MITOCHONDRIAL PRODUCTION OF SUPEROXIDE ANIONS AND ITS RELATIONSHIP TO THE ANTIMYCIN INSENSITIVE RESPIRATION

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1. Introduction

Superoxide anions are produced in the oxidation by molecular oxygen of a multitude of biomolecules [1]. Iron-sulfur flavoproteins like xanthine oxidase [2,3], flavoproteins as flavodoxin [4], iron-sulfur proteins like spinach and clostridial ferrodoxins [5], quinols as the reduced form of menadione [4] and some cytostatic agents [6], glutathione [7] and other thiol containing molecules, etc., all of them are effective sources of superoxide radicals. These radical anions dismutate to H_2O_2 either nonenzymatically or by the reaction catalyzed by superoxide dismutase, according to the Mc Cord – Fridovich reaction:

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (1)$$

We would like to report superoxide generation in submitochondrial particles and its relation with the production of mitochondrial hydrogen peroxide.

It has been already reported that H_2O_2 generation accounts for the antimycin insensitive oxygen consumption in pigeon heart mitochondria [8].

2. Material and methods

Rat heart and beef heart mitochondria were isolated according to the procedures of Chance and Hagihara [9] and Blair [10], respectively. Submitochondrial particles from rat and beef heart mitochondria were obtained by sonication in a MSE sonifier model 500 W (Measuring and Scientific Equip. Ltd., London) at an output of 0.7 mA. Cytochrome *c* type VI was purchased from Sigma Chem. Co. Saint Louis, Mo., 63178, USA and superoxide dismutase obtained from Miles Labs., Elkhart, Indiana 46514. USA. Cytochrome *c* peroxidase was a gift from Professor Takashi Yonetani, Johnson Research Foundation, Univ. of Pennsylvania. Dual wavelength spectrophotometry was performed in an Aminco-Chance double beam spectrophotometer (American Inst. Co., Silver Springs, Md., USA).

3. Results

To detect superoxide anions we have used the assay method based on reduction of added cytochrome *c* and its sensitivity to superoxide dismutase. Fig.1-A shows initially the oxidation of the small amount of reduced cytochrome *c* present in the externally added oxidized cytochrome *c* by submitochondrial particles supplemented with antimycin. Antimycin is used at a concentration higher than that utilized to inhibit respiration, in order to minimize electron leaks. After addition of succinate a rapid reduction of cytochrome *c* is observed. The initial rate of cytochrome *c* reduction is directly proportional to the amount of submitochondrial particles present, and also depends on the concentration of added cytochrome *c*. After approximately one minute, a steady state of cytochrome c reduction is reached. At this point, 5–7% of

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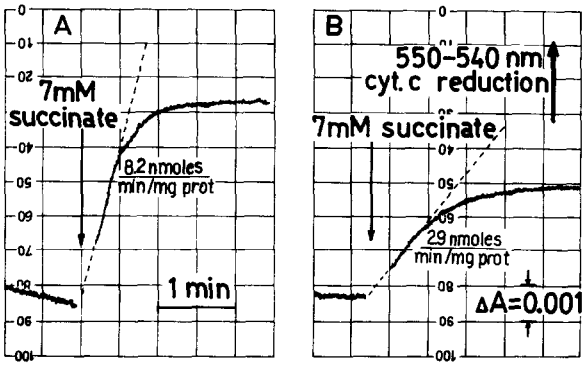

Fig.1. Production of superoxide anions by submitochondrial particles from beef heart mitochondria. Reaction medium: 0.23 M mannitol, 0.07 M sucrose, 30 mM Tris–MOPS (morpholinopropane sulfonic acid) pH 8.2, 0.048 mg mitochondrial protein/ml, 4 μM cytochrome *c*, 0.6 μM antimycin. A: no other additions. B: in the presence of 0.3 μM superoxide dismutase.

the total cytochrome *c* has been nonenzymatically reduced via superoxide anions according to the reaction

$$\text{cyt } c^{3+} + O_2^- \longrightarrow \text{cyt } c^{2+} + O_2 \qquad (2)$$

and via electron leaks through cytochromes b–c_1

$$\text{cyt } c^{3+} + \text{cyt } b^{2+} \longrightarrow \text{cyt } c^{2+} + \text{cyt } b^{3+} \qquad (3)$$

and is oxidized by the cytochrome oxidase reaction:

$$4\ \text{cyt } c^{2+} + 4\ H^+ + O_2 \longrightarrow 4\ \text{cyt } c^{3+} + 2\ H_2O \qquad (4)$$

