SUPEROXIDE RADICALS AS PRECURSORS OF MITOCHONDRIAL HYDROGEN PEROXIDE*

Gerriet LOSCHEN, Angelo AZZI, Christoph RICHTER and Leopold FLOHÉ
Istituto di Patologia Generale e Centro per lo Studio della Fisiologia dei Mitocondri, Università di Padova, 35100 Padova, Italy

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1. Introduction

A component of the mitochondrial respiratory chain located between the sites of the antimycin and rotenone inhibition has been shown to produce considerable amounts of H_2O_2 under certain conditions [1–4]. Since this part of the respiratory chain switches from two electron transfer to one electron transfer H_2O_2 may be formed either directly by a two electron or indirectly by a one electron carrier via O_2^- radicals. This paper tries to indentify a free radical precursor of mitochondrial H_2O_2, which might provide insight into the stoichiometry of the primary process.

The question of a free radical formation in mitochondria may also be relevant to physiopathology, since e.g. O_2^- and one product of its spontaneous dismutation, singlet oxygen, O_2^*, are considered to be more toxic than H_2O_2 itself [5, 6]. In addition, a continuous generation of O_2^- radicals by the respiratory chain would disclose a physiological need for the presence of superoxide dismutase (SOD) in the mitochondrial matrix recently discovered by Weisiger and Fridovich [7].

With the aid of bovine blood SOD as an indicator enzyme, we were able to demonstrate that the superoxide radical, at least under some conditions, is a precursor of H_2O_2 generated by submitochondrial particles (SMP). Some observations, however, revealed an alternative pathway of mitochondrial H_2O_2 formation apparently involving an oxygen species distinct from O_2^-.

* Preliminary results have been reported at the 'First International Symposium on Alcohol and Acetaldehyde Metabolizing Systems' held at Stockholm, July 1973.

2. Materials and methods

Epinephrine and horse radish peroxidase (HRP) were purchased from Sigma Chem. Co., St. Louis, Mo., U.S.A.; scopoletin from Fluka A.G., Switzerland; Catalase from Boehringer, Mannheim. H_2O_2 formation was detected by the scopoletin method [1, 8]. Pure SOD from bovine blood ($\epsilon_{280}/\epsilon_{259}$ = 0.59) was a gift of Dr. U. Weser, Tübingen. The concentration was calculated from the extinction coefficient $\epsilon_{259} = 9840\ M^{-1}\ cm^{-1}$ [9].

2.1. *Epinephrine oxidation test*

About 1 mg protein/ml of membrane fragments obtained by sonication of beef heart mitochondria [10] were supplemented with 1 mM epinephrine, 0.5 μM catalase and 3 mM succinate in 0.25 M sucrose and 50 mM HEPES buffer, pH indicated in the figure. The rate of adrenochrome formation, which was observed after the addition of 0.5 μg antimycin/mg protein, was measured at 480–575 nm with a dual wavelength spectrophotometer (Johnson Research Foundation, University of Pennsylvania) using the difference of $2 \cdot 10^{-7}$ M.

2.2. *Luminol chemiluminescence*

0.5 mg protein/ml of SMP in the same medium, were supplemented with 3 mM succinate and 1 mM luminol (5-amino-2,3-dihydro-1, 4-phthalazinedione, Eastman Kodak Co., Rochester, N.Y., U.S.A.). Light emission observed upon addition of antimycin (0.5 μg/mg protein) was followed in the tritium channel of a Beckman Scintillation counter, model CPM-100, and

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printedcout every 20 sec as counts per minute, Concentration of added SOD was $2 \cdot 10^{-7}$ M.

3. Results and discussion

Misra and Fridovich showed that the inhibition of adrenalin oxidation by superoxide dismutase can be used as an indicator test for O_2^- [21]. Since intact mitochondria have been reported to contain a manganese–zinc-type of superoxide dismutase in the dismutase in the matrix space [7] membrane fragments are to be expected to be largely freed of endogenous SOD.

Membrane fragments from beef heart mitochondria were incubated with succinate epinephrine and catalase. Catalase was added in order to avoid an accumulation of endogenously produced H_2O_2 [11]. Upon addition of antimycin, an oxidation of epinephrine to adrenochrome was observed. The initial rate of adrenochrome formation of 4.5 nmoles/mg protein/min could be slowed down to 0.45 nmoles/mg protein/min after addition of 0.2 μM SOD (fig. 1).

The activity of the superoxide dismutase has been reported to be independent of the pH between 5 and 9, while the stability of oxigen radicals shows a strong pH dependence [12].

