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Kinetics, Control, and Mechanism of Ubiquinone Reduction by the Mammalian Respiratory Chain-Linked NADH-Ubiquinone Reductase[1]

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In mammalian cells the membrane-bound NADH-quinone oxidoreductase serves as the entry point for oxidation of NADH in the respiratory chain and as the proton-translocating unit which conserves the free energy of the enzyme intramolecular redox reactions as the free energy of the electrochemical proton gradient across the coupling membrane. This review summarizes the kinetic properties of the mammalian enzyme. Emphasis is placed on the hysteretic properties of the enzyme as related to the possible control of intramitochondrial NADH oxidation and to the mechanism of the enzyme interaction with ubiquinone. Recent evidence for participation of flavin and the protein-bound ubisemiquinone pair in the enzyme-catalyzed proton translocation mechanism are discussed.

KEY WORDS: NADH-ubiquinone oxidoreductase; ubisemiquinone; flavin; electron transfer; enzyme hysteresis; iron-sulfur clusters; mitochondria.

INTRODUCTION

The formulation of the chemiosmotic rationale of vectorial metabolism and biological energy transfer (Mitchell, 1966) has led to tremendous progress in the understanding of how the intramolecular membrane-bound enzyme redox reactions convert the free energy of electron transfer into free energy of electrochemical ion gradients which is further available for ATP synthesis or other energy-requiring intracellular events. The mammalian respiratory chain consists of four redox-driven proton pumps: membrane-bound NADPH-NAD^+ transhydrogenase (Hoek and Rydstrom, 1988), NADH-ubiquinone oxidoreductase (coupling Site I) (Ragan, 1976), ubiquinol–cytochrome *c* reductase (coupling Site II) (Berry and Trumpower, 1985), and cytochrome *c* oxidase (coupling Site III) (Babcock and Wikstrom, 1992). The NADH-ubiquinone oxidoreductase (EC 1.6.99.3), usually termed Complex I, is perhaps the most complicated and the least understood protonmotive device of the mitochondrial respiratory chain. In fact the term *enzyme* seems hard to apply as a definition of the lipoprotein unit, which is an assembly of at least 41 different polypeptides (Fearnley and Walker, 1992). When the complex operates as an intrinsic component of the inner mitochondrial membrane, it catalyzes the following reversible reaction:

$$NADH_{in} + H^+ + Q \overset{nH^+_{in} \quad nH^+_{out}}{\longleftrightarrow} NAD^+_{in} + QH_2 \quad (1)$$

where the subscripts in and out stand for the mitochondrial matrix and intermembrane space, respectively, and Q and QH_2 are ubiquinone and ubiquinol, respectively. The redox potential gap (ΔE) between the substrate [(−320 mV (Clark, 1960) and acceptor (+60 mV (Erecinska and Wilson, 1976)] pairs at $\Delta\mu_{H^+}$ across the membrane of 210 mV [(the value which would poise the ATP-synthase close to the equilibrium (Nicholls, 1982)], which allows thermodynamic coupling of transmembraneous trans-

