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ENHANCED SUPEROXIDE DISMUTASE ACTIVITY OF PULSED CYTOCHORME OXIDASE

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Summary. The superoxide disumutase (SOD) activity of beef heart cytochrome oxidase, both in the resting (as isolated) and pulsed (reduced and reoxidized) states, has been investigated using their ability to inhibit the autoxidation rate of pyrogallol and epinephrine. Resting oxidase showed variable SOD activity, while in the pulsed state the SOD activity of cytochrome oxidase (CcO) increased by an order of magnitude. These results are discussed in terms of a physiological role for the pulsed oxidase.

Introduction. Cytochrome c oxidase (ferrocytochrome c: O_2 oxido-reductase E.C. 1.9.3.1) is the terminal oxidase of the mitochondrial respiratory chain. It catalyzes the reduction of molecular oxygen to water in a four electron transfer process. This electron transfer produces a proton gradient across the membrane, which in turn drives the production of ATP [1]. CcO contains at least four metal ions in the molecule: two hemes (cytochromes a and a_3) and two associated copper atoms (Cu_a and Cu_{a_3}) Recently it has been reported that CcO contains Zn [2] and Mg [3] as well. CcO as isolated is said to be in the "resting" state. The resting oxidase once reduced by dithionite and reoxidized by molecular oxygen was termed as the "oxygenated" oxidase by Okunuki et al [4,5] and it has been a matter of intensive study since then. Antonini & Brunori [6,7] have approached the difference between the resting and reoxidized states from a kinetic perspective and coined the term "pulsed."

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Abbreviations: CcO: cytochrome c oxidase. EPR: Electron Paramagnetic Resonance. SOD: Superoxide Dismutase

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They found that the pulsed oxidase has an enhanced rate of cytochrome c oxidation in the pre-steady state region. They also reported that the "pulsed" state is present in Keilin-Hartree particles [8].

More recently we have shown that oxygenated oxidase is in fact a peroxide adduct [9,10]. If fully reduced CcO is reoxidized in the strict absence of H_2O_2, the Soret peak moves to 420 nm (from 418 nm) instead of 428 nm for the traditional "oxygenated" oxidase. Upon addition of H_2O_2 to the 420 nm form, the Soret peak shifts to 428 nm, thus establishing that the 428 nm form is indeed a peroxide adduct. It has been proposed that the pulsed oxidase (420 nm form) is a conformational variant of the resting state and most probably is more open than the resting state with a structure resembling that of the peroxidases (9-12). In spite of continuing efforts by several groups, the physiological role (if any) of such a pulsed state is not known.

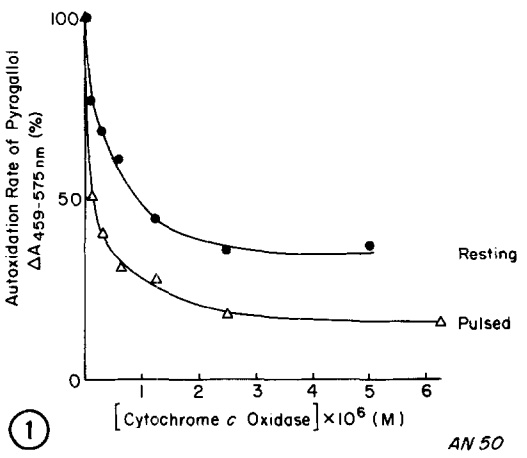
There are indications in the literature that CcO has a small superoxide dismutase (SOD) activity, which is inhibited by cyanide, azide, very high pH and thermal denaturation of CcO [13]. We have studied the SOD activity of CcO in the resting and reoxidized states and report here that the pulsed or the open conformation has a much higher SOD activity compared to the resting state. The results are discussed in terms of a physiological relevance of the pulsed state.

Materials and Methods. CcO from beef heart was prepared according to Yonetani [14] or Volpe-Caughey [15] slightly modified in our laboratory. Specifically, we add 1 mM EDTA in all our buffers to exclude adventitious metal ions from the final preparation. All other reagents were of highest purity available commercially.

The 420 nm pulsed form was prepared essentially according to [9,10]. Stock solutions of pyrogallol (200 mM) and epinephrine (saturated solution) were prepared in 10 mM HCl. Epinephrine solution was prepared fresh every day and kept on ice in an air tight vessel during the experiment.

The SOD activity was measured by two different methods: 1) autoxidation of pyrogallol [16,17] and 2) autoxidation of epinephrine [18,19]. All spectra were run on a Johnson Foundation dual wavelength spectrophotometer.

Results and Discussion. CcO in the resting state inhibits the pyrogallol autoxidation process markedly. In Fig. 1, the rate of autoxidation is plotted as a function of CcO concentration. The curves for the resting and pulsed oxidases are somewhat different for different preparations (not shown).



