Volume 297, number 1,2, 63–66 FEBS 10632 February 1992


# Cytochrome oxidase-catalyzed superoxide generation from hydrogen peroxide

M.Yu. Ksenzenko[a], T.V. Vygodina[a], V. Berka[b], E.K. Ruuge[c] and A.A. Konstantinov[a]

[a]*A.N. Belozersky Institute of Physico-Chemical Biology and* [b]*Faculty of Physics, Moscow State University, Moscow 119 899, USSR* and [c]*Institute of Experimental Physics, Slovak Academy of Sciences, Kosice, Solovievova 47, CSFR*

Received 20 November 1991

Superoxide dismutase is shown to affect spectral changes observed upon cytochrome *c* oxidase reaction with $H_2O_2$, which indicates a possibility of $O_2^-$ radicals being formed in the reaction. Using DMPO as a spin trap, generation of superoxide radicals from $H_2O_2$ in the presence of cytochrome oxidase is directly demonstrated. The process is inhibited by cyanide and is not observed with a heat-denatured enzyme pointing to a specific reaction in the oxygen-reducing centre of cytochrome *c* oxidase. The data support a hypothesis on a catalase cycle catalyzed by cytochrome *c* oxidase in the presence of excess $H_2O_2$ (Vygodina and Konstantinov (1988) Ann. NY Acad. Sci., 550, 124–138):

$$Fe^{III} \xrightleftharpoons{H_2O_2} Fe^{III}\text{-}H_2O_2 \xrightarrow[H_2O]{H_2O_2,\ O_2^-} Fe^{IV}=O \xrightarrow[H_2O]{H_2O_2,\ O_2^-} Fe^{III}$$

Cytochrome *c* oxidase; Superoxide radical; Spin trapping; Oxygen intermediate; Respiratory chain

## 1. INTRODUCTION

Cytochrome *c* oxidase (COX) is a terminal enzyme of the mitochondrial respiratory chain which catalyses the four electron reduction of dioxygen to water [1]. The overall reaction proceeds via a number of intermediates, some of which have been identified by means of time-resolved optical, EPR and resonance Raman spectroscopy (see [2] for a brief review of the recent data and basic references).

An easy way to obtain stable oxygen intermediates of COX consists in the addition of partially reduced oxygen species to the oxidized enzyme. In particular, at least two different spectral intermediates have been observed upon $H_2O_2$ addition to the ferric enzyme [3–11].

At micromolar concentrations, $H_2O_2$ forms a reversible adduct of heme $a_3^{3+}$ with the spectral characteristics closely resembling those of the peroxy intermediate (compound P) [4,7–9]:

$$Fe^{3+} + H_2O_2 \rightleftharpoons Fe^{3+} - H_2O_2 \qquad (1)$$

(the protonation state of the bound peroxide remains uncertain and can be $H_2O_2$, $HO_2^-$ or $O_2^{2-}$ as discussed in [7,8]).

Increasing the $H_2O_2$ concentration above ~$10^{-3}$ M results in a conversion of this initial adduct with a typical high extinction at 607 nm to an oxoferryl complex (compound F) with a peak of the difference spectrum at ~580 nm [4–11]; the reaction was suggested to occur by virtue of a reductive cleavage of the bound peroxide [4,8,12]:

$$Fe^{3+} - H_2O_2 + H_2O_2 \longrightarrow Fe^{4+}=O^{2-} + H_2O + O_2^- + 2H^+ \qquad (2)$$

As proposed in [8], the oxoferryl complex formed in this reaction can be further reduced by $H_2O_2$ to the free ferric enzyme:

$$Fe^{4+}=O^{2-} + H_2O_2 \longrightarrow Fe^{3+} + H_2O + O_2^- \qquad (3)$$

Altogether reactions (1–3) would form a 'catalase' cycle [8] in which COX oxidizes two $H_2O_2$ molecules to two superoxide radicals by a third, heme-bound, $H_2O_2$ which is reduced to 2 $H_2O$ (see eq. 4 in section 4).

Here we show, using a spin trap DMPO, that superoxide radicals are indeed formed when COX is incubated with excess $H_2O_2$.

