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The effect of pH changes on the optical spectrum of oxidised cytochrome oxidase

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The sensitivity to pH changes of oxidised cytochrome oxidase, oxidised oxidase-cyanide and oxidase-azide complexes was investigated by optical difference spectroscopy in the pH region 6.0–8.2. The responses of the optical spectrum of oxidised oxidase and the oxidase-cyanide complex are almost fully developed within some minutes after a pH change of 1–2 units. A blue shift of the spectrum of oxidised oxidase and the oxidase-cyanide complex is observed with decreasing pH, whereas the oxidase-azide complex is almost insensitive to the pH change. From the pH insensitivity of the oxidase-azide complex and the differences in the pH-induced spectral changes of the oxidase-cyanide complex relative to unliganded oxidase, it is concluded that the spectral pH sensitivity in the oxidised enzyme is probably associated with proton binding at or near cytochrome a_3 only. Similar absorption changes are observed with the oxidised oxidase on increasing the pH and on the binding of azide to oxidised oxidase at constant pH. It is suggested that azide binding is probably associated with the deprotonation of some ionizable group(s) in the vinicity of the cytochrome a_3-Cu_B site.

Introduction

Cytochrome oxidase, the terminal enzyme in the mitochondrial electron transport chain, catalyzes the oxidation of ferrocytochrome *c* by molecular oxygen and also proton translocation from the mitochondrial matrix to the cytosol side of the inner membrane [1]. The oxidase contains four catalytically active redox centres: two groups containing haem A, denoted cytochrome *a* and a_3, and two copper ions, called Cu_A and Cu_B. Cytochrome *a* and Cu_A are separate centres, whereas cytochrome a_3 and Cu_B form a binuclear ligand-binding unit.

The electron transfer from reduced cytochrome *c* occurs through cytochrome *a* and Cu_A to the cytochrome a_3-Cu_B binuclear site, where O_2 is reduced to water. The process is associated with a redox-linked formation of an electrochemical gradient, with a probable stoichiometry 2 H^+/e^- [2,3]. One proton is used for the formation of water (called scalar proton transfer), whereas the second one is actively translocated (proton pumping) from the matrix to the cytosolic side of the membrane.

The sites of formation of water and the proton translocation are believed to be distinct ones. The reduction of oxygen to water with the proton uptake takes place at the cytochrome a_3-Cu_B site, but the redox-driven proton translocation has been suggested to be localized at the cytochrome *a* [4,5] or Cu_A [6] redox centre.

The involvement of protons in the redox reaction of cytochrome *a* and a_3 is indicated by the dependence of spectral properties [7–10] and the oxidation-reduction midpoint potentials on pH [11–14]. To explain the cytochrome *a* properties, the presence of three proton wells have been suggested, two connecting cytochrome *a* with the matrix and cytosolic sides, respectively [12,15], and the third one leading to cytochrome a_3-Cu_B from the mitochondrial matrix [16,17].

In the present paper we have investigated in greater detail the effect of pH changes in the medium on three forms of oxidised cytochrome oxidase, unliganded oxidase, the oxidase-cyanide and the oxidase-azide complexes. Our results indicate that pH changes influence the spectra of cytochrome a_3 by proton binding at or

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Abbreviations: Hepes, 4-(2-hydroxyethyl)piperazineethanesulphonic acid; Hepps, 4-(2-hydroxyethyl)-2-piperazinepropanesulphonic acid; E_m, oxidation-reduction midpoint potential.

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near this cytochrome, whereas there is no proton-binding group associated with cytochrome *a*. The pH sensitivity of cytochrome *a* is suggested to be a consequence of the intersite interaction with the cytochrome a_3-Cu_B site.

Materials and Methods

Cytochrome oxidase was isolated from bovine heart by the method of Van Buuren [18]. The concentration of oxidised oxidase (aa_3 unit) was determined from an absorption coefficient of 24 $mM^{-1} \cdot cm^{-1}$ for the α absorption band [19].

For the measurement of the effect of pH on the spectral properties of the oxidase was have used three forms of the enzyme: oxidised oxidase (resting, unliganded form), the oxidised oxidase-cyanide complex and the oxidised oxidase-azide complex. The complexes of oxidase with cyanide and azide were prepared by a 48 h incubation on ice of 130 μM oxidised enzyme in 50 mM Hepes buffer/0.5% Tween 80 (pH 7.4) with 10 mM KCN or 100 mM NaN_3, respectively.

