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Effect of pH on the spectrum of cytochrome *c* oxidase hydrogen peroxide complex

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Hydrogen peroxide binding to ferric cytochrome *c* oxidase in proteoliposomes brings about a red-shift of the enzyme Soret band and increased absorption in the visible range with two prominent peaks at approx. 570 and 607 nm. The molar absorptivity of the H_2O_2-induced difference spectrum is virtually pH-independent in the Soret band and at 570 nm, whereas the peak at 607 nm increases approx. 3-fold upon alkalinization in a narrow pH range 6.0–7.2, the effect being reversible. The pH profile of this transition indicates ionization of two acid-base groups with close p*K* values of 6.7. The lineshape of the peroxide compound difference spectrum is found to respond to pH changes inside the proteoliposomes. It is suggested that peroxide-complexed enzyme can undergo a pH-dependent transition to a form with increased extinction at 605–607 nm, possibly corresponding to the 420 nm (or 'pulsed') conformer of the ferric cytochrome oxidase formed as an early product of the enzyme oxidation. Accordingly, relaxation of the '420 nm' form to the resting state would be linked to an uptake of two protons from the M-aqueous phase. This protolytic reaction might be a partial step of the cytochrome oxidase proton pumping mechanism or it could serve to regulate interconversion between the active 'pulsed' and less active 'resting' states of the enzyme in the membrane.

Introduction

Reaction of H_2O_2 with the oxidized cytochrome *c* oxidase has received considerable attention in the recent years [1–9]. The interest in the subject is inspired by peroxide intermediate formation in the forward (for reviews, see Refs. 10 and 11) and energy-reversed [10,12,13] cytochrome oxidase turnover.

All the earlier studies on H_2O_2 combination with ferric cytochrome oxidase have been carried out with isolated enzyme in detergent solution [1–9]. According to our previous experience with other ligands, such as cyanide or azide, there may be significant differences in the reactivity of the membrane-bound and solubilized cytochrome oxidase [14–16] and whenever possible, it is better to carry out ligand-binding studies with proteoliposome-reconstituted enzyme.

Recently we described H_2O_2 combination with membrane-bound cytochrome oxidase, determined K_d values of the peroxy complex at pH 6–8 and provided evidence for two H_2O_2-binding sites in the enzyme with different affinities for the ligand at acid pH [16–18]. It was found that the magnitude of the peak at approx. 605 nm in the H_2O_2-induced difference spectrum can vary without correlated changes in the Soret or 570 nm absorption bands [2,16–18].

Here we describe effect of pH on the size and shape of H_2O_2-induced spectral changes of cytochrome oxidase in proteoliposomes. The data obtained differ significantly from the results of the earlier work [3] carried out with solubilized enzyme and provide further evidence for 'independent' behaviour of the 605–607 nm band in the H_2O_2-induced difference spectrum. The height of this band appears to depend on pH inside proteoliposomes.

Materials and Methods

Chemicals. Bile acids (Sigma) were crystallized from a charcoal-treated solution in 70% ethanol as described [15]. 30% H_2O_2 (Merck, 'Suprapur' grade) was diluted

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1,1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid.

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to stock solutions of appropriate concentrations before experiments. Concentration of hydrogen peroxide was checked periodically by measuring extinction at 240 nm [19]. Asolectin was purchased from Sigma and monensin from Calbiochem-Behring. Other chemicals were commercial products of the highest purity available from Serva, Sigma, Merck, Fluka and Pharmacia.

Preparations. Cytochrome *c* oxidase (a Fowler-type preparation) was isolated from bovine heart mitochondria and purified essentially according to Refs. 20 and 21. The enzyme was reconstituted in asolectin proteoliposomes by a cholate dialysis method [22] as described earlier [15,23] at a lipid-to-protein ratio of 30 : 60; the latter ratio was preferred for experiments with monensin-catalyzed pH gradient formation.

Four routine experiments proteoliposomes were prepared in a medium containing 2% cholate, 50 mM sodium phosphate (pH 7.5) and 2 mM $MgSO_4$ and dialyzed against the same buffer but without cholate. When monensin-catalyzed perturbation of intraliposomal H^+-activity was to be applied, the procedure was modified as follows.

Na^+-loaded proteoliposomes (for acidification inside). 50 mM phosphate was replaced by 5 mM Hepes-Tris (pH 7.4) and 50 mM Na_2SO_4 in the reconstitution buffer. Before experiments, the proteoliposomes were centrifuged through Sephadex G-25-filled plastic syringes [24] to remove external Na^+.

Na^+-free proteoliposomes (for alkalinization inside). 50 mM phosphate was replaced by 5 mM Hepes-Tris (pH 6.8–7.0), and 0.1 M choline chloride.

Assays. Protein was measured by the biuret method. Cytochrome oxidase concentration was determined from the difference spectra (reduced minus oxidized) using $\Delta\epsilon_{605-630}$ value of 27 $mM^{-1} \cdot cm^{-1}$.

H_2O_2 binding with cytochrome *c* oxidase was studied spectrophotometrically in an Aminco DW-2a or a Hitachi 557 spectrophotometer operated in a double-beam spectra scan mode. Standard 10 mm light pathway cuvettes were thermostatted at 25 °C. If not stated otherwise, the basic reaction medium contained 50 mM Mes, Mops, Hepes or Tris buffer depending on pH (the buffer with pK nearest to the desired pH value was used), 2 mM $MgSO_4$, 100 μM $K_3Fe(CN)_6$ and 1 μM the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone.

Suspension of proteoliposomes in an appropriate medium was divided between the sample and reference cells. Baseline adjusted, saturating concentration of H_2O_2 was added to the sample and difference spectra were recorded one after another for an observation period of up to 1 h. In the visible range, the heights of the peaks at 570 and 607 nm in the H_2O_2-induced difference spectra were measured relative to an empyrically chosen baseline connecting the points at 650 and 500 nm. In the Soret, a peak-to-trough amplitude was measured. In most cases, the values of absorption difference are given in the paper normalized to cytochrome oxidase concentration and are referred to for the sake of brevity as $\Delta\epsilon$ values although they may not necessarily be true extinction coefficients.

Results

When H_2O_2 is added to the oxidized cytochrome oxidase the Soret peak of the enzyme shifts to 427–428 nm (data not shown) and spectral changes occur in the visible range which give rise to the peaks at approx. 570 and 607 nm in the difference spectrum (Fig. 1a and b; Fig. 3). In some works including our own previous publication [5,7,18] the 607 nm peak was found to be located at 605–606 nm; whether this minor discrepancy is due to different enzyme preparations or to some deviations in the spectrophotometer setting is not clear.

