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Catalytic properties of the mitochondrial NADH–ubiquinone oxidoreductase (Complex I) and the pseudo-reversible active/inactive enzyme transition

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1. Introduction

In the comprehensive review entitled 'The DPNH dehydrogenase of the mitochondrial respiratory chain' written more than quarter of a century ago, the authors stated: ''Thanks to the application of sophisticated techniques, the characteristics of this unusual and interesting enzyme are now understood, at least in broad terms, long-standing debates in the literature have been satisfactorily resolved, and the way now seems clear for the solution of the remaining problems ...'' [1]. Five years later another scholar of Complex I has pointed out that ''...since no real breakthrough in the area have occurred since 1963, it is difficult to find a fresh approach to the subject'' [2]. Both cited statements still appear to be correct. Indeed, recent spectacular progress in the structural studies of the mammalian [3], fungal [4] and prokaryotic [5] proton-translocating NADH–quinone reductases reveals an outstanding complexity of this multi-subunit and multi-redox component enzyme. It has been pointed out that the total protein sequence in more than 40 different subunits of the mammalian Complex I exceeds the combined sequences of the constituent polypeptides of prokaryotic ribosome [3]. The enzyme bears up to 10 different redox components: FMN [6], up to seven distinct iron–sulfur clusters [7–10] and at least two bound ubiquinone species [11]. Their operational sequence in electron transfer from NADH to bulk ubiquinone is not known. Thus, the key question of how the intramolecular enzyme redox chemistry is coupled with the vectorial proton translocation remains a matter of speculation compared to the recent detailed understanding achieved for Complex III [12,13] and cytochrome oxidase [14,15].

Reviews during the recent years have focused on the structure [3–5], iron–sulfur clusters [10], possible mechanisms of proton translocation [16] and the comparative molecular biology of the enzyme [3,17]. In the author's opinion, however powerful the modern

Abbreviations: SMP, submitochondrial particles; FP, three subunit iron–sulfur–flavoprotein derived from Complex I; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; Q, ubiquinone-10; QH_2, ubiquinol-10; Q_n, homologues of ubiquinone having *n* isoprenoid units in position 6 of quinone ring; DB, 2,3-dimethoxy-5-methyl-6-decylbenzoquinone; PB, 2,3-dimethoxy-5-methyl-6-pentylbenzoquinone; HAR, hexammineruthenium(III); NEM, *N*-ethyl-maleimide; rhein, 9,10-dihydro-4,5-dihydroxy-9,10-dioxo-2-anthracene carboxylic acid

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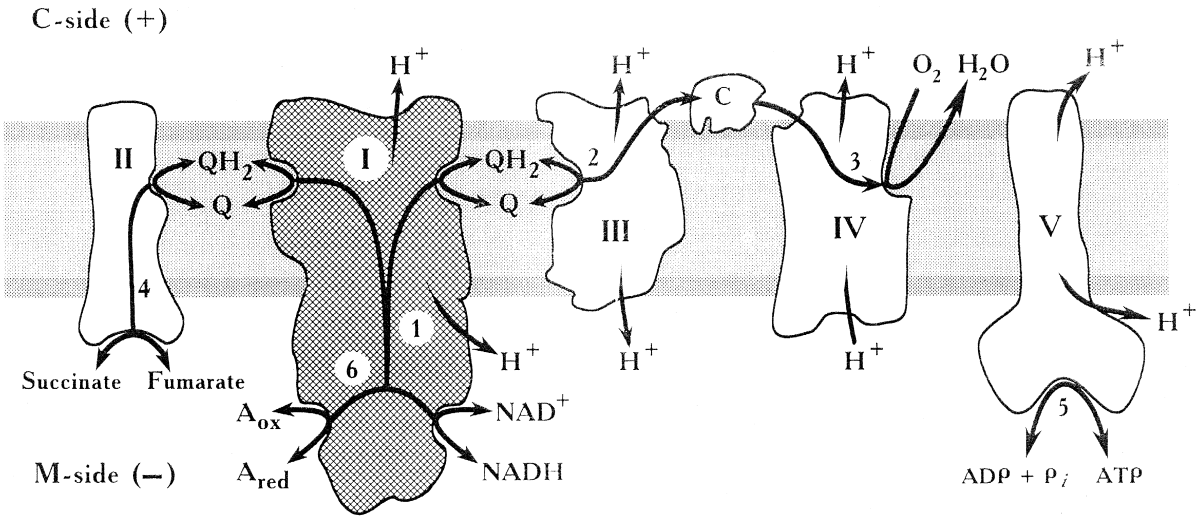

Fig. 1. Schematic presentation of the concerted operation of Complex I (shadowed) within the inner mitochondrial membrane. Thick arrows indicate electron and transmembranous proton transfer pathways. Roman figures stand for numbering of complexes according to the generally accepted nomenclature [18]. The figures indicating thick arrows correspond to the reaction numbers listed in the third column of Table 1.

molecular biology techniques are, only their combination with studies on the functional properties of the enzyme will eventually provide the complete information needed to understand its molecular mechanism and physiological regulation.

This mini-review will attempt to summarize available information on the catalytic properties of the enzyme with a special emphasis on the hysteretic behaviour of the bovine heart mitochondrial Complex I. It is the author's goal to emphasize in this review the complexities in interpretation and difficulties in the reliable catalytic assays of the mammalian Complex I.

2. Activities of the respiratory chain-linked NADH–ubiquinone oxidoreductase

The diagram shown in Fig. 1 depicts the simple text-book presentation of the mammalian respiratory

Notes to Table 1:

[a]Arrows → and ← above figures stand for the forward and reverse reactions, respectively. NADH, succinate, ubiquinol and cytochrome *c* oxidation and ATP synthesis are considered as the forward reactions.

[b]Only rotenone is included as the specific inhibitor acting on Complex I 'Q-junction site'. More than a dozen of other rotenone-like inhibitors are known (see Refs. [19–23] for cross references). Rhein [24,25] and more recent ADP-ribose [26] were reported as the competitive inhibitors of NADH binding, thus all the activities with NADH as the substrate are expected to be sensitive to rhein and ADP-ribose.

[c]The NADH oxidase (two electron equivalents) catalyzed by completely uncoupled AS–SMP at 25°C (pH 8.0) is taken as arbitrary unit (1.00). This corresponds to the average specific activity routinely measured in our laboratory of about 1 μmol of NADH oxidized min^{-1} mg^{-1} protein [27]. The relative figures presented in this column should be considered as provisional. The actual values ($\pm$5–20%) depend on a number of factors, such as ionic composition of the assay mixture, respiratory control ratio of particular preparation, etc. The relative activities are referred to those measured under 'optimal conditions'.

[d]Aerobic NADH oxidation seen either without added electron acceptors (NADH–dehydrogenases) or in the presence of either rotenone or antimycin A, or myxothiasol or cyanide (SMP, Complex I).

