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Biochimica et Biophysica Acta 1412 (1999) 184–189

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Rapid report

Proton linkage of cytochrome *a* oxidoreduction in carbon monoxide-treated cytochrome *c* oxidase

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Received 11 March 1999; accepted 25 March 1999

Abstract

Oxidoreduction of the low spin haem *a* of cytochrome *c* oxidase was recently reported to be coupled to release/uptake of nearly one proton from/to the enzyme at pH 7.5 in the presence of CO to block oxidoreduction of the binuclear haem a_3/Cu_B centre (N. Capitanio et al., Biochim. Biophys. Acta, 1318 (1997) 255–265). This is difficult to reconcile with earlier findings from several laboratories that the pH-dependence of the E_m of haem *a* is ca. 10 mV/pH unit over a wide pH range in such conditions, which implies redox coupling of only ca. 0.17 H^+/e^-. In order to resolve this discrepancy, we have performed careful measurements of proton release coupled to oxidation of haem *a* and Cu_A in CO-inhibited cytochrome aa_3 from bovine heart mitochondria. We find that oxidation of these centres by ferricyanide leads to release of a total of 0.20 protons per enzyme molecule at pH 7.7, increasing to 0.43 protons at pH 6.6, far short of a full 1 H^+/e^-. Using vesicles reconstituted with cytochrome *c* oxidase, we also found that all this proton release occurs towards the outside of the vesicles. The observed dependence can be explained by a model in which oxidoreduction of haem *a* is coupled to uptake and release of ca. 0.17 H^+/e^-, while oxidoreduction of Cu_A is linked to a protonatable group which has a pK_a of 6.2 when Cu_A is in the reduced state. In agreement with existing data, this model predicts that the E_m of Cu_A will only be slightly pH dependent in the pH range of these measurements.

Keywords: Cytochrome *a*; Carbon monoxide; Cytochrome *c* oxidase; Proton linkage

The discovery over two decades ago that cytochrome *c* oxidase functions as a proton pump [1] led early on to an intensive search for a centre in the enzyme whose oxidoreduction could be shown to be directly linked to the proton translocation mechanism. Part of this search focused on establishing which redox reactions in the enzyme are coupled to proton uptake and release. If reduction of a redox centre is linked to proton uptake, and oxidation to proton release, the redox midpoint potential (E_m) of the centre will be pH-dependent. This pH-dependence will be observed in the pH range between the pK_a values of the dissociable acidic site of the oxidised (low pK_a) and reduced (high pK_a) forms of the centre. In the simplest case, when only one acidic group is linked to a one-electron redox centre, oxidation will be associated with release of one proton in this range, provided that the two pK_a values are relatively far apart. Only then will the E_m of such a site exhibit the maximal pH dependence of 60 mV/

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0005-2728/99/$ – see front matter
PII: S0005-2728(99)00053-5