Inspection of the literature reveals considerable variation of the size and shape of H_2O_2-induced cytochrome oxidase spectral changes as reported by different workers [3–9,18]. Therefore, before proceeding to the main issue of this paper, we would point out several complications inherent in the measurements of cytochrome oxidase reaction with H_2O_2 revealed in the course of our preliminary studies [2,16]. These complications demand a careful choice of experimental conditions.

First, the initial peroxide adduct of cytochrome oxidase ('peroxy' complex) can further react with higher concentrations of H_2O_2 to form a spectrally distinct compound (Fig. 1, cf. Refs. 5, 7 and 9), presumably an 'oxyferryl' $Fe^{4+} = O$ complex [13,25]. Accordingly, titration curves of H_2O_2-induced absorption changes in the visible range are biphasic, e.g., the peak at 607 nm first rises with K_d 2–20 μM [18] and then decays with $[H_2O_2]_{0.5}$ of 0.1–1 mM depending on pH and some other conditions [17]. Therefore, care must be taken to select at each pH a minimal H_2O_2 concentration which would be saturating with respect to the peroxy complex formation * and yet would not yield the oxyferryl form to a significant extent. Generation of the oxyferryl complex in the present experiments was further diminished with the aid of ferricyanide included in the reaction medium [17]. This also allowed to avoid reduction of cytochrome oxidase by H_2O_2 at pH > 7 observed in Ref. 5. Under these conditions we were able to employ H_2O_2 concentrations greater than $10 \times K_d$ at

* As shown previously, the cytochrome *c* oxidase absorbance changes induced by H_2O_2 at 605–607 nm saturate at higher H_2O_2 concentrations than those in the Soret band or at 570 nm at pH < 7.5 [18] which might indicate the presence of two different H_2O_2-binding site in the enzyme. The 'saturating' H_2O_2 concentrations used in this work are meant to induce full development of all bands of the peroxy complex difference spectrum at a given pH.

each pH without significant interference from the oxyferryl compound formation or heme reduction.

Second, we found it necessary to work with as low cytochrome oxidase concentrations as possible to minimize interference from the catalase activity of the enzyme. This activity can diminish stability of H_2O_2-induced responses even at submicromolar cytochrome oxidase concentrations (e.g., Fig. 2d) and it can become a serious problem at 5–40 μM of the enzyme as used in Refs. 3, 5 and 7, especially when large excess of H_2O_2 is to be avoided as mentioned above. It is noteworthy in this connection that $K_d = 10$ μM determined for the peroxy complex in experiments with 10 μM cytochrome oxidase [3] is about 4-fold higher than the value found in our experiments with 1–0.5 μM enzyme [18].

Third, spectral responses of cytochrome *c* oxidase to saturating H_2O_2 concentrations often comprise slower secondary changes of absorption (e.g., Fig. 2 and Ref. 16). These can be due in part to a minor proportion of

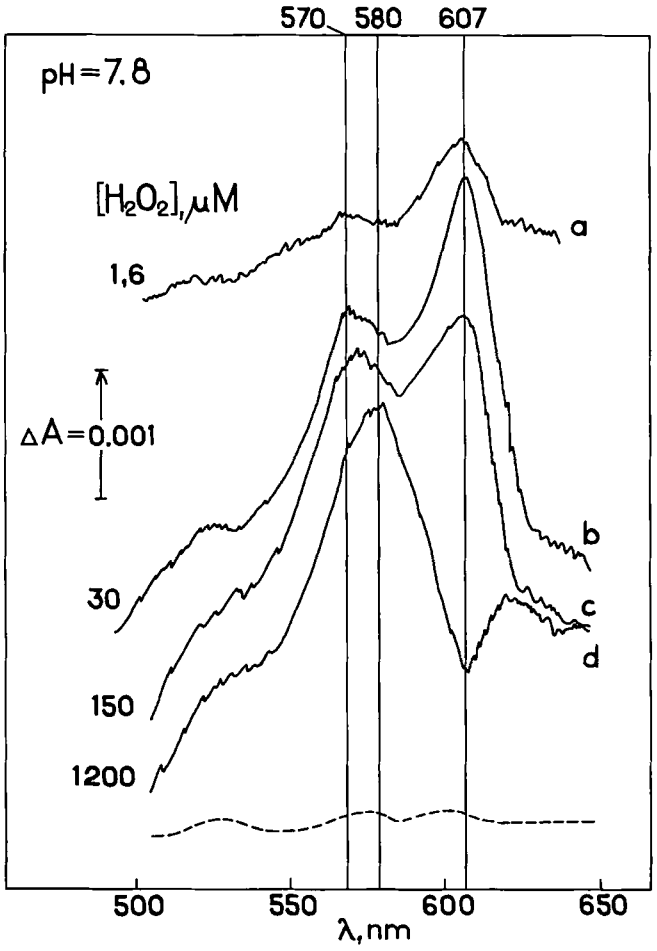


Fig. 1. Formation of two different peroxide compounds of membrane-bound cytochrome oxidase at low and high H_2O_2 concentrations. The sample and reference contained cytochrome *c* oxidase proteoliposomes ($[aa_3] = 1$ μM) in a basic medium (pH 7.8) (see Materials and Methods). After correction of a baseline (dashed line) H_2O_2 was added to the sample at concentrations of 1.6, 30, 150 and 1200 μM (spectra a–d, respectively).

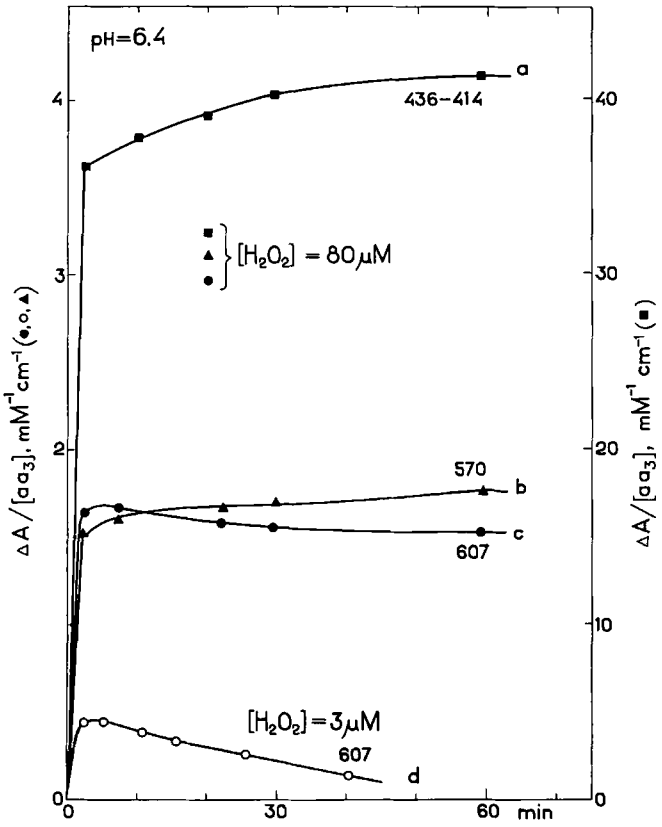


Fig. 2. Time stability of the cytochrome oxidase spectral responses induced by H_2O_2. Cytochrome oxidase proteoliposomes, 0.6 μM in aa_3. At the zero time H_2O_2 was added to the sample at the concentrations indicated in the Figure. Open circles show a typical decay of the H_2O_2-induced ΔA_{607} at $[H_2O_2] \approx K_d$ due to the catalase activity of the preparation.

the enzyme binding H_2O_2 much slower than the bulk of cytochrome oxidase (cf. Ref. 9); however, there appears to be contribution from additional processes as revealed by time-dependent alterations in the form of the difference spectra, especially at pH above 7 (data not shown [16]).

