Biochem. J. (1985) **227**, 163–167
Printed in Great Britain

New insights on the cytochrome *c* oxidase proton pump

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(*Received 14 September 1984/Accepted 5 December 1984*)

Cytochrome *c* oxidase vesicles were used to show that, under appropriate experimental conditions: (1) no net deprotonation of the vesicular membrane or of the incorporated enzyme occurs during the oxidation of ferrocytochrome *c*; (2) the pH equilibration kinetics of a respiration-induced pH gradient across the bilayer are a simple function of the ohmic proton-conductance properties of the membrane; (3) a fairly constant stoichiometry (0.8–0.7) of the numbers of protons pumped per molecule of ferrocytochrome *c* oxidized, i.e. the H^+/e^- ratio, over a wide range of dioxygen molecules reduced (1–12) is observed.

The aerobic oxidation of ferrocytochrome *c* by reconstituted ox heart cytochrome *c* oxidase (EC 1.9.3.1) is accompanied by a transient acidification of the external medium followed by a persistent net alkalinization of the system (Hinkle *et al.*, 1972; Wikström & Saari, 1977; Casey *et al.*, 1979, 1984; Coin & Hinkle, 1979; Prochaska *et al.*, 1981; Papa *et al.*, 1983; Proteau *et al.*, 1983; Thelen *et al.*, 1985). Although it is generally agreed that the reduction of oxygen by cytochrome *c* oxidase involves the net consumption of one proton from the intravesicular space per externally oxidized ferrocytochrome *c* molecule, the nature of the observed transient acidification of the external medium remains a matter of some dispute (see, for review, Mitchell & Moyle, 1983; Casey & Azzi, 1983). Most groups interpret the acidification process to reflect a redox-linked proton-pumping activity in these COVs (Wikström & Saari, 1977; Casey *et al.*, 1979; Coin & Hinkle, 1979; Proteau *et al.*, 1983; Thelen *et al.*, 1985). Alternatively, others, e.g. Mitchell & Moyle (1983), have criticized this interpretation of a vectorial proton translocation by COVs. Although Casey & Azzi (1983) confuted many of these criticisms, there still remain some aspects of COVs studies that require clarification. There are two points that appear to contradict the majority-held opinion that cytochrome *c* oxidase pumps protons. The first pertains to the observed differences of the equilibration kinetics of a pH gradient across the bilayer depending on its origin, i.e. an acid- or base-induced ΔpH or a respiration-induced pH gradient (Krab & Wikström, 1978; Casey *et al.*, 1979). This difference has been proved to be in part due to an inadequate charge-compensation system (Casey *et al.*, 1984). The second is related to the change of the H^+/e^--ratio when higher numbers of turnovers are initiated (Casey *et al.*, 1979; Proteau *et al.*, 1983). We have therefore undertaken a study to clarify these points.

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; COVs, cytochrome *c* oxidase vesicles; b_{in}, b_{out} and $b_{tot.}$, intra-, extra-vesicular and total buffering capacities respectively.

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Methods and materials

Methods

Bovine heart cytochrome *c* oxidase was prepared as described by Yu *et al.* (1975) and had a final haem *a* content of 9.8 nmol/mg of protein (millimolar absorption coefficient, $\varepsilon_{605-630} = 13.5\,mM^{-1}\cdot cm^{-1}$).

Phosphatidylcholine was purified as described previously (O'Shea *et al.*, 1984).

Ferrocytochrome *c* was prepared as described by Casey *et al.* (1979) by gel filtration on Sephadex G-25 in the last dialysis medium given in Table 1. COVs were prepared essentially by the so called 'cholate dialysis' method as follows (all steps were carried out at 4°C and the pH was adjusted to 7.3). Lipid (40 mg/ml) was sonicated with a Branson B15 sonifier (50% duty cycle) for 10 min in 1.5% (w/v) potassium cholate, 185 mM-potassium Hepes under N_2. The lipid/detergent mixture was then centrifuged at 30000 ***g*** for 10 min in Sorvall SS34 rotor equipped with glass tubes. To the supernatant cytochrome *c* oxidase was added to a final concentration of 7.5 μM-haem aa_3. The enzyme was incubated at 0°C for 10 min before the addition

Table 1. *Dialysis procedure for COVs*

The COV suspension was sequentially placed in the dialysis media according to the sequence given on the left. All dialysis media were adjusted to pH 7.3 at 4°C before use.