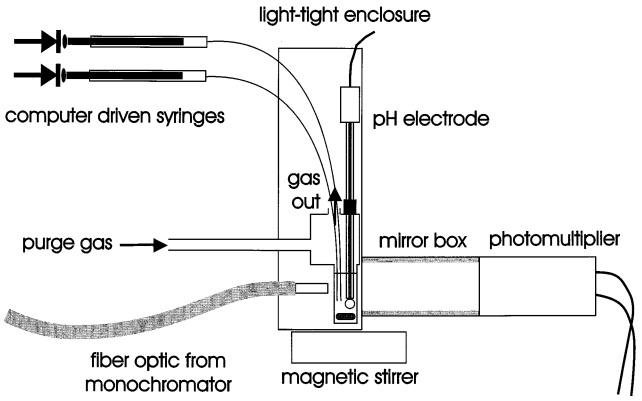

Fig. 1. Schematic drawing of the apparatus to measure the pH and the optical absorbance of enzyme simultaneously, under an oxygen-free atmosphere, with injections of reagents made automatically under computer control. Sample subsystem: The enzyme sample was contained in a cylindrical glass well, approximately 10 mm in diameter, stirred with a magnetic stirrer. At the top of this cylinder, the glass was flared to make a sealed headspace enclosure for gas, with ports for the pH electrode, injection needles, purge gas entry, and access to the sample. All ports were sealed except for the needle ports. These ports were narrow tubes (approx. 10 mm long, 1 mm internal diameter) which also served as the exit ports for the purge gas; the outward flow thus protected against entry of air. Argon (AGA, grade 'S', 99.99% or higher grades) was re-purified to remove any residual oxygen (R&D Separations, OT 3–4 and L-IOT4). CO (Aga grade 2.0, 99%) was used without further purification. In order to minimise oxygen leakage into the purge gas, the entire gas flow path was enclosed in either glass or metal, with the exception of teflon Swagelok ferrules used for the glass-to-metal connections. In order to minimise evaporation of the sample, all gasses were passed through two successive water-filled bubblers. Optical subsystem: A DBS-1 spectrophotometer (Johnson Foundation, University of Pennsylvania) was used. This unit had been retrofitted for computer control (Intel ×86 compatible running MS-DOS; Metrabyte DAS-1800 digitiser; digital rotational position transducer to track 'measurement' monochromator setting T+R Electronics, London, Ontario; software written by N.B.). The sample container and the pH electrode were enclosed in a light-tight box separate from the spectrometer. The probe beam from the spectrophotometer was carried from the sample compartment of the spectrophotometer to the new sample enclosure by means of a large-diameter liquid-filled light guide (Oriel). Light leaving the sample cell was piped to a photomultiplier tube by means of a mirror box (Aminco/SLM) – basically a square tube in which the four walls are mirrors. This allowed the PMT to be placed slightly away from the stirrer in order to avoid magnetic interference. pH measurement subsystem: pH measurements were made using a high-sensitivity pH electrode (CMATL/S7, Russell, Fife, UK) connected to a sensitive pH meter. The pH electrode was situated in the optical path. Acquisition of pH data as well as automatic addition of reactants (below) were handled by a second computer (Intel ×86 compatible running MS-DOS; 24-bit digitiser, Model 201, Lawson Laboratories/Amplicon, Brighton, UK, software by written by N.B.). Injection control subsystem: Injections were made using Gastight syringes (Hamilton). When automated repeated injections were made, the syringes were driven by computer-controlled infusion pumps (Model 200i, World Precision Instruments, Herefordshire, UK). The system included two such pumps.
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pH unit (1 H^+/e^-), but outside this pH range the pH-dependence will be smaller.

In a survey in 1981 [2] it was concluded that the E_m values of both haem groups in the enzyme appear to depend on pH, but that this dependence only approaches 1 H^+/e^- when the companion haem is oxidised, suggesting that both haems may be linked to the protonation state of the same acidic group(s). When CO is bound to the enzyme, clamping both haem a_3 and Cu_B in their reduced states, only haem a and Cu_A can undergo oxidoreduction at reasonable potentials. Consequently, in the presence of CO, the pH-dependence of the midpoint potentials of both haem a and Cu_A in the pH 6–8.5 range have been reported to be very much smaller than the 60 mV/pH unit characteristic of 1 H^+/e^- coupling [3–8].

A recent report by Capitanio et al. [9] that as much as 0.6–1.0 protons are released on oxidation of haem a in CO-inhibited oxidase over the pH range 6.0–8.5, contrasts sharply with these previously reported pH-dependences, which would predict release of only 0.17 H^+. The extent and sidedness of proton coupling to the oxidoreduction of haem a in CO-inhibited cytochrome c oxidase have recently become an important issue in connection with the revival of the idea that oxidoreduction of haem a is directly involved in proton translocation by the enzyme [10], and prompted a careful reinvestigation of this issue. Here, we have determined the number of protons released from the detergent-solubilised enzyme in its CO-bound form, when haem a and Cu_A were oxidised by ferricyanide at a number of pH values covering the range from 6.7 to 7.7. Analogous measurements were also done with oxidase reconstituted in phospholipid vesicles.

