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Evidence for two H_2O_2-binding sites in ferric cytochrome *c* oxidase

Indication to the O-cycle?

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H_2O_2 addition to the oxidized cytochrome *c* oxidase reconstituted in liposomes brings about a red shift of the Soret band of the enzyme and an increased absorption in the visible region with two distinct peaks at ~570 and 605 nm. Throughout pH range 6–8.5, the spectral changes at 570 nm and in the Soret band titrate with very similar pH-independent K_d values of 2–3 μM. At the same time, K_d of the peroxide complex measured at 605 nm increases markedly with increased H^+ activity reaching the value of 18 ± 2 μM at pH 6.0. This finding may indicate the presence of two different H_2O_2-binding sites in the enzyme with different affinity for the ligand at acid pH. The Soret and 570 nm band effects are suggested to report H_2O_2 coordination to heme iron of a_3, whereas the maximum at 605 nm could arise from H_2O_2 binding to Cu_{a_3} followed by the enzyme transition into the 'pulsed' (or '420/605') conformation. Possible implication of the two H_2O_2-binding sites for the cytochrome oxidase redox and proton-pumping mechanisms are discussed.

Cytochrome *c* oxidase; Peroxide compound; O-cycle; Proteoliposome; Spectral characteristic; Proton pump

1. INTRODUCTION

A mechanism by which oxygen reduction to water by mitochondrial cytochrome oxidase is coupled to $\Delta\psi$ generation and proton pumping is one of the challenging problems in molecular bioenergetics.

It is generally agreed that during the catalytic cycle, reduction of certain redox centres of the enzyme is linked somehow to H^+ binding from the matrix aqueous phase and that the protons are released subsequently to the C-phase upon redox centre oxidation [1–7]. Evidence was reported for the presence of proton wells communicating cytochrome oxidase redox centres with the M and C aqueous phases (M- and C-phases, the aqueous compartments corresponding to matrix and cytoplasm, respectively, in the case of the inner mitochondrial membrane) [3,8–11]. However, the nature of the groups involved in the redox-linked proton binding and release is not clear.

In view of an apparent lack of intrinsic hydrogen carriers in cytochrome oxidase, an emphasis was put earlier on the redox-dependent protonation-deprotonation of the protein heme-linked ionizable groups [1–5,12,13]. However it was pointed out recently by Mitchell et al. [6] that the partially reduced oxygen intermediates, notably H_2O_2, may well serve as hydrogen carriers in mitochondrial site 3 similar to ubiquinone in site 2 [14]. Several possible models of energy transduc-

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tion in cytochrome oxidase based on the H_2O_2-mediated hydrogen conduction have been considered [6] under the total name of 'O-loop/O-cycle' mechanism.

One of the important predictions of the O-loop or O-cycle models is the presence of at least 2 separate H_2O_2-binding sites in cytochrome *c* oxidase. Here we show that it may indeed be the case.

2. MATERIALS AND METHODS

Beef heart cytochrome *c* oxidase (a Fowler-type preparation) was isolated, purified and reconstituted into asolectin liposomes as described [11]. Spectrophotometric studies of H_2O_2 binding with the oxidized enzyme in proteoliposomes were carried out in an Aminco DW2a spectrophotometer in a split beam mode, 30% H_2O_2 ('Suprapur', Merck) was diluted to stock solutions of desired concentrations (2–100 mM) before experiments.

Typically, the reaction mixture contained proteoliposomes (0.3–1.2 μM in aa_3) in the basic medium with 50 mM Mes, Mops, Hepes, or Tris buffer depending on pH, 10^{-6} M of the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone and 100 μM ferricyanide. Experiments have been carried out in standard 1 cm optical quartz cells thermostatted at 25°C.

