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Review

Generation of superoxide by the mitochondrial Complex I

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Abstract

Superoxide production by inside-out coupled bovine heart submitochondrial particles, respiring with succinate or NADH, was measured. The succinate-supported production was inhibited by rotenone and uncouplers, showing that most part of superoxide produced during succinate oxidation is originated from univalent oxygen reduction by Complex I. The rate of the superoxide ($O_2^{\bullet -}$) production during respiration at a high concentration of NADH (1 mM) was significantly lower than that with succinate. Moreover, the succinate-supported $O_2^{\bullet -}$ production was significantly decreased in the presence of 1 mM NADH. The titration curves, i.e., initial rates of superoxide production versus NADH concentration, were bell-shaped with the maximal rate (at 50 μM NADH) approaching that seen with succinate. Both NAD^+ and acetyl-NAD^+ inhibited the succinate-supported reaction with apparent K_i's close to their K_m's in the Complex I-catalyzed succinate-dependent energy-linked NAD^+ reduction (reverse electron transfer) and NADH:acetyl-NAD^+ transhydrogenase reaction, respectively. We conclude that: (i) under the artificial experimental conditions the major part of superoxide produced by the respiratory chain is formed by some redox component of Complex I (most likely FMN in its reduced or free radical form); (ii) two different binding sites for NADH (F-site) and NAD^+ (R-site) in Complex I provide accessibility of the substrates-nucleotides to the enzyme red-ox component(s); F-site operates as an entry for NADH oxidation, whereas R-site operates in the reverse electron transfer and univalent oxygen reduction; (iii) it is unlikely that under the physiological conditions (high concentrations of NADH and NAD^+) Complex I is responsible for the mitochondrial superoxide generation. We propose that the specific NAD(P)H:oxygen superoxide (hydrogen peroxide) producing oxidoreductase(s) poised in equilibrium with NAD(P)H/$NAD(P)^+$ couple should exist in the mitochondrial matrix, if mitochondria are, indeed, participate in ROS-controlled processes under physiologically relevant conditions.

Keywords: Superoxide generation; NADH:ubiquinone reductase; Complex I; Respiratory chain (Bovine heart submitochondrial particles)

1. Introduction

The major part of oxygen consumed by aerobic cells is converted into water by four-electron reduction, catalyzed by cytochrome *c* oxidase (respiratory Complex IV). A much smaller part is converted into hydrogen peroxide (H_2O_2) as a result of two-electron reduction, catalyzed by a number of enzymes. In perfused rat liver, the level of hydrogen peroxide, determined at the state of equilibrium with peroxisomal catalase (Compound I), is in the range of 10^{-9}–10^{-7} M, depending on the metabolic state [1]. Superoxide anion ($O_2^{\bullet -}$) is a precursor of H_2O_2, in the reaction: $2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2$, catalyzed by superoxide dismutases (SOD). H_2O_2 and $O_2^{\bullet -}$, although present in very low concentrations, are normal metabolites. These two species along with extremely reactive $OH^{\bullet}$-radical, produced by further reduction of hydrogen peroxide ($H_2O_2 + O_2^{\bullet -} + H^+ \rightarrow H_2O + O_2 + OH^{\bullet}$), are conventionally named Reactive Oxygen Species (ROS). ROS may cause irreversible damage of proteins, nucleic acids, phospholipids and/or participate in signalling mechanisms. It is generally accepted that levels of ROS are fine-tuned by the rates of their formation and decomposition by SOD, catalase and peroxidase. There exists a number of oxygen consuming enzymes, located in mitochondria, peroxisomes, endoplasmic reticulum, nucleus and cytosol, that may produce $O_2^{\bullet -}$ and H_2O_2 (see Refs. [1,2] for comprehensive reviews).

Relative contributions of different subcellular organelles to the total cellular H_2O_2 production in the liver have been estimated under a number of assumptions, approximating the

Abbreviations: ClCCP, carbonylcyanide-*m*-chlorophenyl hydrazone; $\Delta\tilde{\mu}_{H^+}$, difference of electrochemical potentials of protons across the coupling membrane; $O_2^{\bullet}$, superoxide radical; ROS, reactive oxygen species; SMP, submitochondrial particles; SOD, superoxide dismutase

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physiological conditions [3]. The total rate of the H_2O_2 production was calculated to be in the order of 90 nmol/min per g wet wt. of liver; and about 13% of that production (i.e., 12 nmol/min per g wet wt. of liver) was attributed the mitochondria [3]. We were unable to find any other publication in the current literature that describes quantitatively relative contributions of different tissue-specific enzymes located in different organelles to the net H_2O_2 production. The mitochondrial superoxide generation is expected to vary considerably depending on the metabolic state (State 4–State 3 transition) [4].