At present we are not ready to give a fully consistent analysis of these secondary processes. Therefore this paper is confined to initial observation period less than 10 min during which the rapid phase of absorption changes reaches completion (cf. Ref. 9) whereas contribution of the secondary processes remains negligible throughout the pH and H_2O_2 concentration range studied. Most of the data in the paper refer to difference spectra recorded 5 min after addition of H_2O_2.

Effect of pH on the H_2O_2 induced spectral changes

Fig. 3 shows typical difference spectra induced by saturating concentrations of H_2O_2 added to liposome-reconstituted ferric cytochrome oxidase at acid and alkaline pH.

In the Soret band the two spectra are very similar; also the 570 nm peak appears to be unaffected. At the

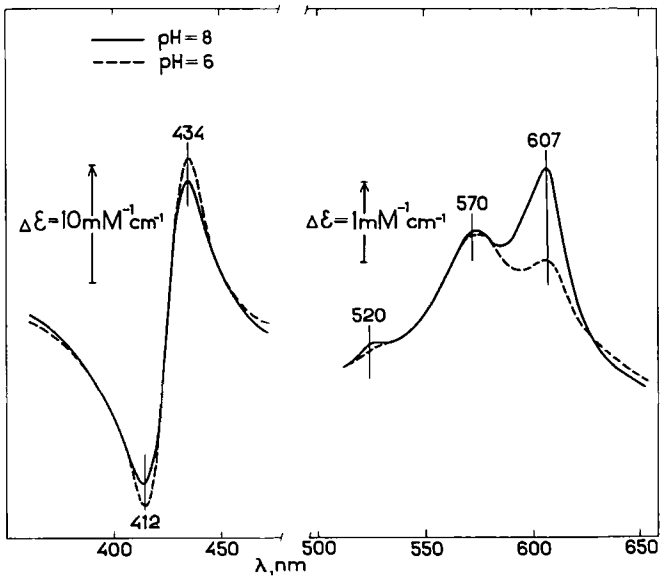


Fig. 3. Effect of pH on the spectral characteristics of cytochrome oxidase peroxy complex. The sample and the reference contain cytochrome oxidase proteoliposomes ($[aa_3] = 0.8$ μM) in the basic medium with pH 6.0 (dashed line) or 8.0 (bold line). Saturating H_2O_2 concentration has been added to the sample (500 μM for pH 6, 30 μM for pH 8).

same time, significant increase in the amplitude of the 607 nm band is observed at alkaline pH.

The effect of pH is reversible (Fig. 4). The same reversible increase of the 607 nm band upon alkalinization was also observed in an experiment analogous to that in Fig. 4 but carried out in a single cuvette so that the difference spectra were scanned in a dual-wavelength mode ($\Delta A_\lambda - \Delta A_{630}$) (data not shown [16]). This result indicates that the effect of pH on the difference

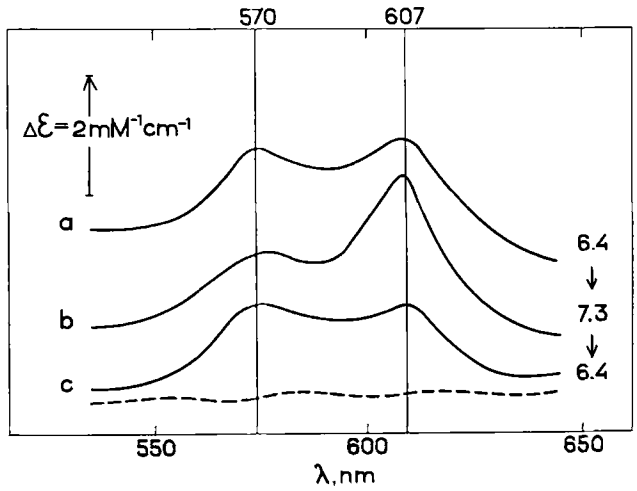


Fig. 4. Reversibility of the pH-dependent changes in the spectrum of peroxy complex of cytochrome oxidase. The sample and reference cells contained cytochrome oxidase proteoliposomes ($[aa_3] = 0.4$ μM) in the basic medium with pH 6.4. 20 μM H_2O_2 added to the sample (spectrum a), pH was adjusted to 7.3 in both cells (spectrum b) and returned back to 6.4 (spectrum c).

