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Review

Proton-translocation by membrane-bound NADH:ubiquinone-oxidoreductase (complex I) through redox-gated ligand conduction

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1. Summary

For the catalytic mechanism of proton-translocating NADH-dehydrogenase (complex I, EC 1.6.99.3) a number of hypothetical models have been proposed over the last three decades. These models are discussed in the light of recent substantial progress on the structure and function of this very complicated multiprotein complex.

Only the high-potential iron-sulfur center N-2 and ubiquinone seem to contribute to the proton-translocating machinery of complex I: Based on the pH dependent midpoint potential of iron-sulfur cluster N-2 and the physical properties of ubiquinone intermediates a novel mechanism is proposed. The model builds on a series of defined chemical reactions taking place at three different ubiquinone-binding sites. Therefore, some aspects of this redox-gated ligand conduction mechanism are reminiscent to the protonmotive Q-cycle. However, its central feature is the abstraction of a proton from ubihydroquinone by a redox-Bohr group associated with iron-sulfur cluster N-2. Thus, in the proposed mechanism proton translocation is driven by a direct linkage between redox dependent protonation of iron-sulfur cluster N-2 and the redox chemistry of ubiquinone.

2. Introduction

The proton-translocating NADH-dehydrogenase (complex I, EC 1.6.99.3) is by far the most complicated and least understood multisubunit enzyme of the respiratory chain in mitochondria and aerobic bacteria [1–3]. The oxidation of one NADH by this integral membrane-protein is considered to be linked to the translocation of 4 $H^+/2\ e^-$ ([4–6], see [7] for a more detailed discussion of this issue) across the energy transducing membrane. The electrons are transferred onto the hydrophobic hydrogen-carrier ubiquinone. Substantial progress has been made during recent years in defining the overall structural organization [8], in determining the subunit structure of eucaryotic [3,9,10] and procaryotic complexes [9,11] and in characterizing the numerous iron-sulfur clusters of complex I [12]. However, little is known about the mechanisms linking electron transfer to vectorial proton-translocation in complex I. Several more or less detailed hypothetical models for proton-translocation have been proposed, which cover a wide range of options.

The purpose of this review is to give an overview over the mechanistic models that have been proposed. The available evidence is summarized to define the structural and thermodynamic constraints the proton-translocating device in complex I has to meet. Possible analogies to other proton-translocating enzymes are explored. This discussion then leads to the proposal of a detailed and thermodynamically feasible model which should be useful to clarify some general principles involved and guide further experimental work.

3. Structural organization, redox groups and ubiquinone binding sites

Mammalian complex I consists of at least 42 different subunits [2] and even bacterial complexes contain 14 subunits [9,11]. Complex I contains one FMN as a prosthetic group and 6 iron-sulfur centers have been identified by EPR spectroscopy [12,13] (Table 1). An L-shaped overall structure was deduced from electron microscopic analysis of the *Neurospora crassa* enzyme at about 8 Å resolution [14]. Different lines of evidence indicate that this L-shaped multiprotein structure can be divided into several subcomplexes [15,16], which are likely to have developed from different evolutionary origins [17]. This issue has been the subject of several recent reviews [2,10,18–20]. Therefore, only aspects of immediate relevance to the localization and function of the

Table 1
Midpoint potentials of the iron-sulfur centers of bovine heart complex I

Iron-sulfur center	Type	E_{m7} (mV)	pH-dependence
N-1a	Fe_2S_2	−370	+
N-1b	Fe_2S_2	−250	−
N-2	Fe_2S_2	−150	+
N-3	Fe_4S_4	−250	−
N-4	Fe_4S_4	−250	−
N-5	Fe_4S_4	−260	−

Assignments and E_{m7} values are taken from [30].

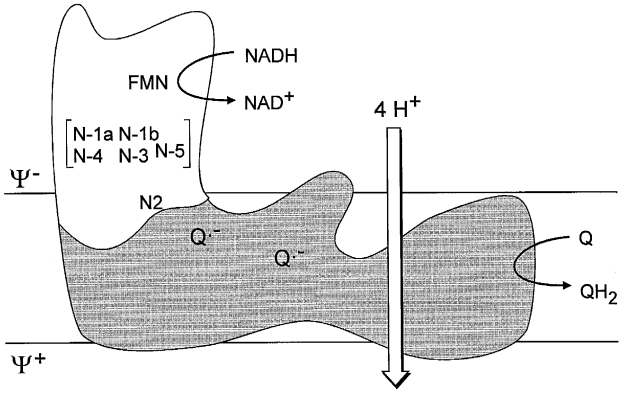

Fig. 1. Diagram of the overall shape and approximate position of the redox-centers and substrate binding sites of complex I. The overall shape was adapted from an reconstruction at about 8 Å resolution using data from electron microscopic analysis of *N. crassa* complex I [14]. The reactions catalyzed by this membrane bound NADH-dehydrogenase are also indicated, namely the oxidation of NADH, the reduction of ubiquinone and a translocation of 4 protons linked to this redox reaction. The white area tentatively indicates the peripheral arm of the enzyme, which should correspond roughly to the Iλ subcomplex of the bovine enzyme [21]. The placement of the NADH-substrate binding site, FMN and the six iron-sulfur centers in this part of the enzyme are arbitrary, as no information is available on their exact positions. Only for iron-sulfur cluster N-2 there is evidence that it resides close to the membrane part [21,30]. The grey area indicates the membrane part of the enzyme which carries the binding sites for ubiquinone and two EPR-detectable semiquinone species [29]. Again the position of the substrate binding site and the semiquinones are given arbitrarily, with the exception that one of the semiquinones has been reported to be 8–11 Å apart from iron-sulfur cluster N-2 [29]. Q, ubiquinone; $Q^{\cdot -}$, ubisemiquinone; QH_2, ubihydroquinone; N-1a, N-1b, N-2,N-3,N-4,N-5, EPR-detectable iron-sulfur clusters of complex I [30].

proton-translocating machinery of complex I will be discussed here.