3. RESULTS

Addition of H_2O_2 to the oxidized cytochrome *c* oxidase reconstituted in proteoliposomes brings about a red shift of the Soret band of the enzyme and an increased extinction in the visible region with maxima at ~570 and ~605 nm (fig.1). These spectral changes are similar to those observed earlier with the solubilized enzyme [15–17], with the difference that in proteoliposomes the molar extinction coefficients of the effect are higher and much more reproducible [18]. At the same time the $\Delta\epsilon$ values of the H_2O_2-induced effect in proteoliposomes (or in beef heart submitochondrial particles, not shown [18]) are still much lower than those reported by Wikström for energy-linked formation of the peroxide compound of cytochrome oxidase in rat liver mitochondria [19,20].

As noticed earlier [15], the peaks at 570 and

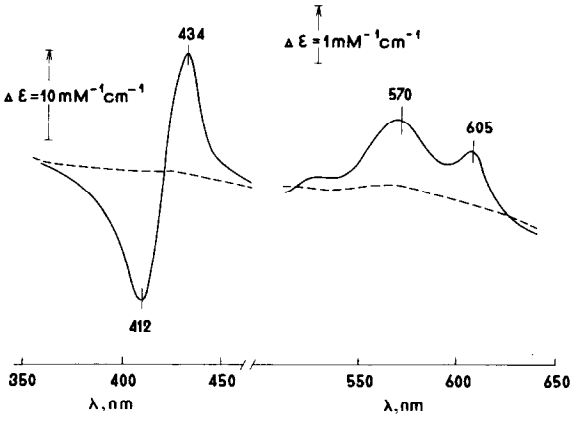


Fig.1. Spectral changes induced by H_2O_2 addition to cytochrome oxidase in proteoliposomes. The difference spectra were recorded 5 min after addition of 0.5 mM H_2O_2 to proteoliposomes ($[aa_3]$ = 1 μM) in the basic medium, pH 6.2.

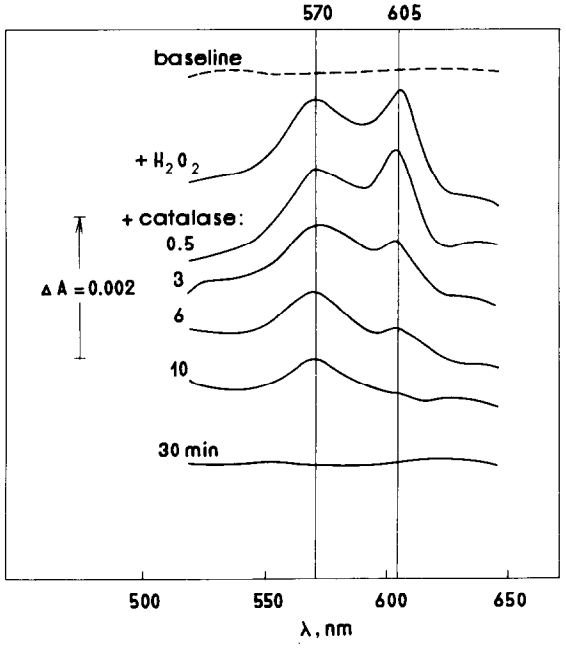


Fig.2. Reversal of the H_2O_2-induced spectral changes by catalase. Cytochrome oxidase proteoliposomes ($[aa_3]$ = 1 μM) in the basic medium with pH 6.4. The baseline recorded, 20 μM H_2O_2 was added to the sample and subsequently 10 nM catalase to both cells. Decay of the H_2O_2-induced spectral changes was monitored scanning the spectra at indicated time intervals after the catalase addition.

605 nm in the H_2O_2-induced difference spectrum behave independently of each other under many conditions. For example, fig.2 shows that when catalase is added to the peroxide complex of cytochrome oxidase at acid pH the peak at 605 nm disappears much more rapidly than that at 570 nm (cf. decay of the 'oxygenated' cytochrome oxidase in [21]). The data in fig.3 provide an explanation for this effect. Clearly, development of the band at 570 nm requires lower concentrations of H_2O_2 than formation of the 605 maximum (fig.3A).