[e]Type 1 dehydrogenases, high molecular mass enzyme preparations solubilized from the membranes by treatment with detergents or phospholipase [28–33]. The preparations are free of phospholipids and ubiquinone and in contrast to Complex I are rotenone-insensitive due to either preparative modification or deficiency in some subunits. Type 2 dehydrogenases, low molecular mass three-subunit iron–sulfur flavoprotein catalyzing oxidation of NADH by artificial electron acceptors [34–36]. The enzyme is evidently a modified fragment of Complex I or Type 1 dehydrogenases.

Table 1
Reactions catalyzed by the preparations of the bovine heart mitochondrial NADH–ubiquinone oxidoreductases different degree of resolution

	Reaction	Reaction pathway according to numbering of partial reactions shown in Fig. 1[a]	Preparations capable of the reaction	Specific inhibitors[b]	Relative specific activity in submitochondrial partial (SMP)[c]
1	NADH–oxidase	$\vec{1} \rightarrow \vec{2} \rightarrow \vec{3}$	Intact mitochondria; SMP	Rotenone; antimycin A; cyanide	1.00[c]
2	NADH–cytochrome *c* reductase	$\vec{1} \rightarrow \vec{2}$	Resolved NADH–cytochrome reductase	Rotenone; antimycin A; myxothiasol	0.04
3	NADH–ubiquinone reductase	$\vec{1}$	Intact mitochondria; SMP; Complex I	Rotenone	1.00
4	NADH–artificial acceptor reductase	$\vec{6}$	SMP; Complex I; Type 1 and Type 2 NADH dehydrogenases[d]	None	
	(a) ferricyanide				9.00
	(b) hexammineruthenium(III);				10.00
	(c) O_2 (superoxide generation)[e]				0.005
5	NADH–acylpyridine NAD^+ (transhydrogenase)	$\vec{6}$ (?)	SMP; Complex I; Type 1 and Type 2 NADH dehydrogenases	None	1.30
6	NADH–fumarate oxidoreductase	$\vec{1} \rightarrow \overleftarrow{4}$	SMP	Rotenone; all the inhibitors of Complex II	0.015
7	Reverse electron transfer				
	(a) ATP-driven reduction of NAD^+ by succinate	$\vec{1} \rightarrow \overleftarrow{4}$ coupled with $\overleftarrow{5}$	Intact mitochondria; coupled SMP in presence of the ubiquinol oxidase inhibitors	Rotenone; all the inhibitors of complex II; inhibitors of F_o-F_1–ATPase; uncouplers	0.25
	(b) ATP-driven rotenone-senstive ferricyanide reductase	$\vec{4} \rightarrow \overleftarrow{6}$ coupled with $\overleftarrow{5}$	Coupled SMP	The same as 7a	0.27
	(c) Succinate-supported NAD^+ reduction ($\Delta\bar{\mu}_{H^+}$-dependent ubiquinol–NAD^+ oxidoreductase; aerobic reverse electron transfer)	$\overleftarrow{1}$ coupled with $\vec{4} \rightarrow \vec{2} \rightarrow \vec{3}$	Intact mitochondria; coupled SMP	The same as 7a except for Fo–F1–ATPase inhibitors	0.25

Table 2
Relative affinities of nucleotides to the enzyme in forward and reverse reactions [37,38,26] (SMP[a], pH 8.0, 25°C)

	Reaction		
	$NADH \rightarrow Q$[b]		$QH_2 \rightarrow NAD^+$[c]
	Coupled	Uncoupled	
K_m (μM)	7 (NADH)	7 (NADH)	7 (NAD^+)
K_i (μM)		1.250 (NAD^+)	40 (NADH)
K_i for ADP-ribose (μM)[d] (competitive inhibition)	24	25	no inhibition

[a]AS-submitochondrial particles [27] with a respiratory control ratio of 7.
[b]In the presence of antimycin A and 0.1 mM Q_1.
[c]ATP-driven, succinate-supported reverse electron transfer.
[d]NADH oxidase reaction.

chain composed of five well-defined complexes (I–V according to the nomenclature by Green's school [18]), which may or may not be involved in the catalytic activity of Complex I per se depending on the assay employed. Some general properties of the complete NADH oxidase and partial reactions catalyzed by Complex I within the inner mitochondrial membrane or by the preparations at different degrees of resolution are summarized in Table 1 and briefly discussed below. The important point to be emphasized is that all the reactions which involve ubiquinone oxidoreduction site(s) (1–3, 6, 7, Table 1) demonstrate abnormal kinetics unless the enzyme preparations are subjected to reductive–oxidative pulse (activation) before assay (see Section 4). The data collected in Tables 1 and 2 are characteristics only for fully activated Complex I.

2.1. NADH oxidase

Oxidation of the intramitochondrial NADH by diatomic oxygen consists of concerted operation of three individual complexes (Fig. 1). The turnover number of Complex I within the complete respiratory chain of uncoupled submitochondrial particles (SMP) at 25°C is around $1 \cdot 10^4$ min^{-1}, assuming an average NADH oxidase activity of 1 μmol NADH oxidized min^{-1} mg^{-1} protein and the enzyme content (FMN-based) of 0.1–0.15 nmol mg^{-1} protein. The iron–sulfur clusters of Complex I are almost completely reduced under the steady-state aerobic coupled or uncoupled NADH oxidation [39] thus indicating that Complex I is not rate-limiting during NADH oxidation by the respiratory chain. The rotenone-sensitive NADH–external Q_1 oxidoreductase activity in SMP is close to or even lower than that of NADH oxidase. Hence, oxidation of the terminal-reduced component of Complex I by the endogenous ubiquinone appears to be the rate-limiting step of the overall NADH oxidase.

Oligomycin, a specific inhibitor of the F_o proton-conductive part of ATP synthase, causes strong inhibition of respiration in some types of SMP which is completely relieved by uncouplers [40] thus inducing a state of 'respiratory control'. NADH oxidase of SMP routinely prepared in the author's laboratory show the oligomycin-induced respiratory control ratio of 5–10, depending on the particular batch of heavy mitochondria from which SMP are prepared.

NADH oxidase of coupled SMP (in the presence of oligomycin) is a useful assay system for studies of Complex I under conditions where the enzyme operates as a proton-pump.

2.2. NADH–cytochrome c oxidoreductase

This activity involves simultaneous operation of Complex I and III and it is seen only in the resolved preparations of the respiratory chain obtained after treatment with the detergents [41]. Cytochrome *c* is located on the external surface of the inner mitochondrial membrane (in the intramembranous space of intact mitochondria) and thus the inside-out SMP do not catalyze the antimycin A-sensitive NADH–cytochrome *c* reductase reaction. The reaction does occur in the classical Keilin–Hartree heart muscle SMP preparations [42] where NADH oxidase activity is stimulated 2–3 times by externally added cytochrome

c [43]. Since the inner mitochondrial membrane is not permeable for NADH, antimycin A-sensitive NADH–cytochrome *c* reductase activity can be taken as a measure of 'open' membrane fragments present in a particular SMP preparation. The antimycin A-sensitive NADH–cytochrome *c* reductase is clearly different from the inhibitor-insensitive NADH–cytochrome *c* reductase activities catalyzed by some soluble preparations of NADH dehydrogenase [28–36]. The latter are evidently the fragments of intact Complex I and in the reactions catalyzed by those preparations cytochrome *c* serves just like other artificial electron acceptors (see Section 2.4.1).

