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# A novel superoxide radical generator in heart mitochondria

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Experimental evidence is presented demonstrating the existence of a potent $O_2^{\cdot-}$ source in heart mitochondria. The novel $O_2^{\cdot-}$ generator is more active than any other known mitochondrial $O_2^{\cdot-}$ generator and also exhibits a higher affinity for molecular oxygen. In contrast to mitochondrial $O_2^{\cdot-}$ sources reported previously [(1974) FEBS Lett. 42, 68–72; (1978) Eur. J. Biochem. 82, 563–567], the $O_2^{\cdot-}$ generator described in this paper is not involved in energy-linked respiration. Superoxide radicals from this source require NADH to initiate their generation, and the radicals formed are released entirely into the extramitochondrial space. NADH-related $O_2^{\cdot-}$ generation was also observed with the solubilized exogenous NADH oxidoreductase of heart mitochondria, an enzyme recently described [(1987) Eur. J. Biochem., submitted]. This finding together with the lack of an NADH-dependent $O_2^{\cdot-}$ source in liver mithochondria suggests that the novel $O_2^{\cdot-}$ generator and the exogenous NADH oxidoreductase of heart mitochondria are identical.

Superoxide generation; Hydrogen peroxide; Mitochondria; Submitochondrial particle; NADH oxidoreductase

## 1. INTRODUCTION

Since mitochondria have been reported to release $H_2O_2$ from decomposing $O_2^{\cdot-}$ during respiration [1,2], the identity of the component responsible for a single electron transfer to oxygen has been a matter of debate.

It is now becoming clear that mitochondrial $O_2^{\cdot-}$ radicals may originate from more than one single source [3,4]. Considering the redox potentials of mitochondrial electron carriers, several compounds of complex I, II and III exhibit thermodynamic properties appropriate to the reduction of $O_2$ to $O_2^{\cdot-}$. In fact, under experimental conditions for the selective reduction of complex I and II, $O_2^{\cdot-}$ formation could be observed [3,4] although the generation rates reported were far below those seen when ubiquinone and *b*-type cytochromes were kept in a high state of reduction. Due to the complexity of electron transfer pathways in this section of the respiratory chain, direct experimental evidence of the main $O_2^{\cdot-}$ generator is still lacking [5–7]. The formation of superoxide radicals as a physiological byproduct of respiration indicates that both, the generator may form an adequate redox couple with molecular oxygen and its interaction with the physiological electron acceptor may exhibits kinetic restraints causing electron deviation from the normal pathway to oxygen at a non-physiological site of the respiratory chain. In normally functioning mitochondria, the latter condition is closely related to mitochondrial coupling, since $O_2^{\cdot-}$ generation was only observed under state 4 respiration and was inhibited during ATP synthesis [8]. This paper provides experimental evidence demonstrating the existence of a potent $O_2^{\cdot-}$ source in heart mitochondria which is more active than previously reported generators and operates independently of the coupling state. Furthermore, in contrast to $O_2^{\cdot-}$ formation associated with state 4 respiration, $O_2^{\cdot-}$ radicals of the new generator can be directly detected without removing superoxide dismutase (SOD) from mitochondria. The requirement exogenous NADH

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to run this source, together with the fact that liver mitochondria do not exhibit this type of $O_2^{\cdot -}$ generator, suggests that the new $O_2^{\cdot -}$ source is identical with the recently found exogenous NADH-oxidoreductase of rat heart mitochondria [10].

## 2. MATERIALS AND METHODS

SOD, ethoxyformic anhydride, *o*-dianisidine dihydrochloride and L-epinephrine were purchased from Sigma (St. Louis, MO). NADH, antimycin A and catalase were obtained from Boehringer (Mannheim). Other biochemicals were from Sigma. Mitochondria were isolated from male wistar rats (250–300 g body wt) according to [12]. Submitochondrial particles were prepared as in [2]. Detection of $O_2^{\cdot -}$ was performed by the inhibitory effect of SOD on the $O_2^{\cdot -}$-induced oxidation of epinephrine to adrenochrome [13]. In addition to this, SOD-sensitive reduction of succinylated cytochrome *c* was followed at 550 nm as a control for $O_2^{\cdot -}$-generation rates inferred from adrenochrome formation. Since formation rates measured with both methods were nearly identical, only the oxidation of epinephrine to adrenochrome is presented here.

## 3. RESULTS

Superoxide radical detection from components of the respiratory chain requires the preparation of SOD-free submitochondrial particles (SMP) to prevent dismutation of these oxygen species by matrix-bound SOD [1,2]. Fig.1a shows that in contrast to these conditions, $O_2^{\cdot -}$ release from intact SOD-containing rat heart mitochondria can be easily followed when NADH is added to the reaction system. Uncoupling of mitochondrial respiration from oxidative phosphorylation (carbonyl cyanide *m*-chlorophenylhydrazone, CCCP) or inhibition of electron transfer through components of the respiratory chain (KCN, rotenone, antimycin, myxothiazol) had no effect on NADH-related $O_2^{\cdot -}$ formation (not shown).