It should be emphasized that in addition to the most active ensemble of oxidoreductases, known as the respiratory chain, intact mitochondria contain a number of oxidoreductases both in the matrix and in the outer mitochondrial membrane potentially capable of superoxide production (see [2] and references cited therein). Thus, attributing generation of hydrogen peroxide solely to the *respiratory chain components*, which is commonly pursued in the current literature, should be done with caution. Production of H_2O_2 by electron transport particles (uncoupled submitochondrial particles, SMP) during residual antimycin-insensitive respiration with succinate and NADH as substrates has been originally reported in 1966 [5]. Hinkle et al. have shown that some component in the NADH-ubiquinone region of the respiratory chain is capable of hydrogen peroxide production during the succinate-supported ATP-dependent reverse electron transfer, catalyzed by cyanide-inhibited loosely coupled bovine heart SMP [6]. Later studies have shown that Complex I [7–12] and Complex III (modified by antimycin) [13–15] are the components capable of the univalent reduction of oxygen into superoxide [16]. The redox component, which reduces oxygen in Complex III is most likely an unstable ubisemiquinone, formed as a result of the concerted ubiquinol oxidation at center P [13]. Which particular redox component in the NADH-ubiquinone region is responsible for the production of superoxide is still a matter of debates. $NAD^{\cdot}$-radical [10], FMN [17–20], iron–sulfur clusters N2 [21] and N1a [22], and Complex I-associated ubisemiquinone (SQ_{Nf}) [23] have been suggested as the species directly reacting with oxygen.

In light of the recent popular theories connecting "oxidative stress" with diseases and aging ([24] and references therein), the interest in the mitochondrial superoxide (and/or hydrogen peroxide) generation has been steadily on the rise. Numerous publications on this subject appeared in the literature during the past decade. For detailed analysis of experimental data and some modern hypotheses in the field the reader is referred to several current reviews [24–27].

2. Methods for superoxide detection

Approaches to quantitative determination of superoxide and hydrogen peroxide produced by enzymatic systems have been extensively reviewed [1,28–30]. Even when a single purified enzyme catalyzes univalent reduction of oxygen, quantitative determination of $O_2^{\cdot}$ as a reaction product is an extremely tedious task. Superoxide is a very unstable particle. The second order rate constant for the spontaneous dismutation of superoxide (pK_a for $HO_2^{\cdot}$ (protonated superoxide) is 4.8 [29]; the predominant form at the physiological pH is superoxide anion) is about 100 M^{-1} s^{-1}, and it greatly increases upon acidification. The half-time of the decay of $\sim 10^{-5}$ M superoxide at pH 7.5 is about 10 s [29], and it increases at lower concentrations. Due to the second order nature of its spontaneous dismutation and the first order nature of the SOD-catalyzed reaction (the enzyme is never saturated by the second substrate) only very low steady-state concentrations of superoxide can be attained.

All conventional methods for superoxide detection are based on the use of redox "dyes" which rapidly react with $O_2^{\cdot -}$ producing optically or ESR-detectable products. Cytochrome *c* reduction, epinephrine oxidation, nitroblue tetrasolium reduction, oxidation of dihydroethidium and a number of spin-trap involving reactions have been used to monitor superoxide production [30]. However, neither of the known $O_2^{\cdot -}$-scavengers are specific for superoxide. Accordingly, if upon generation of $O_2^{\cdot -}$ a "dye" forms (or disappears) at a particular rate, only a fraction of this rate which is sensitive to SOD, can be taken as a measure of $O_2^{\cdot -}$ generation. The alternative approach is to use an excess of SOD as the coupling enzyme, and to detect not superoxide but hydrogen peroxide, either directly or in the coupled peroxidase reactions [1].

When applied to intact mitochondria or preparations derived therefrom both approaches suffer from a number of shortcomings. The rates of the superoxide (and/or hydrogen peroxide) production by mitochondrial preparations are in the range of nanomoles/min per mg of protein, whereas their specific oxidoreductase activities are about 3 orders of magnitude higher. Even unspecific reactions of mitochondrial oxidoreductases with either oxidized or reduced superoxide-sensitive scavengers may thus result in significant measurement errors. Because mitochondria contain SOD both in the matrix and in the intermembrane space, "direct" quantification of $O_2^{\cdot -}$ production by intact mitochondria is virtually impossible. Moreover, quantitative determination of penetrating hydrogen peroxide in the surrounding solution, as a measure of its mitochondrial production, is seriously hampered by the presence of endogenous glutathione (and possibly other substrates), peroxidase [31] and catalase [32].