2.3. NADH–ubiquinone oxidoreductase

Due to insolubility of the natural ubiquinones in aqueous solutions the 'minimal natural' reaction is usually followed using their artificial homologues (Q_0, Q_1, Q_2) or analogues (PB, DB, duroquinone) [44,45] as electron acceptors. The proton translocation coupled with NADH–artificial quinone oxidoreductase was originally observed in intact mitochondria [46], Complex I containing liposomes [47] and recently in SMP [48].

A straightforward interpretation of the initial rate — acceptor concentration dependence (the standard approach in enzymology) for both purified dispersed and membrane-bound Complex I is far from being trivial.

First, it is not known whether the reaction between added acceptor and the enzyme terminal electron donor includes binding of external quinone at the specific site. Reoxidation of the Q_{10} pool (SMP) or of tightly bound ubiquinol (Complex I) by added water soluble quinones or both may equally be operative during steady-state NADH oxidation.

Second, depending on the particular quinone acceptor and its concentration, a significant fraction of the overall reaction is rotenone-insensitive. This is in contrast to the NADH oxidase reaction which shows a residual rotenone-insensitive activity of no more than $\approx 1\%$ (the latter is, at least partially, due to the direct reaction of dioxygen with some redox component(s) of the enzyme). The rotenone-insensitive fraction depends linearly on the concentration of added quinone (within the solubility limits), and thus the true saturation characteristics of the rotenone-sensitive reaction (K_m, V_{max}) cannot be obtained by simple algebraic substraction. Additional difficulties lies in the strong difference between the temperature dependencies of the rotenone-sensitive and rotenone-insensitive reactions. For example, in the presence of 90 μM Q_1 at 20°C NADH oxidation catalyzed by isolated Complex I is 35% rotenone-sensitive and at 38°C the reaction is 92% rotenone-sensitive, whereas the overall NADH–Q_1 reductase activities at those temperatures are 0.34 and 2.8 μmol NADH oxidized min^{-1} mg^{-1} protein, respectively.

The third complication in quantitative and qualitative interpretation of the NADH–quinone reductase reaction is that the diagram shown in Fig. 1 may oversimplify the real operative mechanism of the respiratory chain. Several lines of evidence suggest that the direct interaction between Complex I and Complex III exists [49,50]. The natural ubiquinone reductase reaction may be a result of concerted one-electron reduction of Q or $Q^{\cdot}$ by the dehydrogenase itself which produces either ubisemiquinone or ubiquinol and by cytochrome *b* at center *i* of Complex III, which provide the second electron to complete the two-electron reduction of original quinone to quinol. In fact such a mechanism has been postulated in the original version of Mitchell's Q-cycle [51]. The observations of partial inhibition of NADH–quinone reductase activities [52,53] by several specific inhibitors of Complex III are in line with such a proposal, although the hypothetical structural similarity of the ubiquinone/ubiquinol binding sites shared by Complexes I and III [53,54] can also explain this partial inhibition.

Because of the growing interest in NADH dehydrogenase activity alteration during the ageing process and diseases involving Complex I [19,55] some indirect calculation-based methods for the measurements of true NADH–ubiquinone reductase activity have been offered [56,57]. However, the reliable quantitative measurements of the enzyme activity is still problematic.

2.4. NADH–artificial acceptor reductases

2.4.1. Ferricyanide reductase

SMP, Complex I, Type 1 and Type 2 NADH dehydrogenases catalyze rapid reduction of ferricyanide by NADH. This one-electron acceptor has

been routinely used in the earlier numerous studies directed to isolation of pure enzyme [58]. The reaction is evidently abbreviated compared to the natural electron pathway. What particular component of the enzyme that donates electrons to ferricyanide is not known, although there are strong reasons to believe that FMN is the reactive site since the reaction is readily catalyzed by the simplest catalytically competent fragment of Complex I, Type 2 dehydrogenase [36], containing the iron–sulfur clusters with the midpoint redox potential of −410 mV and −465 mV [59], i.e., not reducible by NADH. The kinetic characteristics of the NADH–ferricyanide reductase reaction differ qualitatively for preparations of different degree of resolution. In SMP and Complex I, the reaction is strongly inhibited by excess of substrate (NADH). The standard graph of the dependence of initial rate on NADH concentration dependence at any fixed ferricyanide concentration appears as a steep curve with the maximum position which depends on the acceptor concentration [60]. Such a behaviour has been interpreted as competition between ferricyanide and NADH for a common redox site (presumably FMN) located in a deep cleft within the quaternary protein structure [60]. Indeed, the soluble FP shows much less pronounced steepness of those curves [61], however, the enzyme turnover number calculated on the basis of FMN content in FP is about 10 times less than that in the parent Complex I or SMP [61]. The specific activity of NADH–ferricyanide reductase catalyzed by FP is significantly increased in the presence of guanidine chloride [62]. It should be noted that the turnover numbers of the enzyme in the NADH–ferricyanide reductase reaction are controversial, because of extreme difficulties in the reliable double extrapolation to V_{max}. The actual kinetic data appear as a set of steep curves which can hardly be approximated as hyperbolaes within any range of substrate and acceptor concentrations. It is worth noting that the non-enzymatic oxidation of NADH by ferricyanide becomes significant when the concentrations of the latter reaches the millimolar range.

2.4.2. Hexammineruthenium(III) reductase [38,63]

Similar to ferricyanide, hexammineruthenium III (HAR) was found to be an efficient electron acceptor for the enzyme in inside-out SMP and isolated Complex I, but not in intact mitochondria [38]. The reaction is rotenone-insensitive and is not coupled with proton-translocation. No inhibition of the reaction by either NADH or by the electron acceptor was revealed in a wide concentration range. For SMP or Complex I the double-reciprocal plots $1/v$ versus $1/\mathrm{NADH}$ at various HAR concentrations give a series of straight lines intersecting in the third quadrant, thus supporting a mechanism in which the reduced enzyme–NAD^+ complex is oxidized by HAR through the operation of a single redox site before NAD^+ dissociation.

Type 2 (three subunits iron–sulfur flavoprotein) dehydrogenase is also highly reactive with this electron acceptor [63]. In fact, HAR is the only artificial acceptor which reveal the same turnover numbers of the enzyme based on FMN content in SMP, Complex I and FP. In contrast to ferricyanide reduction, guanidine does not stimulate but inhibits the NADH–HAR reductase activity of FP competitively with the electron acceptor. Due to the simplicity of quantitation of the kinetic data, the highest reactivity towards the enzyme, absence of spectral overlap with NADH and absence of a non-enzymatic reaction, HAR seems to be a superior choice among a variety of artificial electron acceptors used for the enzyme assay in the resolution studies.