The rate of cytochrome *c* oxidation to be considered for correction of the initial rates seems negligible at this high ratio of oxidized/reduced cytochrome *c*, as it can be seen from the initial part of the spectrophotometric trace before succinate addition. Thus, the reported inhibitory effect of oxidized cytochrome *c* in the isolated oxidase [11] probably also operates in the particulate oxidase tempering the oxidation of the reduced cytochrome *c* and making possible the attainement of a steady state. Fig.1–B illustrates the effect of superoxide dismutase that brings down the initial reduction rate from 8.2 to 2.9 nmol/min/mg protein. Therefore, the generation rate of superoxide radicals is calculated as 5.3 mol/min/mg protein. In our experiments approximately two thirds of the cytochrome c reduction rate are usually sensitive to superoxide dismutase and 0.2–0.3 μM dismutase are necessary to obtain maximal inhibition. From our concentration-effect curves it can be inferred that 0.05–0.1 μM dismutase equals 3 μM cytochrome *c* as a trapping agent for superoxide radicals. The efficiency of the dismutase utilized in this work in competing with cytochrome *c* for superoxide ions is about 25 times lower than the one reported by Forman and Fridovich [12], which we assume is due to the low specific activity of our enzyme.

Fig.2 illustrates the dependence of the reduction rate on cytochrome c concentration. About 4 μM cytochrome *c* is required for half-maximal trapping efficiency. This figure agrees with a similar one given for the xanthine-oxidase generating system of superoxide anions [13]. Fig.2 also shows an analysis of the inhibitory effect of superoxide dismutase on the cytochrome *c* reduction rate. The dismutase acts as a competitive inhibitor with respect to cytochrome *c*.

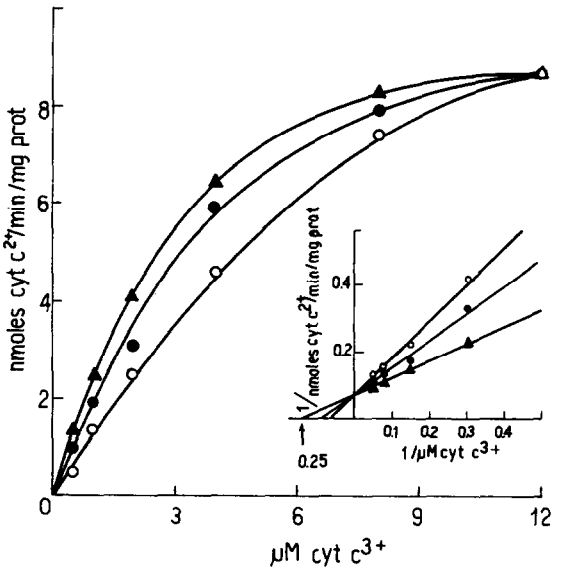

Fig.2. Effect of cytochrome *c* concentration and of superoxide dismutase on the cytochrome *c* reduction rates. Submitochondrial particles from rat heart mitochondria (0.051 mg protein/ml). Other experimental conditions as in fig.1 (▲) in the abscence of superoxide dismutase; (●) 0.05 μM superoxide dismutase; (○) 0.2 μM superoxide dismutase.

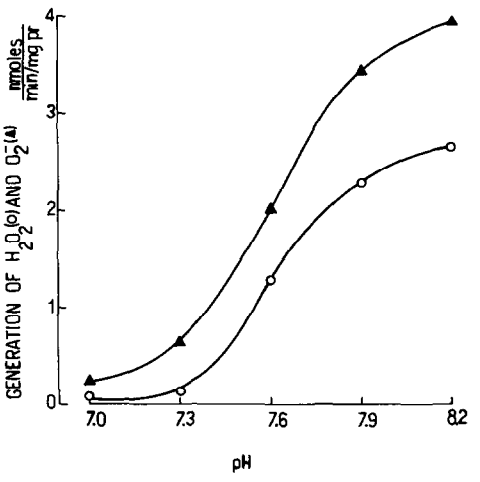

Fig.3. pH dependence of the generation of superoxide anions and hydrogen peroxide by submitochondrial particles from rat heart mitochondria. Reaction medium as in fig.1, variable pH. For superoxide determinations: 0.06 mg mitochondrial protein/ml, 10 μM cytochrome *c* and 0.4 μM antimycin. For hydrogen peroxide determinations: 0.03 mg mitochondrial protein/ml, 0.2 μM antimycin and 0.4 μM yeast cytochrome *c* peroxidase.