The measurements were made in a system where the pH and the optical absorbance of a sample could be measured simultaneously under an atmosphere of oxygen-free CO, and injections of reagents made au-

tomatically under computer control (Fig. 1). Bovine heart cytochrome *c* oxidase was prepared by a modification of the method of Hartzell and Beinert ([11]; cf. [12]). Cytochrome oxidase vesicles were made with the Biobead procedure [12]. Because the measurement cell was a cylinder, and because the pH electrode was situated in the light beam, the effective light path had to be determined by calibration. Thus, each time before an experiment the visible absorbance spectrum of the sample was first measured in a 10 mm path cuvette, using a conventional spectrophotometer (Unisoku, Kyoto, Japan). The sample was then transferred to the cylindrical cell of the pH/optical system (Fig. 1) and the spectrum measured there. The effective path length of the latter system was calculated from the ratio of the absorbance amplitudes.

The enzyme sample was initially reduced by addition of very small amounts of either hexammineruthenium(II) chloride (Aldrich) or potassium ascorbate. When ascorbate was used, the sample included approximately 1 equivalent of equine cytochrome *c* (Sigma). The cytochrome oxidase vesicle experiments also included equine cytochrome *c* but in a substoichiometric amount. Subsequently, CO was added; the difference spectrum of CO binding could be used to verify the concentration of the enzyme. For the measurements, solutions of hexammineruthenium, ferricyanide and HCl for injection were made oxygen-free by exchange with argon using standard vacuum line techniques.

Fig. 2 (left panel) shows the changes in pH, which took place in a sample of solubilised enzyme at pH 7.2, when haem *a* and Cu_A were incrementally oxidised by a series of small additions of oxygen-free ferricyanide solution. Beginning with the fully reduced CO-bound enzyme, each addition of ferricyanide caused a small oxidation of haem *a* and Cu_A, which took place relatively rapidly on the time scale of the measurement (seconds). This was accompanied by acidification of the solution, caused by oxidation-linked release of protons. A subsequent slower drift of the pH trace was often seen, but was not accompanied by visible redox events. In order to follow the redox state of haem *a*, a spectrum was recorded shortly after each ferricyanide addition (Fig. 2, right panel). The additions were continued until haem *a* was fully oxidised. At this point, further injections of ferricyanide failed to cause further changes in absorbance or fast proton release. Finally, a known amount of air-free HCl solution was added to the sample in order to determine the amount of acid corresponding to a given pH change (Fig. 2, left panel).

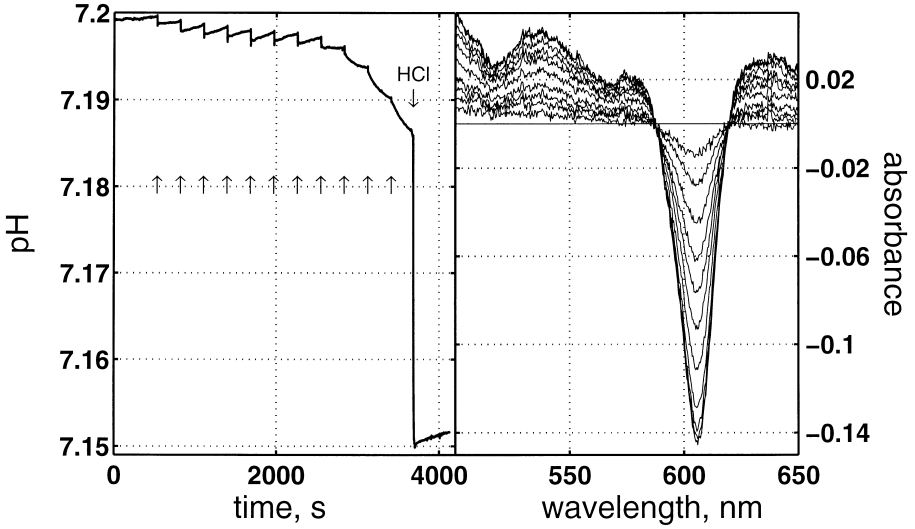

Fig. 2. Proton release on oxidation of CO-inhibited cytochrome *c* oxidase. The reaction medium contained 0.1 M KCl and 10.6 μM cytochrome aa_3. The enzyme was first reduced by discrete 0.1-μl additions of 10 mM hexammineruthenium(II). Then, 0.7-μl additions of anaerobic 3 mM ferricyanide were made (at the arrows) and the pH (left panel) and optical difference spectrum (right panel) recorded, until haem *a* (and Cu_A) were fully oxidised. Finally, a pulse of air-free HCl was added to calibrate the pH trace.