2.4.3. Superoxide generation

Oxygen, strictly speaking, is not an 'artificial' electron acceptor. Direct reduction of O_2 is perhaps the main source of ROS (reactive oxygen species) in mitochondria. The latter have received a considerable attention recently because of their possible participation in destructive damage of mitochondrial DNA, some enzymes and membranes. The production of H_2O_2 by autooxidation at the first coupling site in mitochondria was reported many years ago [64]. $O_2^{\cdot}$ (superoxide anion) is a stoichiometric precursor of the mitochondrial H_2O_2 production [65] and one-electron reduction of dioxygen by some components of the respiratory chain was estimated to account for up to 2% of the respiration under physiological conditions [66]. The superoxide dismutase (EC 1.15.1.1)-sensitive NADH- and NADPH-supported oxidation of adrenaline or reduction of acetylated cytochrome *c* with the specific activity of about 1 nmol of $O_2^{\cdot}$ min^{-1} mg^{-1} protein at 30°C was ob-

served in antimycin A- and rotenone-inhibited SMP [67]. The site of superoxide production in Complex I have not been identified; potentially all the enzyme redox components: FMN [68], iron–sulfur clusters [69] and ubisemiquinone [70] can generate the superoxide radical. In antimycin A-treated SMP the effect of rotenone is biphasic: an increase in $O_2^{\cdot}$ production was observed up to an inhibitor concentration well above the level required to inhibit NADH oxidase (0.3 nmol/mg of protein), and a decrease at higher concentrations [67]. Formation of superoxide radical in both SMP and isolated Complex I with NADH and NADPH is biphasic in the double reciprocal plot (1/rate versus 1/nucleotide concentration) showing two apparent K_m for the substrates [71,72]. Rotenone- and uncoupler-sensitive $O_2^{\cdot}$ generation by ATP-driven reverse electron transfer from ubiquinol to some unidentified component was reported [64,73]. The rate of the energy-dependent reaction was about 20% of that observed in the presence of NADH plus rotenone and it was strongly stimulated (4–5 times) by NAD^+ [73]. Bound $NAD\cdot$ radical was proposed as the source of superoxide in the reverse electron transfer reaction as it has been shown for other NAD-dependent dehydrogenases [74,75].

It worth noting that the specific activity of Complex I in the superoxide generating reactions is about 3 order of magnitude less than that in the NADH–ubiquinone reductase or NADH oxidase (SMP) and the reaction is certainly not suitable for catalytic assay of the enzyme activity. However, $O_2^{\cdot}$ and H_2O_2 (the product of the superoxide dismutase reaction) are potentially involved in numerous physiological and pathophysiological processes such as signal transduction or oxidative damage [76,77]. If the superoxide generation is to be considered as the 'dangerous leak', the functional role of some enzyme subunits which are not directly involved in the electron/proton transfer reaction may be explained: they could serve as a protective 'wrapping' of the low potential redox components to prevent a direct reaction with oxygen. This would also explain why artificial electron acceptors react at the entry point or at ubiquinone reactive site (Q_1, duroquinone) but not at intermediate sites. In addition inhibitors interrupting electron transfer between iron–sulfur centers have not been found so far: apparently the system is well-protected. The superoxide generation by SMP or Complex I is evidently different from that catalyzed by the soluble FP in the aerobic rotenone-insensitive NADH–quinone reductase reaction [78].

2.5. Transhydrogenase and other $NADP^+$/NADPH-dependent reactions

The inner mitochondrial membrane contains two enzymes capable of transhydrogenase activity. One is the proton-pumping transhydrogenase containing no redox prosthetic groups (EC 1.6.1.1) catalyzing a direct hydride transfer between the 4A hydrogen of NADH and 4B hydrogen of NADPH coupled with vectorial translocation of H^+ [79]. In addition to the activities discussed in Section 2.4, Complex I and some simpler preparations catalyze 4B stereospecific FMN-mediated tritium–H_2O exchange [80,81] and oxidation of NADH by acetyl pyridine NAD^+ [62]. It seems pertinent to note that transhydrogenase activity is not a general property of NADH(NADPH)-oxidizing enzymes: for example DT-diaphorase (NADH/NADPH–quinone oxidoreductase (EC 1.6.99.2), a homodimeric FAD containing enzyme catalyzing oxidation of either NADPH or NADH [82,83] is not capable of transhydrogenation. The transhydrogenase activity of Complex I suggests by itself that the redox potential of FMN is close to that of the substrate/product pyridine nucleotide pair. If the reduction of flavin by NADH would be thermodynamically irreversible the large redox gap between the reduced enzyme and oxidized pyridine nucleotide would create considerable restrictions in the subsequent electron transfer from the enzyme to the nucleotide substrate. Indeed, the midpoint redox potentials of FMN measured directly for Complex I [84] and estimated indirectly for FP [85] are close to that for NADH/NAD^+ pair.

SMP catalyze rotenone-sensitive NADPH oxidation in complete absence of transhydrogenase (EC 1.6.11) activity at much lower rate compare to that for NADH [86,87]. NADPH dehydrogenase activity increases at acidic pH (below 6.0) in contrast to NADH oxidation (broad pH optimum at pH 7.5–8.2). NADH → acetyl pyridine nucleotide activity is seen with SMP, Complex I, Type 1 and Type 2 dehydrogenases [62,88]. The Type 2 enzyme catalyzes the NADPH → NAD^+ reaction whereas no NADH →

$NADP^+$ or $NADPH \rightarrow NADP^+$ activities could be detected [62]. Tightly coupled bovine heart SMP catalyze energy-dependent, succinate-supported, rotenone sensitive stereospecific [4B-^{3}H]NAD^+ and $NADP^+$ reduction [79,86,89] which is believed to be a simple reversal of the proton-translocating NADH–ubiquinone reductase reaction (see below). NADPH reduces FMN and Fe–S clusters of the enzyme. The observation of different reduction of some Fe–S clusters and studies on inhibition of NADH and NADPH oxidation by stoichiometric amounts of the inhibitor piericidin has led Bakker and Albracht [90], van Belzen and Albracht [91], and van Belzen et al. [92] to propose a heterodimeric structure for Complex I which is composed of NADH-specific and NADPH-specific protomers [90–92]. More recently, the same experimental results have been reinterpreted to suggest a monomeric structure of the enzyme containing two FMN (and presumably two nucleotide binding sites) [93]. Studies of NADH- and NADPH-induced lipid peroxidation in SMP have led to the conclusion that Complex I is able to distinguish between electron input from NADH and NADPH by differences in the substrate binding sites [94].