## 2. METHODS

Fowler-type cytochrome oxidase was isolated from beef-heart mitochondria [13,14]. $H_2O_2$ ('Suprapur') was from Merck. DMPO (Aldrich) was purified by a charcoal treatment. Other chemicals were

*Abbreviations:* COX, cytochrome *c* oxidase; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; SOD, superoxide dismutase; DETAPAC, diethylenetriamine pentaacetate

*Correspondence address:* A.A. Konstantinov, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119 899, USSR.

commercial products of high purity. Optical measurements were made in a Hitachi 557 spectrophotometer in standard 1-cm cells. EPR measurements were made in a Varian E-4 spectrometer in a standard 0.2 ml flat quartz cell for aquous liquid samples. Standard EPR spectroscopy conditions were as follows. Clystron frequency, 9.13 GHz; modulation frequency, 100 kHz; modulation amplitude, 1 G; microwave power, 10 mW; receiver gain, usually $5 \cdot 10^3$; scan range, 100 G; scan rate, 100 G/min; time constant, 0.3 s; $T$ = 300 K.

## 3. RESULTS

Fig. 1 shows spectral changes induced by $H_2O_2$ addition to isolated cytochrome *c* oxidase at pH 7.5. At low $H_2O_2$ concentration a difference spectrum is observed with a peak at 607 nm typical of the peroxy complex and a weaker band at ~570 nm (A). Increase of the $H_2O_2$ concentration results in the peroxy complex conversion to an oxoferryl state (Fig. 1B).

Interestingly, whereas a complete disappearance of the 607 peak was observed at high $[H_2O_2]$ in experiments with liposome-reconstituted COX [7,8], contribution of this band to the spectrum of the high-peroxide compound of COX remains significant in case of the solubilized enzyme (Fig. 1B, [4,5,15]) indicating admixture of compound P. However, in the presence of SOD, the shoulder at 607 nm is no longer observed in the difference spectrum of the oxoferryl complex (Fig. 1B). A similar effect of SOD was noted earlier for bacterial COX by B. Zimmerman in her PhD thesis. There is no effect of SOD on the spectral changes at low peroxide concentrations (Fig. 1A).

Additional evidence for interference of superoxide with COX interaction with $H_2O_2$ is given in Fig. 2. Whereas the reaction of the liposome-bound enzyme with $H_2O_2$ is fully reversible [8], significant irreversible loss of absorbance was reported in case of the solubilized enzyme [16]. We confirmed the latter observation and found the irreversible changes to increase at low pH. Fig. 2A shows that at pH 6.5, the $H_2O_2$-induced difference spectra are very asymmetric and catalase abolishes only a minor part of the response. In contrast, if the experiment is carried out in the presence of SOD, a symmetrical difference spectrum is observed which is reversed by catalase (Fig. 2B). Presumably, $O_2^-$ radicals generation in the reaction mixture promotes the destruction of heme $a_3$. To probe possible formation of the superoxide radicals we used a conventional spin-trapping technique with DMPO as the spin trap.

