate experiments we observed that at these concentrations of protein and rolliniastatin the ubiquinone reductase activity of Complex I was inhibited by ~90% which is in accordance with the data reported by us previously [25]. Altogether these data indicate that the inhibitor does not block the electron transport between FeS cluster N2 and the presumed, tightly-bound UQ, but does block it between the latter and the loosely-bound UQ (or block the release of tightly-bound ubiquinone if properties of quinone-binding site could be altered upon turnover (see below)).

3.3. UV–vis spectroelectrochemical redox titration

Purified Complex I was electrochemically reduced or oxidized in the OTTLE cell. In this study, the optical redox changes were followed not only in the visible region as before [26] but also in the UV. The data surface is presented in Fig. 6A. The n = 2 redox change of UQ (UQH2 − UQ) is expected to be seen in the spectral region of 270–280 nm. The analysis of redox behavior of the UQ 270–280 nm band is complicated due to (i) potential contribution of n = 2 redox changes of FMN in the same region (Fig. 3); and (ii) overlapping with the redox spectrum of unknown origin that changes in the opposite direction (Figs. 1A, 6A). However, when the absorption points were calculated by the "triangle approach" (Eq. (1)), the titration profile indicates a transition with a midpoint redox potential (E_m) of −200–−180 mV (Fig. 6B). The spectrum of this transition cannot be decomposed due to its complexity. Three FeS clusters with known E_m of −200 (N2) and −235 mV (N1a and N6b) (n = 1 transitions) but unknown optical spectrum shape and extinction coefficients contribute to the spectrum [26]. A possible contribution of FMN at 270–280 can be checked by its prominent band at 450 nm. Although the titration curve at 450 nm where both FMN and FeS clusters contribute, cannot be decomposed due to overlapping absorption from several redox centers, it shows that the majority of these centers have midpoint redox potentials lower than −270 mV (Fig. 6B) in accordance with our data on optical and EPR redox titrations [26]. We did not find a clear two-electron transition at 450 nm at redox potentials around −200 mV that would have been attributed to FMN (in fact, the latter revealed the n = 2 transition at −350 mV) [26,29]. This makes the contribution of FMN to the observed redox changes at 270–280 nm band unlikely. Spectra of DQ (1 μM) and FMN (1 μM) shown in Fig. 3 are close to those expected in Complex I (1.5 μM). Since we do not

Fig. 4. Partial reduction of Complex I upon fast events after mixing with NADH. Spectrum of the fast events (dotted line), taken from Fig. 3. Redox spectra of Complex I at selected redox potentials, as indicated (solid lines). The spectroelectrochemical data in visible region are taken from [26].

Fig. 5. Effect of rolliniastatin on optical changes of Complex I after mixing with NADH. Kinetic curves with (dotted lines) and without (solid lines) 3 μM rolliniastatin (concentration after mixing) were taken at selected wavelengths characteristic for FMN and FeS centers (450 nm), FMN and UQ (see: Eq. (1)), and an unknown band (255 nm). Conditions, as for Fig. 1.

Fig. 6. UV–vis spectroelectrochemical redox titration of Complex I. (A) Spectra taken at a range of redox potentials upon redox titration (E_m = −430 ± +100 mV). (B) Titration curve at 275 nm (data points calculated with a "triangle approach" (Eq. (1)), red circles) reflecting redox transitions of FMN and UQ, and theoretical n = 2 curve (red line, E_m = −200 mV); titration curve at 450 nm (blue circles) reflecting redox transitions of FMN and the majority of FeS clusters; titration curve at 250 nm (black circles) reflecting redox transitions of an unidentified band. In (B), the results of 3 independent titration data sets are combined.
know the size and shape of a large band of unknown nature at approx. 250 nm, with which the ubiquinone band interferes, we cannot estimate the real size of the UQ band in the UV range. Certainly the transition at 275 nm tracked as \( A_{275} = (A_{255} + A_{290})/2 \) (Eq. (1)) reflects only a fraction of the full spectral changes of UQ in the UV range, so that its full extent should be consistent with the expected, concentration-based value.