Superoxide dismutase catalizes the reaction (1) and decreases the steady state values of superoxide anions that effectively collide with oxidized cytochrome *c* to give reduced cytochrome *c*, according to reaction (2).

When submitochondrial particles are assayed at different pH values for production of superoxide anions and of hydrogen peroxide (fig.3), parallel activity curves are obtained. Thus a considerable part of mitochondrial hydrogen peroxide is originated via superoxide anions according to reaction (1) either involving mitochondrial superoxide dismutase [14] or independently of it.

Table 1 gives cytochrome *c* reduction rates extrapolated at infinite cytochrome *c* concentration from double reciprocal plots in order to calculate superoxide generation at the maximal efficiency of the assay system. The rates are corrected by substracting the percentage which is superoxide dismutase insensitive and compared with hydrogen peroxide production rates as measured by the cytochrome *c* peroxidase assay. Apparent stoichiometric relations of 1.6 to 2.1 are observed. Rates lower than two could be explained both by superoxide dismutase contamination in the particles, maximally effective when the dismutase is trapped into the intravesicular space, and by the existence of an H_2O_2 generator independent from the superoxide mechanism. Rates higher than two are due to electron leaks to cytochrome c_1 and erroneous rate calculations. Our measured rates of production of superoxide anions in submitochondrial particles (4.4–6.9 nmol/min/mg protein) in the presence of substrate and antimycin agree with similar value (4.1 nmol/min/mg prot.) previously reported by Loschen and Azzi [15] who utilized adrenochrome formation as the assay system for superoxide anions.

4. Discussion

The identity of the autoxidizable component of the respiratory chain that generates O_2^- is not established at the present time. Superoxide may be produced from the interaction of any of the components bracketed by the rotenone and the antimycin sensitive sites, i.e., succinate dehydrogenase, ubiquinol, cytochromes *b* and iron sulfur centers as indicated and discussed for hydrogen peroxide production by Boveris and Chance [16].

Hydrogen peroxide generation has been shown in a series of vertebrate mitochondria isolated from liver, heart, kidney, etc. [8,16,17]; it has also been shown to occur in mitochondria from yeast [18] and

Table 1
Mitochondrial production of H_2O_2 and O_2^-

Submitochondrial particles from	nmoles cyt c^{2+}/min/mg protein	Sensitivity to SOD (%)	O_2^-	H_2O_2	O_2^-/H_2O_2
			nmoles/min/mg protein		
Rat heart	12.8	54	6.9	3.2	2.1
Rat heart	8.0	62	4.9	3.2	1.6
Beef heart	6.3	70	4.4	2.5	1.7

protozoa [19]. Moreover, hydrogen peroxide production has been detected in perfused rat liver by monitoring the level of the ES-complex of endogenous catalase [20]. As far as it stands, it seems that hydrogen peroxide generation constitutes a physiological event in any aerobic mitochondria. In addition, there are other sources for hydrogen peroxide, as microsomes, peroxisomes and cytosol enzymes [21] that contribute to keep a cellular steady state of H_2O_2. Reaction with intracellular catalase or effusion from tissue to the blood stream constitute the main disappearance processes.

The mitochondrial generation of superoxide anion, if proved to be a physiological event and not an artifact arising from the ultrasonic treatment of mitochondria, provides the second reactant for the Haber-Weiss reaction [22]

$$H_2O_2 + O_2^- \longrightarrow OH^- + OH^{\cdot} + O_2 \qquad (5)$$

giving the toxic radical $OH^{\cdot}$ with ability to destroy membranes, nucleic acids, etc. [23,24]. If we recall that mitochondrial hydrogen peroxide generation is highly dependent on pO_2 values [16], then superoxide anions and hydrogen peroxide may constitute molecular links to explain oxygen toxicity. The role of O_2^- and $OH^{\cdot}$ in oxygen poisoning was advanced by Gerschman et al. [25] in 1954 on the basis of the effect of radioprotective agents and a sinergism in the deleterous actions of high oxygen tensions and X-rays

It appears interesting to speculate about the role of this oxidation mechanism, producing O_2^-, $OH^{\cdot}$ and H_2O_2, which is ubiquitously located as mitochondria are. One possibility is that the continuously generated pulses of mutagenic radicals, modulated by metabolic conditions and by the protective activity of superoxide dismutase and catalase, may contribute to the normal mutation rate and thus to the plasticity of living matter.

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