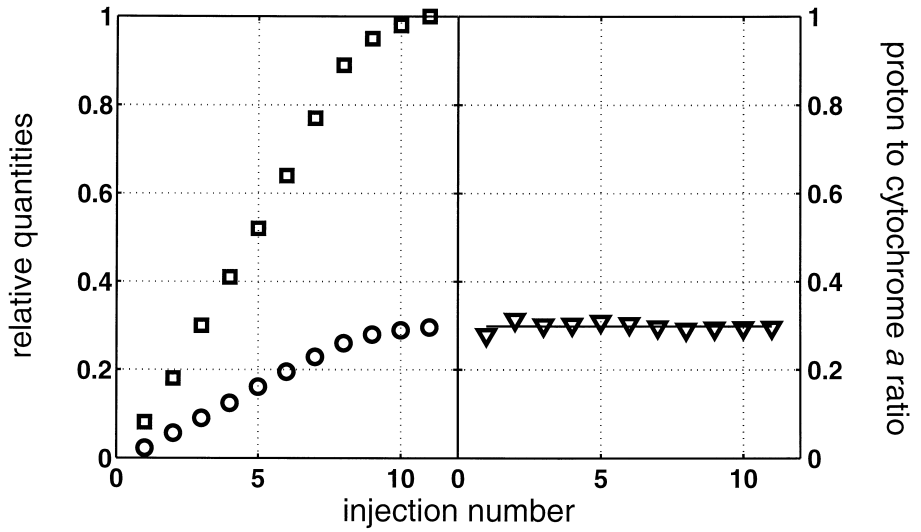

Fig. 3. Proton release on oxidation of CO-inhibited cytochrome *c* oxidase. Result from the experiment of Fig. 2. (Left panel) squares, relative extent of oxidation of haem *a*; circles, cumulative extent of proton release (per aa_3). (Right panel) triangles, extent of proton release normalised to the extent of haem *a* oxidation, for each injection of ferricyanide. The straight line is the mean of these values.

The amount of protons released per aa_3 molecule upon oxidation of reduced haem *a* and Cu_A was then calculated. As illustrated in Fig. 3, this calculation could be made in two different ways. The squares in the left panel show the relative amount of haem *a* which was oxidised after each successive addition of ferricyanide. Over the course of the experiment haem *a* goes from fully reduced (0) to fully oxidised (1). Each oxidation step is accompanied by acidification; the circles show the cumulative acidification (sum of fast changes) after each addition. From these data, the total amount of proton release could be determined by summing all acidification steps which took place as haem *a* was titrated from fully reduced to fully oxidised, the maximum value of the circles. This leads to a net H^+/aa_3 ratio of 0.30. Alternatively, the amount of protons released per haem *a* oxidised could be calculated for each step. These values are shown by the triangles in the right panel of Fig. 3, and their average, again 0.30, is indicated by a horizontal line.

The proton release measurements were repeated in fresh samples at several different pH values. As shown in Fig. 4, oxidation of haem *a* and Cu_A is accompanied by release of 0.20 protons at pH 7.7, rising to 0.43 protons at pH 6.6. The number of protons released throughout this pH range is clearly much smaller than the values reported by Capitanio et al. [9]. Furthermore, in contrast to their report, we