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port of four protons [the value of n in Eq. (1)] per electron pair transferred from NADH to Q. The overall reaction as written in Eq. (1) is reversible and when $\Delta\mu_{H^+}$ is provided by proton-translocating F_1F_0-ATPase or by independent operation of coupling Site II + III, the reduction of NAD^+ by QH_2 is readily observed (Chance and Hollunger, 1960; Klingenberg and Slenczka, 1959; Low and Vallin, 1963; Vallin and Low, 1964). Very little is known on the detailed molecular mechanism of the reaction (1) although rapid progress in the structural-molecular genetic aspects of the mammalian Complex I and its counterpart in other species is being made. Excellent accounts on this subject have recently been published (Fearnley and Walker, 1992; Weiss *et al.*, 1991, Yagi, 1990). In addition to the complexity of the polypeptide composition, Complex I is a multi-redox component enzyme, which bears FMN (Rao *et al.*, 1963; Kumar *et al.*, 1968), 4–6 binuclear and tetranuclear iron-sulfur centers (Beinert and Albracht, 1982; Ohnishi, 1979), and 2–4 moles of tightly bound ubiquinone per mole of FMN (Hatefi and Rieske, 1967; Suzuki and King, 1983). The redox components of Complex I have been discussed in detail in several reviews (Beinert and Albracht, 1982; Ragan, 1976) and in the original papers (Ohnishi, 1975; Ohnishi *et al.*, 1981). An extensive discussion of mostly speculative recent hypotheses on the mechanism of the proton-pumping activity (Ragan, 1987, 1990; Weiss and Friedrich, 1991) and on the historical (Cremona *et al.*, 1963; King *et al.*, 1966) and medical (Singer and Ramsay, 1992) aspects of the field development containing much more specific information than is feasible to present in this review may be found in the respective references. At the invitation of the editor, this paper gives an essay on the recent progress achieved in the author's laboratory. In the first section the catalytic properties derived mainly from the steady-state kinetics will be discussed. The second section deals with an unusual hysteretic behavior of Complex I, i.e., the slow active/inactive enzyme transition as related to the possible regulation of the input of reducing equivalents into the respiratory chain. In the third part some hypothesis on the participation of enzyme redox components in the proton-pumping activity will be formulated. In the conclusion, some avenues which, in the author's opinion, are worthy of pursuit for better understanding the enzyme's mechanism and control will be briefly mentioned.

THE STEADY-STATE KINETIC PARAMETERS

Direct (NADH-Ubiquinone Reductase) Reaction. When coupled or uncoupled NADH-ubiquinone reductase is catalyzed by either submitochondrial particles or isolated Complex I, the complete kinetic mechanism of the overall reaction (i.e., the sequence of the elementary steps, the rates and equilibria constants) is hardly expected to be defined due to the obvious complexity of the enzyme. However, some useful information concerning the substrates/products binding affinities can be obtained if the reactions in the presence of artificial electron acceptors such as ferricyanide (Dooijewaard and Slater, 1976a, b) or hexammineruthenium (III) (Sled and Vinogradov, 1992) are compared with the complete rotenone-sensitive quinone reductase. Both ferricyanide and hexammineruthenium (III) reveal abbreviated rotenone-insensitive (i.e., uncoupled) NADH oxidation. The steady-state kinetics of Complex I revealed that the reaction mechanism with ferricyanide is ping-pong bi-bi with strong double substrate inhibition (Dooijewaard and Slater, 1976a,b). The latter appears to be the reason for the great variation of the enzyme turnover numbers reported in the literature and the lack of reliable information of the apparent affinities of NADH and NAD^+ for the enzyme substrate binding site(s). The simple ordered steady-state kinetics of the NADH-$Ru(NH_3)_3^{3+}$ oxidoreductase reaction has been demonstrated for the particulate and isolated Complex I (Sled and Vinogradov, 1992). A comparison of the kinetic parameters of the enzyme as revealed with $Ru(NH_3)_6^{3+}$ and ubiquinone homologue Q_1 is given in Table I. (NADH-oxidation part). Several conclusions can be made. (i) Uncoupled particles and isolated Complex I show very similar affinities for NADH and NAD^+. (ii) The differences between K_m^{NADH} and K_s^{NADH} (extrapolation to zero V_{max} by variation of the electron acceptor concentration) and the strong difference between the K_m^{NADH} values determined with $Ru(NH_3)_6^{3+}$ and Q_1 as electron acceptors show the significant contribution of the kinetic term to the apparent affinity of the enzyme to the substrate. (iii) The almost identical K_m^{NADH} values for coupled and uncoupled NADH-Q_1 reductase catalyzed by submitochrondrial particles strongly indicate that $\Delta\mu H^+$ itself does not affect the enzyme–substrate interaction, as might be expected due to the −2 charge on the NADH molecule. The same conclusion is apparently valid for NAD^+

Table I. Steady-State Kinetic Parameters for Direct and Reverse Electron Transfer Catalyzed by NADH-Ubiquinone Reductase (30°C, pH 8.0)