The largest subunit of FP which contains FMN and the iron–sulfur cluster N-3 has been identified to contain a single NADH-binding site in the mammalian and bacterial Complex I [95–98]. On the other hand, since FP is capable of transhydrogenase activity it was suggested that FP contains two closely related active sites: site 1 for NADH and NADPH dehydrogenation and site 2 for binding a second nucleotide for transhydrogenation [62]. Most recently a model suggesting two different nucleotide binding sites operating in the direct and reverse electron transfer reactions has been proposed [26]. This model is based on the finding of 'unidirectional' effect of ADP-ribose, which inhibits NADH oxidation in SMP, Complex I and FP competitively and stimulates the aerobic succinate-supported energy-dependent reverse electron transfer [26]. It is pertinent to note that in addition to the conserved primary sequences of the bovine 51 kDa subunit [3] and its *Paracoccus denitrificans* [5] and *N. crassa* [99] counterparts recognized as the most likely candidates for NADH-binding site, another segment of sequence that fits to the nucleotide binding site has been identified in 39 kDa subunit of bovine Complex I [3] and in the homologues 40 kDa subunit of *N. crassa* complex [100].

Summarizing the discussion on NAD and NADP reactivity it should be emphasized that the questions of how many nucleotide binding sites are present in Complex I and (if more than one) their specificity and cooperation (if any) remain and should be experimentally verified.

2.6. NADH–fumarate oxidoreductase

Since the midpoint redox potential of succinate/fumarate couple (≈ 0.00 V) is much more positive than that of $NADH/NAD^+$ (≈ -0.32 V) [101] it is expected that fumarate must be an efficient electron acceptor for NADH oxidation provided that catalytic mechanism for the equilibration exists. Indeed it has been shown that when oxidation of ubiquinol is blocked, SMP exhibit NADH–fumarate reductase reaction which is sensitive to all the inhibitors of Complex I and Complex II. In coupled SMP oxidation of NADH by fumarate was shown to drive ATP formation [102]. The absolute specific activity of SMP in this reaction is quite low (0.015 moles min^{-1} mg^{-1} protein at 25°C in the presence of myxothiasol which is about 1.5% of NADH–ubiquinone reductase, and comparable with the rotenone-insensitive NADH-induced superoxide generation) and it is not stimulated by uncouplers. There are several apparent reasons for such low activity. First, the kinetic properties of the mammalian Complex II which is capable of fumarate reductase activity, is evolutionally adjusted for the catalysis of the physiologically significant direction i. e. succinate oxidation [103]. An interesting diode-like behaviour of succinate dehydrogenase has been demonstrated and interpreted as the kinetic restrictions for substrate binding (or product release) under the conditions of overpotential driving force [104]. Second, in the steady-state NADH–fumarate reductase reaction the ubiquinone pool is reduced. Not only does this decrease the availability of an electron acceptor for Complex I, but also ubiquinol inhibits NADH–ubiquinone reductase (unpublished observation from our laboratory). It appears that the ubiquinone/ubiquinol ratio within the membranous bulk lipid phase regulates the activities of Complexes I and II not only by the availability of the electron

acceptors (Q) but also by a product (QH_2) inhibition [105,106] and by the redox potential-controlled gate mechanism [104].

2.7. Reverse electron transfer

The reversibility of the electron transfer reactions at the first coupling site of the respiratory chain was discovered almost forty years ago when Chance and Hollunger [107] and independently Klingenberg and Slenczka [108] have shown that addition of succinate or α-glycerophosphate to mitochondria in State 4 respiration causes an uncoupler-sensitive reduction of the intramitochondrial pyridine nucleotides. An excellent account of the earlier classical works on the subject can be found in Refs. [109,110]. The general properties of the reaction which is now widely used for studies of Complex I as well as for the reversibility of proton translocating F_o–F_1 ATP synthase are briefly summarized below.

2.7.1. ATP-driven reduction of NAD by succinate

As shown in diagram presented in Fig. 1 and Table 1 the overall reaction in SMP is the result of simultaneous operation of three enzyme complexes: Complex II which provides electrons for ubiquinone reduction, Complex V (reversible F_o–F_1 ATPase) which generates $\Delta\mu_{H^+}$, and Complex I which utilizes $\Delta\overline{\mu}_{H^+}$ for the ubiquinol–NAD^+ reductase reaction under the conditions where the ubiquinol oxidase activity is blocked by inhibitors of Complex III or Complex IV. In contrast to the relatively invariable specific activities of different SMP in the NADH–ubiquinone reductase or NADH oxidase reactions considerable variations in the specific activity of ATP-dependent reverse electron transfer are scattered in the literature. Those variations are evidently due to the differences in proton leakage for different SMP preparations [111] and perhaps even more significantly to the properties of each enzyme participating in the reaction. Succinate dehydrogenase (Complex II) is known to exist, at least partially, in the tightly bound oxaloacetate-deactivated form in most preparations of SMP [112,113]. Prolonged incubation (10–30 min) in the presence of either the substrate (succinate) or a competitive inhibitor (malonate) at rather high temperature (20°–30°C) prior to assay is needed to displace the tightly bound oxaloacetate and to reach full catalytic capacity of the enzyme [112–114]. F_o–F_1 ATPase is also present in most types of SMP in the ADP(Mg^{2+})-deactivated state and shows considerable lag-phase of the 'initial' rate of ATP hydrolysis in the presence of an ATP-regenerating system added in the 'kinetic' excess [115], phosphoenol pyruvate and pyruvate kinase (EC 2.7.1.40) are routinely used to prevent accumulation of strongly inhibitory ADP [116]). It should be noted that most commercial preparations of pyruvate kinase are contaminated with lactate dehydrogenase (EC 1.1.1.27). The latter enzyme, if present, uses stoichiometrically pyruvate formed in the pyruvate kinase reaction to oxidize the NADH formed during ATP hydrolysis thus leading to an underestimation of the reverse electron transfer rate. Complex kinetic behaviour of ATP hydrolysis is also expected for those SMP preparations which contain an ATPase protein inhibitor [117], an intrinsic component of ATP synthase which interacts with F_1 in very complex strongly ionic strength-, pH- and time-dependent fashion [118]. Also as it will be discussed in Section 4, Complex I itself in its deactivated form shows complex kinetic behaviour. In the author's opinion, the previously reported abnormalities of the ATP-dependent reverse electron transfer reaction [89,119] were due to an interplay of the factors listed above. It appears that the preparation of choice for quantitative studies of the reverse electron transfer is so-called AS-SMP [120] which are essentially free of protein inhibitor and partially deficient in F_1. These particles can be artificially coupled by either substoichiometric amount of oligomycin [40] (for the ATP-dependent reaction) or by the excess of the antibiotic (for the aerobic ATP-independent reaction). Under appropriate conditions coupled AS-SMP catalyze ATP-dependent reverse electron transfer at the rate equal to about one-fourth of that for forward NADH–ubiquinone or NADH oxidase [27].