Complex I is composed of two major parts (Fig. 1), which in the *N. crassa* enzyme have been called the membrane arm and the peripheral arm [10]. The peripheral arm seems to correspond roughly to subcomplex I λ of the bovine enzyme [21]. Both contain no mitochondrially coded, hydrophobic subunits and comprise two smaller subcomplexes that have been known for a long time [15,22,23], the so called flavoprotein (FP) and the so called iron-sulfur protein (IP). However, while bovine subcomplex I λ contains all known prosthetic groups, namely FMN and all EPR detectable iron-sulfur clusters [21], iron-sulfur cluster N-2 was not found in the peripheral arm of the *N. crassa* enzyme [10]. Remarkably, compared to the intact enzyme, the EPR spectrum of iron-sulfur cluster N-2 was broadened in subcomplex I λ [21]. As subcomplex I λ is also not capable of transferring electrons onto ubiquinone [21], it can be concluded that the membrane arm contains no known prosthetic group, but has to be the part of the enzyme which carries the ubiquinone binding sites. The above mentioned biochemical evidence and the fact that iron-sulfur cluster N-2 titrates at a much higher midpoint potential than all other iron-sulfur clusters of complex I (Table 1) further suggests that this redox group is located at the interface between the two major parts of the enzyme catalyzing electron transfer from the peripheral part onto the membrane part [21,24,25].

Based on kinetic studies using specific inhibitors of complex I like rotenone and piericidin A [26] and ubiquinone derivatives with varying hydrophobicity [27,28] it has been suggested that there are at least two ubiquinone reactive sites within the membrane domain of complex I. This notion has received further support from the recent observation of two rotenone-sensitive and energy-dependent semiquinones by EPR-spectroscopy with tightly-coupled submitochondrial particles oxidizing NADH [29]. Taking advantage of the spin-spin interaction between one of the semiquinones (SQ_{Nf}) with iron-sulfur cluster N-2 a distance of 8–11 Å between the two paramagnetic species has been estimated [29]. This fits well with the idea of iron-sulfur cluster N-2 being the electron donor to ubiquinone, but it seems to make it even more difficult to understand how a second or even a third ubiquinone could participate in electron transfer and possibly proton-translocation. This is true in particular, if one considers the apparent absence of redox groups in the membrane part of complex I.

At present for none of the redox-centers or substrate binding sites of complex I the exact positions within the overall structure and relative to the membrane are known. However, the bipartite structure of a water soluble peripheral part that contains all known prosthetic groups and a membrane part that carries the binding sites for ubiquinone (Fig. 1) provides a framework that imposes important constraints onto the proton-translocating machinery.

4. Synopsis of proposed mechanisms

The presence of at least 6 iron-sulfur clusters [30], one FMN and at least two quinone binding sites in principle allows a remarkable number of permutations for the sequence of electron and proton-transfers within complex I. Over the last three decades many of these options have been incorporated into a number of quite different mechanistic models. A comparison of these models seems worthwhile, as this should provide a useful basis for the incorporation of current knowledge about the functional properties of complex I and the actual arrangement of its redox-centers and binding sites.

Fig. 2 gives an overview over 8 representative mechanistic models that have been proposed over the years. The purpose of this synopsis is to stress the fundamental similarities and differences using a consistent set of symbols rather than to reproduce every detail of each model for which the reader should refer to the respective original publications.

Peter Mitchell's original proposal [31] for a redox loop at 'coupling site I' (Fig. 2A) did not take into account even the existence of a multiprotein complex conferring this activity. However, already this model pinpointed the hydrogen carrier FMN as potential proton-translocator and the iron-sulfur clusters as electron wires. After 'coupling site I' had been identified as NADH-ubiquinone oxidoreductase [32], Lawford and Garland [33] modified the loop as shown in Fig. 2B.

When a rotenone sensitive semiquinone radical was detected by EPR spectroscopy [34] the idea that a bound $Q/QH^{\cdot}$ couple could be involved in proton-translocation [35] became more attractive. Stimulated by the protonmotive Q-cycle that had been proposed for the cytochrome bc_1 complex [36] this concept led Suzuki and King [34] to formulate a Q-cycle type mechanism for complex I (Fig. 2C). This proposal essentially just changes the proton carrier from FMN to ubiquinone and, different from the Q-cycle operating in the cytochrome bc_1 complex, does not increase the $H^+/2e^-$ stoichiometry above 2. However, it was shown later that this stoichiometry is higher for the complex I segment [4–6] and is now considered to be $4\ H^+/2e^-$ by most authors [7,37,38]. The experimen-