NADH-dependent $O_2^{\cdot -}$-formation rates were higher in SMP freed from SOD as compared to intact rat heart mitochondria (RHM) (fig.1b). However, inhibition of the endogenous NADH-ubiquinone oxidoreductase (EC 1.6.99.3) upon ad-

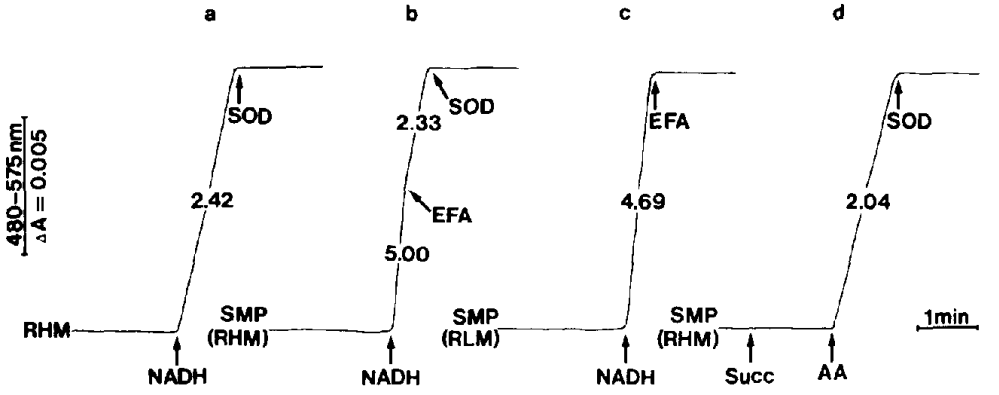


Fig.1. Demonstration of the existence of an NADH-dependent $O_2^{\cdot -}$ source in heart mitochondria releasing radicals into the cytosolic space. Initiation of $O_2^{\cdot -}$ generation by exogenous NADH by (a) SOD-containing RHM; (b) SOD-free SMP of heart and (c) liver mitochondria; (d) $O_2^{\cdot -}$-formation rates of heart SMP under classical conditions of antimycin A (AA)-inhibited succinate respiration. 1 mg protein mitochondria or SMP was suspended in 1 ml of the reaction medium (250 mM sucrose, for RHM and SMP and 300 mM sucrose for RLM, 2 mM ETDA and 20 mM triethanolamine HCl respectively, pH 7.4). The reaction medium was air-saturated and adjusted to 25°C. Final concentrations of added compounds: NADH, 20 $\mu$M; succinate, 4 mM; EFA, 2 mM; SOD, 2 $\mu$M; AA, 0.5 $\mu g \cdot ml^{-1}$. Formation rates of $O_2^{\cdot -}$ were derived from the absorbance increase at 480–575 nm in the presence of epinephrine (1 mM final concentration) using a Shimadzu UV-3000 spectrophotometer in the dual-wavelength mode. The formation of adrenochrome from epinephrine was tested for its sensitivity to SOD to demonstrate the involvement of $O_2^{\cdot -}$. The values on the curves indicate nmol $O_2^{\cdot -}$ radicals formed $\cdot min^{-1} \cdot mg^{-1}$. The curves represent typical results of 6 experiments.

dition of ethoxyformic anhydride (EFA) [9] again decreased $O_2^{\cdot -}$ release to levels found with intact RHM. When using SMP of rat liver mitochondria (RLM) instead of RHM, $O_2^{\cdot -}$ formation following the addition of NADH was completely inhibited by EFA (fig.1c). This finding is in agreement with the earlier observation that RLM do not possess the EFA-insensitive exogenous NADH oxidase of RHM [10]. $O_2^{\cdot -}$ radical formation of SMP in the presence of succinate and antimycin A (AA) was also performed to compare the activity of the new $O_2^{\cdot -}$ generator with $O_2^{\cdot -}$ release under 'classical conditions' (fig.1d). The rates measured (fig.1a,d) clearly indicate that the NADH-dependent $O_2^{\cdot -}$ source is more active.

$H_2O_2$-generation from NADH-respiring RHM was very low due to spontaneous dismutation of $O_2^{\cdot -}$ released. A clear increase in $H_2O_2$-formation rates could be observed when dismutation of $O_2^{\cdot -}$ was catalysed by the addition of SOD (fig.2). The stoichiometry of $O_2^{\cdot -}$ to $H_2O_2$ formation was approx. 2:1, indicating the total dismutation of NADH-related $O_2^{\cdot -}$ radicals by added Cu,Zn-SOD. Since the native SOD protein used cannot penetrate biological membranes, the latter observation reveals that in contrast to $O_2^{\cdot -}$ release from respiring RHM [2], $O_2^{\cdot -}$ release from the new source is directed entirely towards the extramitochondrial space.