The inside-out submitochondrial particles (SMP), lacking the matrix enzymes, but still capable of artificially induced (by oligomycin [33]) tight coupling (respiratory control ratio 2.5–3.5 with succinate and 5.0–7.0 with NADH), seem to be the optimal experimental system for studying superoxide generation by the mitochondrial respiratory chain. Bovine heart SMP, prepared by the routine procedure [34], were used in our studies. Initial rate of acetylated cytochrome *c* reduction served as a quantitative trap for "direct" detection of superoxide generation [35]. To minimize possible artifacts due to interaction of acetylated cytochrome *c* with b–c_1 complex and/or cytochrome oxidase several control experiments briefly described below have been conducted [18]. (i) The addition of SMP did not interfere with the standard assay system when superoxide was produced by xanthine oxidase and detected as SOD-sensitive cytochrome *c* reduction. (ii) Neither acetylated

nor native cytochrome *c* stimulate NADH- or succinate-supported respiration of SMP. It should be mentioned that in our hands a significant fraction of the succinate- or NADH-supported reduction of acetylated cytochrome *c* was insensitive to SOD. It is known that acetylation greatly decreases the activity of natural cytochrome *c* in reactions catalyzed by the respiratory chain [35]). Unspecific interactions of acetylated cytochrome *c* with respiratory chain components could be diminished by adding potassium phosphate or potassium chloride in relatively high concentrations (100 mM, pH 8.0). On the other hand, high concentrations of K^+ caused partial uncoupling (stimulation of State 4 respiration), e.g., the respiratory control ratio with NADH as a substrate decreased to 3.3 in the presence of 100 mM potassium phosphate against 5.1, observed in the absence of salts. Interestingly, the respiratory control ratio with succinate as a substrate was not affected by the presence of potassium phosphate. Although this phenomenon was not yet studied in greater detail, we would like to mention that the actual rates of superoxide generation in State 4 as reported here may be higher if the respiratory control ratio would approach its maximal value. We also observed a significant SOD-sensitive "nonenzymatic" rate of acetylated cytochrome *c* reduction by NADH in the absence of SMP (34 nM/min in the presence of 1 mM NADH and 20 μM acetylated cytochrome *c*) when high concentrations of NADH (in millimolar range) were used. All data reported here were corrected for this "nonenzymatic" rate.

It has been shown that protonated superoxide oxidized NADH bound to the active sites of lactate- and 3-phosphoglyceraldehyde dehydrogenases thus resulting in chain oxidation of NADH in the presence of xanthine oxidase-generated radical [36,37]. It was of interest to check whether superoxide would oxidize NADH bound to Complex I or it would serve as "artificial" electron acceptor interacting with some intrinsic redox component (reduced FMN or iron–sulfur centers). We were unable to detect either of these interactions in the mixture composed of rotenone-treated SMP, NADH, hypoxanthine and xanthine oxidase. Autocatalytic oxidation of NADH was however observed after the mixture having been incubated for longer time. The same phenomenon was seen in the absence of SMP. The spontaneous "non-enzymatic" oxidation of NADH (in the absence or presence of SMP) was prevented by either SOD or catalase. We concluded that delayed SOD- and/or catalase-sensitive oxidation of NADH in the presence of hypoxanthine and xanthine oxidase was due to accumulation of hydrogen peroxide and further formation of extremely reactive hydroxyl radical. NADH (1 mM) did not interfere with the superoxide-mediated hypoxanthine:cytochrome *c* oxidoreduction catalyzed by xanthine oxidase. The experimental details of these "control" studies will be published elsewhere.

3. Relative contributions of different respiratory chain components to the overall production of superoxide

The maximal rates of superoxide production (about 1 nmol/min per mg) by respiring SMP were very small (about 0.1%) compared to their uncoupled or coupled oxidase activities with succinate or NADH as respiratory substrates. About two thirds of the total superoxide production in State 4 with succinate as a substrate were generated in the NADH-ubiquinone region, as evidenced by the sensitivity of the reaction to rotenone (Fig. 1). This was also evident from almost complete quenching of the reaction by uncoupling, which prevents the $\triangle\tilde{\mu}_{H^+}$-dependent reverse electron transfer from ubiquinol to Complex I. The residual activity observed with succinate in the presence of rotenone (also uncoupler-sensitive) can be attributed to the combined superoxide generation by Complex II and Complex III. If Complex I is the predominant superoxide generating component when succinate is oxidized in State 4, it could be expected that the same or higher rate of the superoxide production would be detected with NADH as a substrate. However, this was not the case, and, in fact, the superoxide production with 1 mM NADH (relatively high concentration of NADH was used to reach prolonged steady-state oxidation) was significantly lower than that with succinate. The low activity observed with 1 mM NADH was also greatly inhibited by uncoupling, but it was almost insensitive to rotenone.