The contribution of the \( n = 1 \) transition of UQ (for example, UQ/UQH\(_2\)) to the observed UV optical spectral changes is also unlikely; in the cryo-EPR redox titration of Complex I no radical species was observed at the potentials at least as low as \(-350 \text{ mV} \) [26,29].

UQ and a fraction of FMN released from the enzyme (the latter with a higher \( E_m \approx -220 \text{ mV} \)) could both be responsible for the optical changes at 270–280 nm. However, the most important result is that no redox changes of the UQ band were observed at relatively high ambient redox potentials (+100–100 mV), where the \( E_m \) of a loosely bound, exchangeable UQ is expected. (Note that the \( E_{m,n} \) of pool UQ is +80 mV). On the other hand, all observed redox transitions in the enzyme-UQH\(_2\) occur at much lower redox potentials with the midpoint at approximately \(-200 \text{ mV} \).

4. Discussion

Under aerobic condition \( E. \ coli \) cells contain less than 10% of menaquinone in comparison to ubiquinone [30]. By unknown reason Complex I from aerobically grown \( E. \ coli \) cells is incapable to reduce menaquinone. Since we were using the bacterial cells grown at high aeration for Complex I isolation we did not consider any effect of menaquinone. The literature data vary on the amount of enzyme-bound UQ in different Complex I preparations; the UQ:FMN ratio found was to be 0.2–0.4 (yeast mitochondria, [20]) and 1 (bovine mitochondria, [21]). We found 1.3 ± 0.1 UQ molecules per FMN in our purified Complex I preparations from \( E. \ coli \). In this study we investigated the kinetic and thermodynamic properties of this UQ. We found that its reduction and re-oxidation correlates kinetically with the majority of the FeS clusters. This finding proves that the presence of UQ in the preparation is not a result of unspecific binding or contamination due to hydrophobicity of the enzyme and retained lipids; on the contrary, the bound UQ, indeed, directly participates in the Complex I turnover.

Binding of a UQ species to a binding site in the enzyme leads to a shift in its apparent midpoint redox potential with respect to the potential of the unbound (free) quinone in the membrane pool:

\[
e_{m}^{\text{bound}} \approx E_{m}^{\text{pool}} - 30 \times \log K_{D}^{UQH_{2}}/K_{D}^{UQ}
\]

where \( E_{m}^{\text{bound}} \) and \( E_{m}^{\text{pool}} \) are the midpoint redox potentials for the 2-electron reduction/oxidation of the enzyme-bound and free UQ, respectively; \( K_{D}^{UQH_{2}} \) and \( K_{D}^{UQ} \) are the dissociation constants for the enzyme-UQH\(_2\) and enzyme-UQ complexes, respectively. The ratio \( K = K_{D}^{UQH_{2}}/K_{D}^{UQ} \) indicates the directionality of the enzyme’s catalytic activity: when \( K \gg 1 \), the enzyme preferentially binds the oxidized UQ, which is optimal for a UQ reductase reaction; when \( K \ll 1 \), the enzyme preferentially binds UQH\(_2\), which is optimal for UQH\(_2\) oxidation. It has been shown that the bacterial Complex I is a nearly reversible enzyme with respect to UQ/UQH\(_2\) (see e.g. [31]); a similar situation was extensively studied with a loosely-bound quinone in e.g. bacterial reaction centers and many other weak binding sites, where \( K \) is close to unity (see e.g. [32]). A typical value for the redox midpoint potential of UQ freely dissolved in the lipid bilayer membrane or bound to the detergent micelles when the membrane enzyme is isolated (ubiquinone pool) has been established for different membrane systems and falls within the range +60++100 mV vs. NHE (pH 7, e.g. [32–35]). Noteworthy, in the redox titration experiments, we failed to observe any such transition in the UQ absorption band at 275 nm (Eq. (1)). On the other hand, in many cases a tightly bound UQ has the \( n = 2 \) redox transition at much lower potentials (see e.g. [36,37]) indicating preferential binding of the oxidized form. Such preference for the oxidized form is natural when the bound UQ species acts not as a terminal electron and proton acceptor but rather as an electron transport element, even if the proton charge compensation can occur within the time frame of UQ reduction/re-oxidation. In the present study the apparent midpoint potential of the observed transition at 275 nm characteristic for the UQ/UQH\(_2\) transition (Eq. (1)) was found to be lower than \(-200 \text{ mV} \), although its exact value could not be determined. Such a low value indeed indicates a tight and specifically bound species.