2.7.2. ATP-driven succinate-supported ferricyanide reduction

This reaction is a variant of that previously discussed where ferricyanide instead of NAD^+ serves as the final acceptor for the electrons provided by ubiquinol and delivered to an unidentified low midpoint potential redox component of Complex I as a result of the $\Delta\overline{\mu}_{H^+}$-dependent reverse electron flow [64]. In inside-out SMP the only component which

react with ferricyanide (0.5–1 mM) at significant rate is Complex I as is evident from the about 90% sensitivity of the ATP-dependent succinate–ferricyanide reductase reaction to rotenone and uncouplers [27,64]. It is generally believed that ferricyanide accepts electrons from the same component which participates in NADH–ferricyanide reductase reaction (presumably FMN).

2.7.3. Aerobic succinate-supported NAD^+ or ferricyanide reduction

Although this reaction was in fact the first experimental proof for the reversibility of coupling site 1 [107,108] it has not been demonstrated in SMP until recently [27]. In tightly coupled SMP oxidation the ubiquinol produced by operation of Complex II creates $\Delta\overline{\mu}_{H^+}$ (through subsequent operation of Complex III, cytochrome *c* and cytochrome *c* oxidase) which can be used to drive the reverse electron flow from ubiquinol to NAD^+. The initial rate of this uncoupler- and rotenone-sensitive reaction is the same as that measured under conditions where ubiquinol oxidase is blocked and $\Delta\mu_{H^+}$ is provided by ATP hydrolysis [27]. Depending on the concentration of added NAD^+ the constant steady-state $NADH/NAD^+$ ratio reached (as in intact mitochondria [107–110]), in a first approximation, is a measure of the steady-state $\Delta\overline{\mu}_{H^+}$. The situation, however is not as simple as might be expected if Complex I is considered as the catalytic unit which simply equilibrates $NADH/NAD^+$ pair with Q/QH_2 pool and $\Delta\overline{\mu}_{H^+}$. It has been shown that the steady-state $NADH/NAD^+$ ratio can be *increased* by the addition of rotenone (low concentrations) [121], an observation which led to a proposal that interaction of rotenone with Complex I is $\Delta\overline{\mu}_{H^+}$-dependent and the increase of $NADH/NAD^+$ ratio is due to preferential inhibition of NADH oxidation by the uncoupled 'subpopulation' of SMP [121]. More detailed quantitation of the affinity of Complex I to rotenone has revealed no difference for coupled or uncoupled SMP, although about 10-times difference in the sensitivity of the forward and reverse electron transfer reactions to the inhibitor was found [122]. It has been proposed that different binding sites for ubiquinone and ubiquinol with different sensitivity to rotenone operates during catalytic turnovers in NADH oxidation (forward reaction) or NAD^+ reduction (reverse reaction). This proposal is corroborated by the recent observation of the 'unidirectional' effect of ADP-ribose (the competitive inhibitor of NADH oxidase) which induces an increase of $NADH/NAD^+$ steady-state ratio during aerobic succinate-supported reverse electron transfer [26] similar to rotenone.

3. Steady-state kinetic parameters

Table 2 summarizes the apparent affinities of the nucleotides to the membrane-bound Complex I (SMP) as determined from the initial rate vs. substrate concentration dependencies for the rotenone-sensitive reactions. No deviation from the simple hyperbolic variation in the rate with substrate concentration is seen for either forward or $\Delta\mu_{H^+}$-dependent reverse reactions, indicating that either a single substrate binding site operates, or more than one site with no kinetic cooperativity, or two (or more) sites operate with absolute cooperativity (flip-flop mechanism [123]). The striking difference between the apparent affinities for NAD^+ as a competitive inhibitor of NADH oxidation (1.2 mM) and as a substrate for the reverse electron transfer (7 μM) suggest that different substrate binding sites may be involved in the forward and reverse reactions. This is in accordance with a unidirectional inhibition of NADH oxidation by its analogue ADP-ribose [26].

4. Pre-steady-state kinetics (active / inactive transition)

4.1. Historical

As already mentioned, all the activities and parameters described in the previous sections refer to the 'activated' NADH–quinone reductase. As will be outlined in this section most, if not all the preparations of the mammalian enzyme are heterogeneous mixtures of active and deactivated forms as evident from complex kinetics of the initial rate of NADH oxidation. The abnormal time-course of the reaction was originally reported in 1950 by Slater who has observed a lag-phase in the NADH–cytochrome *c* reductase catalyzed by Keilin–Hartree heart muscle preparation in the presence of cyanide (reaction 2,

Table 1) [124]. Twelve years later Morrison and King have claimed their observation on a pronounced lag in the NADH–cytochrome *c* reductase activity of the same preparation which was increased upon thermal denaturation [125]. Further studies have revealed that the reaction between NADH dehydrogenase and Complex III (i.e., ubiquinone reduction) is responsible for the observed time lag [126]. The thermally induced inactivation of particulate and reconstituted NADH oxidase which was completely restored by the addition of NADH under aerobic conditions have been confirmed by Luzikov's group [127,128], who also found that preheated particles are more sensitive to the destroying effects of chymotrypsin, oleate and phospholipase [127,128]. They have interpreted their observations to suggest that the respiratory chain components exist in activated and non-active forms in a ratio which depends on the electron transfer rate [128,129]. In the papers to resolve the controversial reports on the sensitivity of NADH oxidase to NEM [130,131], Estabrook et al. concluded that after incubation at 37°C ''... the particles remember that they have been treated at the higher temperature and in that way retain their sensitivity to inhibition by NEM'' [132]. Although the pioneering works of Estabrook et al. have clearly indicated a heterogeneity of Complex I within the inner mitochondrial membrane the problem was somehow ignored up till recently when the phenomenon was studied in more detail in our laboratory. A brief account of those studies is given below and is summarized in a scheme shown in Fig. 3.

4.2. Phenomenology

Fig. 2 shows schematically a time-course of the rotenone-sensitive substrate consumption or product formation in the forward or reverse reactions catalyzed by two different preparations of SMP: thermally deactivated (curve 1) and thermally deactivated and further treated with NADH either under aerobic conditions or in the presence of added quinone (curves 2). Several points need to be emphasized before further discussion. All the preparations capable of the rotenone-sensitive NADH oxidation which assayed in our laboratory such SMP, Keilin–Hartree particles [43], purified NADH–cytochrome *c* reductase [41] and Complex I [133] show qualitatively the same pattern as depicted in Fig. 2A, whereas neither the ferricyanide nor HAR–reductase activities of those preparations and none of the activities of the Type 2 dehydrogenase show lags in their NADH dehydrogenase capacity. Also, when Q_1 is employed as electron acceptor only the rotenone-sensitive part of the overall reaction demonstrates such a behaviour. The zero-order rate of NADH oxidation or reduction (v^a, curves 2) are uncoupler-sensitive when tightly cou-

