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# The mechanism of proton and electron transport in mitochondrial complex I

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## 1. Introduction

About one-third of cellular ATP is synthetized during the oxidation of intramitochondrial NADH by NADH:ubiquinone reductase (complex I [1–6]). Complex I is the most complicated redox enzyme which couples electron transport to proton translocation across biological membranes [2–8]. How proton transport is connected to electron trasport from NADH to ubiquinone (Q) is poorly understood [2,4,6–8] as compared to the established mechanism of ubiquinol oxidation by complex III [9]. Although the number of protons translocated per NADH oxidized has been reported to be two [10–12], there is now a consensus of opinion for four [3–7,13–16]. Lower values [11–13,17] may also be true because the stoichiometry of proton pumping by complex I appears to be *variable* [2,4,13], which in principle would exclude [4,7,13,14] a Mitchellian loop mechanism [10].

A novel mechanism is introduced here for the proton and electron transport of complex I that can rationalize also the variable stoichiometry. The basic concept is that complex I possesses two proton pumps connected in series, each of which is gated by a ubiquinone-binding site [18].

## 2. A dual Q-gated pump model for the mechanism of complex I

Contrary to previous mechanisms advanced for complex I [1–4,10–16,19–21], it is proposed that electron transfer through the FMN and iron-sulphur cofactors is not responsible for proton translocation (Fig. 1). The oxidoreduction of protein-bound ubiquinone at sites A and B in the membrane is considered to be the major protonmotive process. One of the iron-sulphur clusters transfers an electron to a tightly-bound ubiquinone, named A by analogy with $Q_A$ of bacterial reaction centers [22], and simultaneously a proton is moved from the negative side of the membrane to the site binding ubisemiquinone A. Here the proton is translocated to the other side of the membrane in a reaction linked to the stabilization of ubisemiquinone A. The electron is then transferred to ubiquinone coming from the pool to form another protein-stabilized ubisemiquinone, called B again for analogy with $Q_B$ of photosynthetic reaction centers [22]. Site B is considered to be wide enough for accommodating both ubiquinone and stabilized ubisemiquinone at the same time, as well as several quinone antagonists such as rotenone. The ubisemiquinone formed at site B would correspond to the rotenone-sensitive radical [19], whose level depends upon the protonmotive force [20]. We postulate that formation and stabilization of this ubisemiquinone B is coupled to the pumping of protons across the membrane by a device physically distinct from that working at site A. The transport of the second electron from NADH to Q could be coupled to proton translocation at both sites A and B as for the first electron with the formation of another ubisemiquinone in the part of site B where normally Q is bound. If ubiquinone does not already occupy this site, however, the second electron from site A will fully reduce the first ubisemiquinone B to quinol, in a reaction which does not elicit proton translocation. When quinone is present at site B, formation of the second ubisemiquinone will be coupled to proton

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; MOA, methoxyacrylate; Q, ubiquinone; UBQ, 2,3-dimethoxy-5-methyl-6-n-undecyl-*p*-benzoquinone.

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translocation. Subsequently, the two ubisemiquinones consecutively present at site B would dismutate (cf. [21]) yielding a quinone product that rebinds to the site and ubiquinol product that is transferred to an adjacent site to be released from the complex. We consider that ubiquinol is released from a site different from site B (labelled C in Fig. 1), because inhibitors such as myxothiazol and stigmatellin [23] are mutually non-exclusive with rotenone [24] but compete with ubiquinol as product inhibitors (results not shown). Proton translocation is not expected to be linked to the dismutation of the ubisemiquinones at site B, contrary to previous interpretations [2,20,21]. This proposal is sustained by the evidence that myxothiazol, stigmatellin and ubiquinol are much more efficient as inhibitors of electron transport than of proton translocation under steady-state conditions (results not shown).

## 3. Predictions and tests of the dual Q-gated pump model

The model just described, named 'dual Q-gated pump', is rather hypothetical, but leads to predictions that can be experimentally tested. For instance the maximal stoichiometry of complex I is considered to be two protons per electron provided that site B is constantly occupied by ubiquinone during the catalytic turnover. The degree of quinone occupancy of site B influences the stoichiometry, because we postulate that formation of ubisemiquinone is coupled to proton pumping whereas full reduction or dismutation of the same ubisemiquinone are not. Consequently, it is expected that the proton translocating efficiency of complex I is enhanced by increasing the concentration of exogenous ubiquinones that are hydrophobic enough to fill site B during turnover.