Noteworthy, for a tightly-bound UQ species there is another factor lowering its \( E_m \) rather than just preferential binding of the oxidized form: the protein surrounding can hamper the access of protons to UQ so that its reduction is not accompanied by protonation. In this case semiquinone anion, UQ\(^-\) (\( n = 1 \)), and deprotonated quinol species, UQH\(_2\) or even UQ\(^-\)-(\( n = 2 \)), can be formed at rather low redox potentials, the way it occurs in the aprotic solvents (see e.g. [38,39] and references therein). Protonation is indeed not required for a tightly-bound UQ to serve as an electron transfer element. Note however, that the lack of protonation of the UQ reduced species (i) usually requires hydrophobic protein surrounding and (ii) can only be observed transiently on short time scales (see e.g. [32,36,37]). The hydrophilic nature of the putative location of the UQ binding site in Complex I and the equilibrium conditions of the redox titration experiments make the formation of unprotonated, reduced UQ species unlikely, since the enzyme should also bind a high redox-potential UQ exchangeable with the quinone pool, there can be two explanations of the results.

(a) There is one quinone-binding site, within which UQ can occupy two positions: tightly bound and exchangeable. UQ in the “as prepared” Complex I is “frozen” in the tightly-bound state characterized by a low redox potential. The arrival of the first electron(s) should release it (e.g. by protonation) and displace to the high redox-potential, exchangeable, position. This situation can either (i) be applicable to Complex I turnover when the ubiquinone movement initiates the conformational changes that result in protons translocation or (ii) it can be a peculiarity of the resting-state enzyme and concern only the first turnover.

(b) Complex I contains two quinone binding sites. Since no other redox centers except FMN, FeS clusters and UQ are found in Complex I it is evident that UQ should be located within the electron tunneling distance from N2 cluster providing reasonable electron transfer rate (<15 Å, edge-to-edge distance). The first estimation of the latter distance was performed by EPR studies based on the spin–spin interactions between N2 and ubisemiquinone in Complex I in closed membrane vesicles from bovine mitochondria [17,40]. The values of 8–11 Å was obtained from the EPR signal splitting data for N2 [17] and 10–13 Å from the power saturation profiles of semiquinone species [40]. With another approach, extensive studies of the mitochondrial Complex I inhibitor resistance caused by point mutations in the 49 kDa and PSST subunits (see [7] for a review) led the authors to conclude that the UQ binding site is located close to the interface between these two subunits and the residue responsible for binding interaction is a conserved tyrosine (Y144 in 49 kDa, \( Yarrowia lipolytica \) enzyme) in the immediate proximity to the N2 cluster [7]; therefore, the distance between N2 and the quinone ring is ~10 Å [41]. All these results are in a good agreement; however, since the location of N2 is known, they indicate a very unusual position of UQ in the hydrophilic part of the enzyme, far away from the membrane dielectric.
Replacement of close to N2 (6–8 Å) Tyr144 which abolish Complex I activity with some quinones but retains with another [42] argues to exchangeable quinone in the vicinity of N2. However, similar effect was observed when a number of residues in 49 kDa and PSST were substituted [43]; these residues reside at the distance of 11–19 Å from N2, they are closer to the membrane and cover a significant area judging the resolved structure of hydrophilic domain of *Thermus thermophilus* (PDB ID: 3M9S) up to 20–22 Å between some of them. (Met88 (Met 127 in PSST): Phe150 (Phe207 in 49 kDa) 24 Å) what could indicate the importance of the whole structure in this area for ubiquinone reduction and does not contradict second quinone binding site.