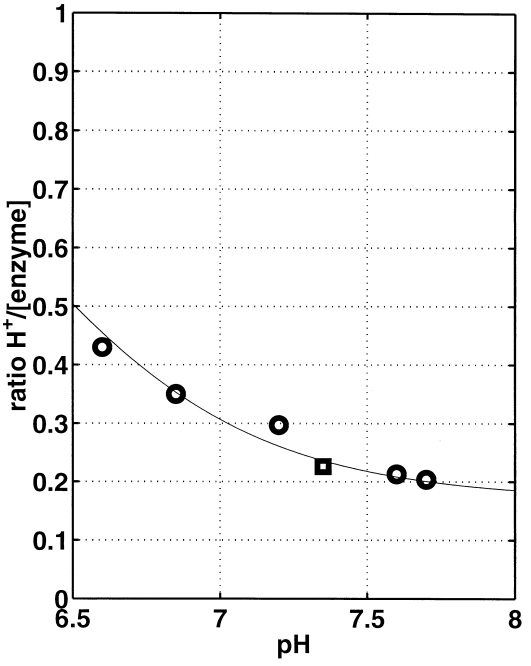

Fig. 4. Proton release on oxidation of CO-inhibited cytochrome *c* oxidase as a function of pH. The circles are data points from experiments like that of Figs. 2 and 3 at different pH values. The square is a data point from a similar experiment with cytochrome oxidase vesicles. The solid curve is drawn with the assumptions that oxidation of haem *a* is associated with release of 0.17 H^+, which is independent of pH in this range (6), and that the pK_a of an acidic group linked to oxidoreduction of Cu_A is 6.2 when this centre is reduced (and well below 5 when oxidised).

observe that the number of protons released on oxidation is decreased and not increased when the pH is raised from 6.5 to 7.5 (Fig. 4). Fig. 4 also shows a data point (square) obtained at pH 7.35 from a similar experiment with cytochrome *c* oxidase reconstituted in liposomes. Fast release of ca. 0.23 H^+/aa_3 was observed on oxidation of the enzyme. This occurred in the absence of either valinomycin or uncoupler, showing that the protons were released into the extraliposomal medium (P side). Since the number of protons released on the external surface of the vesicles on oxidation of haem *a* and Cu_A is virtually the same as that released from the enzyme in solution, we conclude that the acid/base group(s) linked to these centres communicate(s) exclusively with the P side of the membrane in the CO-inhibited enzyme.

As described above, when the reduction and oxidation of a redox centre is coupled to proton uptake and release, respectively, this will also be reflected as a pH dependence of the E_m of that centre. In the CO-bound enzyme, the E_m of haem *a* was reported to have an almost linear pH-dependence of about 10 mV/pH unit, over the range from pH 6.0 to 8.5 [6]. This indicates that oxidation of haem *a* is linked to release of only 0.17 H^+ throughout this range. In the current experiments the number of protons released on oxidation is close to this value (0.20) at high pH, but as the pH decreases the measured amount of proton release becomes larger. The difference is presumably due to proton release linked to oxidation of Cu_A, the only other site which undergoes a redox change in these conditions. This additional proton release can be modelled as arising from a single acidic group which has a pK_a of 6.2 when Cu_A is reduced. The total proton release would then be the sum of the 0.17 H^+ coupled to haem *a*, and the pH-dependent number of protons coupled to Cu_A. The predicted sum of these is shown as a curve in Fig. 4, and is in good agreement with the data. We note that Mitchell [8] also concluded from modelling his careful studies of the effect of pH on the E_m of haem *a* in CO-inhibited mitochondria that there may be a significant, albeit small, proton linkage of oxidoreduction of the Cu_A centre in the neutral pH range. If this is correct, the E_m of Cu_A should be pH-dependent. Van Gelder et al. [5] studied the effects of pH on the redox properties of the haems and Cu_A in cytochrome *c* oxidase. Although they concluded that 'the half-reduction potential of copper is hardly affected by pH', their Cu_A data do in fact show a small pH dependence. The E_m of Cu_A increases by about 20 mV as the pH decreases from about 8.1 to about 6.2 [5]. This result is consistent with what would be expected for a redox-coupled group where the pK_a of the reduced form was 6.2, as in the model above.

Our data (Fig. 3) thus reveal that both haem *a* and Cu_A are involved to a similar extent in weak proton linkage to oxidoreduction, in good agreement with the conclusion by Mitchell [8] for intact mitochondria. On the other hand, the amount of protons that we find to be released on oxidation of haem *a* and Cu_A is very much less than that attributed to haem *a* by Capitanio et al. [9], but consistent with previous reports of the pH-dependence of the E_m of haem *a*. We do not know the reason for this discrepancy, but inadvertent proton release from oxidation of ubiquinol in the broken rat liver mitochondria that were used to facilitate reduction of cytochrome *c* oxidase by succinate [9] might provide a possible explanation.

