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pH changes associated with cytochrome *c* oxidase reaction with H_2O_2

Protonation state of the peroxy and oxoferryl intermediates

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pH changes associated with the mitochondrial cytochrome oxidase reaction with H_2O_2 have been studied. In the presence of ferricyanide or Tris-phenanthroline complex of Co^{III} as electron acceptors, reaction of H_2O_2 with the oxidized cytochrome oxidase is accompanied by a steady proton release with a rate constant of ca. 3 $M^{-1}\cdot s^{-1}$ at pH 6.8. The acidification is completely inhibited by superoxide dismutase and its pre-steady-state kinetics correlates with that of the oxoferryl compound (F) accumulation. Apparently, the proton release is linked to superoxide generation by cytochrome oxidase under these conditions. In the presence of superoxide dismutase and without the electron acceptors, the H_2O_2-induced transitions of cytochrome oxidase from the oxidized to the peroxy (P) and from the peroxy to the oxoferryl state are not associated with any significant proton release or uptake. The results point to the following mechanism of O_2^- generation and protonation states of the cytochrome oxidase compounds P and F:

$$Fe^{3+}\text{–}OH^- \xrightarrow{H_2O_2} Fe^{3+}\text{–}OOH^- \xrightarrow{H_2O_2} Fe^{4+}{=}O^{2-} + O_2^- + H^+$$
$$Cu^{2+}\text{–}OH^- \qquad\quad Cu^{2+}\text{–}OH^- \qquad\quad Cu^{2+}\text{–}OH^-$$

Cytochrome oxidase; Proton pump; Oxygen intermediate; Hydrogen peroxide; Reaction mechanism; Superoxide generation

1. INTRODUCTION

During catalytic turnover mitochondrial cytochrome *c* oxidase (COX) passes through a number of intermediate states in which partially reduced dioxygen remains bound to the enzyme (reviewed in [1,2]). Two of these intermediates, the so-called peroxy (**P**) and oxoferryl (**F**) compounds, corresponding to transfer of 2 and 3 electrons to dioxygen, respectively, are of particular interest as their redox transitions may be linked to proton pumping by COX [2,3]. Elucidation of the structure of compounds **P** and **F** could give a clue to the mechanism of the H^+-translocation coupled to oxygen reduction by cytochrome *c* oxidase.

Of special importance would be to know protonation states of **P** and **F** since the atoms of oxygen bound to the metal ions in the binuclear O_2-reducing centre of COX have been considered as the electron/proton coupling elements of the proton pump [4–6].

Not much is known on this matter. Usually it is assumed that in compound **P**, the bound peroxide is doubly ionized [1,2,6–8]. Accordingly, the reduction of O_2 to the peroxy state will not be linked to $\Delta\mu_{H^+}$ generation, all the events involved in pumping out protons from the mitochondrial matrix being associated with the subsequent steps of the catalytic cycle [2,8]. However, the pH-dependence of H_2O_2 binding with COX [9] as well as essentially the same reaction pattern of COX with H_2O_2 and with alkyl peroxides [10] unable to carry out a second ionization are not consistent with the fully ionized state of peroxide in **P** (cf. also [11,12]).

Protonation of intermediate redox states of COX can be probed directly by monitoring proton release or uptake from the solution associated with their formation [13,14]. Here we attempted to measure pH changes linked to generation of compounds **P** and **F** during the reaction of the oxidized enzyme with H_2O_2.

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Abbreviations: COX, cytochrome oxidase; Co(phen), tris-phenanthroline complex of Co^{III}; FeCy, ferricyanide; Ox, **P** and **F**, the oxidized, peroxy and oxoferryl states of cytochrome oxidase; SOD, superoxide dismutase.

2. MATERIALS AND METHODS

COX was purified from beef heart mitochondria according to [15]. 30% H_2O_2 Suprapur was from Merck. $Co(phen)_3Cl_3$ was synthesized [16]. Other chemicals were commercial products of high purity.

Simultaneous recordings of pH and absorbance changes were made as described earlier [17] in a thoroughly mixed cell placed in a Johnson Foundation dual-wavelength spectrophotometer. A semi-microglass combination electrode (Beckman Instr. Int., Geneve, no. 39030; response time < 1.5 s) was fed into a Keithley differential electrometer (model 604). The output signals were plotted on a two-channel pen recorder. The pH changes were calibrated with 1–2 μl aliquots of 10 mM HCl before and/or after each probe. Static absorption spectra were recorded in a Perkin-Elmer Lambda 5 UV/vis double-beam spectrophotometer. Extinction coefficients of 12 $mM^{-1} \cdot cm^{-1}$ at 607–650 nm [3] and 5 $mM^{-1} \cdot cm^{-1}$ at 582–650 nm [18] have been used to evaluate concentrations of compounds **P** and **F**, respectively.