Aerobic incubation of DMPO with ferric COX or

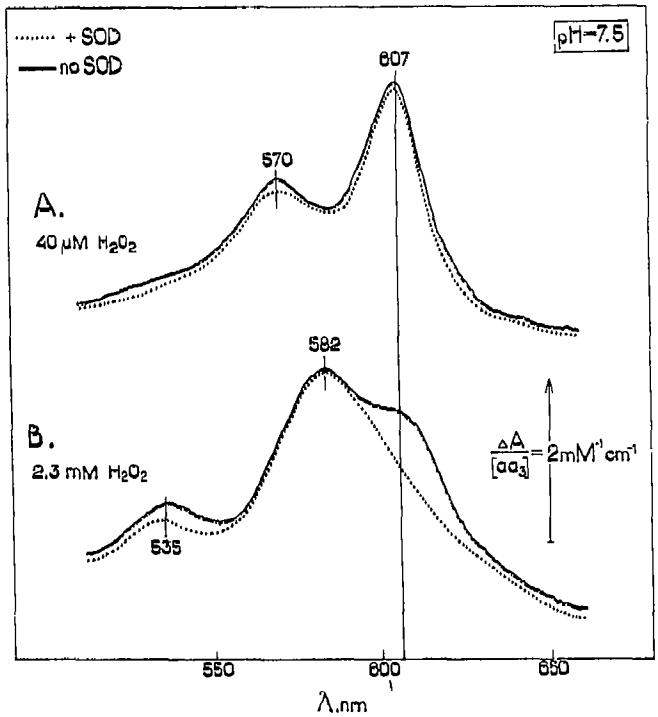



Fig. 1. Effect of SOD on the spectrum of cytochrome oxidase peroxide complex. 1 $\mu$M COX in a basic medium containing 0.5% Tween 80, 50 mM HEPES-KOH pH 7.5, 0.1 mM EDTA, 0.1 mM ferricyanide and, where indicated, 100 $\mu$g/ml of Cu,Zn-superoxide dismutase. $H_2O_2$ has been added to the sample at the concentrations indicated.

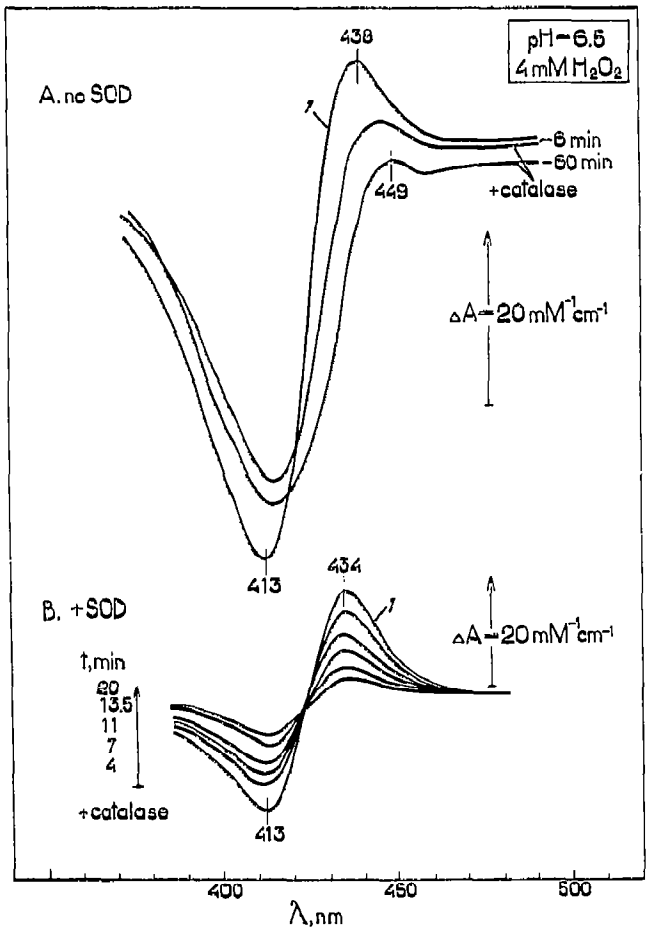



Fig. 2. $O_2^-$-dependent irreversible spectral changes of cytochrome oxidase at acid pH. 1 $\mu$M COX in the basic medium containing 0.5% Tween 80, 50 mM MES pH 6.5, 0.1 mM EDTA, 0.1 mM ferricyanide and, in (B), 100 $\mu$g/ml of SOD. 4 mM $H_2O_2$ is added to the sample (spectra *1*). Subsequently 2 nM catalase was added to both sample and reference cells and difference spectra recorded at the indicated time intervals after the addition.

