SUPEROXIDE DISMUTASE DOES NOT INHIBIT THE OXIDATION OF CYTOCHROME C AND CYTOCHROME OXIDASE

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SUMMARY

The effect of superoxide dismutase on the autoxidation of cytochrome c in phosphate buffer as well as in the presence of cardiolipin and Tween-20 was studied and compared with its effect on enzymic oxidation by cytochrome oxidase. In these systems superoxide dismutase did not inhibit oxidative processes. Also no inhibition of the oxidation of reduced cytochrome oxidase by superoxide dismutase was observed.

INTRODUCTION

The oxidation of cytochrome c by cytochrome oxidase (EC 1.9.3.1, ferrocytochrome c : oxygen oxidoreductase) involves transfer of four electrons from cytochrome c to oxygen. It is not yet clear whether this enzymic reaction actually is a concerted transfer of four electrons or perhaps a succession of four one-electron transfers. One would expect that if terminal oxidative reaction has four-step mechanism then the intermediate of the reaction should be semi-reduced oxygen in the form of anion-radical $O_2^{\bullet -}$, which is the substrate of superoxide dismutase (1,2). Therefore inhibition by SOD of oxidative reactions can be regarded as an evidence of a consecutive one-electron mechanism. However, since cytochrome c is

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Abbreviations used:
CO - Cytochrome oxidase
SOD- Superoxide dismutase

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inherently a one-electron substrate, its oxidation should not be inhibited by SOD even if it has a one-electron mechanism:

$$\text{Cyt } \underline{c}^{+2} + O_2 \longrightarrow \text{Cyt } \underline{c}^{+3} + O_2^{\bullet -} \qquad (1)$$

If SOD nevertheless would be found to inhibit the oxidation of such one-electron substrate this would mean that the superoxide radical formed in reaction (1) is also capable of oxidizing the substrate:

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

In the present study the data are reported on the effect of SOD on cytochrome c oxidation by CO and on the autoxidation of cytochrome c in non-enzymic systems. The effect of SOD on autoxidation of reduced CO was also checked.

MATERIALS AND METHODS

Bovine cytochrome c was electrophoretically homogeneous and had optical purity index of reduced state A_{550}/A_{280} of 1.25. CO was obtained as described by Kuboyama et al.(3). SOD from erythrocytes was prepared as described by McCord and Fridovich (1) and further purified by two-fold chromatography on DEAE-Sephadex, A-50, to give an electrophoretically homogeneous preparations with optical purity index A_{260}/A_{680} of 26. Concentrations of the proteins were calculated from molar extinctions of $2.77 \times 10^4 M^{-1}cm^{-1}$, $3.0 \times \times 10^2 M^{-1}cm^{-1}$ and $2.4 \times 10^4 M^{-1}cm^{-1}$ for the band at 550 nm of reduced cytochrome c, for the band at 680 nm of oxidized SOD and for differential band at 605 nm of CO, respectively. The reduction of CO was carried out as described by Lemberg and Gilmour (4) and its oxidation was followed measuring intensities of maxima at 605 nm and 444 nm. Cytochrome c was dissolved in 0.05 M phosphate buffer pH 6.0, containing 10^{-4}M EDTA. Cytochrome oxidation was followed spectrophotometrically at 550 nm in 10 mm cells at 23°C. Aliquots of SOD (0.05 ml) were added to 3.4 ml of cytochrome solutions three minu-

tes before addition 0.05 ml aliquots of ethanolic cardiolipin, Tween-20 in phosphate buffer or CO. Cardiolipin in the form of ethanolic solution (8 mg/ml) was purchased from Sigma Chemical Company, Tween-20 was a product of Merck-Schuhardt.

RESULTS AND DISCUSSION

Fresh and highly purified preparations of cytochrome c at pH 6.0 in the presence of EDTA are not subject to ready autoxidation, though the aeration somewhat enhances this process. Both in aerated and anaerated systems the addition of SOD was found to accelerate but not inhibit autoxidation process. Even with SOD concentrations higher than concentration of cytochrome c no inhibition was observed. If superoxide radical is indeed formed in the course of cytochrome c autoxidation (Eq.1), then this finding actually excludes the possibility that these radicals can oxidize another molecule of cytochrome c too (Eq.2).

