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Protonation states of the catalytic intermediates of cytochrome *c* oxidase

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Protonation changes accompanying conversion of oxidised (O state) cytochrome *c* oxidase to the 2-electron-reduced P state, and 3-electron-reduced F state at pH 8.0 have been measured. It was found that 2 and 3 protons, respectively, were taken up. The fourth proton required for the reduction of O_2 to H_2O must therefore be consumed in the remaining F → O portion of the catalytic cycle.

Introduction

Cytochrome *c* oxidase catalyses the transfer of electrons from cytochrome *c* on the outer, cytoplasmic side of the mitochondrial membrane to oxygen, along with four protons from the inner side to form water. These processes are accompanied by the transfer of additional protons, probably four, from the inner to the outer side. While a measure of agreement now exists on the spectral characteristics and reaction sequence of some of the oxygenated enzyme intermediates, and several models have been proposed to account for the proton translocation across the membrane, published data that would assist identification of the reaction steps involved in proton uptake and release are scanty.

An essential preliminary requirement for identifying the proton uptake associated with translocation appears to be to establish the steps in the catalytic cycle where the water protons are taken up. This should also be a valuable aid in elucidating the chemical structures of the intermediates. The measurements of protonation changes reported here provide new information, particularly when considered in the context of previously published data [1–3].

Materials and Methods

Fast beef-heart cytochrome *c* oxidase was prepared by a modified Kuboyama method (as preparation C of Ref. 4, except final concentration of bicine buffer was only 5 mM). All reagents were of analytical grade and were obtained from Sigma or other commercial sources, except methyl hydrogen peroxide, which was kindly donated by Dr A. Konstantinov, Moscow.

Optical measurements were carried out with a single-beam spectrophotometer as previously described [4].

Results

When fully-oxidised (O-state) oxidase is mixed with carbon monoxide, a slow 2-electron reduction of the binuclear centre occurs according to the reaction [5]:

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^- \quad (1)$$

Each CO_2 molecule passing into solution at pH 8.0 releases almost one (approx. 0.98) proton owing to hydration and dissociation. Thus very nearly 3 protons per oxidase should be released. If oxygen is present, the two-electron-reduced oxidase immediately reacts to form the 607-nm compound (P state). Fig. 1 shows the time-course of P formation measured at 606–500 nm and the corresponding pH change measured at 578–622 nm in the presence of cresol red. Based on an extinction coefficient of 12 mM^{-1} cm^{-1} at 607–630 nm [1], conversion to P appears to be 86% complete. Proton release, averaging 8 similar experiments, was 0.48 ± 0.07 (S.E.) H^+ per oxidase present, i.e., approx. 0.6 H^+ released per P formed. (Identical results were obtained with carbonic anhydrase present.) Although some uncertainty exists regarding the extinction coefficient for P, and therefore regarding its precise concentration, it

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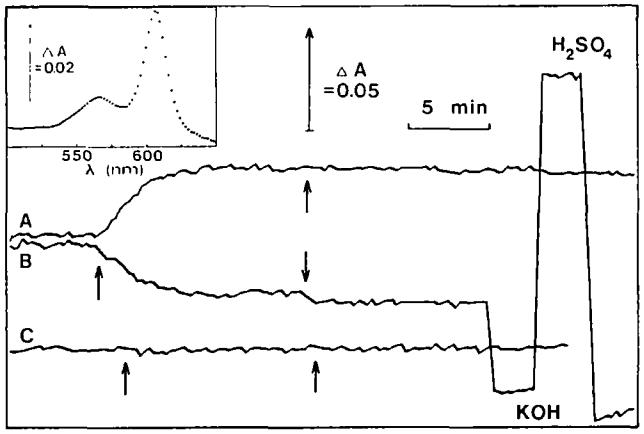


Fig. 1. Proton release associated with CO-induced O → P transition. 1.0 ml 3.0 μM cytochrome oxidase in 50 mM K_2SO_4 at pH 8.0 ± 50 μM cresol red was bubbled approx. 10 s with CO (arrows). The 1.0 μl calibrations of 5 mM H_2SO_4 and 10 mM KOH caused pH changes of about 0.09 unit. Trace A, 606–500 nm; B, 578–622 nm; C, 578–622-nm no-indicator control. Inset is the CO-induced difference spectrum of the no-indicator control.