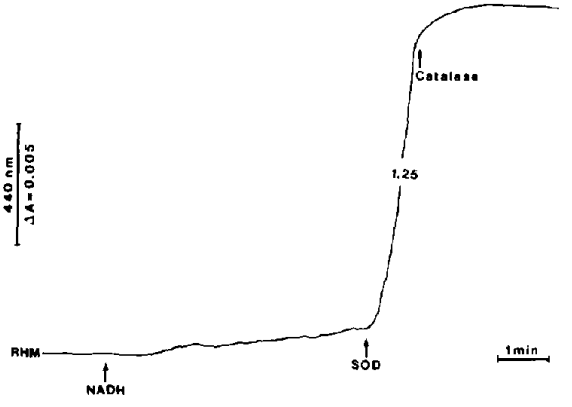


Fig.2. SOD-dependent $H_2O_2$ formation in RHM (2 mg/ml) supplemented with NADH (20 $\mu$M final concentration). $H_2O_2$ formation was measured with the *o*-dianisidine method according to Bernt and Bergmeyer [11]. Catalase (5 $\mu$M final concentration) was added to demonstrate the formation of $H_2O_2$. The value on the curve represents nmol $H_2O_2$ formed $\cdot$ ml$^{-1}$ $\cdot$ mg$^{-1}$ mitochondrial protein. The trace represent a typical curve similar to those obtained in 4 other experiments. The reaction medium was air-saturated and adjusted to 25°C.

Assuming that monovalent reduction of oxygen is an enzyme-catalysed reaction of the exogenous NADH oxidoreductase, $O_2^{\cdot -}$-formation rates may follow first-order kinetics with respect to oxygen. Thus, changing oxygen concentration also changes rates of $O_2^{\cdot -}$ formation in the same direction. This relationship expressed by the Michaelis constant ($K_m$) was determined from the slope of the Lineweaver-Burk plot and compared with the respective plot obtained for $O_2^{\cdot -}$ release of succinate-respiring SMP in the presence of AA (fig.3). The $K_m$ value for NADH-related oxygen activation was calculated to be 526 $\mu$M oxygen,

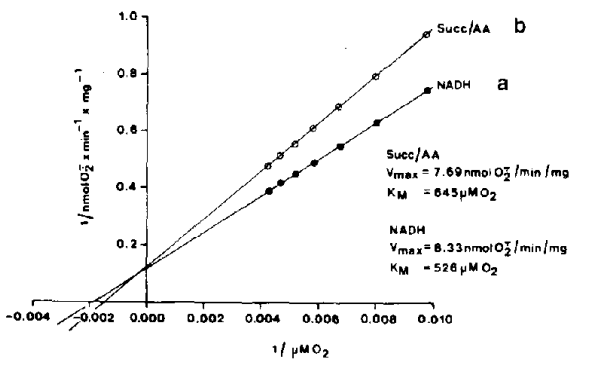


Fig.3. Comparison of Michaelis-Menten kinetics for $O_2^{\cdot -}$ generation of exogenous NADH oxidoreductase and antimycin A-inhibited succinate respiration. Determination of Michaelis constant for the monovalent reduction of molecular oxygen by (a) exogenous NADH oxidoreductase of RHM and (b) AA-inhibited succinate respiration of SMP. Experimental conditions: the continous decrease in oxygen concentration of an air-saturated suspension of RHM or SMP (1 mg/ml), respiring endogenous substrates at 25°C, was followed by a micro-Clark-type electrode of our own design. The respective values of oxygen concentration were taken as one parameter for the determination of $K_m$. Simultaneously, corresponding $O_2^{\cdot -}$-generation rates were measured as described in the legend to fig.1. For technical reasons measurements of oxygen concentrations and $O_2^{\cdot -}$-formation rates were performed simultaneously with aliquots of the same suspension under identical conditions. The data of the 2 plots represent mean values of 5 experiments. The SE of all measured points ranged between 0.00 and 1.03% of the mean. Further experimental conditions were those of fig.1.