The observed "stimulation" of autoxidation in the presence of SOD can be fairly well understood if we assume an equilibrium in reaction (1):

$$\text{Cyt } c^{+2} + O_2 \rightleftharpoons \text{Cyt } c^{+3} + O_2^{\bullet -}$$

As SOD inhibits ferricytochrome reduction by $O_2^{\bullet -}$ (1) then the equilibrium of this reaction would move to the right in the presence of SOD resulting apparent stimulation of cytochrome autoxidation.

CO-catalyzed oxidation of cytochrome c also was not observed to be inhibited by SOD, though no stimulation effect was noted in this system either (Fig.1).

Autoxidation of cytochrome c was found to be considerably promoted by small aliquots of ethanolic cardiolipin. The blank experiments performed with similar and with ten times larger amounts of ethanol did not induce cytochrome oxida-

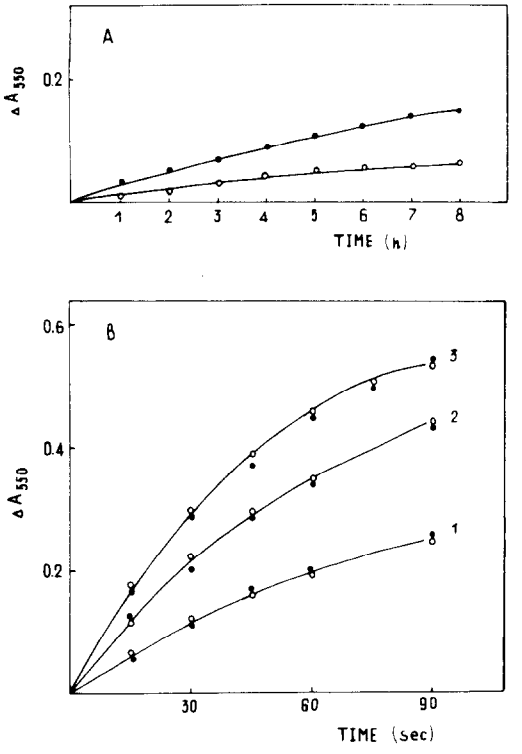

Fig.1. Autoxidation of cytochrome c (3.4 x 10^{-5}M) and its enzymic oxidation in the presence of SOD. (A) kinetics of autoxidation process. (B) kinetics of enzymic oxidation. o-o - without SOD; ●-● - in the presence of SOD. Concentrations of SOD were: 4.2 x 10^{-5}M for (A) and 10^{-6}M for (B). Concentrations of CO were: 1.6 x 10^{-9}M, 2.6 x 10^{-9}M and 5.3 x 10^{-9}M for 1, 2 and 3, respectively.

tion. The kinetics of the autoxidation of cytochrome c in the presence of cardiolipin is biphasic with more fast first step. At low molar ratio cardiolipin/cytochrome the contribution of the fast step increases linearly with increasing of the concentration of cardiolipin. If molar ratio lipid/cytochrome was 3.5 and more, then the slow step was not observed at all. Thus, e.g. in the presence of 1.6 x 10^{-4}M cardiolipin 3.5 x x 10^{-5}M of cytochrome c is completely oxidized in 15 seconds.

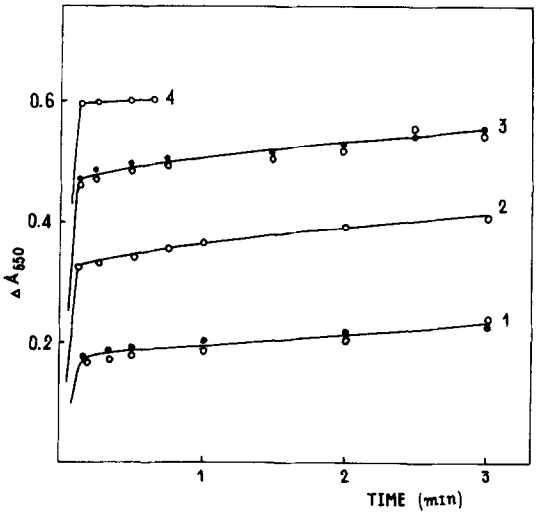

Fig.2. Autoxidation of cytochrome c (3.5×10^{-5}M) in the presence of cardiolipin. Concentrations of cardiolipin were: (1) 0.2×10^{-4}M, (2) 0.4×10^{-4}M, (3) 0.8×10^{-4}M and (4) 1.6×10^{-4}M. o-o - without SOD; ●-● - in the presence of 10^{-6}M SOD.