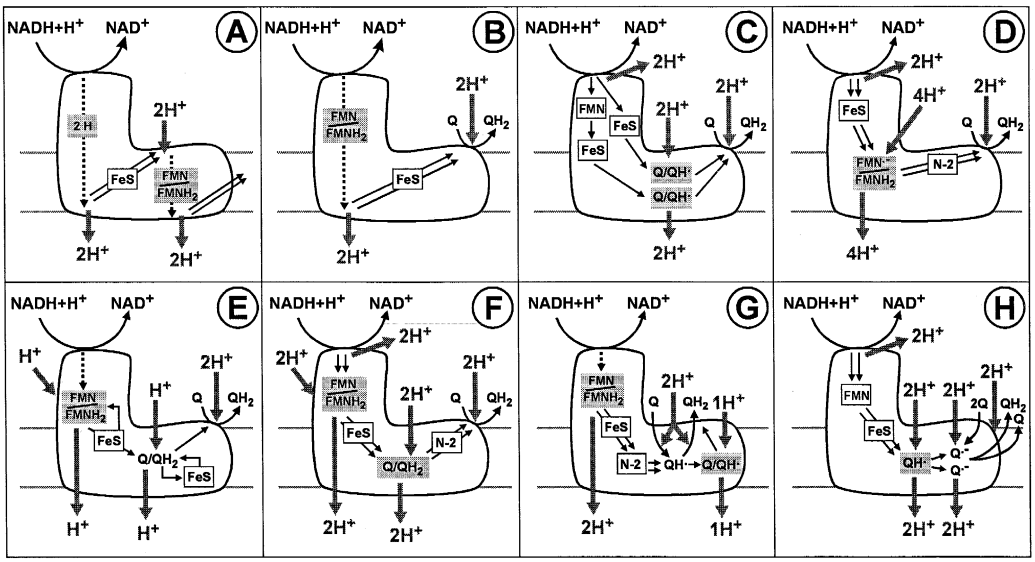

Fig. 2. Synopsis of eight representative proposals for redox-linked proton-translocation by complex I. A, Mitchell, 1966 [31]; B, Lawford and Garland, 1972 [33]; C, Suzuki and King, 1983 [34]; D, Krishnamoorthy and Hinkle, 1988 [40]; E, Ragan, 1990 [45]; F, Weiss et al., 1991 [7,14,46]; G, Vinogradov, 1993 [25,47]; H, Degli Esposti and Ghelli, 1994 [39]. See text for details. Straight arrows indicate electron transfer reactions, dotted arrows hydrogen transfer. Curved arrows show substrate exchange. Bold gray arrows are used to show proton uptake or release. Bound electron carriers are boxed and bound hydrogen carriers are shown on gray background. FeS, iron-sulfur center(s); N-2, iron-sulfur center N-2; Q, ubiquinone; $Q^{\cdot -}$, $QH^{\cdot}$ ubisemiquinone; QH_2, ubihydroquinone.

tal evidence for this value as the mechanistic stoichiometry of complex I is rather solid. The lower stoichiometries observed under some conditions [5,39] could be due to 'slip' of the protonmotive mechanism.

Krishnamoorthy and Hinkle went back to the idea that the key component for proton-translocation was the flavin [40]. Their proposal accounts for a higher proton-translocation stoichiometry by assuming that the FMN changes only between the deprotonated semiquinone and the fully protonated reduced form, with protonations occurring towards the negative and deprotonations towards the positive side of the membrane. In the variant of their mechanism shown in Fig. 2D electrons instead of hydrogens are transferred from NADH to FMN via iron-sulfur centers which results in the release of substrate protons on the negative side of the membrane. The electrons are transferred via iron-sulfur cluster N-2 onto ubiquinone where substrate protons are taken up. In this model cluster N-2 was proposed to lie behind the proton-translocating machinery as its midpoint potential was taken as -30 mV, but is now considered to have an E_{m7} of -150 mV in bovine heart complex I (cf. Table 1). Aside from this discrepancy this intriguingly simple model is likely to be incorrect for other reasons of general significance: This mechanism requires that the flavin-semiquinone becomes fully ionized and the reduced flavin fully protonated during each catalytic cycle. As stated by the authors themselves [40], taking the pK_a values of 8.5 for free $FMNH^{\cdot}$ and 6.5 for free $FMNH_2$ a priori such a protonation/deprotonation behavior is not expected. However shifting the effective pK_a values of one and the same redox group into opposite directions depending on its redox state is not a trivial task and requires a specific mechanism promoting it. In fact, recently pK_a values of 7.7 for the semiquinone form and 7.1 for the reduced form of FMN bound to complex I have been reported [41]. A good example of what is necessary to operate a mechanism based on redox-linked protonation/deprotonation is the histidine-cycle [42,43] which has been proposed by Wikström as the proton-translocating rationale for cytochrome *c* oxidase. Another problem arises from the postulate that a single electron redox change would *force* the uptake or release of two protons, as this leaves one charge uncompensated. However, charge compensation is of central importance for a proton-translocating machinery, as has been worked out by Rich also for cytochrome *c* oxidase [44].

The proposal by Ragan (Fig. 2E, [45]) includes the possibility of branching the electron path at the level of flavine and ubiquinone and cycling it back via iron-sulfur clusters. Variations of this scheme can account for $H^+/2e^-$ stoichiometries between 2 and 6. Its major setback is that it offers many possible variations without specifying the actual reaction sequences during normal turnover. Also at last some variants of the scheme seem to be difficult to reconcile with thermodynamics for similar reasons as discussed in the previous paragraph.