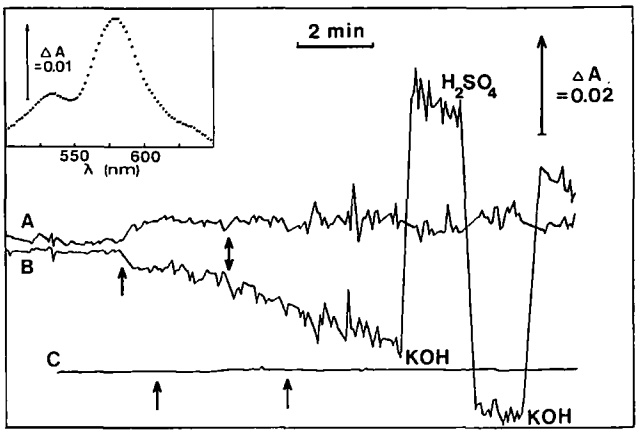


Fig. 2. pH change accompanying H_2O_2-induced O → F transition. As Fig. 1, except 60 U/ml superoxide dismutase present, 2.0 μl additions of M H_2O_2/160 μM KOH as shown by arrows. Trace A, 580–564 nm; B, 570–590 nm; C, 570–590-nm no-indicator control. The inset shows the H_2O_2-induced spectral change in a no-indicator control.

seems clear that not all of the 3 released protons are consumed in the formation of P, but that at least 2 protons are taken up.

In Fig. 2 we show the case where reduction of the oxygen complex is taken a step further to the three-electron-reduced F state by addition of a high concentration of hydrogen peroxide to the O-state enzyme. Since the redox midpoint of the P/F couple is high [1], hydrogen peroxide is able to reduce P to F with formation of superoxide, which then dismutates, particularly in the presence of superoxide dismutase, with the overall release of one proton [6]:

$$H_2O_2 \rightarrow H^+ + e^- + HO_2\cdot \quad (2)$$

$$HO_2\cdot \rightarrow 1/2H_2O_2 + 1/2O_2 \quad (3)$$

Assuming that P formation from oxygen requires about 2 protons (see above), so that the peroxide hydrogens are retained, if no additional protons are taken up as F is formed, we expect to see release of about 1 H^+ per F. In fact, the mean of 7 experiments was only 0.18 ± 0.03 (S.E.) H^+ released per oxidase present, indicating uptake of a further proton. Conversion to F, assuming an extinction coefficient of 6.0 mM^{-1} cm^{-1} at 580–630 nm [1], was about 80 % with fresh enzyme. Since the cresol red and F absorbance peaks overlap considerably, the wavelength pairs required to avoid cross-interference do not measure the full extent of the peaks. Hence the decreased signal-to-noise ratio in these traces.

In order to check the deduction that no pH changes accompany P formation with hydrogen peroxide, lower concentrations of hydrogen peroxide were used, giving a mixture of P and F. Typically the yields of P and F formed within the first minute by addition of 0.15 mM H_2O_2 were about 48 and 46% respectively (see inset spectrum), based on the extinction coefficients given above and the spectra obtained under the conditions of Figs. 1 and 2. This was followed over a period of several minutes by a gradual decline of P and slight increase of F. No significant pH changes occurred (Fig. 3). Six experiments gave a mean value of 0.01 ± 0.02 H^+ per oxidase present, confirming that 2 and 3 protons respectively are taken up on reduction of O to P and F. The wavelength pair used to measure pH, although isosbestic for P formation, was sensitive to F. The no-indicator control therefore shows a small ab-

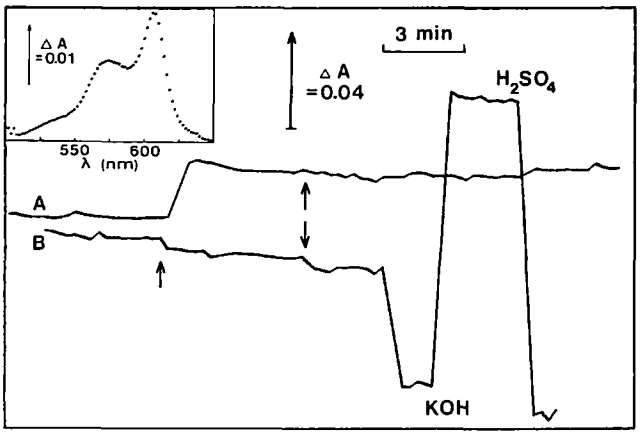


Fig. 3. pH change accompanying H_2O_2-induced formation of P and F. As Fig. 1, except 3.0 μl additions of 50 mM H_2O_2 as shown by arrows. Trace A, 606–500 nm. The no-indicator control has been subtracted from the 578–622-nm trace B.

sorbance increase following the first peroxide addition, which has been subtracted from trace B of Fig. 3.