3. RESULTS

Addition of low H_2O_2 concentration to the oxidized COX as prepared in the Bari group gives rise to significant amounts of the peroxy state, the effect reaching saturation at approx. 30 μM of the peroxide. Subsequent addition of 0.5 mM or more H_2O_2 results in a virtually complete conversion of the enzyme to the **F** state in agreement with [12,19]. The pattern of pH changes associated with the H_2O_2 reaction with COX depends critically on the type of experimental conditions, namely, the presence of electron acceptors and SOD.

3.1. *Experiments in the presence of electron acceptors*

Ferricyanide is often used in the studies of COX reaction with ligands including H_2O_2 to ensure the oxidized state of the enzyme metal centres. We found that reaction of H_2O_2 with COX in the presence of ferricyanide results in a steady acidification (Fig. 1) the rate of which depends linearly on the concentration of COX and of H_2O_2 (results to be described separately).

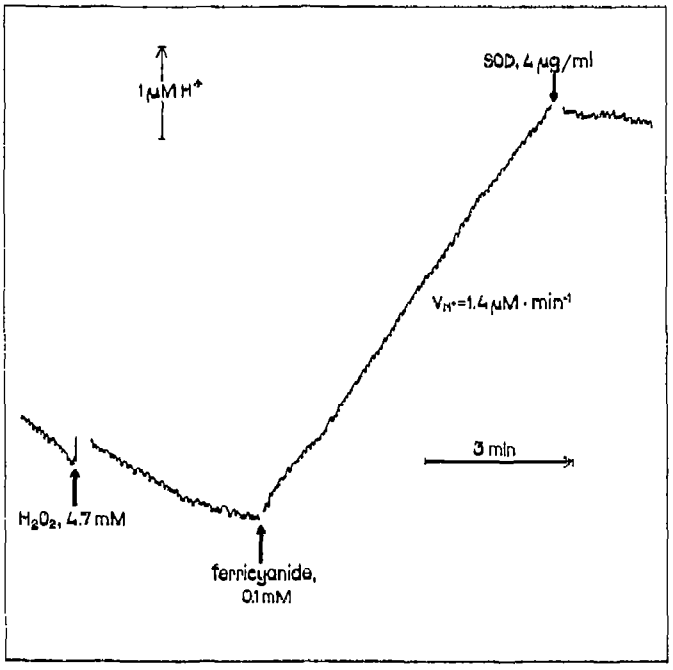


Fig. 1. Proton release associated with the H_2O_2-dependent superoxide generation by cytochrome oxidase. 2 μM COX in the medium containing 0.15 M KCl, 0.5% Tween 80, 50 μM EDTA, pH 6.8.

The acidification is inhibited by SOD and is not observed to a comparable extent in the absence of ferricyanide (Fig. 1). $Co(phen)_3$ can well substitute for ferricyanide (e.g. Fig. 2).

Under appropriate conditions, an initial rapid phase of acidification can be revealed upon mixing of COX with H_2O_2 which is much slower than formation of compound **P** but rather correlates with the pre-steady-state accumulation of **F** (Fig. 2A,B).

3.2. *Experiments in the presence of SOD*

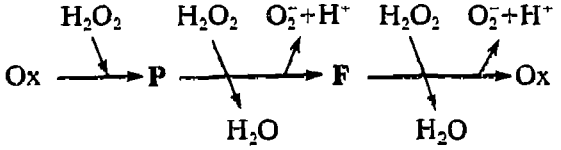
A typical experiment is shown in Fig. 3. Addition of 40 μM H_2O_2 to COX is followed by generation of stable compound **P** with an apparent rate constant of 10^3 $M^{-1} \cdot s^{-1}$ at pH 7.5 which agrees with [21,22]. Adding 40 μM H_2O_2 more does not entail any further changes at 607 nm (not shown) in agreement with the K_d value of 3–5 μM measured for the peroxy compound under similar conditions [9,21]. Subsequent addition of a large excess of H_2O_2 results in a loss of absorbance at 607 nm due to conversion of **P** to **F** as checked by recording the static spectra of the sample.