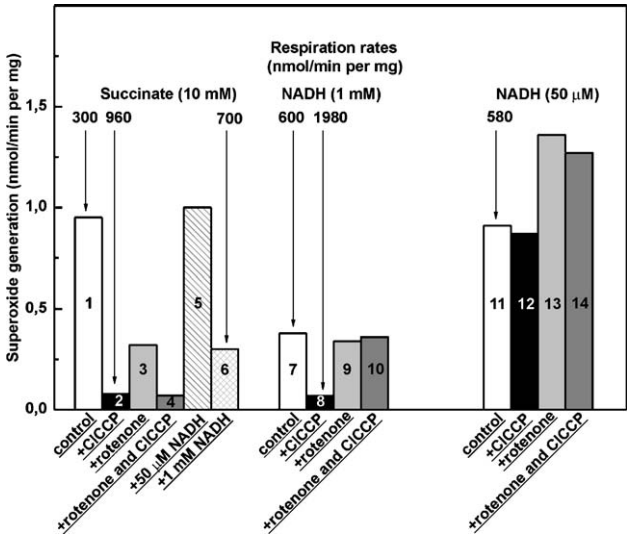

Fig. 1. Generation of superoxide by coupled submitochondrial particles. Bovine heart submitochondrial particles (SMP) were treated by oligomycin, activated and stored as described earlier [38]. Superoxide generation was measured photometrically at 550 nm by tracing the SOD (3 units/ml)-sensitive reduction of 20 μM acetylated cytochrome *c* [35] at 30 °C in the standard mixture comprising 0.25 M sucrose, 50 mM Tris/Cl^-, 0.1 M K-phosphate, 0.2 mM EDTA (pH 8.0) and SMP (0.1 mg/ml). The reaction was initiated by addition of NADH or potassium succinate. The respiratory activities of SMP, measured under exactly the same conditions as superoxide production (except when acetylated cytochrome *c* was added), are depicted in the upper part of the figure. The NADH oxidase activity was measured photometrically at 340 nm (ε^{340}_{mM}=6.22) or at 380 nm (ε^{380}_{mM}=1.25) when NADH concentration was higher than 150 μM in the presence or absence of 5 μM ClCCP (state 3 and state 4, respectively). The succinate oxidase activity was measured either with oxygen-sensitive electrode or by increase in absorption at 278 nm (fumarate formation, ε^{378}_{mM}=0.3). Rotenone (10 μM) was added where indicated. All the activities correspond to the representative experiments made with the same batch of SMP. The absolute values for different preparations of SMP were variable within 20% margin, and relative sensitivities of the activities to the inhibition were always the same.

Moreover, simultaneous oxidation of 10 mM succinate and 1 mM NADH in State 4 resulted in a much lower superoxide generation rate than with succinate alone (samples 1 and 6).

Attempting to reconcile these puzzling observations we measured the rate of the superoxide production at a much lower NADH concentration (50 μM). Under these conditions, the superoxide generation was significantly increased (sample 11), approaching the level observed in the case of succinate. The reaction rate was increased further in the presence of rotenone (samples 11 and 13) and it was almost insensitive to uncoupling. In contrast to the case of high NADH (1 mM, sample 6), 50 μM NADH did not inhibit the succinate-supported reaction (sample 5).

The results shown in Fig. 1 prompted us to look more closely at the dependence of the superoxide generation on NADH concentration. This dependence was found to be bell-shaped with apparent maximum at 50 μM NADH (Fig. 2A). The data reported in the literature [36,37,39] and observations described above (see previous section) excluded the possibility that inhibitory effect of high NADH was due to "direct chemical" quenching of superoxide by the reduced nucleotide. Previous studies have shown that the rotenone-insensitive NADH: ferricyanide reaction, catalyzed by either purified Complex I or SMP, is strongly inhibited by the substrate [40,41]. This phenomenon was also observed under a particular set of conditions employed in this study, and the dependencies of the NADH-supported superoxide generation and ferricyanide reduction were very similar to the ones observed earlier (Fig. 2). Moreover, guanidine, which is known to activate NADH: ferricyanide reduction in the presence of NADH dehydrogenase component of Complex I (FP) [42,43], was found to stimulate the NADH-supported superoxide generation (3.5 and 1.5 nmol/min per mg of protein at 50 μM NADH with and without 50 mM guanidine chloride, respectively). It is possible that that the mechanisms of the inhibition of superoxide generation and ferricyanide reduction by NADH at high concentrations are similar.

