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MINI-REVIEW

Redox-Linked Proton Translocation by NADH-Ubiquinone Reductase (Complex I)

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Introduction

The respiratory chain NADH-ubiquinone reductase (EC 1.6.5.3) of mitochondria, which is also called complex I, links electron transfer from NADH to ubiquinone with translocation of 4 or 5 protons across the inner membrane. The enzyme comprises some 30 different subunits, one FMN, a still not extactly defined number of iron-sulfur clusters, and probably one form of bound ubiquinone as internal redox groups. It is because of this enormous complexity, that NADH-ubiquinone reductase has remained the least understood among the proton translocating complexes of mitochondria (for reviews see Hatefi, 1985; Ragan, 1987; Weiss *et al.*, 1991).

More recent biochemical investigations in combination with electron microscopic studies (Friedrich *et al.*, 1989; Wang *et al.*, 1991; Tuschen *et al.*, 1990; G. Hofhaus, H. Weiss, and K. Leonard, unpublished results) have revealed that complex I is constructed of two distinct parts. They are assembled and probably emerged in evolution independently of each other, are arranged in a different manner with regard to the mitochondrial inner membrane, and contribute distinct segments to the electron pathway in the complex (for review see Weiss *et al.*, 1991). Furthermore, by sequence comparison, related electron transfer segments have been discovered in completely different bacterial enzymes (Friedrich *et al.*, 1990; Pilkington *et al.*, 1991;

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Böhm *et al.*, 1990). This subdivision of complex I has opened a new way of studying the complex. This review will, therefore, preferentially focus on the discussion of the two parts of complex I in terms of their protein and redox components and their possible role in the redox-linked proton translocation of the complex.

The Two Parts of Complex I

Complex I of bovine heart and the filamentous fungus *Neurospora crassa* has been characterized in more detail (for review see Hatefi, 1985; Ragan, 1987; Leonard *et al.*, 1987; Weiss *et al.*, 1991). Both preparations have a molecular mass of approximately 700 kDa and consist of 20–25 different nuclear and seven (the *N. crassa* complex, at least six) mitochondrially encoded subunits (Chomyn *et al.*, 1985, 1986; Ise *et al.*, 1985; Tuschen *et al.*, 1990). One FMN and the four EPR-detectable iron-sulfur clusters N-1, which is binuclear, and N-2, N-3, and N-4, which are tetranuclear, are now generally believed to participate directly in the electron transfer (Ohnishi *et al.*, 1985; Kowal *et al.*, 1986; Burbaev *et al.*, 1989; Wang *et al.*, 1991; for reviews see Beinert and Albracht, 1982; Ohnishi and Salerno, 1982). In the bovine complex I, clusters N-1, N-3, and N-4 form a nearly isopotential group with the midpoint redox potentials ($E_{m,7}$) around -250 mV, whereas in the *N. crassa* complex their potentials are in the range from -330 to -230 mV (Wang *et al.*, 1991). Cluster N-2 has a much more positive redox potential of -50 mV to -150 mV (Kotlyar *et al.*, 1990; Wang *et al.*, 1991). Most authors believe that the clusters are present at concentrations comparable to FMN, but it is also reported (van Belzen and Albracht, 1989; van Belzen *et al.*, 1990) that cluster N-1 is present at half the concentration of the other clusters. The existence in bovine complex I of a fifth, considerably more negative ($E_{m,7}$ -400 to -500 mV) cluster, called N-1a, and proposed to be binuclear, has been reported (Ohnishi and Salerno, 1982; Ohnishi *et al.*, 1985). There is EPR spectroscopic evidence for an internal ubiquinone in complex I (Suzuki and King, 1983; Burbaev *et al.*, 1989).

Treatment of the bovine heart complex I with chaotropic agents such as NaBr leads to degradation into three fractions called flavoprotein fraction, iron-sulfur protein fraction, and hydrophobic fraction (Hatefi, 1985; Ragan, 1987). The flavoprotein fraction, which consists of three subunits and contains the FMN and one to two iron-sulfur clusters, can dehydrogenate NADH and transfer the electrons to a variety of water-soluble electron acceptors (Hatefi, 1985). The iron-sulfur protein fraction comprises six subunits and a not well-defined number of iron-sulfur clusters. Most of the remaining subunits and one iron-sulfur cluster are found in the water-

