Ammonium-dependent hydrogen peroxide production by mitochondria

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Abstract NADH-supported generation of H₂O₂ by permeabilized rat heart mitochondria was partially prevented by the specific complex I-directed inhibitor, NADH-OH, and was significantly stimulated by ammonium. Ammonium did not affect H₂O₂ production by complex I in coupled submitochondrial particles. The soluble mitochondrial matrix protein fraction catalyzed NADH-dependent H₂O₂ production, which was greatly (~10-fold) stimulated by ammonium. We conclude that complex I is not the major contributor to mitochondrial superoxide (hydrogen peroxide) generation and that there are specific ammonium-sensitive NADH:oxygen oxidoreductase(s) in the mitochondrial matrix which are responsible for mitochondrial H₂O₂ production.

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Keywords: Hydrogen peroxide; Mitochondria; Complex I; Ammonium

1. Introduction

Hydrogen peroxide, a relatively inert non-enzymatic oxidant, superoxide anion (O₂⁻), an immediate precursor of H₂O₂ in a superoxide dismutase-catalyzed reaction [1] and harmful hydroxyl radical (OH⁻) which can be produced by the one-electron reduction of H₂O₂ are conventionally named as reactive oxygen species (ROS). It has been generally accepted that ROS are directly involved in a number of physiological processes and it has been suggested they are involved in metabolic pathways that contribute to aging, phagocytosis, necrosis, and apoptosis. The biochemistry of ROS production and utilization [2,3] as well as the holistic concept of oxidative stress and antioxidants has been extensively reviewed [4–6]. Mitochondria are significant contributors to the cellular production of H₂O₂ [7], for example, ~3% of oxygen consumed by pigeon heart mitochondria during coupled (state 4) succinate plus glutamate-supported respiration is converted to hydrogen peroxide [8]. Superoxide radicals have been recognized as precursors of the respiratory chain produced hydrogen peroxide [9]. The vast majority of studies on mitochondrial ROS production are based on explicit or implicit assumptions that the respiratory chain components, particularly NADH:ubiquinone oxidoreductase (complex I) and ubiquinol:cytochrome c oxidoreductase (complex III) are responsible for superoxide production by a “leakage” of electrons from their main pathways thus resulting in univalent oxygen reduction. Under certain experimental conditions both complex I in inside-out submitochondrial particles (SMP) [10–14] or in purified form [15,16] and complex III (especially in the presence of antimycin A) [9,10,17,18] are capable of superoxide production. Quantitative evaluations suggest that complex I is the major contributor to superoxide production by submitochondrial particles during the NADH- or succinate-supported coupled respiration [14].

The characteristic features of complex I-catalyzed superoxide generation are briefly summarized below. In tightly coupled SMP complex I catalyzes NADH-supported superoxide production which is rotenone- and uncoupler-insensitive [14]. The succinate-supported reaction is sensitive to rotenone and uncouplers [11,13,14], and superoxide production proceeds in oxygen first-order dependent reaction [19]. Under “optimal” non-physiological conditions NADH- or succinate-supported reactions show approximately the same rates (the latter is strongly dependent on the coupling of the particular SMP preparation) which are about 0.1% of the enzyme turnover in uncoupled NADH oxidase activity [14]. Superoxide production by complex I is prevented [20] by the competitive active site-directed inhibitor, substrate analog NADH-OH [21]. Both NADH- or succinate-supported generation of superoxide gradually decline when NADH concentration is increased from “optimal” (~50 μM) to higher concentrations and are also inhibited by low concentrations of NAD⁺ (Kᵢ = 10 μM) [14]. This and other findings have led us to conclude [14] that complex I is not a primary source of mitochondrial ROS production under physiological conditions, where the mitochondrial matrix NADH/NAD⁺ pool is in the millimolar concentration range [22]. We have hypothesized that “…the specific NAD(P)H:oxygen superoxide (hydrogen peroxide) producing oxidoreductase(s) poised in equilibrium with the NAD(P)H/NAD⁺⁺⁺ couple should exist in the mitochondrial matrix, if mitochondria are, indeed, participate in ROS-controlled processes under physiologically relevant conditions” [14]. Here, we present evidence strongly supporting this proposal and demonstrate for the first time that mitochondria are capable of ROS production catalyzed by the soluble matrix protein stimulated by ammonium.
2. Materials and methods

Rat heart mitochondria and bovine heart SMP were used as “intact” right side-in and inside-out preparations, respectively, assuming that no significant difference in mitochondrial enzyme location and catalytic properties exist for these species. Rat heart mitochondria were prepared essentially as described by Jacobus and Saks [23]. Bovine heart submitochondrial particles (SMP) were prepared according to our standard protocol [24] and were coupled by oligomycin, and activated as described [25]. The soluble matrix protein fraction was obtained essentially as described [26] (the details will be published elsewhere).