4. Effects of specific ligands on the superoxide generation by Complex I

Intact coupled mitochondria are capable of reduction of endogenous $NAD(P)^+$ when succinate or other substrates donating electrons to ubiquinone, are oxidized in State 4 [44]. Loosely coupled inside-out SMP catalyze ATP- and succinate-dependent reduction of added NAD^+ when their oxidase activity is blocked [45]. Tightly coupled inside-out SMP are capable of aerobic succinate-supported NAD^+ reduction, coupled with $\Delta\tilde{\mu}_{H^+}$-generating succinate-oxidase activity [34]. Numerous data have shown that the succinate-supported reduction of endogenous or added NAD^+ is due to the reversal of $\Delta\tilde{\mu}_{H^+}$-generating NADH:ubiquinone reductase (Complex I). Under optimal conditions the maximal rate of the reverse electron transfer, catalyzed by Complex I in tightly coupled SMP, approaches about 1/4 of the uncoupled NADH oxidation [34]. Interestingly, the kinetic parameters and the inhibitor-sensitivity of the Complex I-catalyzed NADH oxidation and NAD^+ reduction can hardly be reconciled using a simple reversibility model. The substrate in the reverse electron transfer, NAD^+ (K_m ~7 μM [46]), does not inhibit NADH oxidation unless it is added in millimolar concentrations (apparent K_i for the competitive inhibition is 1 mM for both coupled and uncoupled NADH oxidations [46,47]). The apparent K_m for NADH oxidation is about 2 μM, and it only slightly depends on the coupling state. On the other hand, NADH does competitively inhibit $\Delta\tilde{\mu}_{H^+}$-dependent NAD^+ reduction with apparent K_i of 40 μM [46,47]. More significantly, ADP-ribose, a competitive inhibitor of NADH oxidation, does not inhibit the reverse electron transfer, catalyzed by either SMP [47] or tightly coupled vesicles, derived from *Paracoccus denitrificans* [48,49]. Unidirectional inhibition of the forward and reverse electron transfer by the ubiquinone-junction site-directed inhibitors have also been reported [48–50]. Some of these observations perhaps could be explained by marked differences in the catalytic rates of the forward and

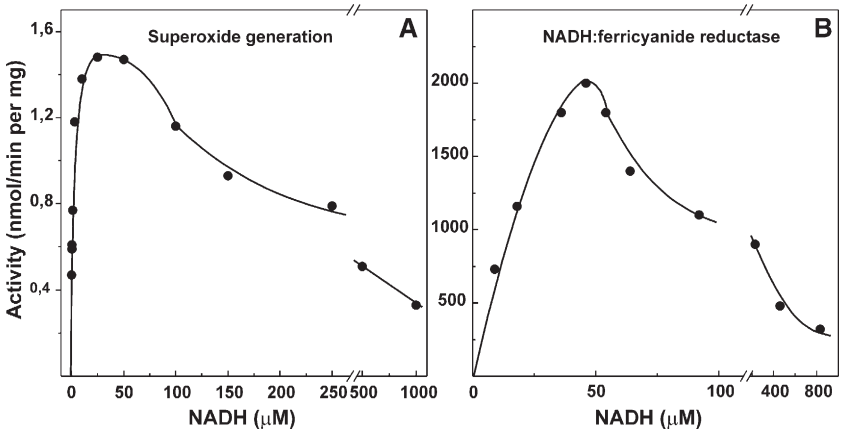

Fig. 2. The dependence of NADH-induced superoxide production (A) and NADH:ferricyanide reductase activity (B) on NADH concentration. (A) Generation of superoxide by SMP (0.1 mg/ml) was measured in the standard mixture (see Legend to Fig. 1) in the presence of 10 μM rotenone. (B) NADH:ferricyanide reductase activity was measured in the standard mixture containing 10 μM rotenone, 0.5 mM potassium ferricyanide, and SMP (17 μg/ml). Note, that the specific activities in superoxide generation and ferricyanide reduction were different by about 3 orders of magnitude.

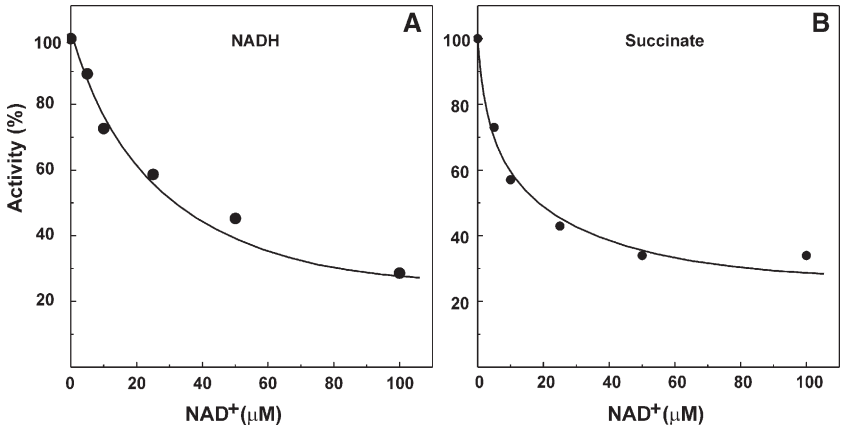

Fig. 3. Inhibitory effects of NAD^+ on NADH- (A) or succinate- (B) supported superoxide generation. The superoxide production by SMP (0.1 mg/ml) was measured as described in Fig. 1 in the presence of 50 μM NADH and 10 μM rotenone (A) or 10 mM succinate (B). One hundred percent corresponds to the specific activities of 1.5 (A) and 1.0 (B) nmol/min per mg of SMP.