	NADH oxidation				NAD^+ reduction	
	Complex I	Submitochondrial particles			Submitochondrial particles	
		coupled[a]	uncoupled		coupled[a]	
Electron acceptor or donor	$Ru(NH_3)_6^{3+}$	Q_1	Q_1	$Ru(NH_3)_6^{3+}$	$Q_{10}H_2$	
K_m^{NADH} μM[b]	60	7.6	7.2	35	$K_m^{NAD^+}$ μM	7.2
K_s^{NADH} μM[c]	40			10	K_i^{NADH} μM	40
$K_i^{NAD^+}$ μM	2,000		1,250	1,000	$K_s^{NAD^+}$ μM	0.9

[a] Submitochondrial particles treated with oligomycin having a respiratory control ratio of 5–7.
[b] The value of K_m depends on the concentration of the electron acceptor. The values indicated were obtained at 0.5 mM $Ru(NH_3)_6^{3+}$ and 0.1 mM Q_1.
[c] Obtained by extrapolation to zero maximal rate.

(−1 charge) binding. (iii) A difference of about two orders of magnitude exists between the affinities of the enzyme to NADH and NAD^+. The simplest explanation for such a difference is that a positively charged group exists (or appears after reduction of the enzyme by the substrate) in a close vicinity to the site where the nicotinamide ring interacts with the protein.

Reverse ($\Delta\mu_H^+$-Dependent Ubiquinol-NAD^+ Reductase) Reaction. The only preparation which shows significant turnover of the enzyme in the reverse electron transfer is tightly coupled submitochondrial particles, and under appropriate conditions as much as about one-fourth (in terms of the catalytic activity) of the uncoupled NADH-ubiquinone reductase can be revealed as the rotenone-senstivie ubiquinol-NAD^+ reductase (Kotlyar and Vinogradov, 1990). Unfortunately, only limited experimental approaches are available for the analysis of the reaction, and some kinetic parameters are given in Table 1 (NAD^+ reduction part). The most striking phenomenon is that the apparent affinity for NAD^+ is about three orders of magnitude higher than the $K_i^{NAD^+}$ value revealed in NADH oxidation reaction. This difference cannot be explained by the $\Delta\mu_{H^+}$ effect (see point (iii) in the previous paragraph). One possible explanation may simply be "purely kinetic". Assuming a ping-pong bi-bi reaction mechanism for NAD^+ and $\Delta\mu_{H^+}$, one may expect that the apparent $K_m^{NAD^+}$ varies from zero to a higher level with a linear dependence on V_{max}, which is evidently a function of $\Delta\mu_{H^+}$. However, simple calculations show that to obtain an apparent $K_m^{NAD^+}$ in the millimolar range one should assume the rate of the reverse electron transfer to be about 100 times higher than the enzyme activity in the "direct" reaction. Such a relation between enzyme turnovers for direct and reverse electron transfer seems rather unlikely because of the physiological function of the enzyme and also because of thermodynamic reasons. To explain the apparent discrepancy between the affinities of the enzyme to NAD^+ as revealed in the direct and reverse reactions, we would like to suggest that the site where NAD^+ interacts with the enzyme during the steady-state reverse electron transfer is different from that where NADH binds during the direct electron transfer. This suggestion is somehow in accord with the data on the enzyme nucleotide binding site(s). It is well established that the 51 kDa subunit of Complex I and its corresponding counterparts from *P. denitrificans* and *N. crassa* (Chen and Guillory, 1981, 1984; Yagi, 1987) bear a single nucleotide-specific site which evidently participates in NADH binding during its oxidation. Another nucleotide binding region in the bovine 39 kDa subunit and its *N. crassa* homologue have been identified from sequence analysis (Fearnley and Walker, 1992). Three possible explanations have been offered to account for the presence of a nucleotide-binding site in the 39 kDa subunit of Complex I (Fearnley and Walker, 1992). Our hypothesis, which adds a fourth possibility, predicts that unidirectional inhibitors for the direct and reverse electron transfer should exist. It is worth noting that should this proposal be true, the precise mechanism of the direct and reverse electron

transfer are different. Such a possibility does not seem far-fetched due to the extremely complex enzyme structure, the possible heterodimeric arrangement (Van Belzen *et al.*, 1990, 1992), and the obvious need for refined physiological control of the enzyme activity.