insoluble, hydrophobic fraction. Because the resolution of the enzyme caused a considerable alteration of EPR-spectroscopic properties of the iron-sulfur clusters, their clear assignment in the three fractions was not possible (Ohnishi *et al.*, 1985). A fragment that in terms of subunit composition resembles the hydrophobic fraction of the bovine enzyme was also obtained by NaBr treatment of the *N. crassa* complex I (Friedrich *et al.*, 1989). All mitochondrially encoded subunits are found in this fragment (Tuschen *et al.*, 1990) and hence, all subunits of the flavoprotein and iron-sulfur protein fractions are nuclear encoded (Friedrich *et al.*, 1989; Tuschen *et al.*, 1990; Chomyn *et al.*, 1988; Gibb and Ragan, 1990).

In *N. crassa*, when the mitchondrial protein synthesis is inhibited by chloramphenicol, a smaller form of complex I is made instead of the large complex I normally found (Friedrich *et al.*, 1989). This small complex I has only about half the molecular weight of the large complex and consists of approximately 13 merely nuclear-encoded subunits which appear to be identical to nuclear-encoded subunits of the large complex I (Weiss *et al.*, 1991). The small complex I contains FMN and the iron-sulfur clusters N-1, N-3, and N-4, but is devoid of cluster N-2 (Wang *et al.*, 1991). The complex has the same high affinity to NADH (or deamino-NADH; T. Friedrich, unpublished result) as the large complex, but differs in the site for ubiquinone, which has a lower affinity and is insensitive to rotenone or piericidin A (Friedrich *et al.*, 1989), well-known inhibitors of the ubiquinone site of the large complex (Singer, 1979). Most remarkably, none of the subunits of the small complex I is found in the hydrophobic fraction, i.e., with regard to their protein components and redox groups the small complex and the hydrophobic fraction are complementary parts of the large complex. The small *N. crassa* complex I corresponds to the bovine heart flavoprotein and iron-sulfur protein fractions together.

The two complementary parts of complex I, namely the small complex I (or the flavoprotein and iron-sulfur protein fractions together) and the hydrophobic fraction, have also been recognized by electron microscopy. Averaging of single particles of large *N. crassa* complex I revealed an unusual L-shaped structure with two arms arranged at right angles (G. Hofhaus, H. Weiss, and K. Leonard, unpublished results). The hydrophobic fraction corresponds in size and shape to one of these arms. A three-dimensional reconstruction from membrane crystals of the large complex I shows a clear peripheral part, protruding from the membrane, with weak unresolved protein density within the membrane. This peripheral part could be removed by washing the crystals with NaBr, after which the membrane buried arm was clearly visible. A three-dimensional reconstruction from membrane crystals of the small complex I shows only the peripheral part. These results suggest that the membrane protruding arm corresponds to the small complex I, whereas the arm lying within the membrane is the hydrophobic fraction.

Bacterial Enzymes Related to the Two Parts of Complex I

Alcaligenes eutrophus, a facultative lithoautotroph that can assimilate CO_2 and use H_2 as an energy source, contains a soluble NAD^+-reducing hydrogenase. This enzyme consists of four different subunits, α (66.8 kDa), β (54.7 kDa), τ (26.0 kDa), and δ (22.9 kDa). According to Tran-Betcke *et al.* (1990), the β and δ subunits together contain one [4Fe-4S] cluster, one [3Fe-3S] cluster, and Ni. They are involved in the generation of protons and electrons from hydrogen. The τ subunit carries two [4Fe-4S] clusters, and the α subunit one [2Fe-2S] cluster, FMN, and the NAD^+ binding site. This $\alpha\tau$ dimer is concerned with the NAD^+ hydrogenase activity. The sequence of the α ([2Fe-2S], FMN) subunit of the NAD^+ hydrogenase is closely related to the 50-kDa and 24-kDa subunits of mitochondrial complex I, and the sequence of the τ (two [4Fe-4S]) subunit of the NAD^+ hydrogenase is related to the N-terminal part of the 75-kDa complex I subunit. (Pilkington *et al.*, 1991; Tran-Betcke *et al.*, 1990). The three complex I subunits are part of the small form of the *N. crassa* complex or the flavoprotein and iron-sulfur protein fractions together of the bovine enzyme.