Although the spectrum of cytochrome oxidase is unstable on the long time-scale (see Results), the spectral response to pH changes is essentially reversible on the short time-scale. We have found that the addition of concentrated acid or base, calibrated to shift the pH by 1–2 units, results in a spectral change which is almost complete in 5 min. Immediate return to the initial pH results in 10–20% spectral irreversibility only in the pH range 6.0–8.0.

A typical measurement of the pH-transition effect on the spectrum of cytochrome oxidase was carried out in the following way. Stock enzyme (resting oxidase or the complexes of the enzyme) was dissolved to 2–6 μM concentration in the medium 5 mM Tris-maleate/0.5% Tween 80/167 mM K_2SO_4/0.1 mM $K_3Fe(CN)_6$ at pH 7.0 and room temperature. Then the pH of the sample was changed with 0.6 M NaOH or 0.5 M H_2SO_4 to the desired starting pH (pH 8.2 or 6.3), and the solution was incubated for 10 min at room temperature. After the incubation the spectrum of the sample was measured and stored in the computer memory of the spectrophotometer (Shimadzu UV 3000). The pH transition of the sample was induced by the addition of calibrated amounts of concentrated citric acid (1 M, pH 5.0) or concentrated Tris (1 M, pH 9.5). The optical difference spectra were then recorded, usually 5 min after the pH change.

The pH difference results in a detectable aggregation of the enzyme, which increases with increasing the pH change and is more pronounced at low ionic strength. The slight aggregation of the enzyme causes some uncertainty in the precise determination of the zero absorption line in the difference spectra. We take the absorption value at 700 nm as the zero value in the range between 500 and 700 nm, as there should be a minimal spectral change at this wavelength.

Results

The optical spectrum of the oxidised enzyme is not stable at constant pH (Fig. 1). After dissolution of the enzyme a slow decrease of the absorption at the γ band is observed, with a minimum at 417 nm. The spectral change is not affected by the following factors: increasing the concentration of ferricyanide from 0.1 mM to 2 mM; changing the ionic strength between 0.05 and 0.5 by K_2SO_4; the addition of asolectin; changing the detergent Tween 80 to cholate or dodecyl maltoside; using buffers Tris, Hepes or Hepps; diluting and reconcentrating of the enzyme; and measuring under anaerobic conditions. The spectral change is pH-dependent, becoming faster when the pH is increased from 7.0 to 9.0. With the oxidase-cyanide complex, however, the spectral change was found to be slower.

We suppose that the observed slow spectral change at constant pH is probably a pH-modulated, slow conformational change of the enzyme. The position of the γ band minimum at 417 nm in the difference spectrum indicates that the main change is associated with cytochrome a_3.

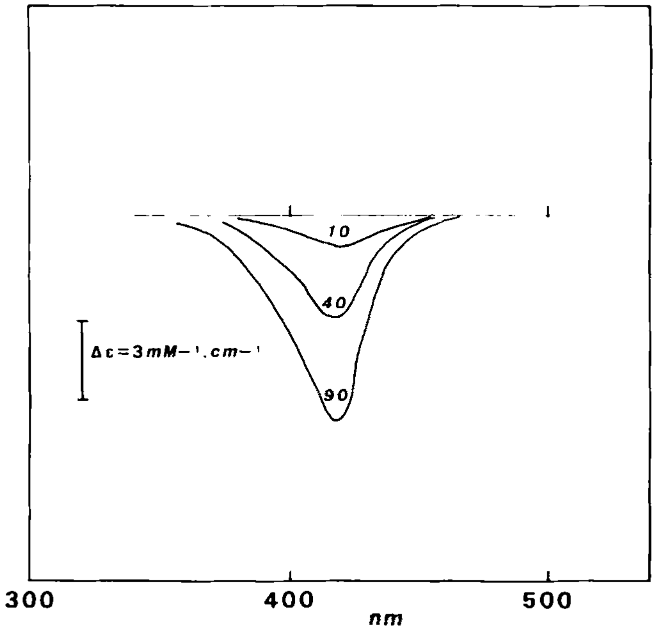

Fig. 1. Time evolution of the spectrum of oxidised oxidase at pH 8.0. The pH of the dissolved oxidase (3.8 μM) in 5 mM Tris-maleate/0.5% Tween 80/167 mM K_2SO_4/0.1 mM ferricyanide (pH 7.0) was changed by the addition of a small amount of 0.6 M NaOH to pH 8.0. After the 10 min incubation at pH 8.0 and room temperature the spectrum of the enzyme was stored in the computer memory of the spectrophotometer, which is taken as the zero-time reference spectrum. Then at times indicated (10, 40 and 90 min) after the storing the difference spectra were recorded.