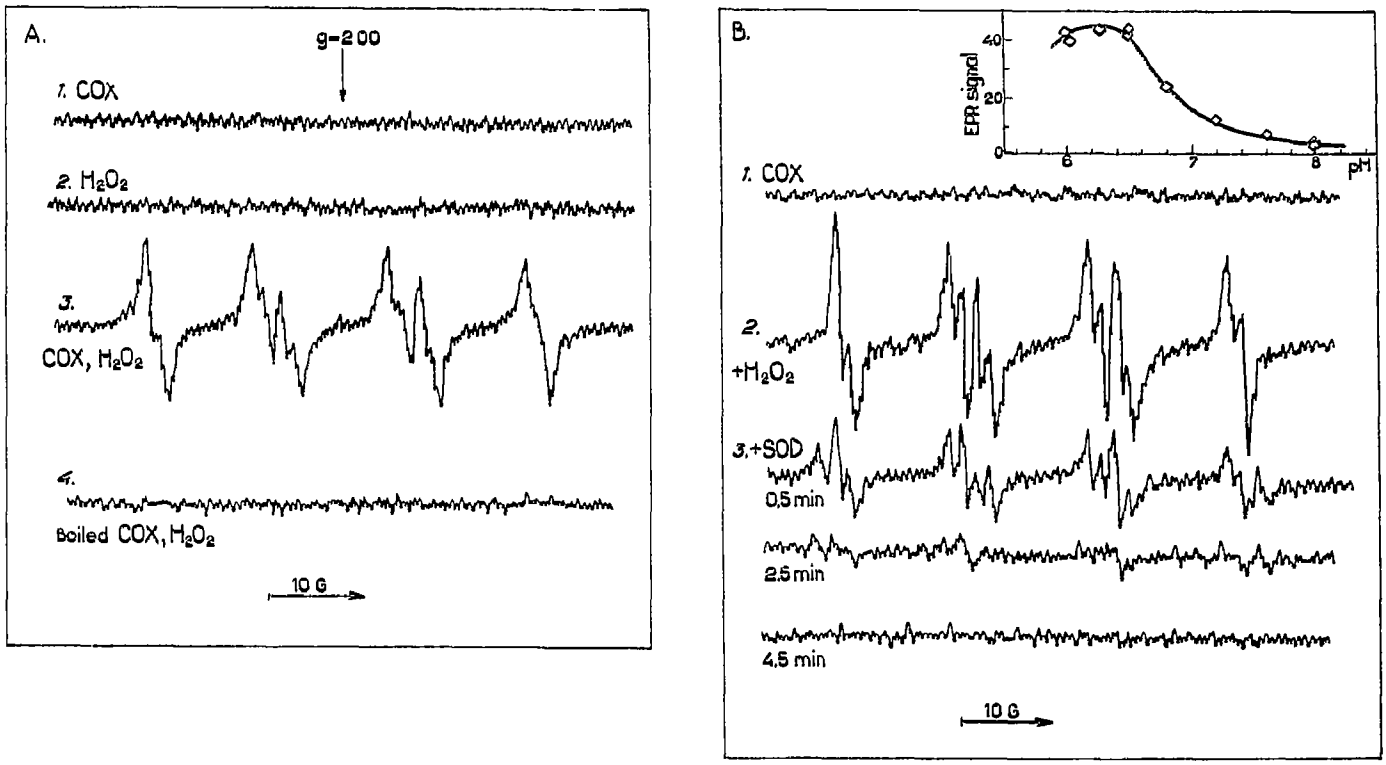



Fig. 3. Superoxide generation by cytochrome oxidase. The basic reaction medium contains 0.5% Tween 80, 25 mM of MES and HEPES pH 6.5, 2.5 mM DETAPAC and 100 mM DMPO. (A) Additions: (*1*) 10 $\mu$M COX; (*2*) 7.3 mM $H_2O_2$; (*3*) COX + $H_2O_2$; (*4*) heat-denatured COX (10 min at 100°C) + $H_2O_2$. The spectra shown were recorded 4 min after the additions. (B) The following additions to the sample were made in sequence: (*1*) 10 $\mu$M COX; (*2*) 7.8 mM $H_2O_2$; (*3*) 150 $\mu$g/ml of SOD; the spectra were recorded 4 min after the additions (*1,2*) or as indicated (*3*). Inset: pH-dependence of the cytochrome oxidase-catalyzed superoxide generation. $H_2O_2$ concentration, 7.8 mM. The peak-to-trough amplitude of the low-field component of the DMPO-OOH EPR signal recorded in 13 min after $H_2O_2$ addition is plotted vs. pH.

with $H_2O_2$ does not result in radical generation (Fig. 3A, *1,2*). However, addition of $H_2O_2$ to DMPO in the presence of COX gives rise to an EPR signal typical of the superoxide adduct of the spin trap (Fig. 3A, *3*). The signal grows with time reaching a plateau level in 4–10 min. Generation of the DMPO-OOH signal is prevented by 5 mM cyanide (not shown) and is not observed with COX inactivated by heat treatment (Fig. 3A, *4*).