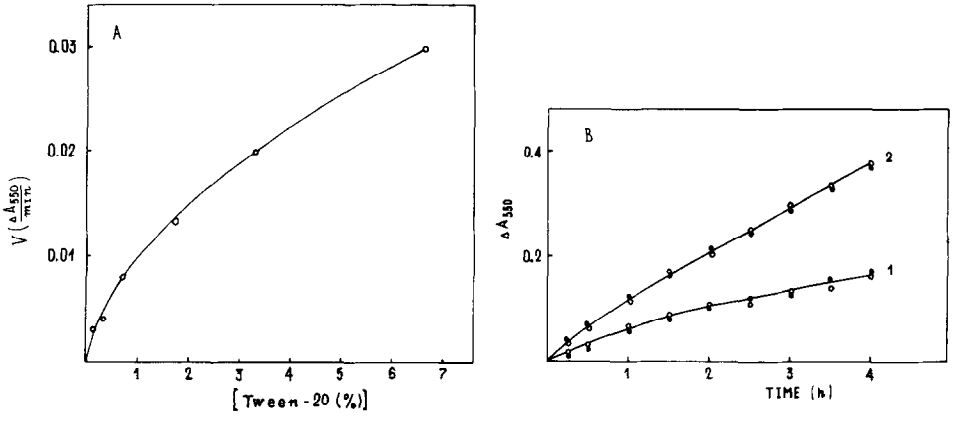

Fig.3. Autoxidation of cytochrome c (3.5×10^{-5}M) in the presence of Tween-20. (A) dependence of initial rate on the concentration of Tween-20. (B) oxidation without SOD - o-o and in the presence of 10^{-6}M SOD - ●-●. Concentrations of Tween-20 were: 0.25% (1) and 1% (2).

Cardiolipin is known to form the complex with cytochrome c of molar composition 4:1, the binding of the lipid inducing

the conformational changes in the protein (5,6). These changes, probably, facilitete the autoxidation of cytochrome c. It was found, that in the same way with CO-catalyzed reaction, oxidation of cytochrome c in the presence of cardiolipin was neither enhanced nor inhibited by SOD (see Fig. 2). Tween-20, like cardiolipin, promoted the autoxidation of cytochrome c, the rate of the reaction depending on the detergent concentration (Fig. 3A). Probably, the effect of Tween-20 is to a certain extent similar to that of cardiolipin as the detergents is known to induce the conformational changes in the proteins (7). SOD also did not affect oxidation of cytochrome c in the presence of Tween-20 (Fig. 3B). Thus it is obvious that neither model nor enzymic systems give any indication of cytochrome c oxidation being inhibited with SOD.

If the oxidation catalyzed by CO, as well as cytochrome

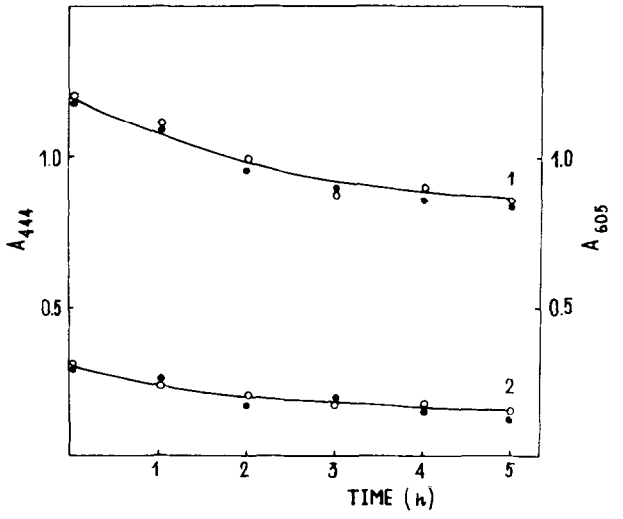

Fig.4. Autoxidation of reduced CO (4.0×10^{-6}M) in the absence SOD - o-o and in the presence of 1.5×10^{-6}M SOD -●-●. (1) data for Soret band at 444 nm; (2) data for α-band at 605 nm.

autoxidation in the presence of lipids or detergents follow the one-electron mechanism then the absence of the reaction stimulation or inhibition by SOD would indicate that in these systems $O_2^{\cdot -}$ formed in reaction (1) is not acceptable for SOD. The alternative explanation of these observations is non-one--electron mechanism of electron transfer in these systems in contrast to autoxidation in water systems.

It is important to note that no stimulation or inhibition effects of SOD on autoxidation of reduced CO were also observed (Fig.4), indicating that the oxidation of a-type heme has the same feature as autoxidation cytochrome c in the presence of cardiolipin or Tween-20.

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