Sequence of dialysis	Time (h)	Volume of buffer per volume of COV suspension	Composition of medium
1.	4	20	0.68% Potassium cholate/200 mM-potassium Hepes
2.	4	40	0.3% Potassium cholate/200 mM-potassium Hepes
3.	14	80	200 mM-Potassium Hepes
4.	5	80	200 mM-Potassium Hepes
5.	5	100	200 mM-Potassium Hepes
6.	16	800	0.25 mM-Potassium Hepes/50 mM-KCl/ 200 mM-sucrose

of 1.5% potassium cholate. The suspension was dialysed as shown in Table 1. The COVs taken from the dialysis tubing were then centrifuged as described above to remove any large lipidic particles which may have formed during the dialysis process.

The respiratory-control index of all COV preparations was greater than 8.5 as measured spectrophotometrically essentially as described by Thelen *et al.* (1985). 81% of the cytochrome *c* oxidase molecules incorporated into the vesicles could be reduced with externally added ascorbate in the presence of catalytic amounts of cytochrome *c* and 2 mM-KCN, whereas the residual 19% was only reducible with $Na_2S_2O_4$. This difference was taken to reflect the actively orientated proportion of the total enzyme population. Proton-translocation measurements were carried out potentiometrically with a Philips CA14/02 electrode and a Radiometer pH-meter connected to a custom-built five-step Butterworth amplifier for noise reduction and traced on a Bryans BS271 recorder.

All experiments were carried out at 20°C in a total volume of 1.5 ml.

Materials

Phosphatidylcholine, CCCP and Hepes were obtained from Sigma, and valinomycin was purchased from Boehringer. All other chemicals were of analytical grade.

Results and discussion

A typical recording of the potentiometric measurements of the pH changes after the additions of ferrocytochrome *c* to a COV suspension in the presence and the absence of large amounts of the protonophore CCCP is shown in Fig. 1. In the presence of only a tiny quantity of CCCP (see below), after the addition of ferrocytochrome *c*, a rapid acidification of the extravesicular space was observed, followed by a slow net alkalinization of the system, corresponding to the consumption of one H^+/e^- for the reduction of O_2 to water. The semi-logarithmic back-extrapolation of the alkalinization phase to zero time gives the total number of protons translocated across the membrane during the oxidation of ferrocytochrome *c* (Fig. 2, upper trace). The monophasic kinetics with a half-time identical with that of an acid pulse (Fig. 2, lower trace), together with the H^+/e^- stoichiometry, are consistent with the description of Mitchell & Moyle (1983) of a redox-linked proton pump. It excludes irreversible deprotonation of the lipid or of the incorporated enzyme as a consequence of ferrocytochrome *c* binding.

It is worthwhile to point out that the final pH change is a function of the total buffering capacity of the system ($b_{tot.}$). However, the pH changes as a consequence of the proton translocation by the COVs are a function only of the extravesicular buffering capacity (b_{out}). As Mitchell & Moyle (1967) have discussed for whole mitochondria, b_{out} is obtained through the back-extrapolation of the pH changes after an acid/base addition from its final value to zero time. Such an acid/base addition is shown in Fig. 1 and analysed in Fig. 2 (lower trace).

After the complete oxidation of ferrocytochrome *c* (within 1–2 s) by the COVs in the presence of an optimal charge-compensation system, only a pH gradient across the vesicular membrane is present. It has been demonstrated that the rate constant of the equilibration of a ΔpH across the bilayer in the absence of any transmembrane potential depends only on the ohmic H^+-conductance properties of the membrane involved (Mitchell & Moyle, 1967; O'Shea *et al.*, 1984). Thus the origin of the pH gradient, either by respiration or by an acid/base addition, should have no influence on its equilibration characteristics. The previously observed differences in the half-times of the equilibrations of the pH gradients generated by different processes (Casey *et al.*, 1979, 1983; Krab & Wikström,