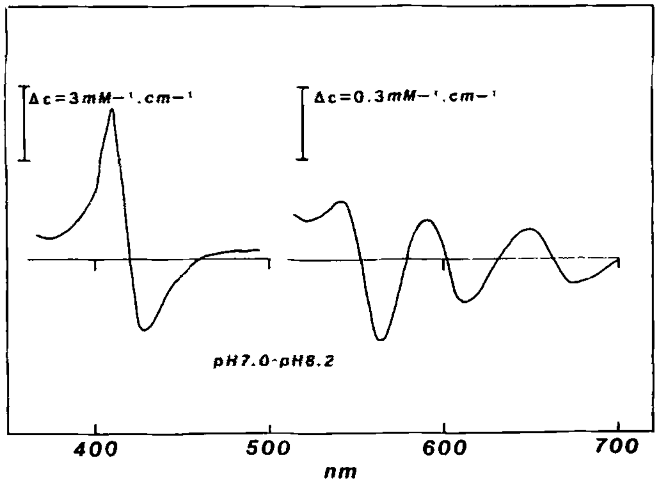

Fig. 2. The difference spectrum of oxidised oxidase induced by the pH change from 8.2 to 7.0. The spectrum of oxidised oxidase (5.5 μM) at pH 8.2 was stored in the computer memory of spectrophotometer. pH change to pH 7.0 was induced by the addition of a calibrated amount of citric acid (1 M, pH 5.0), and after 5 min the difference spectrum was recorded. Conditions as in the legend to Fig. 1.

The spectral change produced by a pH decrease from 8.2 to 7.0 (Fig. 2) leads to a blue shift of the absorption bands of oxidised cytochrome oxidase. The observed minima are at 428, 563, 612 and 674 nm, and the maxima at 410, 542, 590 and 650 nm. The pH-induced absorption changes increase with the size of the pH decrease between pH 8.2 and 6.0. Raising the concentration of ferricyanide to 4 mM does not influence the spectral response to the pH change, as measured in the visible region.

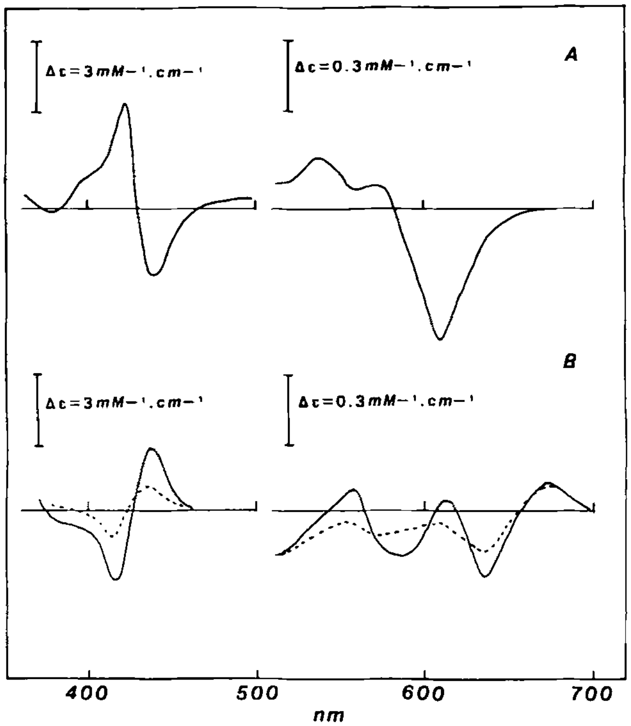

Fig. 3. The difference spectra of oxidised oxidase-cyanide and azide complexes induced by a pH change from 8.2 to 7.0. (A) Oxidised oxidase-cyanide complex. Conditions as in the legend to Fig. 2, but 3 mM cyanide is present in the sample. (B) Oxidised oxidase-azide complex. The same conditions as in the legend to Fig. 2, but 0.05 M K_2SO_4 is used, and 0.3 M NaN_3 (dashed line) or 0.1 M NaN_3 (solid line) are present in the sample.