reverse reactions and by different redox states of the enzyme catalyzing either process. An alternative and more likely explanation is that different nucleotide specific sites operate in the forward and reverse electron transfer reactions, catalyzed by Complex I as it has been proposed earlier [51].

Because both the reverse electron transfer (in the absence of NADH) and the oxidation of NADH, but only at its low concentrations, were able to produce superoxide with significant rates, it was of interest to examine how occupation of putatively different nucleotide binding sites would affect superoxide generation. Fig. 3 shows how NAD^+ at the concentrations that do not affect NADH oxidation causes inhibition of the NADH- and succinate-supported superoxide generation. The apparent K_i for NAD^+ in this reaction was equal to K_m in the reverse electron transfer, measured under the same conditions. The same inhibitory effect was evident for acetyl-NAD^+ (Fig. 4), whose apparent K_i's for superoxide generation were similar to the K_m's, determined for the rotenone-insensitive NADH:acetyl-NAD^+ transhydrogenase reaction and for the succinate-supported electron transfer to acetyl-NAD^+, catalyzed by Complex I. The latter findings are in accord with the observation that a substantial fraction of the Complex I- and FP-catalyzed transhydrogenase proceeds via ternary complex formation mechanism [52].

5. The model

The model we propose to explain the data obtained in these and in some other previously reported studies [18] is shown in Fig. 5. Two different substrate–nucleotide binding sites operate in the forward (oxidation of NADH, F-site) and reverse (reduction of NAD^+, R-site) NADH(NAD^+):ubiquinone (ubiquinol) oxidoreductase reaction, catalyzed by Complex I. NADH binds at the F-site with the primary affinity of

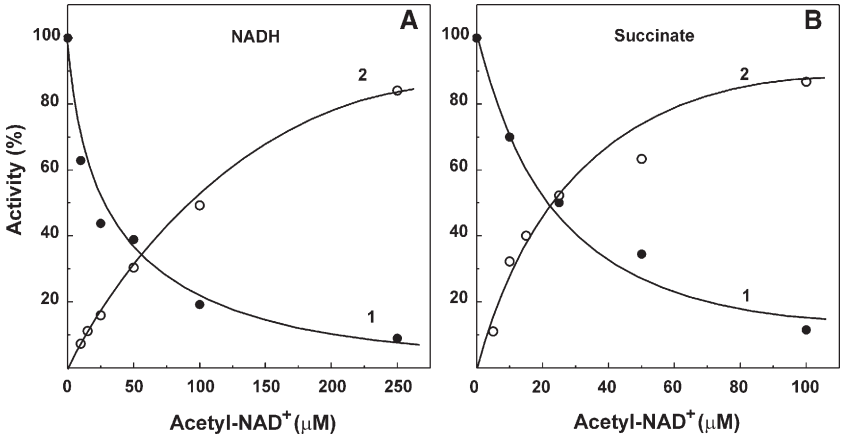

Fig. 4. Inhibitory effects of acetyl-NAD^+ on NADH- (A) or succinate- (B) supported superoxide generation. The superoxide production by SMP (0.1 mg/ml, closed circles) was measured as described in the legend to Fig. 3. NADH:acetyl-NAD^+ transhydrogenase activity (A, curve 2, open circles) was measured photometrically at 375 nm (ε^{375}_{mM}=5.1) in the presence of 50 μM NADH and 10 μM rotenone. The reaction was initiated by the addition of SMP (50 μg/ml). The maximal rate of the reaction (100%) and K_m for acetyl-NAD^+ as determined from double-reciprocal linear graph (not shown) were 0.7 μmol/min per mg and 100 μM, respectively. The succinate-supported reverse electron transfer to acetyl-NAD^+ (B, curve 2, open circles) was monitored photometrically at 363 nm (ε^{363}_{mM}=9.1) in the presence of 10 mM succinate. The reaction was initiated by the addition of SMP (25 μg/ml). The maximal rate of the reaction (100%) and K_m for acetyl-NAD^+ as determined from double-reciprocal linear graph (not shown) were 0.1 μmol/min per mg and 20 μM, respectively.