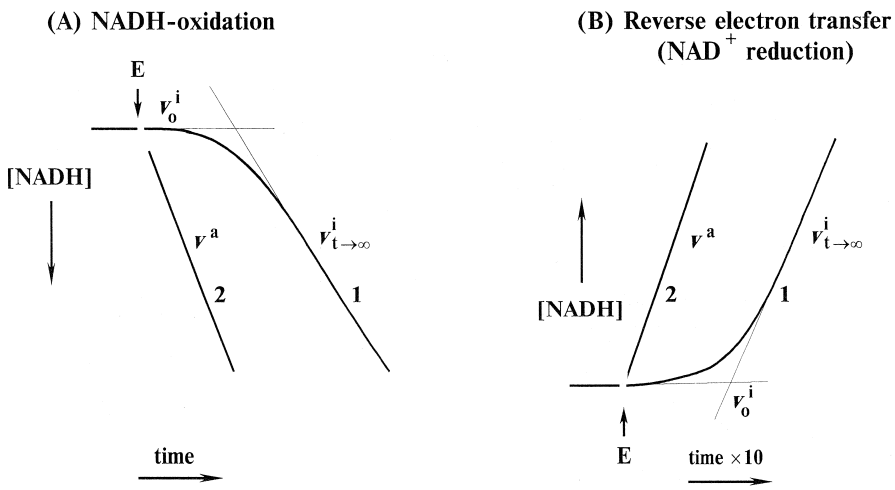

Fig. 2. Time-course of the reactions catalyzed by active (curves 2) and deactivated (curves 1) preparations of SMP (schematic presentation). The final levels of the rates reached during the reactions ($v_{t\to\infty}^i$) are equal to v^a. Note the difference in the time scale in panels (A) (NADH oxidase or NADH–Q_1 reductase, forward reaction) and (B) ($\Delta\overline{\mu}_{H^+}$-dependent ubiquinol–$NAD^+$ reductase, reverse reaction).

pled particles are assayed, however the activation half-times are the same for coupled or completely and partially uncoupled systems. Thus it is safe to conclude that the activation is $\Delta\bar{\mu}_{H^+}$-insensitive. The initial rates of the reactions catalyzed by the preparations deliberately subjected to thermal deactivation (v_0^i) are very close to zero, whereas those for SMP or Complex I as prepared according to the published procedures show a variety of the initial rates (between those shown by curves 1 and 2) depending on the particular preparation. The completeness of activation by aerobic NADH pretreatment is crucially dependent on the protein content in the samples. Very low concentrations of NADH (5–10 μM) are needed for complete activation of diluted (5–50 μg of protein ml^{-1}) samples and much higher NADH is required (up to millimolar range) when concentrated suspensions are subjected to activation. This is simply because a fraction of the activated enzyme formed during preincubation in concentrated samples rapidly consumes added NADH (and/or oxygen or quinone) thus preventing further transformation of the residual inactive enzyme. It should be emphasized that the reduction per se does not induce the transformation of inactive enzyme into its active form. No activation by NADH takes place when the ubiquinone pool is completely reduced by succinate (anaerobically or in the presence of cyanide). Thus slow initiating redox cycle is needed to reach full catalytic capacity of the enzyme. Finally, it is worthwhile to mention that the activation half-time under the conditions usually employed for the NADH oxidase or NADH–ubiquinone reductase assay (pH ≈ 7.5, 20–27°C) is rather short (5–20 s), thus the phenomenon could be easily overlooked when the slowly responding registration system such as a membrane-coated oxygen-sensitive electrode (NADH oxidase) or exclusively computerized spectrophotometers are used.

4.3. Deactivation

The only factor strongly affecting transformation of active enzyme into the inactive form is temperature. NAD^+, reduction of endogenous ubiquinone, NADH under conditions when ubiquinone pool is reduced, the presence of bivalent cations, addition of the competitive inhibitor ADP-ribose [26], dilution or concentration of the enzyme preparations, anaerobiosis, ferricyanide, the presence of the sulfhydryl reagents separately or in combinations (when possible) do not significantly affect the rate of deactivation. On the other hand the effect of temperature is dramatic. An activation energy as high as 270 kJ/mol was determined from the linear Arrhenius plot within the temperature range of 25–40°C [27,133]. A decrease of the initial rate (v^a, Fig. 2) of the forward or reverse electron transfer reactions follows the same first-order time course with $t_{1/2}$ of about 15 min at 30°C. The thermally induced deactivation was originally described as an irreversible process [27]. Closer examination revealed a constant residual activity of the thermally deactivated SMP [122]. Therefore the deactivation process should be considered as spontaneous equilibration between two forms of the enzyme

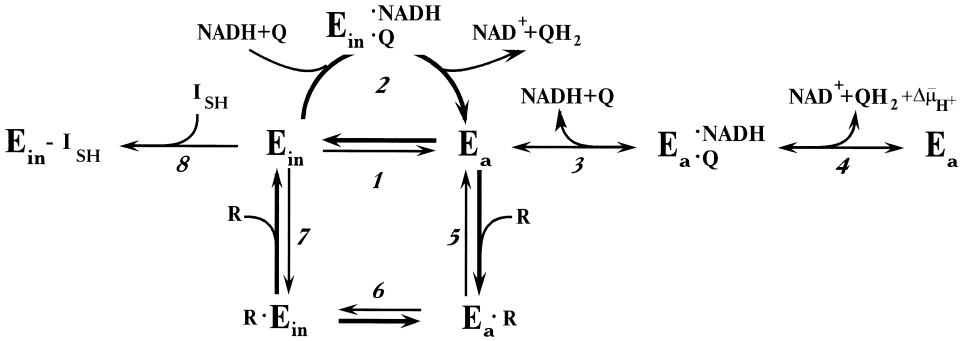

Fig. 3. Hypothetical reaction pathways describing the slow active/inactive enzyme transitions. Figures in italic correspond to the number of particular step as discussed in the text. E_{in}, E_a, I_{SH} and R stand for deactivated enzyme, active enzyme, sulfhydryl reagents and rotenone, respectively. Different thickness of the arrows emphasize shifts in equilibria of the corresponding reactions. See text for further explanations.

(reaction 1, Fig. 3). The equilibrium position does not significantly depend on temperature, whereas the rate of equilibration strongly does. The only factor found so far which influences the spontaneously established equilibrium is the specific Complex I inhibitor rotenone [122] which partially protects and partially reverses the thermally induced deactivation. It has been shown that rotenone has almost two order of magnitude higher affinity for the active form of the enzyme. An extremely tight binding of rotenone to the active enzyme suggests that the inhibitor acts as a transition state analogue [122].

