Evidence for two $\text{H}_2\text{O}_2$-binding sites in ferric cytochrome $c$ oxidase

Indication to the O-cycle?

Tatyana Vygodina and Alexander A. Konstantinov

A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR

Received 17 April 1987; revised version received 19 May 1987

$\text{H}_2\text{O}_2$ 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 $\approx 570$ 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 $2-3 \, \mu\text{M}$. At the same time, $K_d$ of the peroxide complex measured at 605 nm increases markedly with increased $\text{H}^+$ activity reaching the value of $18 \pm 2 \, \mu\text{M}$ at pH 6.0. This finding may indicate the presence of two different $\text{H}_2\text{O}_2$-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 $\text{H}_2\text{O}_2$ coordination to heme iron of $a_h$, whereas the maximum at 605 nm could arise from $\text{H}_2\text{O}_2$ binding to $\text{Cu}_{a_3}$ followed by the enzyme transition into the ‘pulsed’ (or ‘420/605’) conformation. Possible implication of the two $\text{H}_2\text{O}_2$-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 $\text{H}^+$ binding from the matrix aqueous phase and that the protons are released subsequently to the $\text{C}$-phase upon redox centre oxidation [1–7]. Evidence was reported for the presence of proton wells communicating cytochrome oxidase redox centres with the $\text{M}$ and $\text{C}$ aqueous phases ($\text{M}$- and $\text{C}$-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 $\text{H}_2\text{O}_2$, may well serve as hydrogen carriers in mitochondrial site 3 similar to ubiquinone in site 2 [14]. Several possible models of energy transduc-
tion in cytochrome oxidase based on the 
H$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$ binding 
with the oxidized enzyme in proteoliposomes were 
carried out in an Aminco DW2a spec-
photometer in a split beam mode, 30% H$_2$O$_2$ 
('Suprapur', Merck) was diluted to stock solutions 
of desired concentrations (2–100 mM) before ex-
periments.

Typically, the reaction mixture contained pro-
teoliposomes (0.3–1.2 µM in aa$_3$) in the basic 
medium with 50 mM Mes, Mops, Heps, or Tris 
buffer depending on pH, 10$^{-6}$ M of the uncoupler 
carbonyl cyanide m-chlorophenyl hydrazone and 
100 µM ferricyanide. Experiments have been car-
rried out in standard 1 cm optical quartz cells ther-
mostat-ed at 25°C.

3. RESULTS

Addition of H$_2$O$_2$ 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 
Δε values of the H$_2$O$_2$-induced effect in pro-
teoliposomes (or in beef heart submitochondrial 
particles, not shown [18]) are still much lower than 
those reported by Wikström for energy-linked for-
mination of the peroxide compound of cytochrome 
oxidase in rat liver mitochondria [19,20].

As noticed earlier [15], the peaks at 570 and
605 nm in the \( \text{H}_2\text{O}_2 \)-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 \( \text{H}_2\text{O}_2 \) 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 \( \mu \text{M} \) for 605 nm and 2.5 ± 0.4 \( \mu \text{M} \) for the 570 nm and Soret band titrations.

The pH dependence of the peroxide complex \( K_d \) measured at different peaks of the \( \text{H}_2\text{O}_2 \)-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 \( \text{H}^+ \) 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 \( \text{H}_2\text{O}_2 \)-induced spectral changes. Basic conditions, as in fig. 1. (A) Difference spectra observed upon addition of increasing \( \text{H}_2\text{O}_2 \) concentrations to cytochrome oxidase proteoliposomes. (B) Typical titration curves of the \( \text{H}_2\text{O}_2 \)-induced spectral effect at the 3 principal bands of the difference spectrum (cf. fig. 1); the Soret band effect has been measured as \( \Delta A_{434-412} \). 3–5 min was allowed for equilibration after each \( \text{H}_2\text{O}_2 \) addition. The amplitudes of the spectral changes at various wavelengths are given in % to the maximal responses induced by an excess (0.7 mM) \( \text{H}_2\text{O}_2 \). 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 $\lambda_{max} = 580$ nm [4,20] which is easily formed at high $H_2O_2$ concentrations at pH above neutral [18] (cf. [17,36]).

2–3 $\mu$M 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$_{a3}$ and Cu$_{a3}$. 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$_{a3}^{2+}$.

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 $a_5$ (cf. [30]), Cu$_{a3}$ [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$$_{a3}$, 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$_a$ (presumably, Cu$_a$) 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.:

\[
E + H_2O_2 \rightarrow E - H_2O_2 \rightarrow E - (H_2O_2)_2 \rightarrow H_2O_2
\]

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

\[
O_2 + 2e^{-} + 2H_2O = H_2O_2 + 2H_2O (2)
\]

\[
O_2 + 2e^{-} + 2H^+ = H_2O_2 + 2H_2O (3)
\]

\[
H_2O_2 + H_2O_2 = 2H_2O + O_2 (4)
\]

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.

\[
\begin{align*}
&2\text{H}^+ \\
\text{H}_2\text{O}_2 &\xrightarrow{2\text{e}^-} (\text{Cu}_a) \xrightarrow{2\text{e}^-} \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}_2 \\
&2\text{H}^+ \\
\end{align*}
\]

Such an energy-linked H$_2$O$_2$ 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$_2$O$_2$ 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 ox-
idase shows rather low catalase activity ([16,17]
and refs therein). Second, the sequence of reac-
tions 2–4a gives a q/e ratio of 1.5 and H$_2$O$_2$/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 ex-
genous and intrinsically generated H$_2$O$_2$, and the
H$_2$O$_2$/e ratios published for cytochrome oxidase by
different authors show significant deviations [37].
In particular, the H$^+$/e ratios of 0.5 have been
reported for subunit III-deficient enzyme [38–41].

Finally, the suggested protonmotive mechanism
based on H$_2$O$_2$ formation and dismutation does
not exclude the possibility of additional elec-
trogenic 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

Thanks are due to Professor V.P. Skulachev for
his interest in this work and discussion of the
results.

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$_2$O$_2$ in site 3 protonmotive mechanism had
been discussed by Baum et al. in an edition
unavailable to us [42].

REFERENCES

SSSR 237, 713–716.
811, 1–12.
Baum, H. and Wrigglesworth, J.H. (1985) FEBS
Lett. 188, 1–7.
Konstantinov, A.A. and Skulachev, V.P. (1979)
Flux Across Biomembranes (Mukohata, Y. and
York.
Biochemistry 22, 452–461.
Mitochondria: Mechanism of Energy Coupling and
Regulation (Kondrashova, M.N. ed.) p.23,
Pustchino-na-Oke.
[16] Bickar, D., Bonaventura, J. and Bonaventura, C.
715–719.
Bound Cytochrome Oxidase with Ligands, PhD
Thesis, Moscow State University.
USA 78, 4051–4054.