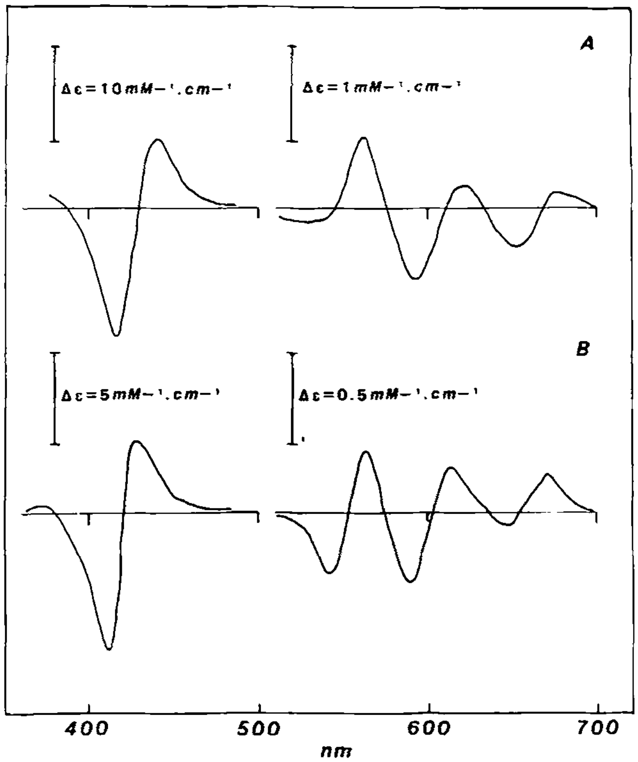

Fig. 4. The difference spectra of oxidised oxidase induced by the azide at constant pH 7.3 and by the increasing pH from 6.3 to 8.0. (A) Azide-induced spectral change. The spectrum of oxidised oxidase (2.2 μM) in 50 mM Hepes/0.5% Tween 80/0.05 M K_2SO_4/0.1 M ferricyanide (pH 7.3) is the reference spectrum which was stored in the computer memory. The difference spectrum was recorded 10 min after the addition of azide (0.1 M). (B) The pH-induced spectral change. The spectrum of oxidised oxidase at pH 6.3 is taken as the reference spectrum. The pH transition was induced by concentrated Tris (1 M, pH 9.5), and after 5 min the difference spectrum was recorded. Conditions as in the legend to Fig. 2, but oxidase 4.3 μM.

The pH effect on the complexes of oxidised oxidase with cyanide and azide is shown in Fig. 3. In the case of the oxidase-cyanide complex (Fig. 3A) minima are observed at 440, 560 and 610 nm, and maxima at 423 and 540 nm. The spectral effect is independent of cyanide concentration between 3 and 20 mM. The difference spectrum of the oxidase-cyanide complex shows, relative to the unliganded oxidised oxidase, the following characteristics: the response of the γ band is red-shifted about 12 nm, the minimum of α band is blue-shifted about 2 nm with an enhancement of the α absorption decrease, and the spectral change around 650 nm is lost.

Some samples of the oxidase-cyanide complex were more stable with respect to time. Restoring the initial pH about 1 h after the pH titration from pH 8.2 to 5.8 results only in 10% irreversibility of the spectral change. Therefore, it was possible to determine a pK value of

6.7–7.0 for the group influencing the α band. During the titration there is the linear relationship between ΔA (610–640 nm) and ΔA (440–500 nm) or ΔA (423–500 nm).

The spectral change of the oxidase-azide complex following a decrease in pH is shown in Fig. 3B. For this complex the spectral change is dependent on the concentration of azide. When the concentration is 0.1 M, the spectral change has minima at 416, 510, 586 and 636 nm, and maxima at 437, 558, 613 and 674 nm. Increasing the azide concentration to 0.3 M at constant oxidase concentration decreases the size of the pH-induced spectral response (Fig. 3B). We performed the measurement also at 0.1 M concentration of azide but with 3-times lower concentration of the enzyme to avoid the high concentration of azide. The result (not shown) is the same as in the case of 0.3 M azide. The enzyme is almost insensitive to the pH decrease.