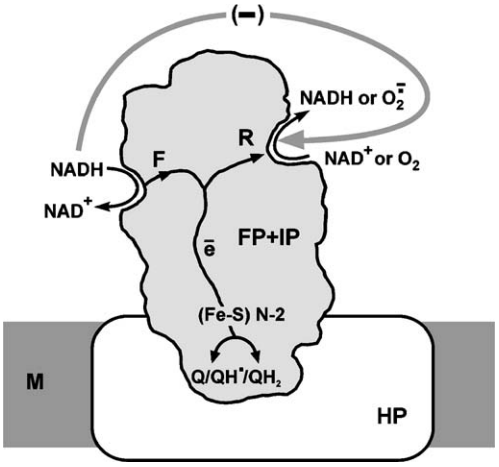

Fig. 5. A model for electron transfer pathways in NADH oxidation, NAD^+ reduction and superoxide generation catalyzed by Complex I. M, the inner mitochondrial membrane; hydrophilic part exposed to matrix (FP+IP) is shown in grey. HP, hydrophobic membranous integral part is shown in white. See text for details.

10^{-5} M, and an intramolecular redox reaction between the bound NADH and the primary electron acceptor (FMN) results in apparent K_m of $\sim 10^{-6}$ M for NADH oxidation. This reaction is considerably shifted towards flavin reduction (see Ref. [41] for more detailed discussion of the kinetics of NADH oxidation by Complex I).

NAD^+ and ADP-ribose [47] bind at the F-site with affinities of 10^{-3} M and $3 \cdot 10^{-5}$ M, respectively, resulting in competitive inhibition of the NADH oxidation. The R-site binds NAD^+ with apparent affinity of 7 μM (K_m in the energy-dependent NAD^+ reduction), but also binds NADH, although with lower affinity. Either ferricyanide anion or oxygen accepts electrons during the NADH oxidation only at the R-site.

The analysis of superoxide generation by Complex I using inhibitors suggests that FMN (fully reduced or semiquinone) is the electron donor in the univalent oxygen reduction. We have shown previously that the rate of superoxide generation during the succinate-supported reverse electron transfer depends linearly on oxygen concentration, i.e., a bimolecular interaction of the reduced enzyme with oxygen takes place [18]. Approximately equal rates of superoxide formation are seen in the case of succinate and at low concentrations of NADH (Fig. 1). However, in the case of NADH, the rate is insensitive to uncoupling and it increases upon addition of rotenone, whereas the steady-state level of the iron-sulfur center N-2 associated ubisemiquinone (SQ_{Nf}) decreases greatly upon either uncoupling or rotenone inhibition [53]. Thus, it seems unlikely that the Complex I-specific ubisemiquinone is the specie that participates in the bimolecular reaction between the reduced enzyme and oxygen. Participation of the iron–sulfur centers as one-electron donors in superoxide generation, however, cannot be excluded.

Superoxide generation by the FP fragment of Complex I, which contains FMN and only one strongly modified iron–sulfur cluster N-3, has been documented in the literature [54,55]. Although superoxide generation by FP is indicative of flavin being the primary oxygen reducing agent, artificial autooxidation of FMN, possibly due to the harsh preparative procedure used for isolation of FP, should be considered as an alternative cause of superoxide-generating activity. Other arguments for FMN being an immediate electron donor in the univalent oxygen reduction by Complex I have been presented [17,20].

The mechanistic implications of the F- and R-sites, depicted in Fig. 5, can hardly be envisioned given the present state of knowledge about the molecular structure of Complex I [56]. Up to five nucleotide-binding sites have been detected in the mammalian Complex I by labeling of the protein with photoactivated NAD^+-derivatives [57]. Two nucleotide-binding motifs, one in the 51 kDa subunit and one in the 39-kDa subunit (homologous *Neurospora crassa* 40 kDa), have been detected in eukaryotic enzymes [58]. Occurrence of two FMN molecules per mol of the mammalian enzyme has been deduced from the stoichiometric analysis of iron, sulfur, flavin and protein contents in different preparations of mammalian Complex I [59]. It should be mentioned, that qualitatively similar patterns of superoxide generation have been observed in the case of tightly coupled vesicles derived from prokaryote *Paracoccus denitrificans* (Grivennikova and Vinogradov, unpublished observations), which contain structurally much simpler although functionally similar [60] homologue of the mammalian Complex I, i.e., an enzyme lacking the 39 kDa subunit. The unidirectional inhibition of the reversible NADH:ubiquinone reaction by ADP-ribose and rotenone have also been documented for *P. denitrificans* enzyme [48,49]. The bell-shaped dependence of the NAD(P)H-induced lipid peroxidation in the presence of iron–ADP complex in submitochondrial particles, reported a number of years ago [61], can also be explained by the model shown in Fig. 5.