Papa et al. [10] recently proposed a key role for haem *a* in proton translocation, in part on the basis of the large proton–electron linkage reported for CO-inhibited cytochrome *c* oxidase [9]. The present results demonstrate, however, that oxidation of haem *a* is linked to the release of much less than one proton when the oxidase is complexed with CO, in agreement with earlier redox titrations [3–8]. We also show that this proton release occurs exclusively towards the positively charged P side of the membrane in cytochrome oxidase liposomes (Fig. 4), in agreement with data reported for intact mitochondria [8]. The suggested involvement of haem *a* in proton translocation was also based on the report [13] that reduction of haem *a* by cytochrome *c* in CO-treated cytochrome oxidase liposomes is associated with uptake of protons from the negatively charged N side of the membrane. This would require the redox-linked acidic group to switch orientation relative to protonic access to the P and N sides of the membrane, when the haem is oxidised and reduced. Such reorientation is hard to reconcile with the data of Michell [8], who concluded from experiments with CO-inhibited mitochondria that a change in the pH of the N phase has no detectable effect on the redox poise of haem *a* (cf. [14]). Our data on anaerobic

reduction of cytochrome aa_3 in liposomes, before CO was added, also indicated that a similar number of protons is taken up from the P side of the membrane on reduction of haem *a*, as the number of protons released to that side on its reoxidation.

This work was supported by grants from the Sigrid Jusèlius Foundation, the University of Helsinki and the Academy of Finland (MRC). We thank Martti Heikkinen and Pekka Pihkala for help with design and construction of the measuring systems.

References

[1] M. Wikström, Nature 266 (1977) 271–273.
[2] M. Wikström, K. Krab, M. Saraste, in: Cytochrome Oxidase – A Synthesis, Academic Press, New York, 1981, pp. 111–115.
[3] M. Erecinska, B. Chance, D.F. Wilson, FEBS Lett. 16 (1971) 284–286.
[4] D.F. Wilson, M. Erecinska, C.S. Owen, Arch. Biochem. Biophys. 175 (1976) 160–172.
[5] B.F. van Gelder, J.L.M.L. van Rijn, G.J.A. Schilder, J. Wilms, in: K. van Dam, G.F. van Gelder (Eds.), Structure and Function of Energy-Transducing Membranes, Elsevier/North Holland Biomedical Press, Amsterdam, 1977, pp. 61–68.
[6] W.R. Ellis, H. Wang, D.F. Blair, H.B. Gray, S.I. Chan, Biochemistry 25 (1986) 161–167.
[7] P. Hinkle, P. Mitchell, J. Bioenerg. 1 (1970) 45–60.
[8] R. Mitchell, The Nature and Significance of the pH-Dependence of Electron Equilibration in the Cytochrome *c*–Cytochrome *c* Oxidase System, Ph.D. Thesis, King's College, London, UK, 1991.
[9] N. Capitanio, T.V. Vygodina, G. Capitanio, A.A. Konstantinov, P. Nicholls, S. Papa, Biochim. Biophys. Acta 1318 (1997) 255–265.
[10] S. Papa, N. Capitanio, G. Villani, FEBS Lett. 439 (1998) 1–8.
[11] C.R. Hartzell, H. Beinert, Biochim. Biophys. Acta 368 (1974) 318–338.
[12] A. Jasaitis, M.I. Verkhovsky, J.E. Morgan, M.L. Verkhovskaya, M. Wikström, Biochemistry 38 (1999) 2697–2706.
[13] N. Capitanio, G. Capitanio, E. De Nitto, S. Papa, FEBS Lett. 414 (1997) 414–418.
[14] M. Wikström, K. Krab, in: Y. Mukohata, L. Packer (Eds.), Cation Flux Across Biomembranes, Academic Press, London, 1979, pp. 321–329.