Recent data on photoaffinity labeling of Complex I by azidoquinazoline showed that this compound binds at two subunits of mitochondrial Complex I, namely 49 kDa (NuoCD in *E. coli*) and ND1 (NuoH in *E. coli*) [44,45]. Quinazolines, strong inhibitors of Complex I and structurally similar to UQ, presumably occupy the quinone binding site(s). The N-terminus of subunit 49 kDa (Asp41-Arg63) and the loop between the 5th and 6th TMH in subunit ND1 have been determined as the location of the bound azidoquinazoline [44]; however, it does not allow to determine whether these two subunits form a common quinone binding site or there are two separate sites. Unfortunately, in the crystal structure of the entire *T. thermophilus* Complex I (PDB ID: 3M9S) the area between hydrophilic and hydrophobic domains, which should be filled with Nqo8 (NuoH in *E. coli*) and small membrane subunits loops is not resolved [11]. However, the location of the binding site on subunit Nqo4 (NuoCD in *E. coli*) can be estimated using the 3M9S structure coordinates. The residues Asp41 and Arg63 (49 kDa subunit) flanking the quinone binding site region in bovine mitochondrial Complex I correspond to Asp20 and Arg42, respectively, in subunit Nqo4 in the *T. thermophilus* enzyme. Fig. 7 shows the resolved residues, Met26-Gly31 and Gly39-Arg42 in *T. thermophilus*, correspondent to the Asp41-Arg63 region in the bovine enzyme. The residues closest to N2 (Gly39 and Leu41) are located 14–15 Å away from N2; the most remote residue (Met26) is ~36 Å away from N2, much closer to the membrane surface and probably forms a contact with the 3rd loop of subunit Nqo8.

Therefore, there is a possibility of two binding sites as shown in Fig. 7: one, in the vicinity of N2 and the other, for loose binding in the interface of hydrophilic and hydrophobic domains and how it was found in other enzymes. Loosely bound, exchangeable UQ can be easily washed out upon Complex I purification that includes intensive enzyme treatment with detergent; hence we observe only tightly bound ubiquinone with oddly low midpoint potential indicating its very specific coordination in the protein. Such rendering of the two quinone binding sites can explain the efficient electron transfer from N2 (and thus, from NADH) to the membrane-exchangeable UQ without having to propose (see: [8]) the energetically unfavorable movement of the UQ isoprenoid chain between the membrane and hydrophilic domains of Complex I. However, the probability that there could be two states of a single ubiquinone-binding site, for tight and loose binding, and observed ubiquinone behavior is a result that “as prepared” enzyme is “frozen” in one particular mode cannot be yet ruled out.

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References


Fig. 7. Hypothetical UQ-binding sites in Complex I. Shown by numbers, are the estimated distances between the FeS cluster N2 and the most proximal (Leu41 and Gly39) and the most distal (Met26) residues, respectively, resolved in the subunit Nqo4, located within the limits of the quinazoline-binding site [44]. The corresponding distances in Å are indicated. Subunits Nqo4 (ochre), Nqo6 (gray), and Nqo9 (tan), are shown. White dotted line indicates the approximate position of the membrane surface. Below the line, the TMH from the membrane subunits Nqo8, Nqo7, Nqo10, and Nqo11, are partially shown in green. Two probable ubiquinone headgroup binding groups are indicated by the red circles. Amino acid residues important for the UQ binding are also shown as licorice (see: Discussion). The figure is produced using the X-ray coordinates for the entire Complex I from *Thermus thermophilus* (PDB ID: 3M9S).