PRESTEADY-STATE KINETICS

The parameters discussed in the previous section are characteristic for the enzyme operating under steady-state conditions. However, complex hysteretic behavior is seen when NADH oxidation is continuously followed after mixing submitochondrial particles or Complex I with NADH. Thirty years ago, Minakami *et al.* (1964) reported that a considerable lag in the appearance of NADH oxidase and NADH–cytochrome *c* reductase activities was observed in some preparations of submitochondrial particles. This lag was not seen in the NADH-ferricyanide reductase or when a second portion of NADH was oxidized after the complete oxidation of a small amount of the substrate. The phenomenon has not been understood, although it was proposed that the reduction of some component in the NADH–cytochrome *c* region of the respiratory chain is a prerequisite for activation of NADH oxidation (Tyler *et al.*, 1965). The results of our recent studies (Kotlyar and Vinogradov, 1990; Kotlyar *et al.*, 1992) on the slow activie/inactive transition of the NADH-ubiquinone reductase, which are qualitatively the same and quantitatively similar for isolated or membrane-bound Complex I, are briefly summarized below.

Complex I within particular preparations of submitochondrial particles or in its isolated form is always a heterogeneous mixture of completely inactive and active forms of the enzyme. The ratio of these forms depends on the "history" of the particular preparation. Complete "irreversible" deactivation can be achieved by a simple incubation of the enzyme in the absence of NADH. The deactivation is a slow reaction (within tenths of a minute) at 30°C and with an apparent activation energy of 270 kJ/mol. The deactivated enzyme is inactive in both direct and reverse electron transfer reactions. Only the rotenone-sensitive activities are subject to the slow active/inactive transition. The irreversibly deactivated enzyme can be reactivated in the course of slow (compare to the steady-state turnovers) enzyme turnover(s). The presence of NADH and oxidized quinone is needed for transformation of the deactivated form to the active one. The deactivation rate is insensitive to NADH, QH_2, and divalent cations and is only slightly pH-dependent, whereas the substrate-induced activation process is strongly pH- and divalent cation-dependent. Significant conformational rearrangement of the enzyme molecule apparently occurs during the slow active/inactive transition, since only the deactivated enzyme is a target for *N*-ethylmaleimide irreversible inactivation.

The parameters of the slow active/inactive transition of the enzyme are summarized in Table II and the scheme which apparently accounts for all the experimental observations is given in Fig. 1.

Several points relevant to the molecular mechanism and possible physiological role of the slow active/inactive transition seem worthy of emphasis. The rotenone-sensitive ubisemiquinone(s) interacting with iron-sulfur cluster N-2 are seen during the steady-state NADH oxidation in the coupled submitochondrial particles (Burbaev *et al.*, 1989; Kotlyar *et al.*, 1990). This suggests that ubisemiquinone bound at the specific enzyme site(s) is an intermediate in the steady-state electron flow from NADH to quinone pool. Thus, it seems reasonable to visualize

Table II. Deactivation/Activation Parameters for the Membrane-bound and Isolated Complex I (28°C, pH 8.5)

Parameter	Submitochondrial particles	Complex I
Deactivation half-time (min)[a]	16	13
Activation energy for deactivation (kJ/mol)	270	245
Activation energy for activation (kJ/mol)		168
Apparent first-order rate constant for NADH/Q-induced activation (min^{-1})	1.0	1.0
pK_a (see Fig. 1)	8.1	
$K_D^{Ca^{2+}}$ mM (see Fig. 1)	1.3	1.6

[a] At 30°C.