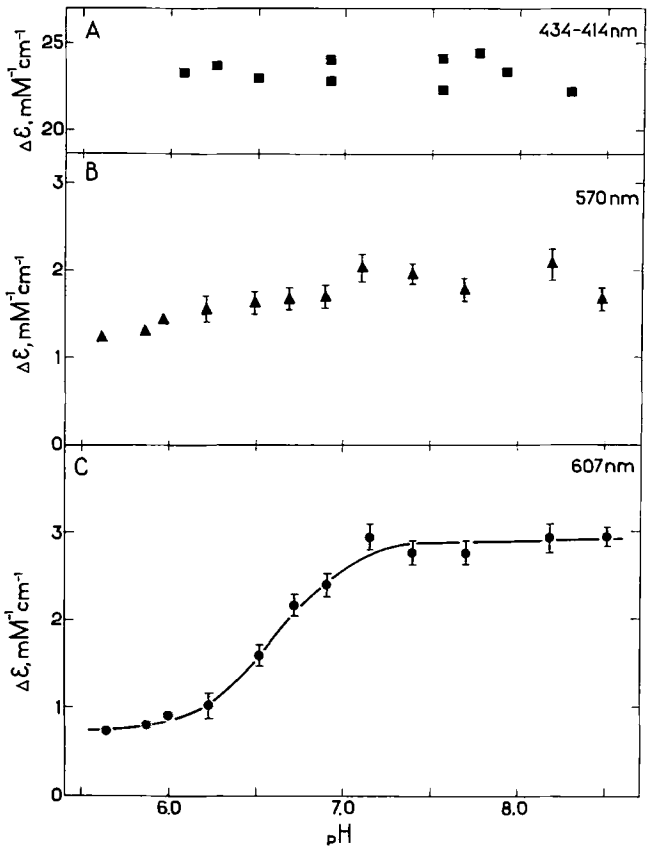


Fig. 5. pH dependence of absorption changes at three principal bands of the cytochrome oxidase peroxy complex difference spectrum. Cytochrome oxidase proteoliposomes were suspended in the basic medium at indicated pH values. Saturating amount of H_2O_2 was added to the sample and the spectral changes were measured 5 min after the addition. The final H_2O_2 concentrations at different pH were 300 μM (6.2), 100 μM (6.2–6.8), 50 μM (6.8–7.2) or 30 μM (7.2–8.5). These concentrations were chosen so as to provide for minimal interference from the secondary processes. Absorption changes in the Soret and visible range were measured in separate experiments. Where indicated, the points are mean values ± mean deviations for 3–6 experiments with different preparations of proteoliposomes. The bold line in panel C is a theoretical curve for a transition linked to ionization of two acid-base groups with the same pK of 6.7.

spectrum is due to pH-dependence of the absorption of the peroxide compound rather than of the free enzyme.

pH-dependence of the cytochrome oxidase peroxy complex absorptivity at the three principal bands of the difference spectrum is shown in Fig. 5. In contrast to Ref. 3, we found no significant pH-dependence of the Soret band effect amplitude (panel A). The size of the 570 peak proved fairly constant under these conditions as well (panel B). At the same time, the amplitude of the 607 nm band increases several-fold as pH is raised above 6 reaching a plateau at approx. 7.2 (panel C).

Thus, the peroxy complex of cytochrome oxidase is likely to undergo a reversible transition to a state with increased intensity of the 607 nm peak, the transition being associated with deprotonation of two ionizable groups with very close pK values of 6.7. As suggested in

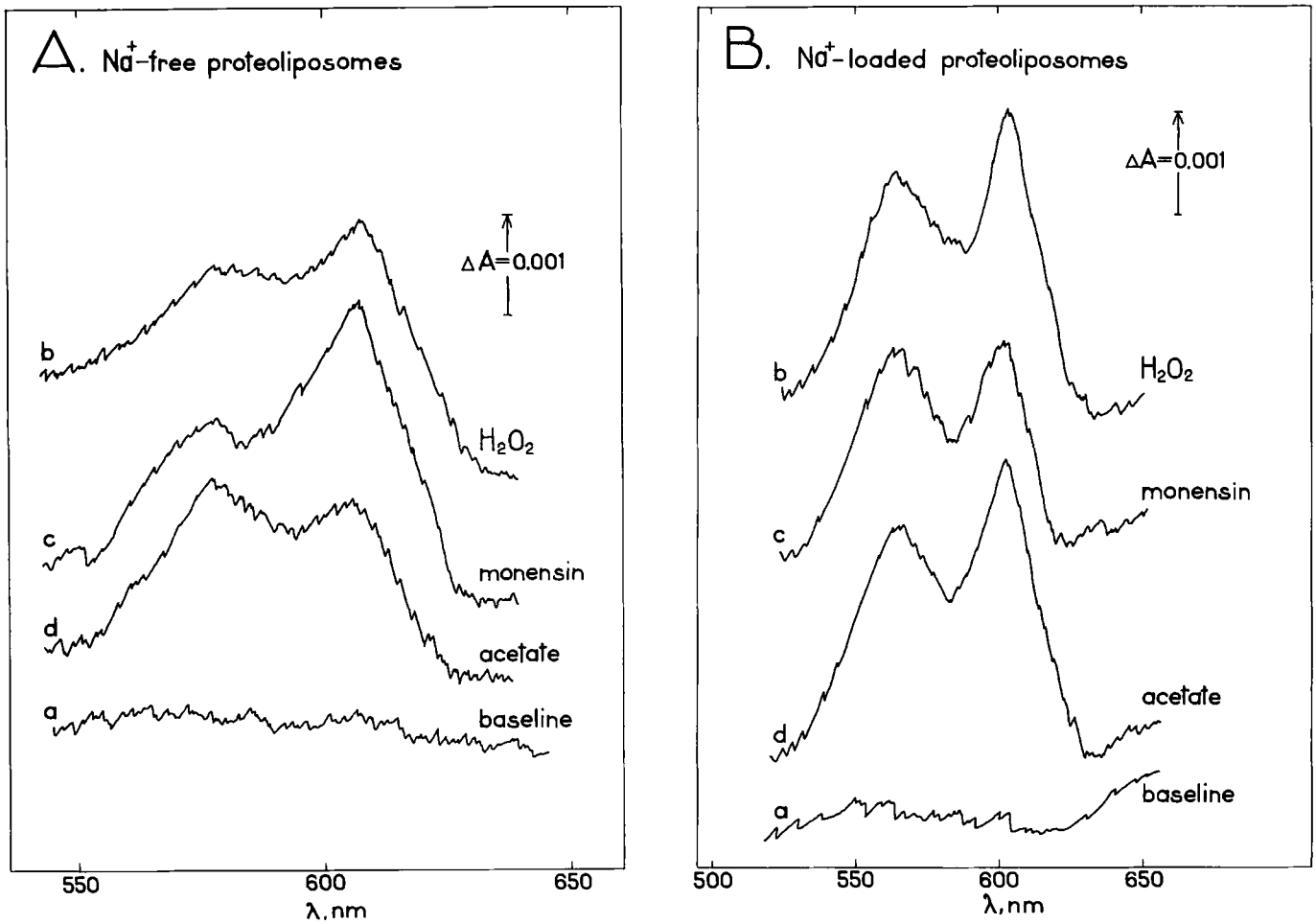


Fig. 6. Effect of alkalinization (A) and acidification (B) inside proteoliposomes on the spectral characteristics of cytochrome oxidase eroxy complex. (A) Na^+,K^+-free proteoliposomes ($[aa_3] = 0.5$ μM) with low internal pH-buffering capacity were suspended in a buffer containing 25 mM Mes-KOH (pH 6.4), 50 mM Na_2SO_4 and 100 μM ferricyanide and preincubated for 20 min. a, baseline; b, 30 μM H_2O_2 added to the sample; c, 5 min after addition of monensin (5 ng/ml) to both cells; d, spectrum c recorded, 20 mM NH_4CH_3COO was added to the sample and reference. (B) Proteoliposomes ($[aa_3] = 0.6$ μM) loaded with 50 mM Na_2SO_4 were suspended in a K^+,Na^+-free buffer containing 50 mM Mops-Tris buffer (pH 7.4), 100 mM choline chloride and 100 μM potassium ferricyanide. a, baseline; b, 20 μM H_2O_2 added to the sample; c, 5 min after addition of monensin (5 ng/ml) to both cells; d, spectrum c recorded, 20 mM NH_4COOH was added to the sample and reference cells.