The abolition of the pH-sensitivity of cytochrome oxidase in the oxidase-azide complex led us to compare the spectral changes produced on increasing the pH with those found on the addition of azide at constant pH (Fig. 4). The spectral responses to the addition of azide (Fig. 4A) and to the pH increase from 6.3 to 8.0 (Fig. 4B) are very similar. For the azide-induced change, the minima are at 416, 594 and 654 nm, and the maxima at 440, 563, 610 and 676 nm. The difference spectrum for the pH increase has minima at 410, 542, 590 and 646 nm, and maxima at 428, 563, 613 and 672 nm.

Discussion

The spectral changes in oxidised cytochrome oxidase induced by pH can be divided into two separate categories: the slow spectral changes at constant pH (Fig. 1) and the fast spectral changes, which are almost fully developed within 5 min after the pH change (Fig. 2 and 3). The fast spectral changes are probably caused by protonation/deprotonation of ionizable groups in the enzyme, whereas the slow ones correspond to a pH-induced denaturation. Here we consequently focus our attention on the rapid spectral changes induced by changes in pH.

Samples of cytochrome oxidase often contain small amounts of endogenous reducing agents, which could be redistributed on the pH change, thereby inducing the spectral change. This effect can be excluded because the spectral pH responses are independent of the concentration of ferricyanide (up to 4 mM) in the sample. In addition, a pH-induced change in the redox state of cytochrome a_3 or cytochrome a should be connected with the spectral changes at 444–445 nm (maxima for reduced cytochrome a_3 and cytochrome a) [19], which is not observed (Fig. 2 and 4B).

In addition, the pH-induced spectral change (Fig. 2 and 4B) cannot be assigned to a transformation of Cu_A to a 'blue' copper site, because the spectral band around 610 nm is considerably more narrow than it would be in the case of Cu_A transformations [20].

The observed spectral change in oxidised cytochrome oxidase upon the pH transition corresponds to a change in haem absorption. The spectral maxima in the absorption spectrum of the oxidised oxidase are around 420 nm (Soret or band), 562 nm (β band) and 600 nm (α band) with a shoulder at 650 nm [19]. Decreasing or increasing the pH shifts all the bands to shorter (Fig. 2) or longer wavelengths (Fig. 4B), respectively. The spin state of cytochrome a_3 is not changed, because in the transition from the high- to low-spin state, the γ maximum in the difference spectrum should be shifted up to 432 nm [21], which is not observed (Fig. 4B). In addition it has been established by magnetic circular dichroism that oxidised cytochrome a_3 remains high-spin even with a pH change from 7.3 to 11, at least over the first 30 min at high pH [10].

The interesting question remains of the assignment of the pH-induced spectral changes to the individual cytochromes. In attempts to solve this problem, we have used two complexes of oxidised cytochrome oxidase, that with cyanide and azide, respectively.

The optical spectrum of the oxidase-cyanide complex is sensitive to the pH change (Fig. 3A), but the spectral response differs from that of unliganded oxidase (Fig. 2). The differences between the pH-induced spectral changes of the oxidase-cyanide complex and the unliganded oxidase (see Results) correspond to an alteration in the absorption spectrum of cytochrome a_3 after the coordination of cyanide [21]. Therefore, we ascribed the observed pH-induced spectral change of the oxidase-cyanide complex to the pH sensitivity of cytochrome a_3-CN^- only.

The assignment of the pH-sensitivity of the oxidase-cyanide complex to cytochrome a_3 is also supported by the following facts: (i) the pH titration leading to the absorption decrease at 610 nm in the oxidase-cyanide complex (not shown) gave a pK of 6.7–7.0 for the group which influences the α spectral transition; this is the same pK value as has been determined for the group which governs the binding of cyanide to oxidised cytochrome a_3 [16,22]; (ii) during the pH titration, there is a linear relationship between the increasing absorption at 423 nm or decreasing absorption at 440 nm and the absorption decrease at 610 nm, which supports the conclusion that the sensitivity of the spectrum to pH is associated with one haem only.