4.4. Activation

As shown in Fig. 3, there are two routs for the transformation of deactivated enzyme into its active form. One, which is substrate-independent, includes slow equilibration within the closed reaction sequence 1-5-6-7 and proceeds to significant extend only in the presence of the rotenone [122]. The other (reaction 2), which is also slow proceeds via a turnover-dependent mechanism. Oxidation of NADH by the active enzyme (the reaction rates v^{a} and $v^{i}_{t\rightarrow\infty}$ shown in Fig. 2) is insensitive to NEM or other sulfhydryl reagents and to the presence of bivalent cations. In contrast, the reactions catalyzed by the deactivated preparations are blocked when the enzyme is treated with SH-reagents prior to the addition of NADH. The lag-phases are considerably prolonged in the presence of bivalent cations or at alkaline pH [134]. Thus, the activation process (reactions 1 and 2, Fig. 3), not the catalytic mechanism of the steady-state NADH oxidation (reactions 3 and 4, Fig. 3), are sensitive to those effectors. Deactivated enzyme retains its capacity to catalyze all NADH–artificial acceptor reductase reactions and shows the same pattern of the iron–sulfur clusters reduction by NADH as seen in the active form [70]. Injection of reducing equivalents from the substrate–nucleotide (NADH or NADPH) is an absolute prerequisite for further transformation of the enzyme into its active form via reaction 2. On the other hand, prolonged preincubation under the condition where the ubiquinone pool is completely reduced (anaerobically or in the presence of succinate and antimycin A or cyanide) does not result in activation [27]. No activation was observed when the enzyme was preincubated with NAD^{+} or ADP-ribose thus showing that binding of nucleotide and oxidized quinone per se (with no further oxidoreduction) does not induce the conformational transformation into the active form. On the other hand, complete activation was observed when deactivated SMP were incubated in the presence of sub-stoichiometric concentrations of NADH and a NADH regenerating system [27]. Taken together these findings suggest that at least one slow half-turnover is needed for transformation of thermodynamically stable inactive enzyme into the thermodynamically unstable but catalytically competent form. When active enzyme catalyzes oxidation of NADH by ubiquinone the redox potential gap between the substrates/products pairs (-320 mV [101] and $+60$ mV [135], respectively) provides free energy for transmembranous proton transfer and creates $\Delta\overline{\mu}_{H^{+}}$. The same reaction when performed by the deactivated form results in conservation of free energy as a constrained enzyme conformation. In other words, the existence of the equilibria 1 and 2 (Fig. 3) suggests that part of the free energy of NADH oxidation is used to maintain the catalytically competent state of the enzyme. This conclusion leads to further consequences.

First, both spontaneous deactivation and redox-dependent activation are slow processes compared to the active enzyme turnover in reactions 3 and 4 (the first order rate constants are in a minute scale), hence it seems unlikely that a combination of the reactions 2 and 1 contributes significantly to the steady-state rate of NADH oxidation. It does not, however seem unlikely that this ‘futile’ cyclic transformation contributes to the fine tuning of the main entry point for the energy-producing respiratory chain. It has been demonstrated that a different degree of enzyme activation is attained during the overall NADH oxidation by SMP at different levels of ubiquinone reduction or by Complex I in the presence of exogenous Q_1 and different level of the bacterial quinol oxidase [136]. In contrast to the spontaneous deactivation and to the reaction catalyzed by the active enzyme, the redox-dependent activation was shown to be sensitive to a number of factors of the potentially physiological significance, such as pH and bivalent cations [134]. The appearance of a highly reactive sulfhydryl in the deactivated form can also be considered as a chemical prerequisite for the possible modulation of the total enzyme activity via naturally existing SH–/S–S

compounds such as intramitochondrial glutathione [137].

The second point relevant to the redox-dependent activation is the reversibility of the Complex I-catalyzed reactions (see Section 2.7). Since maintenance of the active enzyme needs energy provided by the NADH–ubiquinone oxidoreduction it is hard to accept that the enzyme operates as a simple catalytic unit equilibrating $NADH/NAD^+$, Q/QH_2 and $\Delta\mu_{H^+}$, unless the reaction 2 is reversible. The observations of different rotenone-sensitivity of the direct and reverse electron transfer [122] and of unidirectional competitive inhibition of NADH oxidation [26] suggest that different binding sites for substrates (products) operate in either reaction. In a strict sense this would mean that the mechanisms of the direct and reverse reactions are different as has been suggested for another 'reversible' energy-transducing complex—F_o–F_1 ATP synthase [138].

The molecular mechanism of the enzyme active/inactive transition is a 'black box'. Besides the complete change in the catalytic capacity, several other features such as strong temperature-dependence, dramatic change in the sulfhydryl reactivity and affinity to rotenone indicate that gross conformational changes are involved in the specific alteration in the ubiquinone reactivity. Since ubisemiquinone(s) are now accepted as intermediate(s) of the steady-state $NADH \rightarrow Q$ and $QH_2 \rightarrow NAD^+$ activities [39,70,139,140] the reaction 2 (Fig. 3) mechanistically can be visualized as follows:

$$E^{in}_{red} \overset{Q}{\longleftrightarrow} E^{in}_{red}\cdot Q \longleftrightarrow E^{a}_{ox}\cdot Q^{\cdot} \xrightarrow[\text{dismutation}]{QH_2} E^{a}_{ox}\ ,$$

$$|\leftarrow \text{fast} \rightarrow| \longleftarrow \text{slow} \longrightarrow|$$

where indexes red, ox stand for the reduced, oxidized, deactivated and active enzyme, respectively. The deactivated, NADH–reduced enzyme is oxidized by quinone in one-electron reaction (iron–sulfur cluster *N*-2 is most likely involved [11,39,140]) coupled with a slow rearrangement of the protein resulting in the formation of ubisemiquinone(s) at specific binding site(s). In the absence of further electron flow through the enzyme the ubisemiquinones dismutate leaving constrained active enzyme conformation.

It is of interest that the 'turnover-dependent' activation appears not to be a unique property of Complex I; it has been reported that heterodimeric Fe–S, Ni-containing hydrogen–quinone oxidoreductase of *Alcaligenes eutropus* [141], *Azotobacter vinelandi* [142] and *Brodhyrhizobium japonicum* [143] demonstrate quite similar phenomena.

5. Conclusion and perspectives

Despite recent revived interest in molecular structure and mechanisms of the mammalian, microbial, fungal and plant Complex I, it is hard to deny that little progress has been achieved over the last twenty years in answering several key questions such as the sequence of the intramolecular electron transfer reactions, the number of proton-translocating sub-sites the minimal unit retaining the catalytic activities seen in intact systems. In the author's opinion much more work is needed in isolation of 'pure unmodified' enzyme capable of the redox-linked proton translocation in proteoliposomes. The lack of specific inhibitors and artificial electron acceptors/donors interacting at the sites different from those of nucleotide and quinone binding severely hampers the creation of new models and delineation of the path as was done for Complex III. Simpler counterparts of the mammalian 42-subunit Complex I such as, for example, *P. denitrificans* 14–20-subunit NDH-1 [5] are now available for molecular genetic manipulation. The comparative investigation of the mammalian and simpler bacterial enzymes would be a great help in understanding of the individual subunits functions. It is beyond doubt that further studies of the catalytic properties of the presently available and future preparations will facilitate progress in the field.

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