In fig. 2 the rate of adrenochrome formation was measured under the same conditions as described in fig. 1, at various pH-values, in the presence (lower curve) and in the absence (upper curve) of superoxide dismutase. Over the whole pH-range tested the superoxide dismutase acted as a potent inhibitor. The deline of adrenochrome formation at pH > 8 is most

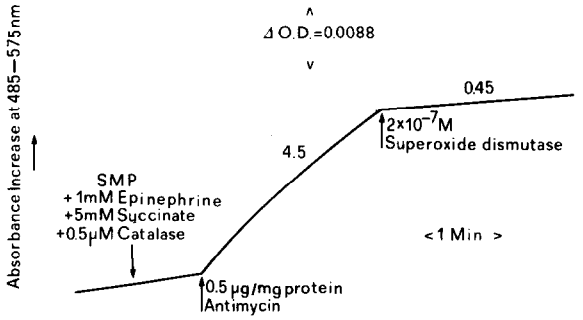


Fig. 1. Antymicin induced oxidation of epinephrine by SMP and its inhibition by SOD (bovine erythrocuprein). Sucrose–HEPES buffer pH 8.1.

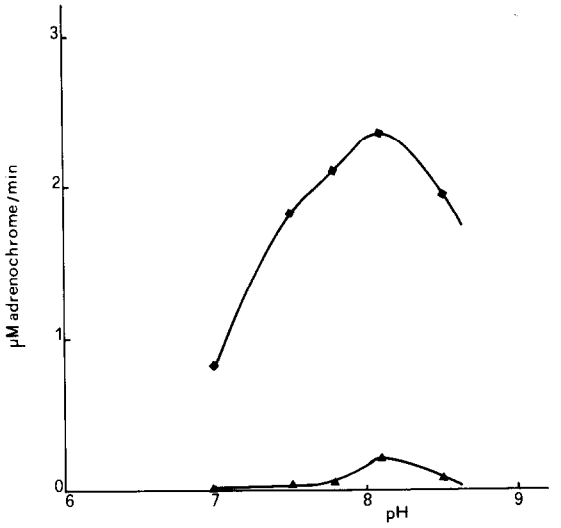


Fig. 2. The dependence of the rate of adrenochrome formation on the pH in the absence (top curve) and in the presence (bottom) of SOD.

probably due to inactivation of succinate dehydrogenase resulting in a decreased respiration [13].

In fig. 3 the effect of superoxide dismutase on the rate of mitochondrial H_2O_2 formation was measured [1–4]. SMP were incubated with succinate, horse radish peroxidase and scopoletin. The rate of the scopolrtin fluorescence decrease observed after the addition of antimycin indicates the rate of H_2O_2 formation (0.12 nmoles/min). This rate is nearly doubled (0.22 nmoles/mg protein/min.) in the presence of 0.2 μM SOD. The effect of superoxide dismutase on the rate of mitochondrial H_2O_2 formation has also been measured at various pHs, (fig. 4). Surprisingly, a stimulation of the rate of H_2O_2 formations is observed only at pH values below 7.2, while beyond this value SOD inhibits. Possible mechanisms of this effect will be discussed later on.

Further strong evidence for the generation of highly reactive oxygen species in the mitochondrial respiratory chain comes from the luminol test. This sensitive method for the detection of reactive oxygen species unfortunately yields qualitative results, since the time and pH dependence of luminol luminescence is rather complicated [14]. In addition, it is not yet clear to what extent the luminol-dependent chemiluminescence induced by O_2^*, O_2^- and/or H_2O_2 [5, 14]. In any case,

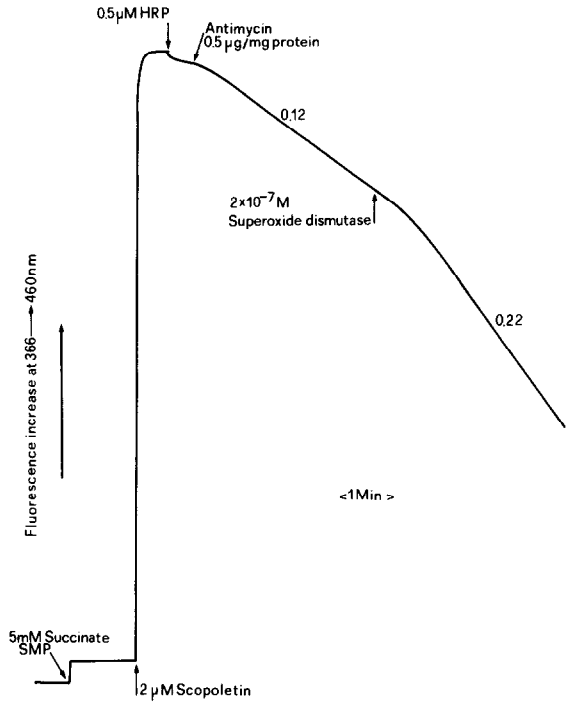


Fig. 3. Effect of bovine erythrocuprein (SOD) on the rate of mitochondrial H_2O_2 formation. H_2O_2 was detected by the scopoletin method [1, 8] in an Eppendorf fluormeter. The scopoletin fluorescence decrease indicates directly the rate of mitochondrial H_2O_2 formation and is indicated by the numbers over the trace in nmoles H_2O_2/mg protein/min. 1 mg/protein/ml of SMP was suspended in sucrose–HEPES buffer pH 7.0.