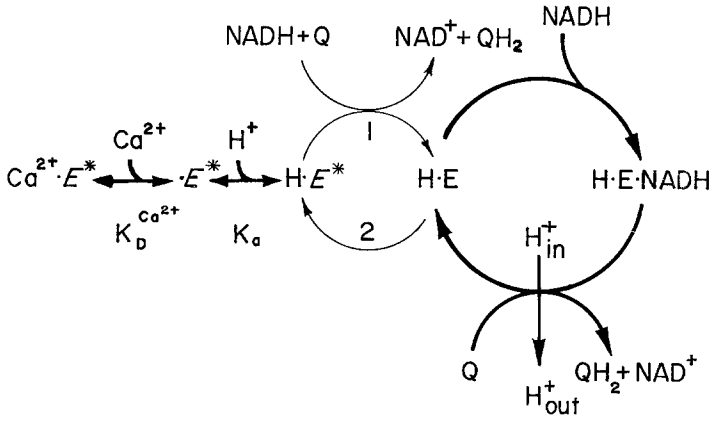

Fig. 1. The equilibria and futile cycle involved in the active/inactive transition of the mammalian Complex I. Thick arrows describe rapid equilibria and rapid steady-state enzyme turnover. Thin arrows 1 and 2 describe slow reactions. E and E^* stand for active and inactive forms of the enzyme, respectively.

the slow activation of the enzyme as a slow reduction of oxidized Q to semiquinone coupled with the formation of the ubisemiquinone binding site which further operates as the enzyme acceptor site during the steady-state catalysis.

An important point depicted in Fig. 1 is that a combination of the reactions 1 and 2 gives a slow futile cycle of NADH oxidation, where the free energy of the $NADH + H^+ + Q \rightarrow NAD^+ + QH_2$ reaction is accumulated within the protein structure and dissipates during the spontaneous irreversible deactivation. The turnover of this futile cycle is evidently very slow (but strongly temperature-dependent) compare with further turnovers of the active form in the steady-state catalysis. The importance of the futile cycles at the substrate or enzyme (e.g., protein phosphorylation–dephosphorylation) level is now recognized as one of the key mechanisms of metabolic regulation (Koshland, 1984). The sensitivity of the slow activation to pH and divalent cations (in particular to Ca^{2+}) may also be of physiological importance, since mitochondria from a variety of tissues are able to accumulate considerable amounts of Ca^{2+} via an electrogenic $\Delta\mu_{H^+}$-dependent mechanism (Bygrave, 1977). When massive Ca^{2+} uptake occurs in the presence of inorganic phosphate, an alkalinization of the mitochondrial matrix would considerably increase the sensitivity of the activation/deactivation futile cycle to Ca^{2+}. Whether deactivated Complex I does exist in mitochondria under some physiological conditions or is just an artifact arising as the result of the experimental manipulation is not known. The kinetic parameters of the enzyme-active/inactive transition (Table II) certainly excludes the interconversion as part of the catalytic turnover; they do not, however, exclude the possibility that for any given set of parameters such as pH, Ca^{2+}, temperature, and ubiquinone/ubiquinol ratio, the steady-state level of enzyme activity in the mitochondria is determined by the equilibrium between the inactive and active enzyme forms.

ON THE POSSIBLE MECHANISMS OF THE REDOX-LINKED PROTON PUMPING ACTIVITY

The key point concerning the intramolecular events involved in the coupling of electron transfer between enzyme-bound redox groups with vectorial proton translocation remains largely a matter of guesswork. Several more or less detailed schemes which include NADH (Mitchell, 1966; Krishnamoorthy and Hinkle, 1988), FMN (Mitchell, 1966; Ragan, 1990; Krishnamoorthy and Hinkle, 1988; Ragan, 1987), and ubiquinone (Mitchell, 1979; Weiss and Friedrich, 1991) as specific proton-conducting enzyme components have been published mostly in an attempt to account for the variation of the H^+/e stoichiometry between 2 and 6 depending on whether the electron transfer is arranged in a classical loop or a cyclic sequence (Mitchell, 1966; Ragan, 1987). Here we will focus on the discussion of the possible involvement of classical hydrogen carriers, FMN and Q, in the proton translocation activity of Complex I.