To test key features of our model, we have measured the proton pumping function of complex I in coupled inverted particles [25] from mammalian heart mitochondria by using the fluorescent probe 9-amino-6-chloro-2-methoxyacridine (ACMA) [26]. The particles are treated with oligomycin or venturicidin to block proton leak through the ATP-synthase complex [20], and with antimycin plus methoxy-acrylate(MOA)-stilbene (which, unlike other center 'o' inhibitors such as myxothiazol [23], does not inhibit complex I) to prevent reoxidation of ubiquinol. The undecyl-analog of ubiquinone (UBQ) has been routinely used as the quinone substrate for complex I, mainly because its reactions are fully sensitive to rotenone [27] and suffer of little interference from complex III [23]. Addition of UBQ to submitochondrial particles reduced by NADH induces rapid acidification of their internal compartment as monitored by the quenching of ACMA fluorescence (Fig. 2). The different quinone dependence of

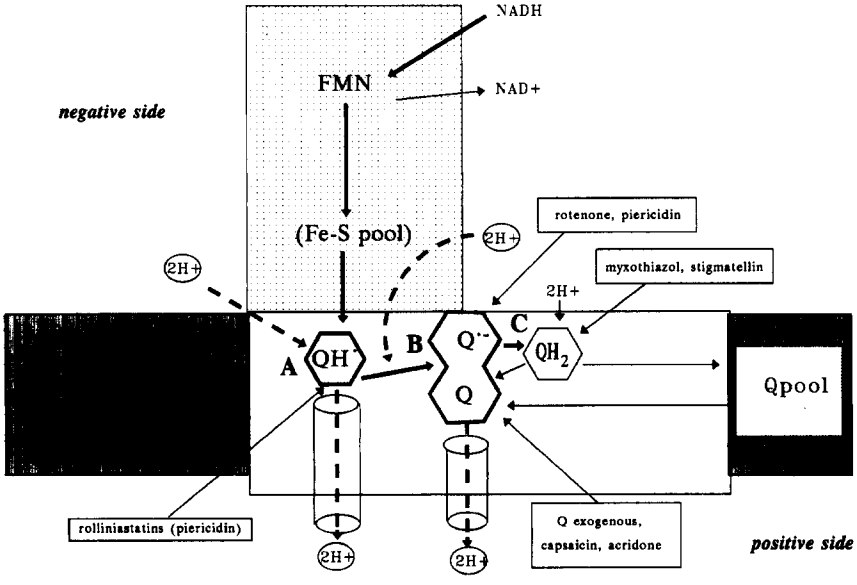


Fig. 1. The dual Q-gated pump model for the mechanism of complex I. Electron transfer from NADH to the Fe-S clusters is not specified in view of the uncertainties in the number and redox sequence of these clusters [1–4,7,8]. Each electron from the Fe-S pool flows to a tightly bound ubiquinone at site A and the formation of the semiquinone is coupled to proton pumping. Ubisemiquinone A is labelled QH because it could correspond to the pH-independent and rotenone-insensitive radical seen in complex I [19]. Exogenous quinones or endogenous Q enter site B and get an electron from semiquinone A, thus forming the ubisemiquinone anion B that corresponds to the rotenone-sensitive radical seen by EPR [19,20]. The formation and/or stabilization of ubisemiquinone B is also coupled to proton pumping across the membrane. The second electron from NADH via site A forms another semiquinone in the part of site B where Q is normally bound (Q). Subsequently, ubiquinol ($QH_2$) is formed, presumably by dismutation of the two semiquinones at site B, and released from the complex at the adjacent site C. These reactions are considered to be uncoupled from proton translocation. Inhibitors of complex I that are believed to bind to site A, B and C are shown in the boxes (see Refs. [24,27]). Thick solid arrows label electron transfer steps, whereas thin arrows indicate binding reactions.

the redox and proton pumping function of complex I (Fig. 2A) suggest that the proton/electron stoichiometry increases by increasing the UBQ level, as previously reported with ubiquinone-1 [13]. With more than 25 μM UBQ the extent and rate of fluorescence quenching of acridines (either ACMA or 9-amino acridine, wich is more accurate for large changes in pH [25]) are about 70% of those seen during oxidation of ubiquinols in the same particles (without complex III inhibitors). This indicates complex I with an adequate supply of exogenous Q can pump about two protons per electron, since ubiquinol oxidase theoretically yields