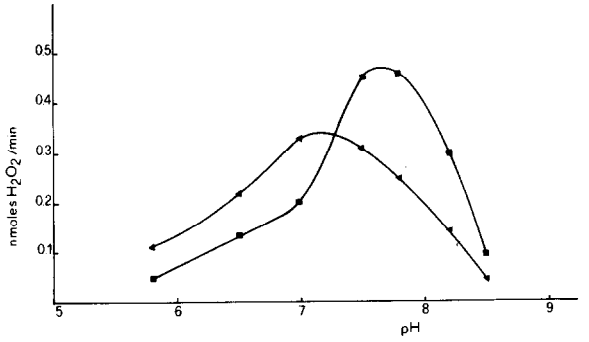


Fig. 4. Dependence of the rate of mitochondrial H_2O_2 formation on the pH in the presence (▲) and absence (■) of SOD. Experimental conditions as in fig. 3.

however, a substrate of SOD must be responsible for the luminescence, because this enzyme acts as a potent inhibitor. In fig. 5 SMP were incubated with succinate, catalase and luminol in sucrose–HEPES buffer, pH 8.1. In the absence of antimycin (no H_2O_2 formation) the chemiluminescence was indentical to the background is (= 35 cpm ± 10). Addition of antimycin (curve A), which induces H_2O_2 formation, results in strong chemiluminescence. In the presence of superoxide dismutase (and antimycin) however, (curve B) the luminescence is completely quenched.

Table 1 shows the luminol chemiluminescence induced by SMP measured under various conditions. Since the chemiluminescence is strongly time dependent and goes through a minimum after about 2 min (see fig. 5) the values given in this table correspond to the minimum values of the trough of each curve. This table shows that succinate alone does not induce any detectable luminol chemiluminescence over the whole

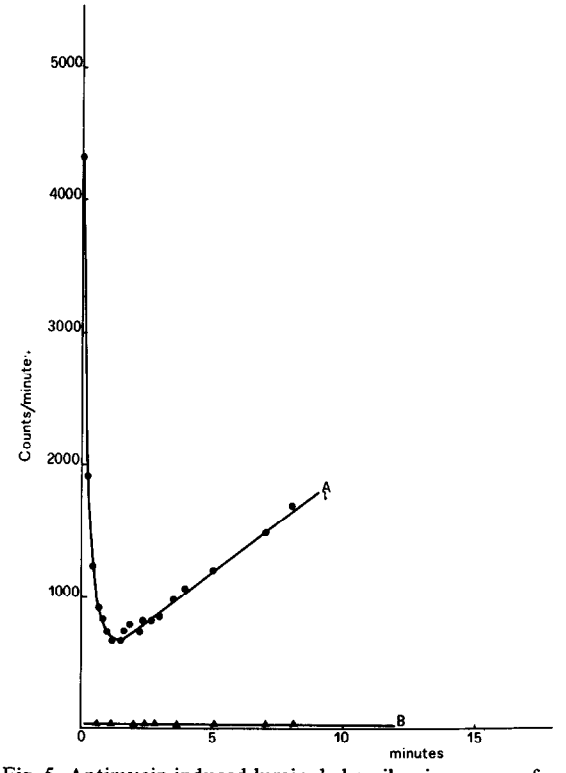


Fig. 5. Antimycin induced luminol chemiluminescence of SMP (curve A) and the effect of SOD (curve B). 0.5 mg protein/ml of SMP in sucrose–HEPES buffer pH 8.1. Other experimental conditions as descibed under Materials and methods.

Table 1
Dependence on the pH of luminol chemiluminescence induced by SMP under various conditions

	Counts per minute			
pH	+ Succinate	Antimycin + succinate	Antimycin + succinate + erythrocuprein	Blank
6.9	35	40	–	25
7.5	35	100	–	35
7.8	35	220	–	35
8.0	40	1000	35	35
8.5	40	5000	40	35

Experimental conditions as described under Materials and methods. The background counts per minute were 35 ± 10. The blank contained antimycin, succinate and SOD but SMP were omitted.

pH-range measured. In the presence of antimycin a chemiluminescence is observed, which strongly depends on the pH, SOD quenches the chemiluminescence completely over the whole pH range measured.