Flavin. FMN, which is believed to be the most likely immediate oxidant of NADH, has not been characterized in any detail due to the spectral overlap with iron-sulfur chromophores. It is generally accepted that since NADH binds to the 51 kDa subunit (Chen and Guillory, 1981, 1984; Yagi and Dinh, 1990) and the three-subunit fragment derived from Complex I (FP) is still capable of NADH oxidation (Chen and Guillory, 1985; Galante and Hatefi, 1978; Hatefi and Stemplel, 1969), FMN is also bound to the same polypeptide; the only other possibilities left are the 24 kDa and 9 kDa subunits. It is clear that FP, being the simplest water-soluble component of Complex I, is not able to catalyze any proton conductive activity. However some electrochemical and binding properties of FN within the FP fragment may shed some light on the flavin participation as a hydrogen atom carrier across the osmotic membranous barrier. We found that the reduction of FP by NADH results in $FMNH_2$ dissociation in the diluted (10^{-9} M) enzyme solutions (Sled and Vinogradov, 1993). The dissociation is protected by externally added FMN or NAD^+. The apoprotein formed is unstable and rapidly and irreversibly inactivated; therefore an apparent midpoint redox potential of FMN could be determined from the dependence of the inactivation rate on the NADH/NAD^+ ratio. The value of −325 mV ($n = 2$), which is considerably more negative than that for the free FMN/$FMNH_2$ pair (Clark, 1960), was found from a simple $2e^-$ titration curve. Therefore, it appears that no electrochemical gap (i.e., no energy is available for proton translocation) exists between NADH and FMN, although the results should be taken with some reservations because of possible alterations of the flavin binding site during the drastic procedures used for the preparation of FP. The strong change in FMN affinity to its specific binding site upon reduction provides a clue to the possible movement of flavin within intact Complex I, where FP is apparently sheathed by other polypeptides and FMN release to the surrounding medium is protected. The translocation of the flavin from one site to another (specific for $FMNH_2$) during the redox catalysis is in accord with the basic requirement for the operation of a classical proton/electron conductive loop mechanism (Mitchell, 1966) with a H^+/e^- stoichiometry of 1 (see Fig. 2). The description of FMN-mediated NADH oxidation in a way similar to the Q-cycle mechanism in the b-c_1 region (Mitchell, 1975), where iron-sulfur centers N-1_a, N-3, and N-4 fulfull the functions of two cytochrome b

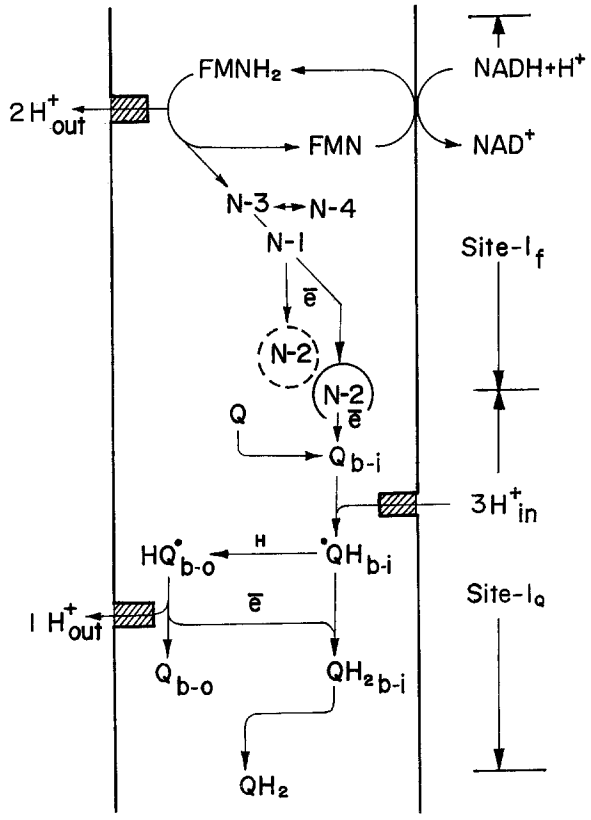

Fig. 2. The model for the cooperative redox-linked proton translocation at two coupling subsites (Site I_f and Site I_Q) in the NADH-ubiquinone reductase region of the mammalian respiratory chain. The sequence of electron transfer between FMN and iron-sulfur center N-2 mediated by the isopotential group of iron-sulfur centers N-1, N-3, and N-4 is not specified. The vertical thick lines indicate the osmotic barrier, where the redox components bound to the specific polypeptides are located. Q and Q_b stand for bulk and protein-bound ubiquinone, respectively. Shaded areas indicate the specific proton-conducting wells. Circled N-2 indicate Q_{b-i}-reactive (solid line) and unreactive (broken line) species of the N-2 sulfur center.