Ref. 18, it is tempting to relate this state to a so-called '420 nm' conformation of the free ferric enzyme characterized by a very similar peak at 605–607 nm in the difference spectrum versus the resting cytochrome oxidase [6,7,26,27] (see Discussion).

Subsequently, we attempted to find out whether it is pH inside or outside proteoliposomes that affects the spectrum of the peroxy compound of cytochrome oxidase. To this end a method of pH perturbation inside proteoliposomes by virtue of monensin-catalyzed Na^+/H^+-antiport was used [23,28].

In Fig. 6A, Na^+,K^+-free proteoliposomes were suspended in a 50 mM Na_2SO_4-containing buffer at pH 6.4. A typical two-band difference spectrum was observed in the visible region upon addition of H_2O_2 (spectrum b). It can be seen that monensin which under these conditions brings about alkalinization inside the vesicles [28], induces marked increase in the height of the 607 nm peak (spectrum c) which is reversed by ammonium acetate (spectrum d). Reversal and prevention of the monensin-induced effect were observed also in the presence of carbonylcyanide *m*-chlorophenylhydrazone (Cl-CCP), the uncoupler (not shown [16]).

In experiments of this kind collapse of ΔpH by NH_4CH_3COO often resulted in the 607 nm peak somewhat lower than in the initial difference spectrum of the peroxide compound (e.g., Fig. 6A, spectra b and d). It has to be mentioned that in order to obtain well-coupled proteoliposomes we found it necessary to prepare the vesicles at pH > 6.6, i.e., above that of the final reaction mixture. Although a 20 min incubation was allowed before the experiment in Fig. 6A for equilibration of proteoliposomes with the medium, it is possible that pH inside the vesicles was still slightly higher initially than the ambient 6.4.

Results of a complementary experiment with Na^+-loaded proteoliposomes suspended in an essentially Na^+/K^+-free medium are shown in Fig. 6B. In this case

monensin brings about acidification inside the vesicles [28] and, accordingly, the 607 nm peak of the peroxy complex difference spectrum declines (spectrum c), the effect being reversed by the permeant base (spectrum d). In the absence of Na^+ gradient (50 mM Na_2SO_4 added to the medium) or in the presence of ClCCP monensin exerted no effect (data not shown).

The experiments in Fig. 6 indicate that the spectral characteristics of cytochrome oxidase peroxy complex depend on pH of proteoliposome interior which is equivalent topographically to the matrix aqueous phase of mitochondria [23,28].

Discussion

The magnitude of H_2O_2-induced spectral changes

In contrast to earlier studies on H_2O_2 combination with the oxidized cytochrome oxidase [1–7,9], this work has been carried out with the membrane-bound (or, more accurately, the liposome-bound) enzyme.

It seems likely that in proteoliposomes cytochrome oxidase becomes more homogeneous with respect to ligand reactivity as compared to the detergent solubilized enzyme (Ref. 16, see also Konstantinov, A. and Musatov, A., unpublished results). For instance, a great scatter (approx. 20-fold) in the magnitude of the H_2O_2-induced cytochrome oxidase spectral changes was reported by Bickar et al. [3] and confirmed by Gorren et al. [9]. The $\Delta\epsilon_{H_2O_2}$ values in our experiments also varied for different preparations but to a significantly lesser extent, i.e., 22–50 $mM^{-1}\,cm^{-1}$ for the Soret peak-to-through amplitude and 2.2–4.5 $mM^{-1}\cdot cm^{-1}$ for the 607 nm peak * (Table I). Notably, these values are markedly higher than the maximal effects observed for the solubilized enzyme, resting or 'pulsed', in Refs. 3 and 5 and are equal to or exceed those obtained by Kumar et al. [6,7] for their '420 nm form' (Table I).

Our data are also in good agreement with the $\Delta\epsilon$ values reported by various authors for the difference spectrum of the 'oxygenated' or 'pulsed' cytochrome oxidase (428 nm form) versus the resting state (Table I).

The reason for the approx. 2-fold variability of $\Delta\epsilon$ values observed in this and earlier works (cf. Table I) could be at least partly due to a well-known lability of the free ferric enzyme spectral characteristics.

At the same time, even the highest of our $\Delta\epsilon$ values for the 607 nm peak are still much lower than the coefficient of approx. 12 $mM^{-1}\cdot cm^{-1}$ reported by Wikström for the 607 nm peak of the peroxy compound ('compound P') formed by an energy-linked reversal of cytochrome oxidase reaction in rat liver mitochondria [12]. A $\Delta\epsilon$ value of approx. 12–20 $mM^{-1}\cdot cm^{-1}$ was also calculated for the 607 nm peak of 'compound C' [10,35].

This discrepancy may indicate the H_2O_2-adduct to be not fully identical with compounds P or C. Several possibilities are considered below.

First, one could suggest only part of cytochrome oxidase to have reacted with hydrogen peroxide in the H_2O_2-adduct. It has to be mentioned here that H_2O_2 may bind with cytochrome oxidase in two sites, tentatively identified as Fe_{a_3} and Cu_B [18]. There are probably no large differences between the H_2O_2 adduct and energy-linked compounds P or F in the Soret band (Table I, cf. Refs. 12, 16, 17, 34 and 36). Therefore, low binding site occupancy is not likely with respect to H_2O_2 coordination at the 6th axial position of heme a_3 iron (possibly, as a bridging ligand between Fe_{a_3} and Cu_B) which is responsible for the red shift of the γ-band of the enzyme [18]. On the other hand, partial occupancy of the second H_2O_2 binding site (Cu_B ?) [18]

TABLE I

Molar extinction values for difference spectra of H_2O_2-complexed cytochrome oxidase and related enzyme species versus the 'resting' state

The figures were taken as given by the authors or were calculated from the drawings in the papers.

Enzyme species, reference	$\Delta A/[aa_3]$ ($mM^{-1}\cdot cm^{-1}$)	
	Soret	605–607 nm
Peroxide complex (428 nm form)		
Bickar et al. [3]	12 [a]	1.6 [a]
Wrigglesworth [5]	–	0.6
Kumar et al. [6,7]	25	2
this work	22–50 [b]	2.2–4.6 [b]
'Oxygenated' enzyme (428 nm form)		
Wharton and Gibson [29]	22	–
Tiesjema et al. [30]	50	3.0
Orii and King [31]	24	2.1
'Pulsed' enzyme (428 nm form?)		
Brunori et al. [32,33]	50	3
'Recently oxidized enzyme' (420 nm form)		
Rosen et al. [26]		3.4
Armstrong et al. [27]		5.2
Kumar et al. [7]		2.1 ± 0.3
Energy-dependent peroxy complex (compound P)		
Wikström [12]	–	12
Wilson et al. [34]	46 [c]	(17) [d]

[a] The maximal values obtained in the paper.