The dose/effect curves at the two wavelengths (fig.3B) have simple hyperbolic forms typical of a single saturating site in each case but with K_d differing several-fold. At the same time the titration curves at 570 nm and in the Soret band are very similar.

These results have been reproduced with different preparations of cytochrome oxidase. At pH 6.0 the mean K_d values obtained in 3–15 experiments are 18 ± 2 μM for 605 nm and 2.5 ± 0.4 μM for the 570 nm and Soret band titrations.

The pH dependence of the peroxide complex K_d measured at different peaks of the H_2O_2-induced difference spectrum is shown in fig.4. It can be seen that the data for 570 nm and Soret band are superimposable throughout the pH range studied and do not depend on H^+ activity.

On the other hand, the K_d values obtained from the titrations of the 605 nm peak decline significantly with alkalinization and at pH above ~7.5 approach the same pH-independent value of

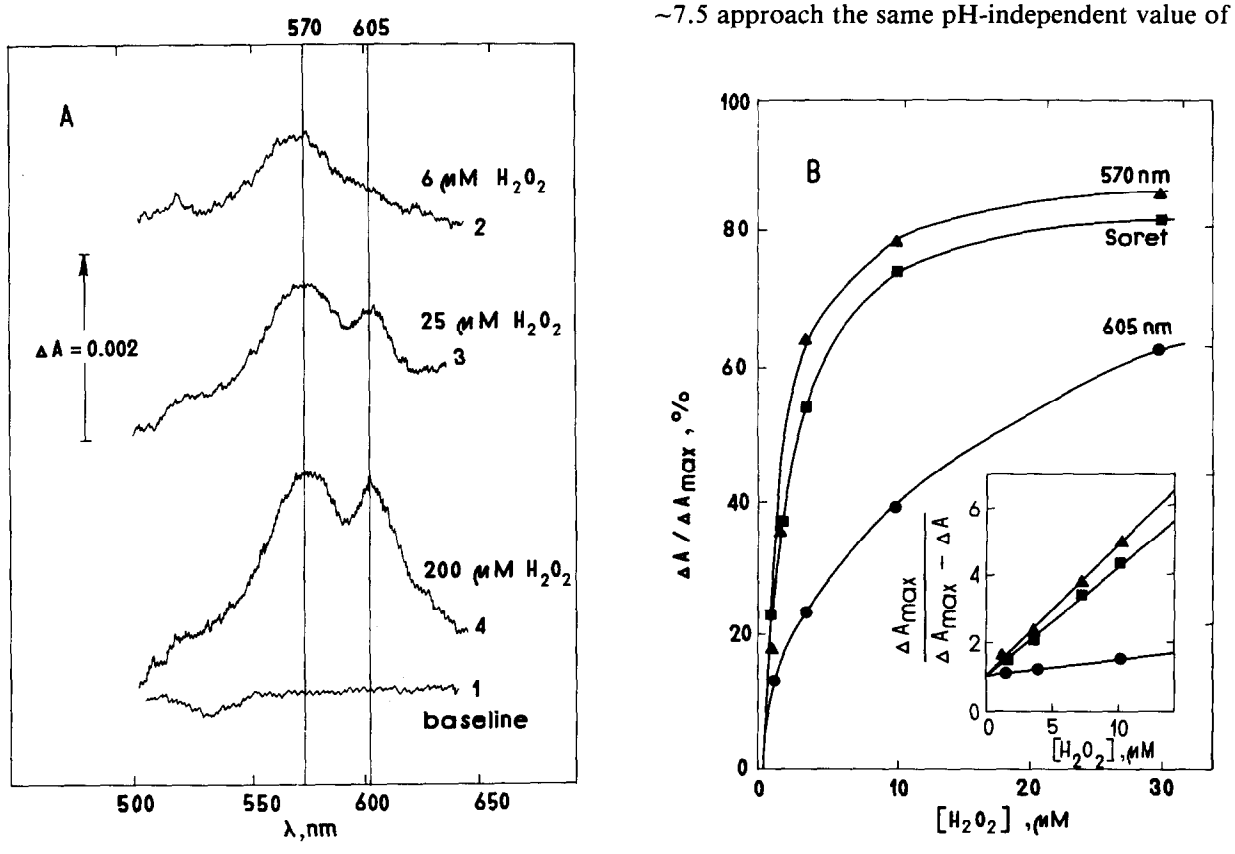


Fig.3. Concentration dependence of the H_2O_2-induced spectral changes. Basic conditions, as in fig.1. (A) Difference spectra observed upon addition of increasing H_2O_2 concentrations to cytochrome oxidase proteoliposomes. (B) Typical titration curves of the H_2O_2-induced spectral effect at the 3 principal bands of the difference spectrum (cf. fig.1); the Soret band effect has been measured as $\Delta A_{434-412nm}$. 3–5 min was allowed for equilibration after each H_2O_2 addition. The amplitudes of the spectral changes at various wavelengths are given in % to the maximal responses induced by an excess (0.7 mM) H_2O_2. The inset in (B) shows linearization of the titration curves in Dixon coordinates.

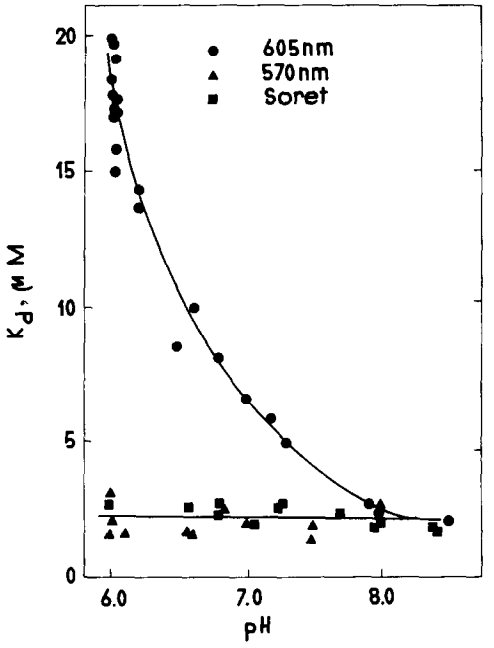