SOD prevents the $H_2O_2$-dependent DMPO adduct formation (not shown) and brings about a loss of the EPR signal when added after COX and $H_2O_2$ (Fig. 3B).

Generation of $O_2^{\cdot-}$ radicals increases greatly with acidification (Fig. 3, inset) which could account for augmentation of the $H_2O_2$-induced irreversible spectral changes at acid pH (Fig. 2).

## 4. DISCUSSION

Our data show that $O_2^{\cdot-}$ radicals are formed from $H_2O_2$ in the presence of COX. The reaction is not likely to be catalyzed by adventitious transition metal ions as neither EDTA nor DETAPAC inhibits the process. Moreover, heat inactivation of COX results in a loss of the radical generation. Therefore we are inclined to think that the process is catalyzed by COX. Since the reaction is blocked by cyanide the $O_2^{\cdot-}$ generation appears to be associated with the $a_3/Cu_B$ site of COX.

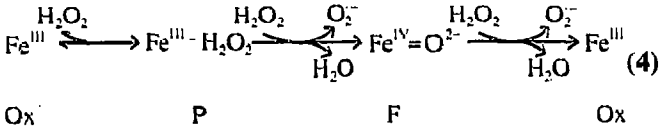
Accurate quantitation of the radical formation rate remains to be done; preliminary evaluation indicates DMPO-OOH adduct concentrations to be in the $<10^{-5}$ M range*. Thus, the reaction is rather slow (about 1 turnover per minute or less) and we do not imply COX to be a physiologically significant source of $O_2^{\cdot-}$ radicals in the cell. Rather, the reaction might be interesting in the context of the enzyme oxygen compound chemistry.

Presumably, the mechanism of the superoxide generation consists in one-electron oxidation of $H_2O_2$ to $O_2^{\cdot-}$. This redox transition is characterized by an $E_m^7$ value of 0.8–0.9 V [19,20]. None of the known redox centres in COX has a midpoint potential sufficiently high to serve as an electron acceptor in such a reaction. However, the peroxy and oxoferryl compounds of COX are supposed to be powerful one-electron oxidants with $E_m^7(P/F) \approx 1.2$ V and $E_m^7(F/Ox) \approx 1.1$ V (Wikstrom and Morgan, in preparation; cf. Ref. [21]), which agrees with

*The amount of $O_2^{\cdot-}$ radicals formed can be underestimated because of the SOD activity inherent in COX [17,18].

the $E_m$ values of ca. 1 V determined for the Compound I/Compound II and Compound II/ferric transitions of peroxidases [22]. Therefore, $H_2O_2$ oxidation by COX compounds P and F (eqs. 2 and 3) should be thermodynamically feasible.

When COX reacts with excess $H_2O_2$, stable levels of compounds P and F are observed which depend reversibly or quasi-reversibly on the $H_2O_2$ concentration, pH and some other factors [6–8]. This could imply that P and F are reaction endproducts in equilibrium with the free enzyme and each other; alternatively, the stable levels of P and F could correspond to steady-state concentrations of the compounds formed as intermediates in the catalase-type cycle run by COX [8] (see eqs. 1–3):




Experimental confirmation of $H_2O_2$ oxidation to $O_2^-$ supports the latter explanation and indicates that relationships between Ox, P and F in the presence of excess $H_2O_2$ would be viewed in terms of steady-state kinetics rather than thermodynamic equilibrium as discussed below.

### 4.1. *Effect of SOD*

At pH>7 and high concentration of $H_2O_2$, SOD decreases the steady-state concentration of compound P (Fig. 1B). Within a framework of scheme (4) this could mean that

(i) SOD promotes the P→F transition removing $O_2^-$ as the reaction product of this step;

(ii) SOD inhibits the F→Ox transition. This might be the case if F reduction to Ox could use $O_2^-$, released at the preceeding P→F step of the cycle, as electron donor in addition to (or instead of) $H_2O_2$ (cf. Ref. [8]). It seems to be a meaningful possibility since $O_2^-$ is a much better reductant than $H_2O_2$ [19,20].