Sequence alignment between the 75-kDa complex I subunit (of bovine and *N. crassa*, Pilkington *et al.*, 1991; Preis *et al.*, 1990) and the α subunit of the NAD^+ hydrogenase (Tran-Betcke *et al.*, 1990) shows three blocks of conserved cysteines which very likely are involved in the coordination of the two [4Fe-4S] clusters. Comparison of the sequences of the 50-kDa complex I subunit (of bovine and *N. crassa*) with the α subunit of NAD^+ hydrogenase exhibits three highly conserved regions. In the one region, four glycines are spaced in consensus with known NADH binding sites (Scrutton *et al.*, 1990). The other region contains a pair of glycine residues with a $\beta\alpha\beta$ structure that might be the FMN binding site (Wierenga *et al.*, 1986), and the third region contains a block of conserved cysteines which might ligate an iron-sulfur cluster. The 50-kDa subunit of complex I was, in fact, shown by photoaffinity labelling (Chen and Guillory, 1981; Deng *et al.*, 1990; Yagi and Dinh, 1990) to contain the NADH binding site. EPR spectroscopy suggested that the subunit contains an iron-sulfur cluster (Ohnishi *et al.*, 1985). Taken together, the relationship between the NAD^+ hydrogenase α and τ subunits and the 75-, 50-, and 24-kDa complex I subunits suggests that the latter constitute a structural and functional unit which contributes the electron pathway from NADH via FMN to the iron-sulfur clusters N-1, N-3, and N-4.

A glucose dehydrogenase found in many aerobic bacteria (e.g., *E. coli*, *G. oxydans*, *A. calcoaceticus*, *Z. mobilis*) oxidizes glucose to gluconolactone in the periplasmic space and delivers the electrons via pyrolloquinoline quinone to ubiquinone (Duine *et al.*, 1979; Matsushita *et al.*, 1987). This

single polypeptide enzyme was found to be inhibited also by piericidin A competitively with regard to ubiquinone like complex I (Friedrich *et al.*, 1990), suggesting that complex I and glucose dehydrogenase contain the same domain motif for ubiquinone reduction. The amino acid sequence is known for the glucose dehydrogenase of *Acinetobacter calcoaceticus* (Cleton-Jansen *et al.*, 1988). Only the N-terminal part of the sequence is hydrophobic enough to span the membrane. In the case of complex I, the subunit encoded by the mitochondrial ND1 gene (36 kDa in the mammalian and 42 kDa in the *N. crassa* complex) was photolabelled by rotenone analogues (Early *et al.*, 1987) and was therefore suggested to contain the ubiquinone binding site. Homology, in fact, was found between a sequence region in this subunit and the N-terminal sequence of the glucose dehydrogenase (Friedrich *et al.*, 1990). This region is predicted to span the membrane with three helices and one helix to be located at the outer membrane surface. The related helices of the two enzymes show good conformities concerning their positions and lengths.

Exactly the same domain motif is found in the gene product of ORF4 in the *E. coli* formate hydrogenlyase operon (Böhm *et al.*, 1990). Most remarkably, this ORF 4 is flanked by ORF3, a homologoue to the mitochondrial ND5 gene. This gene encodes a 67-kDa subunit of the bovine heart complex I (the related *N. crassa* subunit has the molecular mass of 78 kDa), which is predicted by sequence comparison to bear the iron-sulfur cluster N-2 (Ragan, 1987; Weiss *et al.*, 1991). One might therefore expect that the two (ND1 and ND5 encoded) complex I subunits, which are both parts of the the hydrophobic fraction, constitute a functional unit concerned with the electron transfer from cluster N-2 to ubiquinone. (For the formate hydrogenlyase of *E. coli* this would mean that formate oxidation can alternately be coupled to proton or ubiquinone reduction.)

Taken together, the electron input part of complex I, from NADH via FMN to the isopotential iron-sulfur-clusters N-1, N-3, and N-4, and which is contributed by the small form of the *N. crassa* complex I (or the corresponding flavoprotein and iron-sulfur protein fractions of the bovine complex I), is found in the NAD^+-reducing hydrogenase of *A. eutrophus*. The electron output part of complex I, from cluster N-2 to the rotenone-sensitive ubiquinone reduction site, and which is contributed by the hydrophobic fraction, is found in the *E. coli* formate hydrogenlyase and in part in the bacterial glucose dehydrogenase.