Participation of the two hydrogen carriers involved was put together in a more straightforward way by Weiss and coworkers [7,14,46]. According to this proposal 2 $H^+/2e^-$ are transported across the membrane by each, the $FMN/FNNH_2$ couple and an 'internal' Q/QH_2 couple (Fig. 2F). Although this circumvents some of the thermodynamic inconsistencies of other models and gives an overall stoichiometry of 4 $H^+/2e^-$, no explanation is given how the internal ubihydroquinone can be reoxidized by iron-sulfur cluster N-2. However, this is not a trivial question considering a midpoint potential of this redox-center below -100 mV. Also the problem of promoting a cyclic reduction and reoxidation of the rather positive Q/QH_2 couple ($E_{m7} = +70$ mV) in the absence of any high-potential redox site is not addressed by this proposal.

A similar scheme was proposed by Vinogradov [25,47] which accounts for a $H^+/2e^-$ stoichiometry of 3 (Fig. 2G), while based on the available reports on proton stoichiometries [4–6] now a value of 4 $H^+/2e^-$ is considered most likely. The reason for the lower value is that this model, like the 'Q-cycle' variant of Krishnamoorthy and Hinkle (Fig. 2C), employs $Q/QH^{\cdot}$ couples rather than a Q/QH_2 couple. However, Vinogradov's mechanism addresses two important aspects. Firstly, postulating one 'translocating' and one 'substrate' ubiquinone/semiquinone couple which symproportionate to give the product ubihydroquinone implies a thermodynamically feasible reaction sequence capable of translocating 1 $H^+/2e^-$ against the membrane potential. The only points remaining unclear are how the 'translocating' semiquinone gets moved across the mem-

brane or how it is ascertained that protonation and deprotonation occur at opposite sides of the membrane. As discussed earlier for the FMN- dependent mechanism by Krishnamoorthy and Hinkle (cf. Fig. 2D) this and the question of effective pK_a values for the intermediates involved is a central question for any directional protonation/deprotonation mechanism. The second important aspect discussed by Vinogradov [25] is the role of cluster N-2. According to his model, electron transfer from this redox center onto ubiquinone has to be 'blocked' at certain stages of the catalytic cycle to prevent it from reducing semiquinone to hydroquinone. However, no clues are given how such a conditional electron transfer could be controlled. Nevertheless, as cluster N-2 has been found to be only 8–11 Å apart from one of the EPR-detectable ubisemiquinones [29] and thus seems to be the immediate reductant of ubiquinone, it is in fact a likely candidate for the control of a putative ubiquinone-dependent proton-translocating process (see below).

The dual Q-gated pump model by Degli Esposti and Ghelli [39] entirely abandons any contributions to the proton-translocation process by the electron input part of complex I containing FMN and the iron-sulfur clusters. As shown in Fig. 2H, translocation of 4 $H^+/2e^-$ is proposed to be linked to the sequential formation of two types of semiquinones. After two semiquinones have formed in the second site, they are supposed to dismutate resulting in the net formation of one ubihydroquinone. It remains unclear however, how such a 'gated pump' would work in detail. If the translocation process was directly linked to the protonation/deprotonation of the ubisemiquinones involved, the same difficulties as for Vinogradov's mechanism would apply. Indirect 'pump'-mechanisms, on the other hand, are difficult to prove or disprove and seem unlikely in the light of better understood mechanisms operating in other proton-translocating enzymes [43,48,49]. Apart from these more general points, there are two more specific problems associated with the dual Q-gated pump which seem to exclude this mechanism: (i) if two semiquinone anions have to exist in the same ubiquinone-binding pocket at some point during the catalytic cycle to allow dismutation, formation of the second semiquinone is expected to be highly endergonic for electrostatic reasons; (ii) if the immediate donor to the bound quinone is iron-sulfur cluster N-2 with an $E_{m7} \approx -150$ mV, only about half of the potential drop between $NAD^+/NADH$ and the Q/QH_2 couple is available to drive vectorial proton-transport of 4 $H^+/2e^-$. In fact, this is one of the key problems for any mechanism based on ubiquinone as hydrogen carrier which does not employ FMN and the iron-sulfur clusters for proton-translocation.

In recent years Dutton and Ohnishi have emphasized as well that proton translocation of complex I is likely to employ a variation of the protonmotive Q-cycle and employs only ubiquinone as hydrogen carrier (L.P. Dutton and T. Ohnishi, personal communication). A mechanistic model based on these ideas has been presented recently [50]. This model combines a direct reversal of a Q-cycle type mechanism with a semiquinone-gated proton translocator which employs the movement of a bound quinone between two sites and in this respect is somewhat reminiscent to Vinogradov's proposal [47].

5. Localization of the proton-translocating machinery

The most controversial and important question emerging from the comparison of available models given in the previous chapter is, whether both hydrogen carriers FMN and ubiquinone contribute to proton-translocation. There is sufficient evidence to conclude that at least part of the proton-translocating machinery is associated with the reactions of ubiquinone. Most notably, two EPR detectable and rotenone sensitive ubisemiquinone species [29], the selective reactivity with ubiquinones of varying hydrophobicity [27,28] and kinetic evidence for at least two types of binding sites for hydrophobic inhibitors [26] indicate that complex I carries a minimum of two ubiquinone reactive sites. Also the assignment of these sites to the membrane part of the enzyme supports the notion that ubiquinone contributes to proton-translocation.