Protein content was determined by the biuret procedure. NADH oxidase activity was assayed photometrically ($\varepsilon_{562} = 6.22 \text{mM}^{-1} \text{cm}^{-1}$) at 30°C in a standard reaction mixture comprised of 0.25 M sucrose, 50 mM Tris–HCl, and 0.2 mM EDTA with 50 M NADH as the substrate. H$_2$O$_2$ generation was monitored at 30°C at 572 nm (formation of resorufin, $\varepsilon_{572} = 54 \text{mM}^{-1} \text{cm}^{-1}$ [27]) in the standard reaction mixture supplemented with 10 M Amplex Red, horseradish peroxidase (2 U/ml), superoxide dismutase from bovine erythrocytes (6 U/ml), 5 M rotenone and different concentration of NADH. We noted that $\varepsilon_{572}$ for resorufin depends on the NADH concentration and the optical dye response was calibrated at any substrate (NADH) level by the addition of hydrogen peroxide solutions of known concentration. One to one stoichiometry with a precision of ±10% between NADH consumed and resorufin formed was found when NADH-supported hydrogen peroxide was measured by Amplex Red assay. Although the NADH oxidase activity of horseradish peroxidase has been reported [28] and its significance for H$_2$O$_2$ detection by Amplex Red assay was emphasized [29], the resorufin formation seen in the absence of SMP, mitochondria, or the matrix protein fraction at any NADH concentration under our experimental conditions was never more than 15% of the enzymatically catalyzed hydrogen peroxide formation. It is worth noting that the specific activities of both NADH- and succinate-supported hydrogen peroxide formations by SMP determined by Amplex Red assay (in terms of electron equivalents) were significantly (up to three times) higher than those previously reported [14] for superoxide generation directly detected as acetylated cytochrome c reduction under similar although not identical conditions. Several explanations for this apparent discrepancy can be proposed. Potassium phosphate at high concentration (0.1 M) which was used to minimize the natural antimycin-sensitive cytochrome c reduction may directly interfere with the nucleotide binding site of complex I. At fixed acetylated cytochrome c concentration superoxide formed at FMN-site may partially escape in the spontaneous dismutation reaction. More interestingly, the quantitative difference between the assays specific for either superoxide or for hydrogen peroxide can be considered as an indication that both species are the products of direct oxygen reduction by complex I. Discrimination between these possibilities merits special investigation and was not elaborated in these studies.

3. Results

The major point of contention regarding the physiological significance of the well-established ability of complex I to generate superoxide under certain non-physiological experimental conditions [10–16] was that this reaction is strongly depressed by physiologically relevant concentrations of NADH and NADP$^+$ [14]. It could be argued, however, that this inhibition was observed for inside-out SMP where the hydrophilic part of complex I bearing the nucleotide binding site(s) is certainly not in the natural matrix environment which may alter the sensitivity of superoxide production to nicotinamide nucleotides. Thus, it was of interest to determine the NADH concentration dependence of complex I-mediated ROS production in intact permeabilized mitochondria where the matrix is freely accessible to externally added NADH [30]. Fig. 1A depicts the representative experiments on the NADH concentration dependence of H$_2$O$_2$ production by inside-out SMP as compared with that for permeabilized rat heart mitochondria (Fig. 1B). Assayed under exactly the same conditions the patterns seen for these two preparations were not significantly but notably different. H$_2$O$_2$ production pattern by inside-out SMP measured by Amplex Red was essentially the same as that of superoxide anion generation measured previously by acetylated cytochrome c reduction [14]. The mitochondria-catalyzed reaction also showed a peak of activity at “optimum” slightly shifted to higher NADH concentration (about 100 M) and significantly less inhibition was observed at higher NADH concentration (Fig. 1B). Two interpretations of these results seemed possible. The difference in the NADH-dependency pattern could be due to altered catalytic properties of complex I in “natural” (mitochondria) and “artificial” (SMP) environ-