Electron Pathways

Combining the data discussed in the previous sections with more direct functional studies leads us to propose the following scheme of the electron

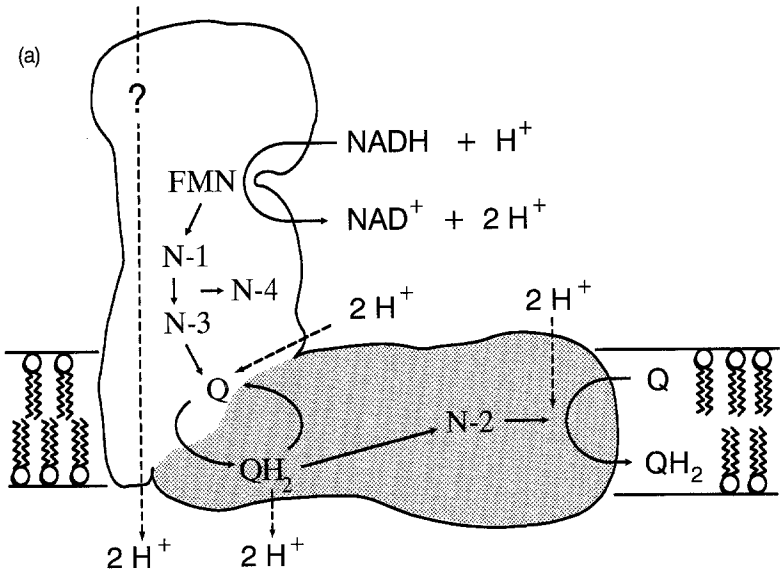

Fig. 1. Two alternate models of the cooperation of the two parts of complex I in electron transfer and proton translocation. In the model (a) the two parts are connected by an internal quinone. The vertical, predominantly peripheral part with the NADH binding site, the FMN, the clusters N-1, N-3, N-4, and the internal (low-affinity) ubiquinone reduction site (Qi) corresponds to the small form of complex I. The horizontal membrane part containing the putative internal ubiquinol oxidation site, cluster N-2, and the rotenone-sensitive site for the substrate ubiquinone corresponds to the hydrophobic fraction. In (b) the electron pathway behind cluster N-3 is branched. Alternatively, electrons can be transferred to cluster N-2 or a ubiquinone reduction site connected with the ubiquinone pool in the membrane. Both parts of the complex have to be proton pumps to account for the thermodynamic limits and the measured $H^+/2e^-$ stoichiometry of 4.

route in complex I (see Fig. 1): NADH binds to the 50-kDa subunit and delivers two electrons to the FMN bound to the same subunit. From $FMNH_2$ the electron transfer occurs in two single steps to the (2Fe-2S) cluster N-1 also bound to the 50-kDa subunit and then to the two (4Fe-4S) clusters N-3 and N-4 bound to the 75-kDa subunit. N-1 was, in fact, suggested from EPR studies to be the first cluster reduced by FMN (Krishnamoorthy and Hinkle, 1988). The view that the clusters N-1, N-3, and N-4 build an isopotential electron pool (Krishnamoorthy and Hinkle, 1988) is supported by the observation of Vinogradov's group (Burbaev *et al.*, 1989; Kotlyar and Vinogradov, 1989; Kotlyar *et al.*, 1990) that complex I has to be activated by several turnover cycles leading to the reduction of the three iron-sulfur clusters. This is not a precondition for the linear electron pathway through the iron-sulfur cluster according to their midpoint potential as suggested (Bakker and Albracht, 1986). Furthermore, the finding that cluster N-4 can