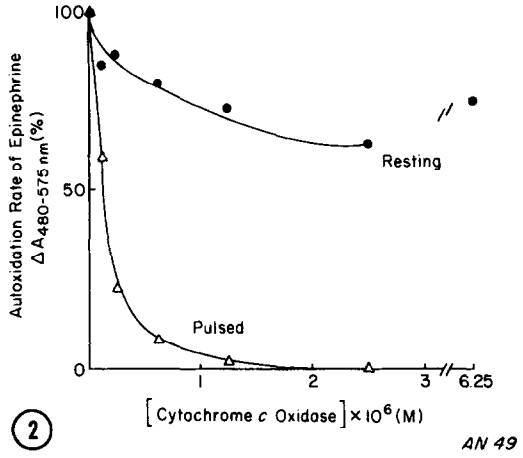


Fig. 1. Inhibition of initial rate of pyrogallol (final concentration 0.2 mM) autoxidation by CcO in resting and pulsed forms. The product of pyrogallol autoxidation was monitored at 459 nm (reference wavelength 575 nm). 0.1m phosphate buffer containing 1mM EDTA, pH 8.2. Temperature 21°C.

Fig. 2. Inhibition of epinephrine (final concentration 15 uM) autoxidation by CcO in resting and pulsed forms. The product of epinephrine autoxidation was monitored at 480 mm (reference wavelength 575 nm). 0.1M carbonate buffer containing 1mM EDTA. pH 10.0. Temperature 21°C.

However, the difference between the resting and pulsed states is always clear when the same preparation is compared. CcO inhibits the autoxidation of epinephrine as well. The results are shown in Fig. 2. It is clear from Fig. 2 that the difference between the resting and the pulsed states are much more pronounced here than in the case of pyrogallol method.

It has been reported that CcO has an intrinsic SOD activity. The authors [13] used the tetrazolium method for determining the SOD activity of CcO and added that "similar results were noted also in epinephrine autoxidation tests." They reported that 50% decline in activity can be achieved at 2.5 x 10^{-7} M of CcO. This is in direct contradiction to our results. We do not even achieve the 50% of the uninhibited rate with the resting oxidase. When the CcO concentration was increased further, we observed a small but reproducible increase instead of a decrease in rate (Fig. 2). However, in the case of pulsed oxidase, the inhibition of the autoxidation rate is quite dramatic and full inhibition can be obtained. Although Markosian et al [13] did not report the conditions at which they used the epinephrine tests, we may be able to reconcile our differences by reported heterogeneity in the CcO preparations

[20,21]. The enhanced SOD activity of the reoxidized C<u>c</u>O is also clear in the pyrogallol method (Fig. 1). The more pronounced change in SOD activity between the resting and pulsed states in epinephrine test may be due to the conditions used (pH 10.0). In an earlier report [22] it was proposed that the Cu atoms of C<u>c</u>O are involved in the SOD activity. Whether the recently reported Zn atom [2] plays any role in this property is an intriguing question.

Now the question arises as to why a SOD activity is necessary for C<u>c</u>O? The natural and simple possibility is that of a superoxide scavanger or "proof reader." This is in accordance with [13] and [12] (proposed for peroxidatic and catalatic activity). The reason being, if the superoxide ion is generated by "mistake" or during turnover, C<u>c</u>O should be able to "take care" of this toxic substance [23, but see 24].

Quantitation of the SOD activity shows that it is about 0.3% (from the pyrogallol method) for resting and 2.5% for pulsed oxidase as judged from the concentration of C<u>c</u>O required to inhibit the autoxidation rate by 50%. If one takes into consideration that SOD is one of the "fastest" enzymes known having a second order rate constant of ~2 x 10^9 $M^{-1}s^{-1}$ [23], 2.5% is still a formidable value of 5 x 10^7 M^{-1} s^{-1}! The differrence in SOD activity between the resting and pulsed forms could be explained by the fact that the resting state has a "closed" conformation whereas the pulsed form is more open, making it easier to "trap" a superoxide ion. The reason for the difference can be the following: if the pulsed form is a more active form, the probability that superoxide ion will be generated at a greater rate (by "mistake" or as a turnover intermediate) is much higher than the "slower" resting state. An eight-fold enhancement seems reasonable because the pulsed oxidase oxidizes cytochrome <u>c</u> five times faster than the resting state [6,10].

<u>Epilogue</u>. Does pulsed oxidase have any physiological relevance? The answer to the question is unknown. The results of this paper are an attempt to answer the question raised above. It can be postulated that pulsed oxidase being a faster turnover species, can indeed produce more superoxide by "mistake" or as an intermediate. Indeed, the radical EPR signal g = 2 (from superoxide) seen

during turnover of CcO is higher under "high turnover conditions" [25] (for a discusion see [26]). Whatever the reason may be, the higher superoxide concentration demands more "protective devices" and more careful "proof reading" or "higher power" to dismutate it. Thus, the enhanced SOD activity of pulsed oxidase may have a physiological relevance.

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