It can be seen that neither generation of compound **P** nor its decay to compound **F** are associated with pH changes except for the small transients during the mixing time. Essentially the same results were obtained at pH 8 and 6.8 (not shown). Occasionally, some small rapid acidification (< 0.2 H^+ per compound P) could be observed, but this response was much more rapid than compound **P** formation and did not correlate with the amount of **P** formed.

4. DISCUSSION

4.1. *The oxidant-dependent proton release*

The SOD-sensitive acidification observed in the presence of FeCy or $Co(phen)_3$ (Figs. 1,2) is obviously due to O_2^- generation catalyzed by COX described recently [22]:



(1)

In the absence of electron acceptors or in the presence of SOD, dismutation of the O_2^--radicals takes back the protons:

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

and no net acidification is observed. However, if the O_2^-

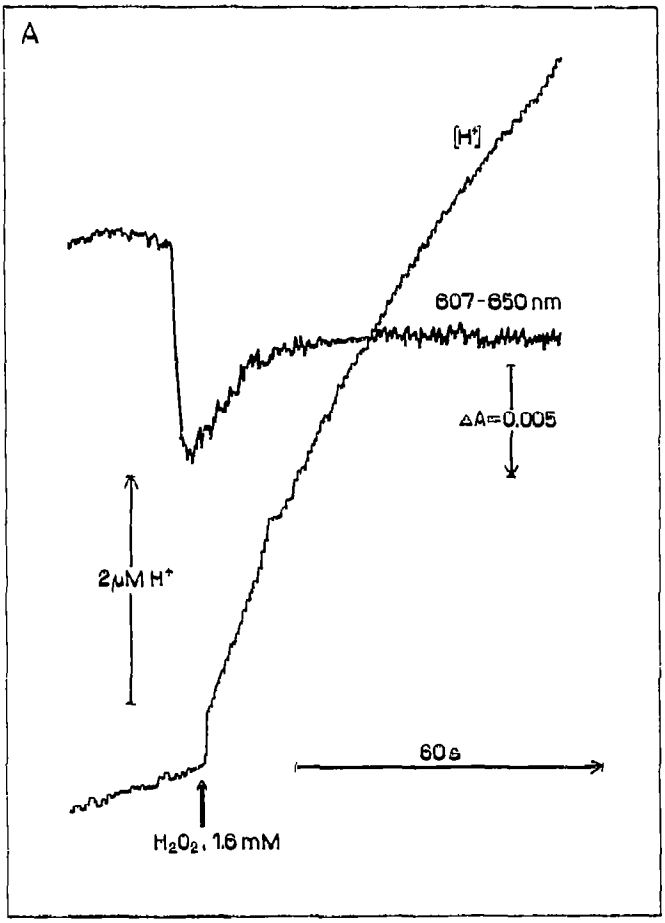


Fig. 2. Pre-steady-state kinetics of the O_2^- generation-linked proton release by cytochrome *c* oxidase. 3.5 μM COX in the medium indicated in the legend to Fig. 1, but pH 7.5 and 20 μM $Co(phen)_3^{3+}$, is present. Generation and decay of the compounds **P** (A) and **F** (B) have been monitored simultaneously with the proton release measurements. About 2 mol of H^+ per mole of compound **F** are released in the pre-steady-state phase of the acidification.

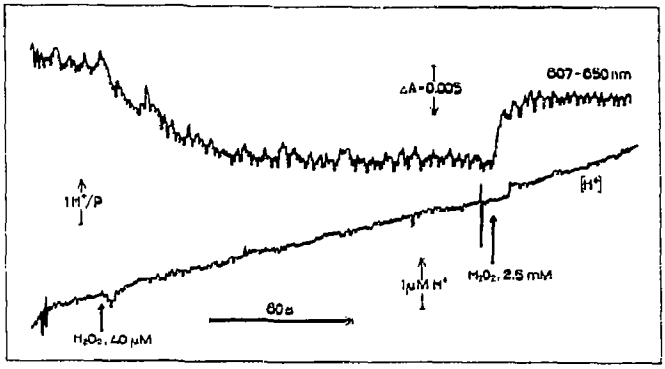


Fig. 3. pH balance of the H_2O_2-induced conversion of the oxidized cytochrome *c* oxidase to the peroxy and oxoferryl states. Conditions, as in Fig. 2, but without $Co(phen)_3^{3+}$ and with 38 μg/ml of SOD present. Amount of compound **P** formed is 0.81 μM.

radicals are oxidized to O_2 by FeCy or $Co(phen)_3$, the protons derived from HO_2 dissociation remain in the medium.