The simplest structural proposition agreeing with the model in Fig. 5 would be that two structurally distinct primary binding sites for NADH and NAD^+ are located at hydrophilic matrix exposed part of Complex I, resulting in different interactions of the substrates with the same FMN, that participates in NADH oxidation, reverse electron transfer, DD-transhydrogenase reaction and superoxide generation. The formation of a ternary complex in which two NADH molecules are simultaneously bound to F- and R-sites at high NADH concentration results in inhibition of the NADH:ferricyanide reductase activity ([41] and Fig. 2 in this paper) and DD-transhydrogenase reaction [62] as well as one-electron oxidation of the reduced flavin by oxygen (these studies). The mechanistic explanation for the latter phenomenon can hardly be offered at present state of knowledge on the structure of mammalian Complex I. It is conceivable that the hydrogen bonds network keeping FMN within the protein structure is changed upon binding of the second NADH molecule at R-site thus making reduced flavin nonreactive to oxygen. The alteration of FMN affinity to FP fragment of Complex I upon its reduction [63] and substantial conformational change of *E. coli* Complex I upon reduction by NADH [64] have been documented. Very recently the atomic structure of the hydrophilic domain of Complex I from *Thermus thermophilus* has been solved at 3.3 Å resolution [65]. In this

structure flavin is visualized at the deep end of a solvent-exposed cavity, which can also accommodate one NADH molecule. No other NADH binding site(s) was detected elsewhere in the structure. The authors have concluded that "the suggestion that Complex I has two different catalytic binding sites for NADH, with second site operating during reverse electron flow, is inconsistent with the structure" [65]. Note should be made, however, that most of the experimental data that led us to propose a "two-binding sites" hypothesis have been obtained from the experiments with mammalian 46-subunits Complex I operating in coupled membranes. To our knowledge neither coupled vesicles from *T. thermophilus* have ever been prepared nor have kinetic properties of Complex I from this organism been characterized. It does not seem judicious to make any speculations based on direct comparison of very different, although evidently evolutionary related enzymes.

We consider our model as a testable working hypothesis. For example, it would be of great interest to see the effects of recently described (although not yet chemically identified) nucleotide site-directed tight binding inhibitor [66] on superoxide generation and on the forward and reverse electron transfer reactions catalyzed by Complex I.

6. Physiologically relevant implications

The model described above suggests that if the "futile" cycle of NADH oxidation at the Site F and NAD^+ reduction at the Site R operates under the physiological conditions, the steady-state level of NADH/NAD^+ in the mitochondrial matrix, which is generally determined by the relative activities of the NAD^+-dependent dehydrogenases and the NADH-oxidizing capacity of the respiratory chain, is also partially controlled by this cycle. Although cycling of NADH seems energetically wasteful, it may have important physiological functions. Any ligand specifically interacting with either R- or F-site would alter the NADH/NAD^+ ratio, thus providing a fine regulation of this key redox couple. Therefore, searching for natural unidirectional inhibitors (activators) in the mitochondrial matrix seems like a worthwhile task.

The total concentrations of NADH and NAD^+ in the mitochondrial matrix are in a higher than millimolar range [67], and significant fractions of these nucleotides are present in their free forms [68]. We have shown that both NADH and NAD^+ inhibit the succinate- and the NADH-supported superoxide generation. Therefore, it seems highly unlikely that Complex I, which under in vitro experimental conditions is the major contributor to the overall superoxide generation in the respiratory chain, serves as the source of the intracellular superoxide under the physiological conditions. On the other hand, as discussed in the Introduction, mitochondria may contribute significantly to the overall intracellular hydrogen peroxide production. To eliminate this apparent contradiction, we propose that there is a specific enzyme(s) in the mitochondrial matrix, which is poised in the redox equilibrium with NAD(P)H/$NAD(P)^+$ couple, and that this enzyme(s), but not the respiratory chain itself, is (are) responsible for the mitochondrial production of hydrogen peroxide. This hypothesis fits well the recent data on the effects of genetic deletions of the respiratory chain components on the superoxide production in *E. coli* [69]. A possibility that other than the respiratory chain enzymes, for example, lipoyl dehydrogenase component of α-oxoacids dehydrogenase complexes, may significantly contribute to the mitochondrial superoxide generation has been proposed [2]. The superoxide and subsequent hydrogen peroxide formation in mitochondria are always considered as a side (leakage) reaction catalyzed by the enzymes that have well defined "main" functions such as participation in Krebs cycle (α-oxoacids dehydrogenase complexes) or in $\Delta\tilde{\mu}_{H^+}$ generation (respiratory chain). In our view, if the mitochondrially produced superoxide and hydrogen peroxide are, indeed, physiologically relevant signalling molecules, the special NAD(P)H:oxygen superoxide (or hydrogen peroxide) generating oxidoreductase would be much better suited for the regulated production of these species.

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