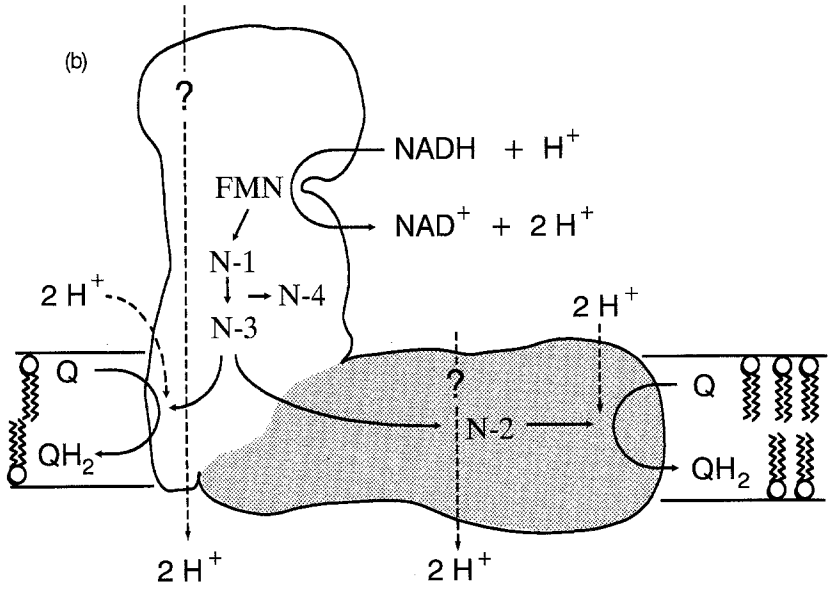

Fig. 1. Continued.

be removed without affecting the electron transfer rate (Krishnamoorthy and Hinkle, 1988) underlines the model of an electron buffer.

As electronic connector between the isopotential iron-sulfur cluster N-1, N-3, and N-4 and the high-potential cluster N-2 we propose an internal ubiquinone. Some evidence for this assumption came from the discovery of the small form of the *N. crassa* complex I, which contains a ubiquinone reduction site different to the site in the large complex I. Since for each subunit of the small complex I an identical (or homologous) counterpart exists in the large complex I, the ubiquinone site of the small complex might serve as a site for an internal ubiquinone in the large complex. A semiquinone/quinone couple as electron mediator between the low-potential clusters N-1, N-3, and N-4, and the high potential N-2 cluster could work as a ubiquinone cycle (Mitchell, 1976). What remains to be established is an internal ubiquinol oxidation site that should be contained in the hydrophobic fraction of complex I.

Electron transfer from cluster N-2 to ubiquinone seems to take place in single one-electron steps leading to the transient formation of a protein-bound ubisemiquinone (Burbaev *et al.*, 1989; Kotlyar and Vinogradov, 1989; Kotlyar *et al.*, 1990). Close functional association of cluster N-2 and the binding site for the substrate ubiquinone was shown by EPR spectroscopy (Kotlyar *et al.*, 1990), and close structural association can be followed from

the direct neighborhood of ORF3 and ORF4, the ND5 and ND1 homologues in the formate hydrogenlyase operon of *E. coli*.

Alternatively, the ubiquinone reduction site found in the small complex I corresponds to the postulated rotenone-insensitive quinone reduction site of the large complex not involved in proton pumping (Ragan, 1978; DiVergilio and Azzone, 1982). This would mean that the electron pathway is branched behind the isopotential iron-sulfur clusters and the electrons may flow either to the rotenone-insensitive ubiquinone reduction site or to cluster N-2 (see Fig. 1b)

H^+/e^- Stoichiometry

Kinetic and thermodynamic approaches were applied to determine the H^+/e^- stoichiometry of complex I, and $H^+/2e^-$ values between 4 (Vercesi *et al.*, 1978; Pozzan *et al.*, 1979; DiVergilio and Azzone, 1982; Scholes and Hinkle, 1984; Wikström, 1984; Beavis, 1987; Brown and Brand, 1988) and 5 (Lemasters *et al.*, 1984; Lemasters, 1984) have been reported. This stoichiometry was equal to the charge per two electrons ratio when it was measured. The value is usually given as a whole number, but it might be a broken number because the protonmotive force is a delocalized form of energy (Ferguson and Sorgato, 1982). Furthermore, proton translocation might occur with variable stoichiometry (Murphy and Brand, 1987). For all recent determinations of the proton translocation stoichiometry of complex I, intact mitochondria or submitochondrial particles were used.

Kinetic measurements were carried out as oxidant pulse techniques using quinones (DiVergilio and Azzone, 1982) or oxygen as oxidant (Vercesi *et al.*, 1978; Pozzan *et al.*, 1979). Alternatively, proton extrusion of the NADH-cytochrome *c* segment was compared to the succinate-cytochrome *c* segment of mitochondria, which has a known $H^+/2e^-$ stoichiometry of 2 (Wikström, 1984). Besides the kinetic measurements, thermodynamic approaches were applied. Equilibrium thermodynamics have the advantage over kinetic measurements, that the calculated stoichiometry is not influenced as much by ion permeabilities. However, reaction equilibrium is not easily achieved in real systems. Nonequilibrium thermodynamics for systems not too far away from equilibrium were applied (van Dam and Westerhoff, 1977; Rottenberg, 1985). The same $H^+/2e^-$ value of 4 was obtained as with the kinetic measurements (Rottenberg and Gutman, 1977; Scholes and Hinkle, 1984; Brown and Brand, 1988). A different thermodynamic approach by Lemasters and coworkers (Lemasters *et al.*, 1984; Lemasters, 1984) based on a net H^+/ATP ratio of 4 gave a $H^+/2e^-$ stoichiometry of 5. In another method based on a simple kinetic model and a linear nonequilibrium