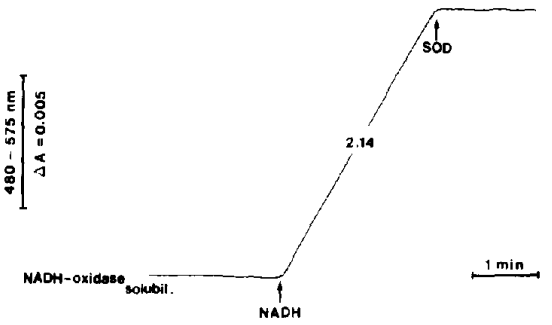


Fig.4. Detection of $O_2^{\cdot -}$ formation by exogenous NADH oxidoreductase solubilized from RHM. The enzyme was solubilized as described in [10]. Experimental conditions were identical to those described in the legend to fig.1.

while the maximal velocity of $O_2^{\cdot -}$ formation was found to be 8.33 nmol · $min^{-1}$ · $mg^{-1}$, both under conditions of excess NADH. AA initiated $O_2^{\cdot -}$ formation of succinate-respiring SMP exhibited a $K_m$ value of 645 $\mu$M oxygen, and a $V_{max}$ value of 7.69 nmol · $min^{-1}$ · $mg^{-1}$.

The identity of the new $O_2^{\cdot -}$ generator was further confirmed by investigating the capability of the solubilized enzyme to form $O_2^{\cdot -}$ radicals when supplemented with NADH. Fig.4 shows that univalent reduction of molecular oxygen also occurs with the solubilized enzyme when removed from the inner membrane of RHM where it is normally localized [10].

## 4. DISCUSSION

The present investigation provides experimental evidence which demonstrates the existence of an as yet unknown superoxide radical source in mitochondria. Although the $V_{max}$ value of the novel $O_2^{\cdot -}$ generator was only slightly higher as compared to classical sources of $O_2^{\cdot -}$ release (see fig.3), the NADH-requiring $O_2^{\cdot -}$ generator was found to be about 20% more active under conditions of atmospheric oxygen pressure (see fig.1). A rationale for this observation is based on the $K_m$ values measured indicating a distinctly higher oxygen affinity of the NADH-driven $O_2^{\cdot -}$ generator as compared to the classical $O_2^{\cdot -}$ source of AA-inhibited succinate respiration. Thus, the novel $O_2^{\cdot -}$ generator may be regarded as the major $O_2^{\cdot -}$ source in heart mitochondria. In contrast to univalent oxygen reduction associated with mitochondrial respiration, the generator described here releases $O_2^{\cdot -}$ radicals directly and entirely into the extramitochondrial space. This can be concluded from: (i) the possibility of detecting this oxygen species in suspensions of intact RHM; (ii) the complete decomposition of $O_2^{\cdot -}$ radicals to $H_2O_2$ when adding exogenous SOD to mitochondria; and (iii) the lack of higher $O_2^{\cdot -}$-formation rates in SMP freed from matrix-bound SOD. The novel $O_2^{\cdot -}$ generator is suggested to be identical with the recently reported exogenous NADH oxidoreductase of RHM [10]. According to the organ distribution of this enzyme, $O_2^{\cdot -}$ formation from exogenous NADH could not be obtained with intact RLM but was found to exist in heart mitochondria. Furthermore, exogenous NADH oxidoreductase was also reported to operate at the cytosolic face of the inner mitochondrial membrane [10], assuming both reductant (NADH) and oxidant (molecular oxygen) to have access to this enzyme from the extramitochondrial compartment. Thus, in harmony with our observations, the reduced oxygen species was also expected to be released from this enzyme into the extramitochondrial space. Despite the capability of endogenous NADH-ubiquinone-oxidoreductase to generate $O_2^{\cdot -}$ radicals in SOD-free SMP (see fig.1b,c) a contribution of the latter enzyme to $O_2^{\cdot -}$ formation from the novel source can be excluded. This is based on the fact that EFA, which inhibits endogenous NADH consumption, was found to decrease $O_2^{\cdot -}$-formation rates of heart SMP to values also obtained with intact RHM (see fig.1a,b), while NADH-related $O_2^{\cdot -}$ release from liver SMP was completely inhibited by means of EFA (see fig.1c). The most convincing experimental evidence supporting our assumption on the identity of the novel $O_2^{\cdot -}$ generator was provided by demonstrating the ability of the solubilized NADH-oxidoreductase to produce $O_2^{\cdot -}$ radicals in the presence of NADH (see fig.4). The solubilized enzyme was only obtained from heart mitochondria, not from liver mitochondria, further indicating that this enzyme and the novel superoxide radical source are identical. Demonstration of $O_2^{\cdot -}$ formation from the solubilized enzyme, together with the observed insensitivity of the NADH-related $O_2^{\cdot -}$ generator towards inhibitors and uncoupling of mitochondrial respiration, clearly

shows that this oxygen activator operates independently of energy-linked respiration.

The existence of an oxygen activator in heart cells also releasing $O_2^{\cdot -}$ radicals into the cytosol may be a rationale for an understanding of the physiological significance of cytosolic SOD in this tissue. Until now this had been unclear, since in contrast to liver cells which contain a great variety of extramitochondrial $O_2^{\cdot -}$ generators [14], most of these $O_2^{\cdot -}$ sources could not be found in heart cells or exhibited very low activities (unpublished).

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