[b] The minimal and the maximal values are indicated.

[c] In Ref. 34 an energy-dependent oxyferryl rather than peroxy complex formation was observed; however, according to our data, the oxyferryl and peroxy complexes have very similar Soret band spectra [17]. The value has been calculated from Fig. 1 in Ref. 34 assuming aa_3 specific content of 0.5 nmol/mg protein, which is probably a lowest estimate for pigeon heart mitochondria; accordingly, $\Delta\epsilon$ values may be in fact lower.

[d] Calculated for the energy-dependent peroxy complex taking the ratio of $\Delta\epsilon^{605}_{peroxy}/\Delta\epsilon^{580}_{oxyferryl}$ of approx. 3 [12].

* These $\Delta\epsilon_{607}$ values refer to the plateau level above pH 7.5 (see Fig. 5C).

cannot be ruled out at present and could account in principle for the low apparent ϵ_{607} value of the peroxide adduct as compared to compound P. Notably, energization was reported to increase capacity of cytochrome oxidase oxygen binding pocket from one to two ligand molecules [37,38]. However, such an explanation implies significant heterogeneity of liposome-reconstituted Fowler type cytochrome oxidase, which has not been revealed so far by our experiments with other ligands. Moreover, the same low $\Delta\epsilon_{607}$ values up to approx. 4 $mM^{-1} \cdot cm^{-1}$ have been observed for H_2O_2 adduct in submitochondrial particles (Ludvig, N., Vygodina, T. and Konstantinov, A., unpublished results) for which cytochrome oxidase heterogeneity is very unlikely.

Second, high extinction of compound P at 607 nm could be a marker of a special thermodynamically unstable conformation of ferric enzyme favoured by both energization and H_2O_2 binding. In such a case even full occupation of the binding sites might be insufficient for complete conversion of the enzyme to the form with $\Delta\epsilon_{607} \approx 12$ $mM^{-1} \cdot cm^{-1}$ by exogenous H_2O_2 under de-energized conditions.

Third, the peak at approx. 607 nm in the difference spectra of various cytochrome oxidase compounds may comprise more than one optical effect and in our opinion it is the most likely explanation. A moderate increase in A_{607} typical of the H_2O_2-adduct or pulsed enzyme ($\Delta\epsilon_{607} \leqslant 5$ $mM^{-1} \cdot cm^{-1}$) could originate in an α-band red shift associated with a conformational transition of ferric cytochrome oxidase. Spectra simulations show that such a shift gives rise to an asymmetric difference spectrum with a major peak at 607–608 nm and a minor trough around 590 nm; for instance, $\Delta\epsilon_{607}$ of 4 $mM^{-1} \cdot cm^{-1}$ corresponds to a shift of the ferric enzyme α-band to the red by approx 2 nm (Konstantinov and Vygodina, unpublished results).

In compounds P or C this effect may be overlapped by a strong charge-transfer band arising from interaction between Fe_{a_3}, Cu_B and peroxy ligand (see pp. 124–127 in Refs. 10 and 35 and references therein). Notably, such a charge-transfer band should be sensitive even to subtle changes in the O_2-binding site and, in particular, to protonation state of the peroxy ligand, which can provide a plausible explanation for the absence of this electronic transition in the H_2O_2-adduct (see next section and Scheme I).

pH-dependence of the H_2O_2-induced spectral changes

We were not able to confirm the observation [3] that the magnitude of the H_2O_2-induced spectral changes in the Soret band increases 5-fold upon alkalinization from pH 6–8. In view of low $\Delta\epsilon$ values obtained by Bickar et al. [3] for their peroxide complex, as compared to this and other works (Table I), it is likely that alkalinization in Ref. 3 simply increased proportion of cytochrome oxidase interacting with H_2O_2 *. At the same time, the amplitude of the 607 nm peak in our experiments increased significantly upon alkalinization. The following questions may be concerned in this connection.

(I) Is it neutral (H_2O_2) or ionized (O_2^{2-}) hydrogen peroxide bound with the a_3/Cu_B site in the peroxy complex?

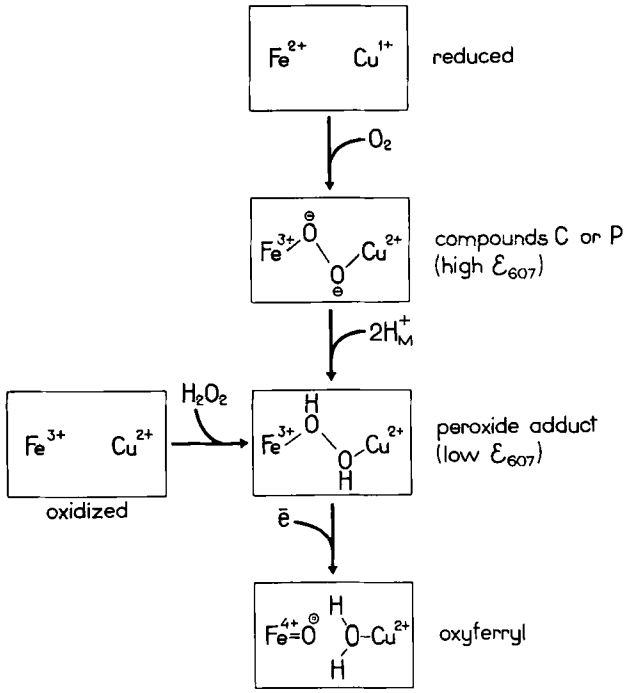
Generally, the pH-dependence of the peroxide complex spectrum could be due to ionization of either the enzyme, or the ligand in either of the two H_2O_2 binding sites. Thus, according to Bickar et al. [3] it might be H_2O_2 which binds cytochrome oxidase below pH 6 but the conjugated base (O_2^{2-}) that reacts with the enzyme at pH above the neutral. Doubly deprotonated state of peroxide bound to the oxygen-reducing centre of cytochrome oxidase has been assumed also by most other workers (e.g., Refs. 10, 25, 35, 39 and 40). However, K_d for hydrogen peroxide binding to heme a_3^{3+} is pH-independent, which indicates H_2O_2 to be the species bound throughout the pH range studied 6–8.5, and there is only a weak pH dependence of H_2O_2 affinity for the second binding site [18]. Incidentally, this fact implies that O_2 reduction to O_2^{2-} during the catalytic cycle of cytochrome oxidase should be coupled to an uptake of two protons, and this protonation of the reduced oxygen intermediate might be one of the partial electrogenic steps of the reaction (Scheme I) [18,41,42]. This is at variance with the mechanism suggested by Wikström according to which the proton uptake takes place at a later step of the peroxy intermediate reduction to the oxyferryl complex [12,13,43].