For FMN the situation is much less clear. Different arguments have been used in favor of a role for FMN as a proton-translocator. For example, a translocation of the flavin from one site to another has been suggested based on the observation that NADH reduction of the flavoprotein-subcomplex results in dis-

sociation of $FMNH_2$ [51]. However, FMN resides in the peripheral part of complex I, which can be separated as a water-soluble subcomplex. Therefore, if the redox change of FMN is linked to proton-translocation there has to be a rather long proton channel connecting it to the positive side of the membrane. This situation would be different from other known proton-translocating enzymes, where the proton-translocating group and the proton channels are found on the same membrane spanning subunit [52,53]. Moreover, oxidation of NADH by ferricyanide in submitochondrial particles was not found to be linked to vectorial proton-transport [39,54], although this reaction has been reported to occur via oxidoreduction of FMN and at least one iron-sulfur cluster [55]. Thus, there seems to be no compelling evidence for FMN being part of the proton-translocating machinery, but several arguments against this possibility.

On the other hand, as the peripheral part of complex I also contains all iron-sulfur centers including the 'high-potential' cluster N-2 with an E_{m7} of -150 mV, the remaining potential gap appears not to be sufficient to transport 4 $H^+/2e^-$. The only obvious way to resolve this dilemma is to postulate that iron-sulfur cluster N-2 is an active part of the proton-translocating machinery. In principle, this could be envisioned in two different ways: cluster N-2 either is involved in a direct proton-translocating mechanism somewhat comparable to the histidine-cycle proposed for cytochrome *c* oxidase [43], or its redox-change is linked to a ubiquinone dependent mechanism. To explore these options, it seems necessary to take a closer look at the properties of iron-sulfur cluster N-2.

6. Role of iron-sulfur cluster N-2

A correlation between the presence of specific iron-sulfur clusters and piericidin A sensitive NADH oxidation was first reported a long time ago [56]. Since then a number of authors have discussed a possible involvement of the redox-Bohr properties of cluster N-2 in proton-translocation [13,30,57,58], but surprisingly redox-linked protonation/deprotonation of this center has not been incorporated into any of the working models for proton-translocation by complex I (see above).

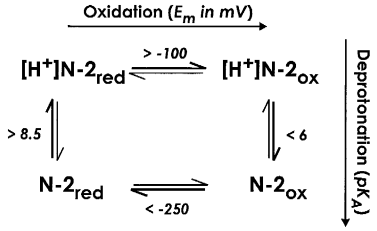

Fig. 3. Protonation- and redox-states of iron-sulfur cluster N-2 of bovine heart complex I. The limits for the pK_a and E_m values were estimated from experimental data given in [13]. N-2, iron-sulfur cluster N-2; ox, oxidized; red, reduced. Thicker arrows indicate the direction the reactions take according to the redox-gated ligand conduction hypothesis.

In bovine heart the midpoint potential of iron-sulfur cluster N-2 is pH dependent following a slope of -60 mV/pH unit over a pH-range from 6 to 8.5 [13]. This means that the pK_a-value for the oxidized species must be below 6 and that for the reduced species above 8.5. In turn, this shift of pK_a by more than 2.5 units reflects a difference in midpoint potential between the deprotonated and the protonated species of at least 150 mV (Fig. 3). In other words, the midpoint potential of deprotonated cluster N-2 is in the same range as, or even lower than that of the other iron-sulfur centers of bovine complex I, except cluster N-1a (cf. Table 1). As another consequence, cluster N-2 in its reduced state should have a tendency to pick up a proton due to its rather high pK_a. Likewise, the oxidized species should be quite acidic and deprotonate upon formation. Thus, it can be concluded that there is a redox-Bohr group associated with iron-sulfur cluster N-2 which has properties making it suitable to be a part of the proton-translocating device. Note that if in fact every redox-change of cluster N-2 is associated with a protonation-change of this group, one of the other iron-sulfur centers would have to reduce the deprotonated, low potential species. This means that there would be practically no potential drop during catalysis, but abstraction of a proton from somewhere else would then shift the cluster N-2 midpoint potential to the positive.

If the redox-Bohr group associated with iron-sulfur center N-2 is an intrinsic part of the proton-translocating machinery of complex I, it should be present in all proton-translocating NADH-dehydrogenases.

Aside for bovine heart complex I, a pH-dependent midpoint potential of iron-sulfur cluster N-2 has been found in *Escherichia coli* [9], *Rhodobacter sphaeroides* [59] and *Paracoccus denitrificans* [60]. A pK_a of 7.7 for the reduced cluster N-2 has been reported for *P. denitrificans* and the pK_a of the oxidized species must be below 6 [60]. This corresponds to a redox-dependent pK_a shift by at least two units. For *Thermus thermophilus* the situation is less clear, as no high-potential iron-sulfur cluster has been found [61]. The tetranuclear iron-sulfur cluster which has been proposed to correspond to mammalian cluster N-2 exhibits no pH dependence of its midpoint potential, but seems to form two species with different midpoint potential above pH 8 [61]. Also, vectorial proton-translocation by complex I from *Th. thermophilus* has yet to be demonstrated. Moreover, this enzyme is a NADH-menaquinone oxidoreductase. Menaquinone, however, has a much lower midpoint potential than ubiquinone and a proton-translocating machinery, if it exists, would have to meet different thermodynamic requirements, than in complex I of other species.