thermodynamic approach, the upper and lower limits of the mechanistic stoichiometry were measured as the K^+/O ratio (Beavis, 1987), leading to a value of 4. Summarizing all experimental data, a mechanistic $H^+/2e^-$ stoichiometry of 4 is the most likely.

Linkage between Electron Transfer and Proton Translocation

Not much is known on the linkage between electron transfer and proton translocation. According to the redox potential gaps, energy transduction could principally occur between FMN and the isopotential clusters N-1, N-3, and N-4, between these and cluster N-2, and between cluster N-2 and the substrate ubiquinone. The last coupling site only exists if the midpoint potential of cluster N-2 is as negative as -120 to -150 mV (Wang *et al.*, 1991; Kotlyar *et al.*, 1990). The effect of energization by ATP on the redox poise of individual iron-sulfur clusters was examined. These early investigations pointed mainly to cluster N-2 (Ingledew and Ohnishi, 1980). This is supported by the finding that cluster N-2 has a pH-dependent midpoint potential (Ingledew and Ohnishi, 1980). Other evidence for the assumption that N-2 is involved in energy transduction arose from the observation that in *C. utilis* and *P. denitrificans* the energy coupling at site I, rotenone or piericidin sensitivity, and the iron-sulfur cluster N-2 occur simultaneously (Cobley *et al.*, 1975; Meijer *et al.*, 1978).

It was often assumed that the FMN takes part directly in proton translocation by performing a "flavin cycle" (Kotlyar *et al.*, 1990; Skulachew, 1986; Krishnamoorthy and Hinkle, 1988). This would imply a flavin reduction site at the negative (matrix) side and a flavin oxidation site at the positive (cytosolic) side of the enzyme. The amino acid sequence of the 50-kDa subunit carring the FMN, however, almost certainly excludes the possibility of a transmembrane operation of the FMN. As a working hypothesis, we would like to propose that the reoxidation of the $FMNH_2$ is linked with the translocation of two protons by a kind of proton channel, which directs them to the cytoplasmic side. If this translocation is coupled with the electron transport through the isopotential cluster N-1, N-3, or N-4, the small form of the complex has to be a proton pump. The small complex is indeed very sensitive to DCCD (U. Sackmann, T. Friedrich, and H. Weiss, unpublished result), and NADH-ubiquinone reductase activity was reported to be inhibited by DCCD only in organisms that possess a proton-translocating NADH-ubiquinone reductase (Yagi, 1987). If the small complex I is no proton pump, this translocation should be coupled with the electron transport via cluster N-2. This means that the ability of proton pumping of complex I

emerged from the association of the two parts of the complex (Tuschen *et al.*, 1990, Weiss *et al.*, 1991).

From the ispotential iron-sulfur clusters the electrons are transfered to the putative internal quinone bound near the inner membrane side. This could occur via the ubisemiquinone or the ubiquinol. Ubiquinone reduction would lead to proton uptake at the matrix side, and ubisemiquinone or ubiquinol oxidation at the outer membrane surface would lead to the release of protons at the cytosolic side. The electrons are then transported to cluster N-2 in an electrogenic step back across the membrane to the reduction site of the substrate ubiquinone. This site is predicted from sequence data to lie at the inner membrane surface (Friedrich *et al.*, 1990). Concomitant with the final reduction of the substrate, two further protons are taken up from the matrix. This speculative mechanism would be in agreement with an overall $H^+/2e^-$ stoichiometry of 4. The prerequisites for this model are, firstly, proton translocation by that part of complex I which corresponds to the small form of the complex I and, secondly, the existence of an internal ubiquinol cycle.

If the ubiquinone reducing site of the small complex I corresponds to the rotenone-insensitive ubiquinone reduction site found in the large complex I, which is not involved in proton pumping (see Fig. 1b), both parts of the complex have to function as proton pumps in order to account for the thermodynamic limits.

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