In this connection it is interesting to speculate that compound C and energy-dependent compound P may indeed contain a deprotonated O_2^{2-} species rather than H_2O_2 (Scheme I). Notably, this could account for the above-discussed higher extinction of these compounds at 607 nm as compared to the H_2O_2-liganded enzyme, since it is the deprotonated O_2^{2-} ligand, much more electronegative than H_2O_2, which can be expected to give a strong ligand-to-metal charge-transfer band (see p. 126 in Ref. 10).

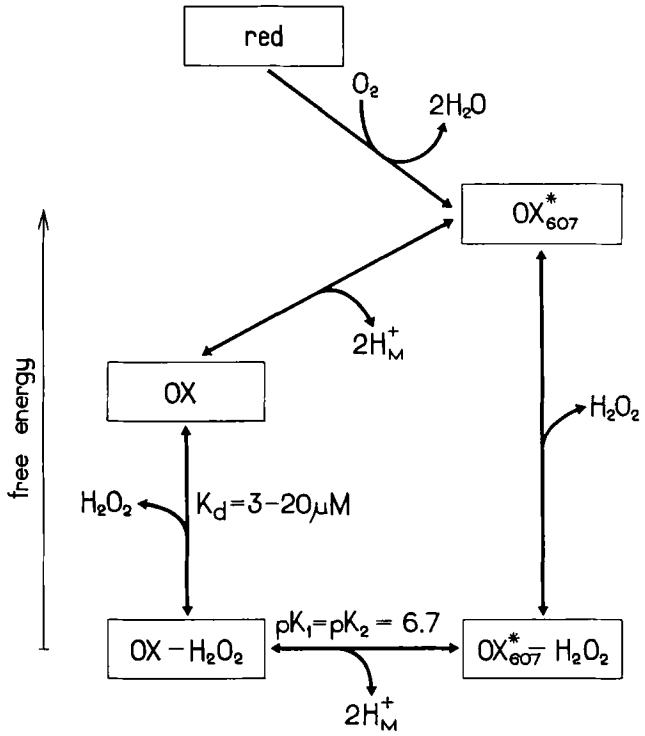
Deprotonation of bound peroxide could be a consequence of a highly energized membrane state in experiments of Wikström [12,13], since $\Delta\psi$ has been shown to raise local pH in heme a_3/Cu_B-surroundings relative to the M-aqueous phase [23,28,43]. As to compound C, protonation of O_2^{2-} is very likely to be prevented in this case due to the frozen state of samples.

It is possible that in a catalytic cycle, protonation of the bound O_2^{2-} is prerequisite for its subsequent rapid

* It has to be pointed out that the pH dependence of $\Delta\epsilon_{Soret}$ values in Ref. 3 was reported for H_2O_2 concentrations as high as 1 mM, which certainly gave rise to a variable mixture of peroxy and oxyferryl compounds depending on pH [17].



Scheme I. Possible relationships between various peroxy compounds of cytochrome oxidase. See text for discussion. Binding of the second H_2O_2 molecule [18] is not included in the scheme.



Scheme II. pH-dependent interconversions of spectrally distinct conformers of ferric cytochrome oxidase. ox, stable 'relaxed' (or 'resting') form of the oxidized cytochrome oxidase. ox^*_{607}, denotes 'tense' (or 'pulsed') conformation of cytochrome oxidase formed as an early product of the enzyme oxidation and characterized by moderately increased extinction at 605–609 nm (a red shift of the α-band?) and by Soret band in the unliganded state at approx. 420 nm [26,27,7]. H_2O_2 ligation at the two putative binding sites [18] has been combined into a single step for simplicity with a maximal difference in the K_d values indicated. Whereas the 'tense' → 'relaxed' transition appears to be thermodynamically favourable for the free enzyme, in the H_2O_2-complexed cytochrome oxidase the two conformations may have comparable stabilities. Conversion of the 'tense' form to the 'relaxed' one is suggested to be linked to an uptake of two protons from the M-phase as found in this work for the H_2O_2-complexed enzyme.

reductive cleavage to the oxyferryl compound. In such a case the proton uptake linked to double electron reduction of O_2 to O_2^{2-} could well look as if being associated with the oxyferryl state generation, as observed by Wikström [44].

(II) pH-dependent 'resting' ↔ 'pulsed' interconversion of ferric cytochrome c oxidase

As concerns pH-dependence of the H_2O_2-induced difference spectrum, we suggest that the hydrogen peroxide-complexed cytochrome oxidase (with both H_2O_2 binding sites occupied) can undergo a reversible transition to a conformation with increased extinction at 605–607 nm (or, as discussed above, with a red-shifted α-band), the transition being linked to deprotonation of two ionizable groups of the enzyme with $pK_1 = pK_2 = 6.7$.

As already mentioned above, it is tempting to suggest that such a transition does exist in the unliganded enzyme as well, since a conformation of the oxidized cytochrome oxidase with increased absorption at approx. 605 nm but essentially unchanged Soret band has been identified as an immediate product of the enzyme oxidation [6,26,27]. Being thermodynamically unfavourable, this 'tense' ('pulsed') conformation under common conditions is known to decay virtually irreversibly to a stable 'relaxed' ('resting') state. Presumably, in the peroxy complex the free energy difference between the two conformations is diminished so that it becomes possible to observe pH-dependent equilibrium interconversion between the 'relaxed' and 'tense' forms (Scheme II and Ref. 18).

This pH-dependence accords with the observations that 'oxygenated' conformation of cytochrome oxidase is stabilized by alkaline pH kinetically [45] (but cf. Ref. 30) and thermodynamically [46].

Implication for cytochrome oxidase functions

As found here, the magnitude of the 607 nm peak in the H_2O_2-induced difference spectrum of cytochrome oxidase depends on pH of the intravesicular aqueous phase corresponding to the matrix phase of mitochondria. Accordingly, Wikström has reported recently compounds P and F to be stabilized by alkaline pH inside rather than outside the mitochondria [43]. These data may be indicative of protonic communication between the oxygen-reducing centre of the enzyme and M-phase and corroborate a conclusion on the existence of a cytochrome a_3-associated input proton well inferred earlier from cyanide binding studies [41,23,28].

In addition, the present results indicate that relaxation of the '607 nm' (pulsed) form of the oxidized cytochrome oxidase to the stable conformation may be associated with an uptake of two protons from the M-phase. Implications of such protonation may be two-fold.

First, as suggested in Ref. 18 it could be a one more step of the cytochrome oxidase protonmotive mechanism.