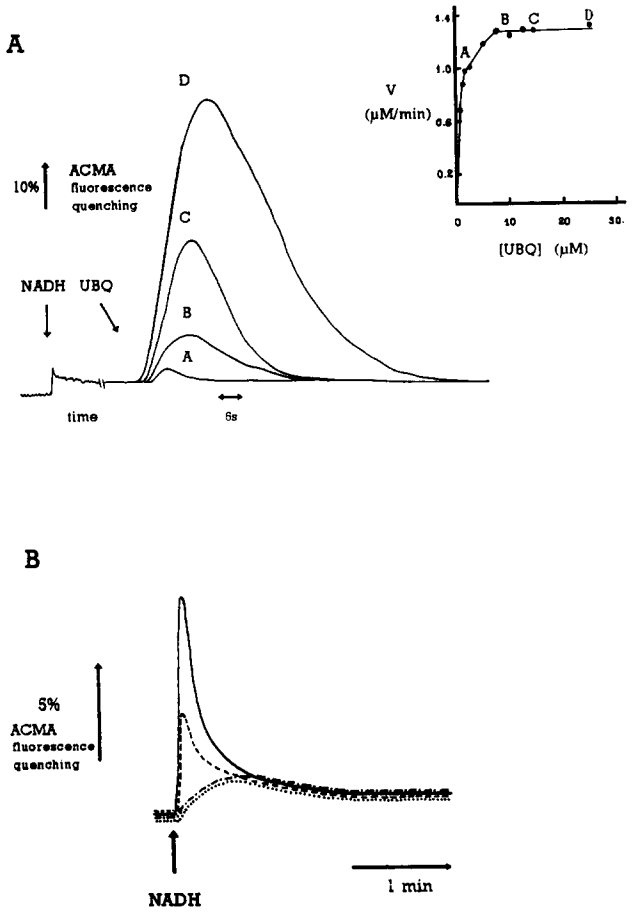


Fig. 2. Proton pumping of complex I. Submitochondrial particles from beef heart [25] were diluted in sucrose 0.125 M, Tricine-Cl 50 mM, $MgCl_2$ 25 mM, KCl 40 mM, pH 8.0 to 5–10 mg/ml of protein and treated with 2 μg/mg oligomycin, 1 nmol/mg antimycin and 3 nmol/mg MOA-stilbene. The fluorescence quenching of ACMA (4 μM) was measured at 412 nm ($\lambda_{excitation}$) and 510 nm ($\lambda_{emission}$) [26] at room temperature in the above buffer containing 2 μM valinomycin. (A) Steady-state proton pumping by UBQ with 100 μM NADH and 2 mg/ml of particles protein. UBQ concentration was: A, 2.5 μM; B, 10 μM; C, 14.5 μM; and D, 25 μM. The titration of the NADH:UBQ reductase activity is shown in the insert and the letters label the same UBQ concentrations as above. (B) Non-steady-state proton pumping under the same experimental conditions as in (A) except that the protein concentration was 0.8 mg/ml. (~~), control without inhibitors; (- - - - -), in the presence of 0.95 μM rotenone; (· · · · · ·), in the presence of 0.6 μM rolliniastatin-2; and (· – · – · –), plus 6 μg/mg of gramicidin to uncouple the particles.

three protons per electron inside submitochondrial particles (one vectorial proton and one scalar proton by complex III and one vectorial proton by cytochrome oxidase [6]).

The concept of two ubiquinone-gated pumps in series (Fig. 1) implies that compounds acting near site A should inhibit proton translocation by complex I under non-steady-state conditions much more than rotenone or other inhibitors acting at site B (Fig. 1). Recently we found that rolliniastatin-2, a potent inhibitor extracted from Annonaceae plants such as Spanish 'cherimoya' [28], acts in a way that is not overlapping that of rotenone [27]. This natural product, which is also called bullatacin [28,29], inhibits the proton pumping function of complex I with similar efficiency under steady-state (i.e., with excess of both NADH and UBQ, cf. Fig. 2A) and under non-steady-state conditions (i.e., with a pulse of NADH alone, Fig. 2B). Rotenone instead inhibits proton pumping by complex I much more efficiently under steady-state than under non-steady-state conditions (Figs. 2B and 3A). The rotenone titre is also 6-fold higher for non-steady-state proton pumping than for steady-state NADH:UBQ reductase and for steady-state generation of membrane potential [30] (Fig. 3A). Conversely, the titres of rolliniastatin-2 are similar for all these functions (Fig. 3B).

The different rotenone potency under non-steady-state and steady-state conditions does not appear to have trivial explanations such as a preferential binding to the partially reduced complex because: (i) the titre of the inhibitor at steady-state is similar if the reaction is started with the reducing substrate NADH or the oxidizing substrate UBQ; (ii) piericidin, which displaces rotenone from its binding site(s) [7], inhibits with equal efficiency the proton pumping of complex I under either non-steady-state or steady-state conditions (results not shown, see Fig. 3B).