To assess a possible role of cluster N-2 in proton-translocation, it would also be important to know to which subunit it is bound and where this subunit is located relative to the membrane. At present these questions cannot be answered completely. Two subunits have been discussed to carry iron-sulfur cluster N-2 [19,38,62] namely TYKY (20.2 kDa) and PSST (20.1 kDa) of the bovine enzyme. Homologues of both subunits are also found in bacterial complex I [9,11].

Subunit TYKY carries eight conserved cysteines arranged in the consensus pattern (CxxCxxCxxxCP-(x_n)-CxxCxxCxxxCP) found in bacterial eight-iron ferredoxins. This suggests two tetranuclear clusters in a similar arrangement as in these ferredoxins. The edge-to-edge distance between the two clusters in *Peptococcus aerogenes* ferredoxin is less than 6 Å [63,64], resulting in strong electromagnetic coupling. Such iron-sulfur clusters are difficult to analyze by EPR spectroscopy [65] and it therefore remains uncertain whether both clusters are present and could be detected by EPR in complex I. At present, it cannot be excluded that the tetranuclear iron-sulfur cluster N-2 is located in subunit TYKY, but the structural similarity of this protein to bacterial low potential ferredoxins, which carry two tetranuclear clusters in close vicinity, makes it not a very likely candidate.

PSST does not carry a motif which would identify this subunit unambiguously as an iron-sulfur protein, but its amino-acid sequence contains three conserved possible cysteine-ligands for an iron-sulfur cluster. Because the third cysteine is immediately followed by a proline, it has been suggested that this subunit carries a Fe_4S_4-cluster and that the fourth ligand might be a conserved glutamate three residues downstream from the first cysteine [38]. Such a cluster would be expected to have unusual properties, making it an interesting candidate for iron-sulfur center N-2. The impressive number of 40–50 charged residues in a protein with about 150 amino acids that can be found in the bacterial and mammalian subunit fits well into the picture of a subunit carrying a redox-group involved in proton-translocation. However, mutagenesis work will be required to answer the question whether PSST is in fact the N-2 iron-sulfur protein.

No matter which of the two subunits carries cluster N-2, both, like cluster N-2 itself, have been assigned to the peripheral part of the enzyme. While this includes the possibility that the subunits might be submerged into the membrane domain to a certain extent, it certainly means that cluster N-2 is located more on the negative side of the membrane and has to play its supposed role in proton-translocation from there.

Summarizing the discussion up to this point, present knowledge on structure and function of complex I gives only a rather crude picture of this large and complicated enzyme. Still, there seems to be sufficient information to indicate where one has to look for the proton-translocating machinery and which components are likely to contribute to this process. At this point, FMN cannot be excluded with absolute certainty as a possible player, but clearly attention should focus on ubiquinone and iron-sulfur cluster N-2 as contributors to the proton-translocating machinery.

Due to the properties of iron-sulfur cluster N-2 described above, this redox-center by itself could operate as the central part of a proton-translocating device [13,50,57,58], accepting a proton from the negative side of the membrane upon reduction and releasing it towards the positive side following reoxi-

dation. However, as indicated also by the $n = 1$ pH-dependence of the cluster N-2 midpoint potential, such a mechanism would contribute only 2 $H^+/2e^-$ or 50% of the total proton-translocation capability of complex I. This means that the same number of protons would have to be translocated by an unknown ubiquinone dependent mechanism for which no supporting redox group is known. Combining a mechanism involving cluster N-2 as a proton-gate and the semiquinone mechanism proposed by Vinogradov [25] would result in a stoichiometry of only 3 $H^+/2e^-$, which is less than the 4 $H^+/2e^-$ suggested by other authors. Moreover, it seems rather arbitrary to postulate that two *adjacent* components of complex I would operate as two essentially independent proton-translocators. The objective then would be to develop a mechanistic model that is based on the combined action of cluster N-2 as a redox-Bohr group and ubiquinone as a hydrogen carrier.

7. Properties of ubiquinone intermediates

At center P of the cytochrome bc_1 complex bifurcated electron flow and thereby vectorial proton-transfer is also based on the combined action of an iron-sulfur center and ubiquinone. A proton-gated charge-transfer mechanism operating at the core of a protonmotive Q-cycle [36,67] has been proposed recently to describe the chemistry of this interaction [48,68]. Despite of the many differences between complex I and cytochrome bc_1 complex, some of the general concepts developed to describe the mechanism of the cytochrome bc_1 complex should provide a useful guideline to develop a mechanistic model for complex I. Most notably, ubiquinone is involved as a hydrogen carrier in proton-translocation by both respiratory chain complexes.