The ligand binding to cytochrome a_3-Cu_B does not influence the spectral state of cytochrome a, since there is only a weak spectral interaction between cytochrome a and the cytochrome a_3-Cu_B site [1,23]. We suppose, therefore, that differences in the pH-induced spectral changes between the oxidase and its complexes arise mainly from the perturbation of cytochrome a_3.

The most striking ligand effect on the pH sensitivity was observed in the case of the oxidase-azide complex (Fig. 3B). The low pH-sensitivity of the oxidase-azide complex could be explained by two processes. Decreasing the pH induces the spectral change of the oxidase-azide complex, which is compensated by the additional azide binding to the remaining unliganded oxidised oxidase. In this case we can expect, for the low-spin oxidase-azide complex, a short-wavelength shift in the γ band induced by the pH decrease with the minimum and the maximum similar to that of the low-spin oxidase-cyanide complex (Fig. 3A). The minimum should be at 437–440 nm and the maximum at 420–423 nm. However, the binding of azide produces a minimum at 416 nm and maximum at 440 nm (Fig. 4A), which could eliminate the pH-induced spectral change at 437–440 nm but not at 420–423 nm. Therefore, we conclude that the observed spectral sensitivity of the oxidase-azide complex on the pH decrease reflects mainly the additional binding of azide to the remaining unliganded oxidised oxidase.

The explanation given is also supported by the similar spectral changes induced by azide binding to oxidised oxidase at constant pH (Fig. 4A) and the spectral changes in the oxidase-azide complex induced by decreasing pH (Fig. 3B). Moreover, the observed additional binding of azide must reflect the pH-dependent dissociation constant of the oxidase-azide complex, which decreases with decreasing pH. The relation between the dissociation constant and pH has already been observed for the oxidase-azide complex, but only in micromolar azide concentrations [24,25].

Raising the pH or binding azide bring about nearly the same spectral changes (Fig. 4). The spectral responses are not identical because the azide binding to the oxidised enzyme causes a spin transition from the high- to the low-spin state, but the pH increase does not change the spin state.

The observation of a pH-dependent rate for azide binding to the oxidase (but only at micromolar azide concentrations) has been explained by two possible effects: (i) the binding species is HN_3 or (ii) the binding of N_3^- is accompanied with the deprotonation of some ionizable group of the oxidised enzyme [24]. Our result, i.e., the similar spectral responses to increasing pH and to azide binding, suggest that the binding species at millimolar azide concentrations is probably N_3^- and its binding is concomitant with the deprotonation of some ionizable group(s).

The conclusion that cytochrome a_3 is pH-sensitive and cytochrome a insensitive is supported by the measurements of several spectral properties of the oxidised oxidase after a pH increase from 7.3 to 11.0 [10]. It was observed that cytochrome a_3 is the spectrally most sensitive component, and the intensity of the EPR signal at $g = 3.0$ of cytochrome a is affected after minutes only at pH values higher than 10.0. The same site in the oxidised oxidase, sensitive to an electrochemical gradient, has been identified from the spectral effect produced on addition of ATP to coupled mitochondria [26]. In this case, the mitochondrial energization induces the spin transition of oxidised cytochrome a_3 from the high- to low-spin state.

It has been shown recently that the protolyzable groups around the cytochrome a_3-Cu$_B$ site are accessible to protons from the mitochondrial matrix, probably through a proton well [16,17]. Thus, the observed dependence of the cytochrome a potential in the oxidase-cyanide complex [12], and also the dependence of the steady-state level of reduced cytochrome a [15] on the pH from the matrix side, are consistent with our explanation, namely pH-dependent intersite interactions and the existence of a proton well from the matrix to the cytochrome a_3-Cu$_B$ site.

According to this suggested explanation cytochrome a in the oxidized enzyme is influenced by pH through the interaction with cytochrome a_3-Cu$_B$ site, and it is not directly accessible to the protons from the medium. In a proton pump there must, however, exist redox-linked conformational changes [4], so that cytochrome a may well be associated with proton channels in other states. Thus, our results in no way exclude the direct involvement of cytochrome a in proton pumping [4,5]. A distinct possibility, on the other hand, is that the coupling is indirect, i.e., the proton-translocating group is not associated with any redox centre. In the case, our results reflect the interaction of the dioxygen reducing site with the scalar protons involved in dioxygen reduction.

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