We have also investigated the reaction of methyl hydrogen peroxide with O-state oxidase (not shown). With 50 μM methyl hydrogen peroxide instead of H_2O_2, conditions otherwise as in Fig. 3, approx. 80% of the enzyme was converted to a state spectrally similar to P, taking 2 min. Subsequent formation of what appeared to be the F state was very slow, taking more than 20 min. As with hydrogen peroxide, net protonation changes of the medium during formation of 'P' were insignificant.

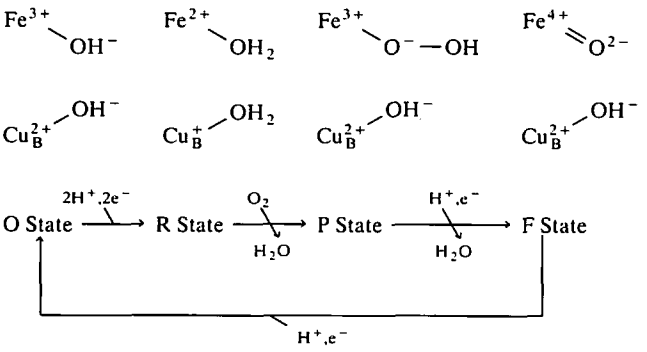
Discussion

Oliveberg et al. [3] found no pH change on flash photolysis of the mixed valence CO compound in the presence of oxygen to form P; an observation that we have confirmed under conditions where O_2 was allowed to displace CO without photolysis (not shown). We found also that anaerobic photolysis of the reduced CO complex caused no pH change (data not shown). At first sight, these results might appear inconsistent with the above findings. However, the initial state is different in the two cases. P formation from the O state appears to be accompanied by the uptake of about two protons at pH 8; whereas when the binuclear centre is first reduced, as in the Oliveberg experiments, no protons are required. We therefore infer that two protons are taken up on reduction of the binuclear centre with two electrons. This is not unexpected in view of the pH sensitivity of the anaerobic midpoints [7,8]. This might involve protonatable groups on nearby amino acid side chains, e.g. the conserved residues Glu-241, Tyr-243 (helix VI) or Lys-318 (helix VIII); or direct ligands of haem a_3 or Cu_B, e.g.:

$Fe^{3+}\text{-}OH^- \rightarrow Fe^{2+}\text{-}H_2O$ and $Cu_B^{2+}\text{-}OH^- \rightarrow Cu_B^{+}\text{-}H_2O$

These protons are, however, not necessarily directly involved in the chemistry of oxygen reduction, and participation of bases on the protein has already been postulated [3]. When the fully-reduced CO complex was photolysed in the presence of oxygen, Oliveberg et al. [3] found that one net proton was taken up. Our data support their suggestion of a protonation associated with formation of F. The fact that only one proton was observed in their experiments indicates that three protons are already taken up on complete reduction of the enzyme.

We suggest the following simplified model for the binuclear site reaction sequence, where the protonations on reduction are presumed directly to involve the metal ligands.



Other possibilities obviously exist, for example the water molecules might be lost earlier in the sequence, or another base might be involved, as postulated above. This might be sufficiently close to provide the hydrogen bond to the ferryl oxygen indicated by resonance Raman data [9]. Since the transition O → F appears to be accompanied by uptake of 3 protons at pH 8, we suggest that the fourth proton in the complete turnover is associated with the final F → O stage of the catalytic cycle.

Wikström [1] found that during ATP-dependent reversal to the F and P states in mitochondria, the external pH dependence of the F/O and P/F equilibria were such as to suggest that all four water protons are associated with the P/F and F/O transitions. However, since these protons communicate with the inner aqueous phase, the validity of the method depends on the maintenance of a constant pH difference across the membrane over the measured range of outer pH. Also, if transmembrane proton translocations are involved in the relevant state changes, as is likely, it is necessary that the protonmotive potential be pH-independent. The underlying concern, which applies also to observations on the effect of pH on P/F ratios in isolated oxidase [10], is that under such steady-state conditions, the result may be distorted by kinetic factors. If our measurements of the number of protons taken up during the P/F and F/O transitions are correct, an important consequence is that the numbers of translocated protons associated with these steps would need to be recalculated [11].

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