hemes and Rieske iron-sulfur protein, would increase the net stoichiometry to the value of 2 (Ragan, 1990). It should be emphasized that since the original suggestion (Mitchell, 1966), FMN was postulated as a hydrogen transmembranous carrier in several schemes describing the coupling mechanism in Site I (Krishnamoorthy and Hinkle, 1988; Ragan, 1987, 1990). At present, neither of them can be accepted or dismissed due to lack of experimental evidence.

Ubiquinone. During $2e^-$ reduction of Q, two protons should be accumulated within the hydrophobic phospholipid bilayer where bulk ubiquinone functions as a mobile carrier between the membrane-bound dehydrogenases and the rest of the respiratory

chain. Such electrochemical properties of quinones (Rich, 1982; Trumpower, 1981) make it somehow difficult to reconcile proton translocation step(s) with the quinone reduction which is the terminal phase of the overall reaction (1). To overcome this difficulty, several schemes have been proposed where $QH^{\cdot}/Q$ or $Q^{\cdot-}/QH_2$ couples were placed between some low-midpoint potential iron-sulfur centers (e.g., N-3) and the most positive N-2 cluster (Mitchell, 1979; Weiss and Friedrich, 1991). Due to thermodynamic reasons, this arrangment would require the redox potential of the quinone couple to be much more negative than that of N-2 [variable midpoint potentials of N-2 from −20 to −130 mV have been reported for different preparations bearing coupling Site I (Ohnishi, 1975, 1979; Yagi, 1990)]. The discovery of Q-binding proteins (King, 1990) greatly increased knowledge on ubiquinone participation as an obligatory component of the respiratory chain. Indeed, when protein-bound quinone/semiquinone/quinol is considered, the thermodynamic restrictions become vague because the specific protein environment can certainly alter the electrochemical properties of the prosthetic group just like what takes place when flavins or hemes are bound to flavoproteins or cytochromes. A 15 kDa Q-binding protein(s) has been isolated from bovine heart Complex I (Suzuki and Ozawa, 1986), and free radicals of Q upon reduction of Complex I by NADH, with EPR properties different from those of free ubisemiquinone, have been detected (Suzuki and King, 1983). The Site-I associated, rotenone-sensitive, N-2 spin-coupled ubisemiquinone(s) have been observed in tightly coupled submitochondrial particles during steady-state NADH oxidation in state 4 (i.e., under the conditions where $\Delta\mu_{H^+}$ inhibits the electron flow through Complex I (Burbaev *et al.*, 1989; Kotlyar *et al.*, 1990). The amplitude of the signal was proportional to the respiratory control ratio, and the signal dissappeared in the presence of uncouplers (Kotlyar *et al.*, 1990). The cross-over type of this behavior suggests that oxidation of $OH^{\cdot}$ or $Q^{\cdot-}$ species stabilized at the specific binding site(s) located close to the rapidly relaxing N-2 center, is coupled with an energy accumulation step. Starting from the pioneering ideas of King on the Q-binding proteins (King, 1990) and taking into account energetically favorable ubisemiquinone dismutation (Kroger, 1976), we have proposed a mechanism for proton translocation at the terminal region of Complex I, which is depicted as Site I_Q in Fig. 2. According to our model two electrons are transferred sequentially from N-2 to bound Q, and two protons are taken up from the mitochondrial matrix through the proton-conducting well to produce two bound protonated ubisemiquinones with different midpoint redox potentials ($QH^{\cdot}_{b-o}$ and $QH^{\cdot}_{b-i}$). At this stage the subsequent reduction of $QH^{\cdot}_{b-i}$ by the next electron delivered by N-2 is blocked. The next step is deprotonation of $QH^{\cdot}_{b-o}$ leaving H^+ outside (again through the proton conducting well) and the vectorial movement of $Q^{\cdot-}_b$ against the electric field driven by the electrochemical gap between $(Q^{\cdot-}/Q)_i$ and $(QH^{\cdot}/QH_2)_o$ pairs bound at the specific sites. The dismutation resulting from bimolecular interaction of the protein-bound semiquinones is accompanied by uptake of a third proton fom the matrix side and generates QH_{2b-i} which further dissociates or interacts with the bulk Q, producting bulk QH_2. At this stage the enzyme bears two bound Q_{b-i} and Q_{b-o} and the cycle can be repeated, starting from the reduction of Q_{b-o} by N-2. The catalytic cycle would translocate one proton per $2e^-$ transferred from N-2 to the Q pool. Operating together, two subsites (flavin-mediated and Q-mediated) would give a net stoichiometry $H^+/2e$ of 3. This value seems to be in accordance with the published data on the stoichiometry of net vectorial H^+ translocation at Site I (Lawford and Garland, 1971; Lemasters, 1984; Scholes and Hinkle, 1984; Wilkstrom, 1984). The scheme, as depicted in Fig. 2, should be considered as our current working hypothesis which provides a basis for experimental verification. The existence of two distinct protonated ubisemiquinone molecules with different locations, different midpoint redox potentials, and different spin–spin interaction with N-2 is an essential part of the proposed mechanism. Some preliminary results on two $\Delta\mu_{H^+}$-dependent Site I-associated ubiquinone species have been reported (Vinogradov *et al.*, 1991). It would be of a great interest to study the spatial distribution between N-2, $OH^{\cdot}_{b-o}$, and $QH^{\cdot}_{b-i}$ during the tightly coupled steady-state NADH oxidation. The scheme also postulates the existence of two specific proton-conductive wells, which may account for the DCCD inhibitory effect on electron transfer and proton-translocation activities of Complex I (Vuokila and Hassinen, 1988; Yagi, 1987).