Fig. 1. NADH-dependence of hydrogen peroxide production catalyzed by inside-out SMP (A) and amethicin-permeabllized mitochondria (B). The standard H$_2$O$_2$ assay mixture (see Section 2) contained 5 M rotenone and 35 M/ml of SMP (A) and 50 M/ml of rat heart mitochondria (B). The reaction was initiated by the addition of NADH.
ments. An alternative explanation is that the mitochondrial matrix contains a component other than complex I which is capable of NADH-supported ROS production at relatively high substrate concentrations and the overall pattern in Fig. 1B is a superposition of at least two catalytic activities catalyzed by different enzymes. To examine the second alternative the active site-directed inhibitor of complex I, NADH-OH [21] which has been shown to avoid both NADH-supported and succinate-supported superoxide generation [20] was used to prevent contribution of complex I activity in the overall H$_2$O$_2$ production by permeabilized mitochondria. NADH-OH, as expected, inhibited almost completely the NADH oxidase activity of mitochondria, but only partial inhibition of NADH-supported H$_2$O$_2$ generation (60%, compare Figs. 2A and B) was seen. The residual NADH-OH insensitive activity was greatly stimulated by 20 mM ammonium chloride. The results shown in Fig. 2 strongly suggest that the mitochondrial matrix contains a component which catalyzes NADH-supported ammonium-dependent H$_2$O$_2$ production. The activity catalyzed by inside-out SMP (complex I) showed no stimulatory effect of ammonium (Fig. 3A). The NADH-supported hydrogen peroxide generation by the rotenone-treated SMP (Fig. 3A) and inhibition of the succinate-supported activity by uncoupler and rotenone (Fig. 3B) witness that H$_2$O$_2$ production was, indeed, catalyzed by complex I. In fact, ammonium significantly inhibited the succinate-supported reaction that most likely was resulted from partial uncoupling of SMP. Next, we measured the NADH-supported reaction catalyzed by a crude soluble protein fraction derived from mitochondria which was free of membranes and found that it was capable of high ammonium-dependent H$_2$O$_2$ production (Fig. 4). The concentration dependencies of the soluble fraction activity on NADH and ammonium concentrations shown in Fig. 5 correspond to apparent $K_{\text{m}}^{\text{NADH}}$ and $K_{\text{m}}^{\text{NH}_4^+}$ of about 25 $\mu$M and 4 mM, respectively. Some properties of the NADH-supported hydrogen peroxide production catalyzed by the soluble protein fraction are summarized in Table 1. The activity was neither significantly inhibited by high (1 mM) NADH concentration, nor it was sensitive to

Fig. 2. NADH-supported H$_2$O$_2$ production by permeabilized rat heart mitochondria. Figures on the curves are the specific rates of hydrogen peroxide production (nmol/min/mg of protein). (A) The standard reaction mixture was supplemented with reagents for H$_2$O$_2$ determination and 5 $\mu$M rotenone (see Section 2). (B) Permeabilized mitochondria were preincubated with 50 nM NADH-OH for 1 min. NADH oxidase activities of permeabilized (control and NADH-OH treated) mitochondria were: 1.3 (100%) and 0.09 (7%) $\mu$mol/min/mg of protein, respectively. The activities were not changed in the presence of 10 $\mu$M FMN or 10 $\mu$M FAD.

4. Discussion

Identification of the specific ROS production site(s) in mitochondria is a matter of numerous and controversial reports in the current literature. What seems to be generally agreed is that the rate of mitochondrial ROS production under various conditions is proportional to the steady-state NAD(P)H/NAD(P) + ratio and independent of whether this ratio is altered by variation of the substrate-donor or by the proton-motive force [32–34]. Succinate has long been known as the most efficient reductant of the intramitochondrial NAD(P)H pool at
state 4 respiration [35] which agrees with numerous observations showing that the succinate-supported ROS generation is higher than that in the presence of NAD+-dependent substrates. Simple model for thermodynamic control of ROS production at complex I associated site(s) where the rate is proportional to Fe–S center N-1a reduction [36] is, however, in disagreement with the hypothetical role of respiratory chain components as the primary sites for superoxide generation because the latter is significantly inhibited by both NAD+ and NADH [14,16]. Remarkably, an *Escherichia coli* strain lacking intracellular ROS scavenging enzymes showed no decrease in extracellular H₂O₂ production by mutants deleted for NADH dehydrogenases I and II [37]. These and other findings led us to propose that some mitochondrial matrix-(or prokaryotic cytoplasm-)located component(s) is (are) responsible for superoxide/hydrogen peroxide generation.

The data reported here strongly reinforce this proposal. Under conditions where superoxide generation by complex I was prevented permeabilized mitochondria produced (under “optimal” conditions, in the presence of ammonium) hydrogen peroxide at a rate which was over three times higher (Fig. 2B) than that seen when complex I was active (Fig. 3A). Moreover, the soluble matrix fraction free of membranes catalyzed H₂O₂ formation (Fig. 4). An obvious question arises: what is the source of ammonium-dependent hydrogen peroxide production catalyzed by the crude soluble protein fraction of the mitochondrial matrix? Although the present studies were not aimed to identify and purify the component(s) responsible for the reaction, some possibilities merit brief discussion.