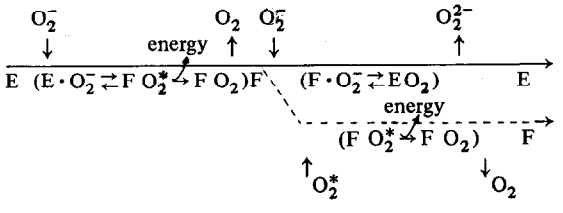
The interpretation of the results presented above suffers from the ambiquity of the test systems. It is generally agreed that superoxide dismutase catalyses the dismutation of O_2^- yielding H_2O_2 and triplet oxygen:

$$2H^+ + 2\,O_2^- \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2 \qquad (1)$$

whereas the spontaneous dismutation partly results in the formation of highly reactive O_2^*. Finazzi-Agrò et al. [5] and Weser et al. [6] provided some evidence that superoxide dismutase also catalyses the transformation of singlet to triplet oxygen:

$$O_2^* \xrightarrow{\text{superoxide dismutase}} O_2 \qquad (2)$$

The 'singlet oxygen decontaminase' [6] activity (Weser) however, of the superoxide dismutase, remains to be established. Although not yet proven, the 'decontaminase' activity nevertheless is likely to be a partial function in the ping-pong mechanism of SOD [15] depicted in the following scheme:



In spite of these dual functions of SOF, some definite conclusions can be drawn from out experiments:

1) The oxidation of epinephrine in the mitochondrial system (fig. 1), which cannot be suppressed by catalase, but almost completely by SOD, reveals that substrates of latter enzyme are primarily generated under these conditions. No decision, however, is possible, whether epinephrine is oxidized by the ordinary substrate O_2^- (eq. 1) or the hypothetical one O_2^* (eq. 2).

2) At low pH (5.8–7.0) O_2^- can clearly be identified as precursor of the H_2O_2 generated by SMP, since quenching of O_2^* cannot account for the stimulation of H_2O_2 formation by SOD.

3) At higher pH-values, surprisingly, superoxide dismutase inhibits H_2O_2 formation. This indicates that H_2O_2 under these conditions cannot be exclusively generated from O_2^-. Contrarily, a different oxygen species results in H_2O_2 formation only in the absence of SOD, may be transformed in its presence into a product different from H_2O_2. This rectant species of course could be O_2^*, which is quenched by SOD according to eq. 2 and thus cannot react with hydrogen donors to form O_2^- radicals and/or H_2O_2.

4) In agreement with this view the luminol test reveals the formation of highly reactive oxygen species especially at high pH (>7.5). These strong oxidants are almost completely eliminated at high pH by SOD as shown in the experiments of fig. 5 and the table.

In summary, H_2O_2 generated by SMP in the presence of antimycin and succinate cannot be considered at least in large part as primary product of the respiratory chain. H_2O_2 precursors could be shown to be

subtrates of SOD. Apparently, the mechanism of H_2O_2 formation depends on the experimental conditions: at low pH the predominant precursor of H_2O_2 is O_2^-, at higher pH another precursor with the following characteristics has to be considered: it is eliminated by SOD, similarly to O_2^-, but without formation of H_2O_2; it oxidizes adrenalin and gives rise to luminol chemiluminescence. Thus it is not unlikely that the alternative pathway of mitochondrial H_2O_2 formation consists in an initial activation of triplet oxygen to singlet oxygen.

This double mechanism of H_2O_2 formation does not necessarily need two different generators, if one assumes that the same electron carrier at low pH binds and reduces oxygen forming O_2^- while at high pH the interaction is restricted to an activation to O_2^*.

The present finding that substrates of SOD, whatever they are, must be considered as precursors of mitochondrial H_2O_2 may well have physiopathological significance. These highly reactive oxygen species are known to be involved in the oxidative alteration of biological membranes and enzymes. A significant protective effect of SOD could be demonstrated for instance in the following systems: decrease of respiratory control of GSH-treated rat liver mitochondria [1]; lipidperoxidation in GSH-treated mitochondrial membranes [16, 17]; dialuric acid-induced lysis of erythrocytes [18]; growth inhibition of bacteria and viruses induced by irradiation and enzymatic O_2^- radical production [19]; oxidative denaturation of ribonuclease and lysin + RNA ligase [19]. Boveris and Chance reported a linear relationship between the rate of mitochondrial H_2O_2 formation and oxygen pressure up to 20 atm. [4]. The rate of H_2O_2 formation under these conditions approaches 20 nmole/mg protein/min. If these large amounts of H_2O_2 are actually formde in free radical reactions, it can be imagined that the biological protective mechanisms may become insufficient. In spite of the relative low toxicity of H_2O_2 itself [20], the precursors of mitochondrial H_2O_2 formation thus may well contribute to the phenomenon of O_2 toxicity.

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