Second, the pH-dependence of cytochrome oxidase conformational transition might serve to control an equilibrium between the active 'pulsed' and inactive 'resting' forms of the ferric enzyme in the mitochondrial membrane [47,48]:

'resting' $\rightleftharpoons$ 'pulsed' (607 nm)
$2\,H^+_M$

Scheme III

Energization (at least, ΔpH component of $\Delta\tilde{\mu}_{H^+}$) can be envisaged to favour the 'pulsed' state. Such an effect could compensate for increased tendency of the enzyme to escape from the catalytic cycle in the energized membrane when electron flow through cytochrome oxidase diminishes.

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References

1 Bickar, D., Bonaventura, C. and Bonaventura, J. (1980) Fed. Proc. Fed. Am. Soc. Exp. Biol. 39, 206.
2 Grigorjev, V.A. and Konstantinov, A.A. (1981) in: Mitochondria: mechanism and regulation of energy coupling (Kondrashova, M.N., ed.) p. 23, Pustshino.
3 Bickar, D., Bonaventura, J. and Bonaventura, C. (1982) Biochemistry 21, 2661–2666.
4 Chance, B., Kumar, C., Powers, L. and Ching, Y. (1983) Biophys. J. 44, 353–363.
5 Wrigglesworth, J.M. (1984) Biochem. J. 217, 715–719.
6 Kumar, C., Naqui, A. and Chance, B. (1984) J. Biol. Chem. 259, 2073–2076.
7 Kumar, C., Naqui, A. and Chance, B. (1984) J. Biol. Chem. 259, 11668–11671.
8 Bickar, D., Lehninger, A., Brunori, M., Bonaventura, J. and Bonaventura, C. (1985) J. Inorg. Biochem. 23, 365–372.
9 Gorren, A.C.F., Dekker, H. and Wever, R. (1986) Biochim. Biophys. Acta 852, 81–92.
10 Wikström, M.K.F., Krab, K. and Saraste, M. (1980/1981) Cytochrome oxidase, Academic Press, New York.
11 Hill, B.C., Greenwood, C. and Nicholls, P. (1986) Biochim. Biophys. Acta 853, 91–113.
12 Wikström. M.K.F. (1981) Proc. Natl. Acad. Sci USA, 78, 4051–4054.
13 Wikström, M.K.F. (1987) Chim. Scr. 27B, 53–58.
14 Andreev, I.M. and Konstantinov, A.A. (1983) Bioorgan. Chem. (USSR) 9, 216–227.
15 Vygodina, T.V. and Konstantinov, A.A. (1985) Biological Membranes (USSR) 2, 261–270.
16 Vygodina, T.V. (1987) Reaction of the oxidized membrane-bound cytochrome oxidase with ligands, Ph.D. Thesis (in Russian), Moscow State University.
17 Vygodina, T.V. and Konstantinov, A.A. (1989) Ann. N.Y. Acad. Sci, in press.
18 Vygodina, T.V. and Konstantinov, A.A. (1987) FEBS Lett. 219, 387–392.
19 Bergmeyer, H. (1974) Methoden in Enzymatischen Analysen, p. 714, Verlag Chemie, Weinheim.
20 Fowler, L.R., Richardson, S.H. and Hatefi, Y. (1962) Biochim. Biophys. Acta 64, 170–173.
21 MacLennan, D.H. and Tzagoloff, A. (1965) Biochim. Biophys. Acta 96, 166–168.
22 Hinkle, P.C. (1979) Methods Enzymol. 55, 748–751.
23 Konstantinov, A.A., Vygodina, T.V. and Andreev, I.M. (1986) FEBS Lett. 202, 229–234.
24 Fry, D.W., White, J.C. and Goldman, I.D. (1978) Anal. Biochem. 90, 809–815.
25 Witt, D.N. and Chan, S.I. (1987) J. Biol. Chem. 262, 1446–1448.
26 Rosen, S.,. Brandon, R., Vanngard, T. and Malmström, B. (1977) FEBS Lett. 24, 26–30.
27 Armstrong, F., Shaw, R.W. and Beinert, M. (1983) Biochim. Biophys. Acta 722, 61–71.
28 Vygodina, T.V., Andreev, I.M. and Konstantinov, A.A. (1986) Biological Membranes (USSR) 3, 563–574.
29 Wharton, D.C. and Gibson, Q.H. (1968) J. Biol. Chem. 243, 702–706.
30 Tiesjema, R.H., Muijsers, A.O. and Van Gelder, B.F. (1971) Biochim. Biophys. Acta 256, 32–42.
31 Orii, Y., King, T.E. (1976) J. Biol. Chem. 251, 7487–7493.
32 Brunori, M., Colosimo, A., Rainoni, G., Wilson, M.T. and Antonini, E. (1979) J. Biol. Chem. 254, 10769–10775.
33 Brunori, M., Colosimo, A., Sarti, P., Antonini, E. and Wilson, M.T. (1981) FEBS Lett. 126, 195–198.
34 Wilson, D.F., Erecińska, M. and Nicholls, P. (1972) FEBS Lett. 20, 61–65.
35 Denis, M. and Clore, G.M. (1981) Plant Phys. 68, 229–235.
36 Wikström, M.K.F. and Saari, H. (1976) Mol. Cell Biochem. 11, 17–33.
37 Harmon, H.J. and Sharrock, M. (1978) Biochim. Biophys. Acta 503, 56–66.
38 Harmon, H.J. and Wikström, M.K.F. (1978) Biochim. Biophys. Acta 503, 67–77.
39 Hill, B.C. and Greenwood, C. (1983) Biochem. J. 215, 659–667.
40 Blair, D.F., Witt, S. and Chan, S. (1985) J. Am. Chem. Soc. 107, 7389–7399.
41 Konstantinov, A.A. (1977) Dokl. Akad. Nauk SSSR 237, 713–716.
42 Mitchell, P., Mitchell, R. Moody, J., West, T., Baum, H. and Wrigglesworth, J.M. (1985) FEBS Lett. 188, 1–7.
43 Wikström, M. (1988) FEBS Lett. 231, 247–252.
44 Wikström, M. (1988) Chem. Scr. 28A, 71–74.
45 Kornblatt, J.A. (1975) Can. J. Biochem. 55, 458–464.
46 Kornblatt, J.A. and Hoa, G.H.B. (1982) Biochemistry 21, 5439–5444.
47 Antonini, E., Brunori, M., Colosimo, A., Greenwood, C. and Wilson, M.T. (1977) Proc. Natl. Acad. Sci USA 74, 3128–3132.
48 Wilson, M.T., Peterson, J., Antonini, E., Brunori, M., Colosimo, A. and Wyman, J. (1981) Proc. Natl. Acad. Sci USA 78, 7115–7118.