Fig.4. pH-dependence of K_d of the cytochrome oxidase peroxide complex. Titrations of the H_2O_2-induced spectral changes were carried out essentially as in fig.3 with the difference that saturating H_2O_2 concentrations were varied for different pH so as to avoid interference from the ferryl compound with λ_{max} = 580 nm [4,20] which is easily formed at high H_2O_2 concentrations at pH above neutral [18] (cf. [17,36]).

2~3 μM as observed for the other two wavelengths.

4. DISCUSSION

The results presented provide evidence for the existence of 2 H_2O_2-binding sites in the oxidized membrane-bound cytochrome *c* oxidase with different affinities for the ligand at acid pH.

What can these sites be? It has been established that many other ligands of the enzyme such as cyanide [22], azide [23,25], NO [25–27], sulfide [28] and CO [29] can bind to both Fe_{a_3} and Cu_{a_3}. It seems reasonable to assume the same 2 binding sites for H_2O_2.

The spectral changes at 570 nm correlate with the red shift of the Soret band, and the latter represents a typical response of a high-spin hemoprotein to ligand binding. Therefore, both effects are likely to report H_2O_2 ligation at the 6-th coordination position of heme a_3^{3+}. Consequently, the 605 nm peak may be associated with H_2O_2 binding to $Cu_{a_3}^{2+}$.

The nature of the electronic transition giving rise to the 605 nm peak remains to be established (cf. discussion of the 'Compound C' spectrum in [4]). Cytochrome *a* heme reduction observed in [17] can be ruled out since careful inspection of the Soret band spectra did not reveal (in contrast to [17]) any signs of the increased absorption at 440–444 nm concurrent with the 605 nm peak development during the titrations of the enzyme with H_2O_2. It is then possible that the peak belongs to heme a_3 (cf. [30]), Cu_{a_3} [3], or to a charge-transfer band of the entire H_2O_2-bridged binuclear centre [31].