CONCLUSIONS AND PERSPECTIVES

Despite the remarkable progress in the structural

studies on Complex I, the functions of the individual subunits in catalysis of the overall reaction are very far from being understood. Undoubtedly, modern biophysical methods together with molecular biological approaches seem to provide promise for better understanding the enzyme mechanism. However, in the author's opinion, the classical biochemical approach, i.e., resolution and reconstitution studies, is still the most powerful tool for further progress in the field. During the last decade one part of this strategy (resolution) has been greatly advanced, whereas the other part (reconstitution) was and still is a challenge for biochemists. In fact, the reconstitution of any original function from resolved components of Complex I is yet to be achieved. It is not surprising, due to the extreme complexity of the native enzyme. However as the outstanding authority in reconstitution studies stated, "...it should be possible under favorable conditions to take out a component from the system and put the component back to reconstruct the original set *in spite of an almost infinite number of other possibilities*" (King, 1966).

Another problem concerning structure–function interrelations in the mammalian Complex I can be formulated as follows: Why is such a complex machinery used for the catalysis of the proton-translocating NADH-ubiquinone reductase reaction, whereas much simpler structures with apparently the same function are operating in prokaryotic cells (Yagi, 1987)? The answer may be very simple: Not all the functions of Complex I are presently known. The surprising discovery of an acyl carrier protein as a subunit of *N. crassa* and mammalian Complex I (Runswick *et al.*, 1991; Sackmann *et al.*, 1991) show that several other new enzymatic activities of Complex I may be expected.

Finally, it is worth noting that the perfection of enzymes resulting from natural selection consists not only of their catalytic effectiveness (Burbaum *et al.*, 1989; Knowles and Albery, 1977) but also (and perhaps more importantly) of their remarkable function as the regulatory device in the cell (Koshland, 1984). It is hard to accept that the main entry point for reducing equivalents into the respiratory chain is not a subject of refined metabolic control. Our knowledge of this aspect remains at the primitive stage, and new discoveries of the regulatory mechanisms of the mammalian Complex I are expected.

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