The steady-state kinetics of the oxidant-dependent proton release (k_v ca. 2–4 $M^{-1}\cdot s^{-1}$ at pH 7) is in agreement with the rate of O_2^- generation by COX measured by Nitro blue tetrazolium reduction (paper in preparation). The pre-steady-state kinetics of H^+-release (Fig. 2) with an apparent second-order rate constant of the initial phase 50 $M^{-1}\cdot s^{-1}$ is very much slower than generation of compound **P**, but is rather close to (although a bit slower than) the kinetics of the **P** ⟶ **F** conversion under these conditions (k_v = 40–120 $M^{-1}\cdot s^{-1}$; [20,21] and our observations) which supports the mechanism of O_2^- generation proposed in [22] (Eqn. 1).

4.2. *Protonation state of the compound* **P**

The O_2^- generation-linked H^+ release eliminated H_2O_2 binding with the oxidized COX is not associated with proton release or uptake which accords with pH-independence of K_d of the reaction above pH 7 [9]. Formally, this could be interpreted as H_2O_2 binding COX in a fully protonated state (e.g. [9,19]):

$$Fe^{3+}_{a_3} + H_2O_2 \longrightarrow Fe^{3+}_{a_3}{-}H_2O_2 \qquad (3)$$

However, mono- (HOO^-) and even doubly ionized state (O_2^{2-}could fit the data as well if deprotonation of the bound H_2O_2 is counterbalanced by proton uptake by ionizable group(s) of the enzyme. In particular, protonation of the OH^- ions bound to the ferric heme a_3 iron [23] and perhaps Cu_B^{2+} as well [24] is a plausible possibility, e.g.:

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

or

$$Fe^{3+}-OH^-/Cu_B^{2+}-OH^- + H_2O_2 \rightarrow Fe^{3+}-O^-\cdots O^--Cu_B^{2+} + 2H_2O \quad (5)$$

Of these equations, Eqn. 4 seems to us the most probable. Indeed, the pK of water shifts by ca. 7 pH units towards the acid upon coordination to ferric heme iron [25]. By analogy, one can expect pK_i of the heme-coordinated H_2O_2 to be below 6 so that H_2O_2 binding heme a_3^{3+} in the undissociated state (Eqn. 3) is not likely.

At the same time, the ability of alkylhydroperoxides to react with COX in much the same way as H_2O_2 [10] argues against the Eqn. 5, since alkylhydroperoxides cannot be doubly dissociated and are not likely to make 1,2-μ-peroxo-bridges between heme and copper in the binuclear centre.

Accordingly, we are left with the model (Eqn. 4) to explain the absence of net protonic changes during the H_2O_2 binding.

Oliveberg et al. [8] reported recently that generation of compound **P** during the oxidation of the mixed-valence COX by O_2 is not associated with H^+ uptake. Their data fit our model, given Cu_B^{2+} in compound **P** is ligated by OH^-, rather than H_2O [1,6]:

$$Fe^{2+}/Cu_B^{1+} + O_2 + H_2O \rightarrow Fe^{3+}-O-OH^-/Cu_B^{2+}-OH^- \quad (6)$$

which puts further constraints on the possible structure of the COX intermediates

4.3. *Protonation state of the intermediate* **F**

Similarly to the Ox → **P** conversion, the H_2O_2-induced transition of **P** to **F** is not associated with any significant pH changes except for those linked to O_2^- generation (Fig. 3). This means that being reduced by H_2O_2 compound **P** receives both an electron and a proton. Accordingly, E_m of the **P**/**F** redox couple may be expected to have a pH dependence of −60 mV/pH unit (but cf. [26]). Assuming the structure $Fe^{3+}-O-OH^-/Cu_B^{2+}-OH^-$ for compound **P** (Eqns. 4 and 6), the only reasonable configuration for compound **F** consistent with the addition of an H atom will be:

$$Fe^{3+}-O-OH^-/Cu_B^{2+}-OH^- + H_2O_2 \rightarrow Fe^{4+}=O^{2-}/Cu_B^{2+}-OH^- + H_2O \quad (7)$$

In particular our data do not fit the structure suggested recently for compound **F** by Babcock and Wikstrom ($Fe^{4+}=O^{2-}/Cu_B^{2+}$ OH_2; [2]). On the other hand, our results and conclusions are in good agreement with the very recent paper by R. Mitchell et al. [27] issued after this work was prepared for publication.

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