Following the original report [38] on NADH oxidase activity catalyzed by the lipoyl dehydrogenase component of 2-oxoacid dehydrogenases, α-ketoglutarate dehydrogenase, and/or free lipoyl dehydrogenase [39–42], which are natural constituents of the mitochondrial matrix [43] have been suggested as ROS producers upstream of the respiratory chain. The most relevant to the present discussion is the report by Starkov et al. [42] who observed α-ketoglutarate-supported H₂O₂ production by permeabilized mouse brain mitochondria at the rate of about 0.4 nmol/min/mg of protein at pH 7.4, 37 °C, a value corresponding to about 1% of the overall enzyme α-ketoglutarate dehydrogenase activity. In our hands the overall α-ketoglutarate dehydrogenase activity (NAD+ reduction) of permeabilized rat heart mitochondria [31] under the conditions as shown in Fig. 2A and α-ketoglutarate-supported H₂O₂ production (in the absence of NAD+ and in the presence of 20 mM NH₄Cl) were 83 and 0.8 nmol/min/mg of protein, respectively. The specific rate of H₂O₂ production for permeabilized mitochondria (Fig. 2A) were significantly higher than those expected to originate from the lipoyl dehydrogenase catalyzed reaction. The most striking observation reported here is a dramatic effect of ammonium on mitochondrial H₂O₂ production. It should be noted that in their original paper Huennekens et al. [38] reported the stimulation of NADH oxidase activity of lipoyl dehydrogenase by ammonium at very high (0.25 M) concentration. An inspection of the literature has pointed to other possible suspects, including a family of FAD, molybdenum, and iron–sulfur center containing oxidoreductases (i.e., aldehyde oxidases, E.C. 1.2.3.1) [44,45] which have been shown to be activated by ammonium at a millimolar concentration range [46]. To the best of our knowledge the exact intracellular location and relevant physiological function, as well as, the ability to oxidize NADH is ill defined for these enzymes, which belong to a family of homodimeric oxidoreductases and show very broad substrate specificity [47]. It appears that further study to identify

**Table 1**

H₂O₂-production by the mitochondrial matrix protein fraction

<table>
<thead>
<tr>
<th>Substrate and inhibitors</th>
<th>Specific activity (nmol/min/mg of protein)</th>
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<tr>
<td></td>
<td>−NH₄Cl</td>
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<tr>
<td>NADH, 50 μM</td>
<td>4.0</td>
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<tr>
<td>NADH, 1 mM</td>
<td>3.1</td>
</tr>
<tr>
<td>NADH, 50 μM</td>
<td>+NAD⁺, 200 μM</td>
</tr>
<tr>
<td>+NADH-OH⁺, 50 nM</td>
<td>4.0</td>
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<tr>
<td>+Dicumarol, 10 μM</td>
<td>4.2</td>
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<tr>
<td>+Rotenone, 5 μM</td>
<td>4.0</td>
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<tr>
<td>+Rubidium chloride, 20 mM</td>
<td>4.0</td>
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<tr>
<td>NADPH, 50 μM</td>
<td>1.0</td>
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The activities were assayed as in Fig. 4.

Acetate, phosphate and sulfate salts of ammonium were equally effective.

Protein fraction was preincubated with NADH-OH for 1 min.

RbCl was added as analog of ammonium cation.

Fig. 5. The substrate (A) and activator (B) dependencies of hydrogen peroxide production by the soluble matrix protein fraction (averaged from three experiments). The activity was measured as described in Fig. 4 in the presence of 20 mM NH₄Cl (A) and 50 μM NADH (B); 13.5 μg/ml of protein was added.

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The activities were assayed as in Fig. 4.

Acetate, phosphate and sulfate salts of ammonium were equally effective.

Protein fraction was preincubated with NADH-OH for 1 min.

RbCl was added as analog of ammonium cation.

formation.
and purify the protein(s) responsible for ammonium-dependent ROS production in the mitochondrial matrix are needed.

The last point to be briefly discussed is the possible physiological significance of ammonium-dependency. The mitochondrial matrix is a compartment where oxidative decomposition of amino acids, particularly glutamate, occurs which produces ammonium further utilized in the first step of urea synthesis. The steady-state concentration of free ammonia in the mitochondrial matrix under various physiological conditions is not known, although the total free ammonia in isolated perfused rat hearts of about 2 µmol/g dry weight (corresponding to approximately 0.4 mM concentration) have been determined [48]. This value corresponds to about one tenth of “activating” \( K_a \) determined here (Fig. 5). The linear hydrogen peroxide production response to variation of ammonia concentration is thus expected. It would be of great interest if ammonium-dependent ROS production by mitochondria as reported here does take place in vivo. Should this be true, it opens a new avenue for studies on interplay between ROS biochemistry and the nitrogen metabolism network.

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