Notably, the effect may not be a direct manifestation of the H_2O_2 ligation of Cu_{a_3}. A similar peak at 605 nm, not accompanied by the 570 nm band or by major changes in the Soret, is characteristic of the difference spectrum of the pulsed (or 420-form) ferric cytochrome oxidase versus the 'resting' state [32–36]. One can suggest that H_2O_2 binding in the site other than Fe_{a_3} (presumably, Cu_{a_3}) brings about partial transition of the enzyme to the '420/605' conformation, which is thermodynamically unfavourable in the absence of H_2O_2, e.g.:

$$E + H_2O_2 \xrightarrow{} E - H_2O_2 \xrightarrow{570} E - (H_2O_2)_2 \xrightarrow{} E^* - (H_2O_2)_2 \quad (1)$$

(605,570 nm; H_2O_2)

The presence of 2 H_2O_2-binding sites in cytochrome oxidase may have important implications for reaction mechanism of the enzyme. For instance, one could consider a possibility of H_2O_2 dismutation involved in oxygen reduction to water

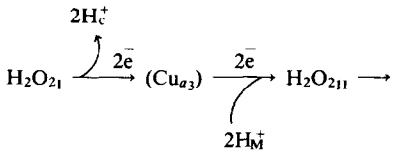
$$O_2 + 2e_C + 2H_M^+ = H_2O_{2_I} + 2q \quad (2)$$

$$O_2 + 2e_C + 2H_M^+ = H_2O_{2_{II}} + 2q \quad (3)$$

$$H_2O_{2_I} + H_2O_{2_{II}} = 2H_2O + O_2 \quad (4)$$

where the subscript indexes M and C denote the corresponding aqueous phases and *q* indicates electrical charges transferred across the membrane. Reaction (4) is thermodynamically favourable and,

if appropriately catalyzed, could be coupled to electrogenic proton translocation.



$$2H_2O + O_2 + 2q \quad (4a)$$

Such an energy-linked H_2O_2 dismutation can be viewed as an O-loop [6] associated with the cytochrome a_3 oxygen-reducing centre; the reactions 4 and 4a may be mechanically easier to understand than the H_2O_2 oxidation by cytochrome *a* suggested by the O-loop/O-cycle schemes in [6].

Two possible arguments against the proposed model can be considered. First, cytochrome oxidase shows rather low catalase activity ([16,17] and refs therein). Second, the sequence of reactions 2–4a gives a $q/\bar{e}$ ratio of 1.5 and $H_C^+/\bar{e}$ stoichiometry of 0.5 instead of the quotients of 2 and 1, respectively, implied by the classical proton pump model [1,3–6]. However, these objections may not be decisive. The catalase activity of cytochrome oxidase can differ drastically for exogenous and intrinsically generated H_2O_2, and the $H_C^+/\bar{e}$ ratios published for cytochrome oxidase by different authors show significant deviations [37]. In particular, the $H^+/\bar{e}$ ratios of 0.5 have been reported for subunit III-deficient enzyme [38–41].

Finally, the suggested protonmotive mechanism based on H_2O_2 formation and dismutation does not exclude the possibility of additional electrogenic steps being associated with the cytochrome oxidase reaction which could raise the $q/2e$ stoichiometry from 1.5 to 2.0. In this context, we would draw attention to relaxation of the 420/605 form of the oxidized enzyme to the stable 418 conformation as a possible 'auxiliary' H^+-translocating step 'indirectly' coupled to the reaction.

ACKNOWLEDGEMENT

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NOTE ADDED IN PROOF

During a visit to Bari Symposium on Cytochrome Systems (April, 1987), one of us (A.K.) became aware that similar ideas on the role of H_2O_2 in site 3 protonmotive mechanism had been discussed by Baum et al. in an edition unavailable to us [42].

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