Reexamination of the biophysical properties of the redox-states of ubiquinone is prerequisite to explore its suggested role in the proton-translocating mechanism of complex I. The E_{m7} and pK_a values of the ubiquinone intermediates determined in 80% ethanol are summarized in Fig. 4. Although these parameters could be significantly different in the bound state, they provide a useful starting point and it should be possible to rationalize any deviation by correlating it with properties of the binding site. Likewise, if a

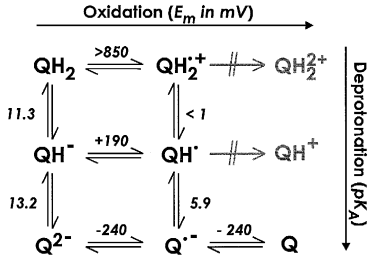

Fig. 4. Ubiquinone intermediates. E_m values and pK_a values were taken from [66] and [48].

determined pK_a or midpoint potential of a ubiquinone intermediate differs from the values in solution, this can give important clues on the environment of the bound species. A useful rule of thumb for an electrostatic change in the environment of ubiquinone intermediates is that an increase of the midpoint is expected to lower the pK_a of the species, and vice versa.

Proton-translocation by ligand conduction is established by ubihydroquinone oxidation with proton-release on one side of the membrane and ubiquinone reduction with proton uptake on the other side of the membrane. Taking the rather negative potential of the iron-sulfur clusters of complex I, reduction of ubiquinone is thermodynamically no problem. Thus, the crucial question is, whether there is a mechanism by which ubihydroquinone can be oxidized even in the absence of a high-potential acceptor. To solve this problem one has to consider that any redox-reaction of ubiquinone is a sequence of electron- and proton-transfers and that the rather positive overall midpoint potential of the Q/QH_2 couple of $+70$ mV is largely due to the high pK_a values for the deprotonation of ubihydroquinone (see Fig. 4). In other words, the fully deprotonated Q^{2-} is a remarkably strong reductant with $E_{m7} = -240$ mV in 80% ethanol. On the other hand, following the rule of thumb given above, lowering the pK_a values increases the midpoint potentials. Therefore, a ubihydroquinone-oxidation site in the membrane domain of complex I has to be designed such that deprotonation is facilitated, but that the deprotonated species generated is still a strong enough reductant to re-reduce a ubiquinone-intermediate on the other side of the membrane.

8. Redox-gated ligand conduction

The mechanistic model of redox-gated ligand-conduction shown in Fig. 5 is an attempt to account for all thermodynamic constraints and experimental evidence available for complex I. In this mechanism a net stoichiometry of 4 $H^+/2e^-$ is realized exclusively by redox-linked protonations and deprotonations of ubiquinone.

Three ubiquinone-binding sites (see Fig. 5) are involved in the proposed reaction sequence, namely one ubihydroquinone oxidation site called center P_I and two ubiquinone reduction sites called center N_A and N_B. The only iron-sulfur center directly involved is cluster N-2. The other iron-sulfur centers as well as FMN serve as electron input pathway and may provide an isopotential pool of reduction equivalents. Upon reduction of cluster N-2 (Fig. 5, step 1) its pK_a increases and it abstracts a proton from ubihydroquinone bound at center P_I through a proton channel. When N-2 donates electrons to ubiquinone bound at center N_A (Fig. 5, step 2), it is reoxidized and the lowered pK_a of the associated redox-Bohr group forces a proton out towards the positive side of the membrane. This step could be promoted by the properties of the proton channel necessary for this proton extrusion.

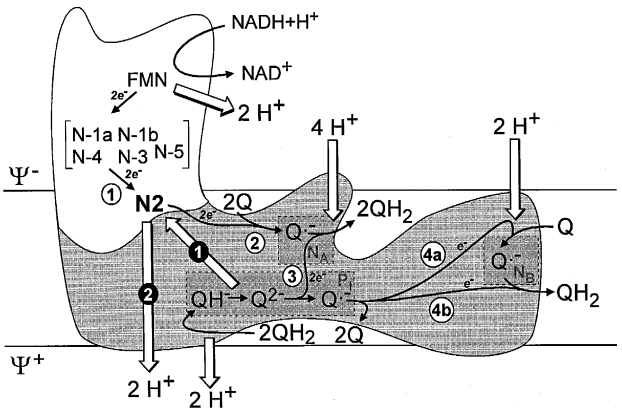

Fig. 5. Redox-gated ligand-conduction mechanism. The path of electrons from the low potential iron-sulfur centers of the peripheral part to ubiquinone is described as a series of numbered reactions shown by solid arrows. Reactions 1 to 3 have to occur twice for a complete reaction sequence. White numbers on black circles indicate proton-movements associated with the respective electron-transfer reaction marked with black numbers on white circles. Thick open arrows indicate proton movement, uptake or release. See text for further details. N_A, center N_A; N_B, center N_B; P_I, center P_I. See also legend of Fig. 1.

Center P_I is proposed to lower the pK_a of the bound ubihydroquinone to some extent. This would allow one spontaneous deprotonation and the abstraction of the second proton by the reduced cluster N-2 even if its pK_a is not very high, and would lead to the generation of Q^{2-}. Q^{2-} is still at a potential negative enough to reduce the semiquinone formed at center N_A (step 2) to ubihydroquinone (Fig. 5, step 3). This results in the uptake of two protons on the negative side of the membrane..

The semiquinone formed at center P_I then reduces ubiquinone bound at center N_B to form ubisemiquinone (Fig. 5, step 4a). Subsequently the whole sequence is repeated with the exception that now the ubisemiquinone bound at center N_B is completely reduced to ubihydroquinone (Fig. 5, step 4b) leading to the uptake of another two protons from the negative side of the membrane.