### 4.2. *Effect of pH*

It is noteworthy that the pH-dependent increase in $O_2^-$ generation (Fig. 3, inset) correlates with a decrease of compound P steady-state concentration (cf. Fig. 5C in Ref. 7). Presumably, the rate of the $O_2^-$-yielding P→F transition increases at acid pH.

The data obtained on COX proteoliposomes [7] and confirmed recently on the solubilized enzyme with both $H_2O_2$ [23] and alkyl peroxides as the reactants (Vygodina et al., in preparation) indicate the P→F conversion to require the uptake of two protons with the apparent p$K$ values of 6.7 in the case of $H_2O_2$ or 7.7 in the case of alkyl peroxides, the protons coming from the matrix side of the membrane [7,24]. These results corroborate the hypothesis that the conversion of P to F is linked to proton pumping by COX [1,21].

*Acknowledgements:* We are much obliged to Dr. Yu. Koksharov for his kind help with EPR measurements. Thanks are due to prof. V.P. Skulachev for his interest in this work and critical reading the manuscript.

## REFERENCES

[1] Wikstrom, M.K.F., Krab, K. and Saraste, M. (1980/1981) Cytochrome Oxidase, Academic Press, New York.
[2] Wikstrom, M. and Babcock, G.T. (1990) Nature 348, 16–17.
[3] Bickar, D., Bonaventura, J. and Bonaventura, C. (1982) Biochemistry 21, 2661–2666.
[4] Wrigglesworth, J.M. (1984) Biochem. J. 217, 715–719.
[5] Kumar, C., Naqui, A. and Chance, B. (1984) J. Biol. Chem. 359, 11668–11671.
[6] Vygodina, T.V. and Konstantinov, A.A. (1987) FEBS Lett. 219, 387–392.
[7] Vygodina, T.V. and Konstantinov, A.A. (1989) Biochim. Biophys. Acta 973, 390–398.
[8] Vygodina, T.V. and Konstantinov, A.A. (1988) Ann. NY Acad. Sci. 550, 124–138.
[9] Wrigglesworth, J.M., Ioannidis, N. and Nicholls, P. (1988) Ann. NY Acad. Sci. 550, 150–160.
[10] Orii, Y. (1988) Ann. NY Acad. Sci. 550, 105–117.
[11] Weng, L. and Baker, G.M. (1991) Biochemistry 30, 5727–5733.
[12] Chance, B., Kumar, C., Powers, L. and Ching, Y. (1983) Biophys. J. 44, 353–363.
[13] Fowler, L.R., Richardson, S.H. and Hatefi, Y. (1962) Biochim. Biophys. Acta 64, 170–173.
[14] MacLennan, D.H. and Tzagoloff, A. (1965) Biochim. Biophys. Acta 96, 166–168.
[15] Fee, J.A., Zimmerman, B.H., Nitsche, C.S., Rusnak, F. and Munck, E. (1988) Chemica Scripta 28A, 75–78.
[16] Gorren, A.C.F., Dekker, H. and Wever, R. (1986) Biochim. Biophys. Acta 852, 81–92.
[17] Markossian, K.A., Poghossian, A.A., Paitian, N.A. and Nalbandian, R.M. (1978) Biochem. Biophys. Res. Commun. 81, 1336–1343.
[18] Naqui, A. and Chance, B. (1986) Biochem. Biophys. Res. Commun. 136, 433–437.
[19] Mitchell, P., Mitchell, R., Moody, A.J., West, I.C., Baum, H. and Wrigglesworth, J. (1985) FEBS Lett. 188, 1–7.
[20] Wood, P.M. (1988) Biochem. J. 253, 287–289.
[21] Wikstrom, M.K.F. (1988) Chemica Scripta 28A, 71–74.
[22] Hayashi, Y. and Yamazaki, I. (1979) J. Biol. Chem. 254, 9101–9106.
[23] Vygodina, T.V., Schmidmeyer, K. and Konstantinov, A.A. (1992) Biol. Membranes (Moscow), in press.
[24] Wikstrom, M.K.F. (1988) FEBS Lett. 231, 247–252.