The effects of potent inhibitors support the mechanism in Fig. 1. First, proton translocation in complex I also occurrs upstream to the rotenone site, since rotenone inhibits the proton pumping of complex I much more under steady-state than under non-steady-state conditions. Secondly, this rotenone insensitive proton pump is sensitive to rolliniastatin-2 (Figs. 2B and 3B) and piericidin, inhibitors which share a common interaction site in complex I [27]. Thirdly, the complete inhibition of proton pumping by quinone antagonists [24] such as rolliniastatin-2 [27] (Fig. 2B) and piericidin [7,31] (results not shown) indicates that proton translocation by complex I is coupled only to its reactions with Q.

We also tested the possible involvement of FMN in proton pumping as postulated in previous models [1–4,10–12,16,19–21]. The oxidation of NADH by ferricyanide proceeds via oxidoreduction of FMN and of at least one iron-sulphur cluster [2,7,21,31]. Hence, addi-

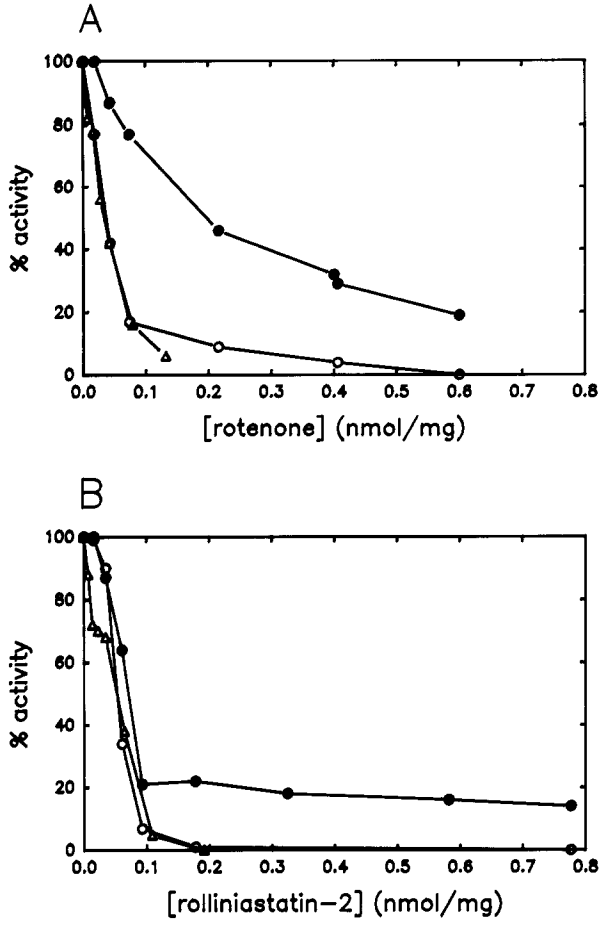


Fig. 3. Titrations of rotenone and rolliniastatin-2 on proton pumping and other functions of complex I. The extent of non-steady-state proton pumping of 0.5 mg/ml of freshly prepared submitochondrial particles (●———●) was measured as in Fig. 2B with 80 μM NADH, except that valinomycin was absent and 25 μM carboxin was added to block complex II. The rate of steady-state proton pumping was measured as in Fig. 2A with 30 μM UBQ (○———○). The rate of membrane potential generation by UBQ was monitored with 3 μM oxonol VI at 630–608 nm [30] with 0.17 mg/ml of particles protein under identical assay conditions (△———△). The inhibitor titrations of the NADH:UBQ reductase activity were similar to those of membrane potential (not shown). (A) Rotenone titrations. (B) Titrations with rolliniastatin-2. Results similar to these have been found with rolliniastatin-1 and also piericidin.

tion of ferricyanide to coupled submitochondrial particles prereduced with NADH should elicit some proton pumping and/or membrane potential if FMN were involved in proton translocation. No uncoupler sensitive quenching of ACMA fluorescence was however detected in this way. A small signal of oxonol VI could be detected upon ferricyanide addition, but this signal was completely abolished by rotenone and strongly inhibited by carboxin, a potent inhibitor of complex II [32]. It thus appears that the oxidoreduction of the FMN cofactor is unlikely to be directly involved in the energy-conserving function of complex I, in agreement with earlier reports [13,31,33] and consistent with the model in Fig. 1.

## 4. Conclusion

The model of dual Q-gated pump (Fig. 1) that we propose for the mechanism of complex I is consistent with current structural knowledge [4,18] and is sustained by the different sensitivity of proton pumping towards various inhibitors (Fig. 3). Much experimentation is needed to clarify several aspects of the NADH:Q reaction, but the proton pumping function of complex I can be studied in native mitochondrial membranes even under non-steady-state conditions (Fig. 2). So, the predictions of our and other models can be tested in detail. Some light has thus been thrown on the mechanism of complex I despite its structural complexity.

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