A complete reaction sequence leads to the oxidation of 1 NADH and the net reduction of 1 ubiquinone. 2 protons are released and 6 taken up on the negative side, while 4 protons are released on the positive side.

Note, that if the ubiquinone binding sites are close enough to each other no electron transferring redox groups are required for this reaction.

The essential feature of this model of proton-translocation by complex I is the linkage between reduction of cluster N-2 and deprotonation of ubihydroquinone at center P_I. By coupling these two reactions the potential drop between the iron-sulfur cluster and a stabilized $Q/Q^{\cdot-}$ couple can be used to drive oxidation of ubihydroquinone on the other side of the membrane. To have the system operate efficiently one has to propose that the redox-Bohr group associated with cluster N-2 is linked to the 'input'-channel, when the iron-sulfur center is reduced and to the 'output'-channel when it is oxidized. Certainly, the 'timing' of the individual proton- and electron-transfer steps is critical for efficient proton-translocation and it is quite likely that the redox-gated ligand-conduction mechanism would tend to 'slip'. In fact, variable proton-translocation stoichiometries have been observed with complex I under certain conditions [5,39]. It is also tempting to speculate that the pH- and cation-dependent transition between active

and deactivated forms of complex I [25,69] might be related to the redox-Bohr group getting trapped in an inactive state. If indeed a transmembrane proton-channel leading from center P_I to cluster N-2 exists, an interesting side effect would be that the deprotonation of ubihydroquinone bound to the ubihydroquinone-oxidation site would actually be promoted by the membrane potential. This could ensure efficient proton-translocation in the energized state due to a decrease of the effective midpoint potential of the ubisemiquinone bound to center P_I. Note that in this case the steady-state reaction would still be inhibited by the membrane potential as deprotonation of iron-sulfur cluster N-2 is predicted to be highly electrogenic.

Although the presentation of the redox-gated ligand-conduction mechanism in Fig. 5 is presented in a way suggesting that all ubiquinones are freely exchangeable with the ubiquinone-pool, this is not a necessary implication of the model. The same mechanism could operate based on the assumption of some sort of substrate channeling between the ubiquinone binding sites as suggested by some experimental reports [70,71].

It should also be stressed that the model shown in Fig. 5 is only one of several possible mechanisms that can be proposed using the same general concepts. For example, if the second ubiquinone-reduction site (N_B) was omitted and both electrons from the ubihydroquinone-oxidation site were transferred to the same ubiquinone reduction site, this would result in a reaction scheme translocating only 2 $H^+/2e^-$. In this case cluster N-2 could assist in the abstraction of both protons from ubihydroquinone, but a second proton-translocating device within complex I would be needed to account for the overall stoichiometry of 4 $H^+/2e^-$.

9. Ubiquinone and inhibitor binding-sites

To test and refine the hypothesis of a redox-gated ligand conduction mechanism it will be essential to determine the number and properties of ubiquinone binding-sites of complex I. As discussed above, there are several lines of evidence indicating that there are at least two ubiquinone binding sites.

Within the redox-gated ligand-conduction mechanism the two types of semiquinone bound to complex I detected by EPR spectroscopy [29] should be associated with centers N_A and N_B, as the ubisemiquinone at center P_I should be the least stable.

The impressive array of different hydrophobic inhibitors of complex I most of which are likely to be ubiquinone-analogs [26,72–75] can be grouped into different classes based on a number of kinetic studies. Friedrich et al. have defined two classes of inhibitors based on Michaelis-Menten type inhibition kinetics with complex I from three different organisms and a comparison with inhibition of glucose dehydrogenase from *Gluconobacter oxidans* by the same set of 12 inhibitors [76]. Class I inhibitors, represented by piericidin A, inhibit complex I in a partially competitive manner and also inhibit glucose dehydrogenase competitively. Class II inhibitors, represented by rotenone, inhibit complex I in a non-competitive manner, and glucose dehydrogenase is not inhibited at all. Degli Esposti et al. have found [77] that stigmatellin and myxothiazol, which are known as very tight binding inhibitors of the cytochrome bc_1 complex [78], inhibit complex I at micromolar concentration without competing with piericidin A or rotenone. With some caveats, this can be taken as an indication for a third ubiquinone binding site of complex I. A more detailed discussion of the large body of literature on the action of different inhibitors of complex I would be beyond the scope of this review. However, if indeed there are three classes of inhibitors of complex I, in terms of the redox-gated ligand-conduction mechanism, a tentative assignment would be that piericidin inhibits center N_A, rotenone inhibits center P_I and myxothiazol/stigmatellin inhibit center N_B.

10. Conclusions

Although knowledge about structure and function of complex I is still rather limited when compared to other respiratory chain complexes, based on the evidence available the contours of the proton-translocating mechanism operating in this complicated multisubunit enzyme seem to emerge. The hypothesis of a redox-gated ligand conduction mechanism proposed here describes proton-translocation by complex I as a series of defined chemical reactions. It makes specific predictions on the role of iron-sulfur cluster and the

properties of the three postulated ubiquinone binding sites. This should make the model easily testable and hopefully stimulate further experimental work. It will be of particular importance to find a way to test whether in fact ubihydroquinone is oxidized by a partial reaction of complex I and whether this reaction is linked to a redox-dependent protonation of iron-sulfur cluster N-2.

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