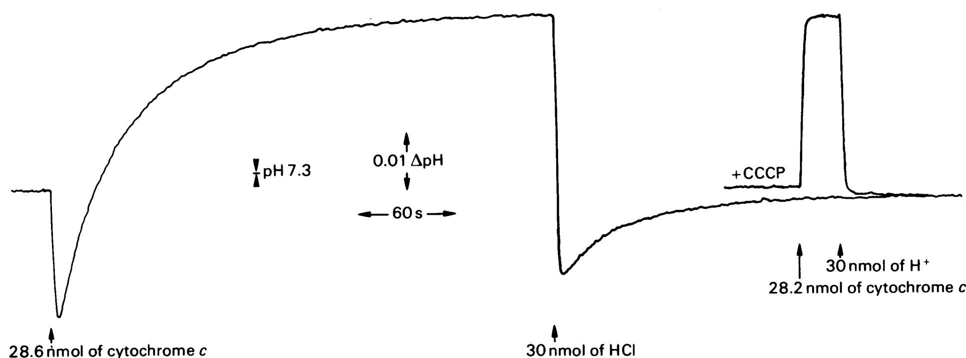

Fig. 1. *Potentiometric measurements of the proton pump of COVs*
COVs were prepared as described in the Methods and materials section. The experimental conditions were as follows: 0.58 nmol of cytochrome *c* oxidase in an active orientation were diluted in the last dialysis medium to a final volume of 1.5 ml; 2.1 nmol of valinomycin/mg of phospholipid and 2.67 nmol of CCCP/litre were added; $T = 20°C$; pH as indicated. The trace marked '+CCCP' was obtained in the presence of 1.33 μmol of CCCP/litre. Acid additions were made with calibrated HCl (10 mmol/litre).

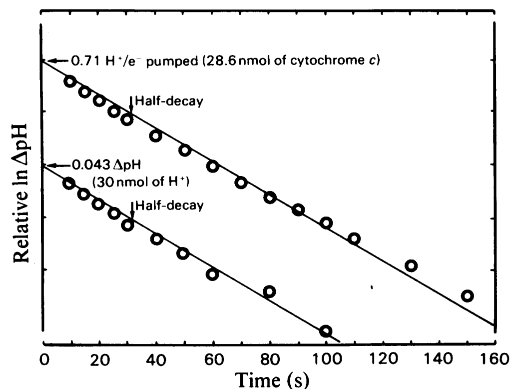

Fig. 2. *Semi-logarithmic presentation of the pH equilibration kinetics*
The slow pH changes shown in Fig. 1 after the ferrocytochrome *c* addition (upper trace) and the acid addition (lower trace) in the absence of the high concentration of CCCP were plotted semi-logarithmically against time.

1978), have been held by Mitchell & Moyle (1983) as evidence for the non-vectorial nature of the extravesicular acidification occurring after a respiratory pulse. We have recently (Casey *et al.*, 1984) demonstrated, however, that, in the presence of an adequate charge compensation system, this discrepancy was almost abolished. Under these conditions the equilibration kinetics of the respiration-induced pH gradient across the COV membranes best fitted by a double-exponential function and not, as expected, by a first-order process (Mitchell & Moyle, 1967). Nevertheless, in the presence of an optimal charge-compensation system, i.e. for each set of experiments an individually titrated valinomycin concentration (usually about 2.1 nmol of valinomycin/mg of phospholipid), a monotonic decay of a respiration-induced pH gradient was observed with a similar half-time value as for an acid addition of the same magnitude to the same sample (Fig. 2). These observations are in agreement with those of Arents *et al.* (1981). Those authors only observed a linear current–voltage relationship with bacteriorhodopsin containing liposomes if more than 1 nmol of valinomycin/mg of lipid was used. In the experiments described below an appropriate amount of the protonophore CCCP, i.e. 2–3 nM-CCCP, was included to allow a more rapid re-equilibration of the protons across the membrane, in a much faster time range than the instrumental drift, thus avoiding baseline artefacts.

It has been suggested that the changes in the bulk-phase pH, as shown in Fig. 1, after the addition of ferrocytochrome *c*, may not only be due to the respiration-induced proton movements across the membrane, but may also include a contribution from pH changes induced by the interaction of cytochrome *c* with the phospholipids or with the incorporated enzyme (Mitchell & Moyle, 1983; Papa *et al.*, 1983). To further exclude this possibility, phospholipid vesicles were prepared by using the same experimental procedure as that described for COVs but excluding any cytochrome *c* oxidase. No immediate acidification of the extravesicular medium after the ferrocytochrome *c* addition was observed (Fig. 3); instead, a slow alkalinization parallel with the oxidation of the ferrocytochrome *c* occurred. However, the total reaction time of the cytochrome *c* with the phospholipids was always 100-fold slower than the time of the enzymic oxidation (about 0.5–2 s) with the

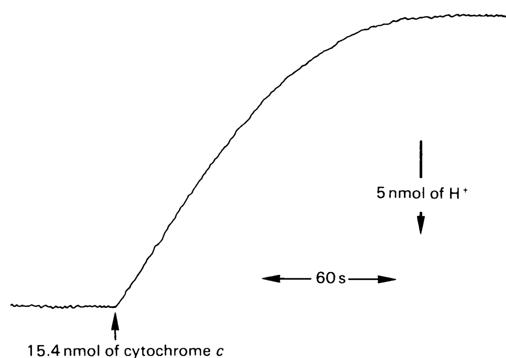

Fig. 3. *Non-enzymic pH changes after a ferrocytochrome c pulse*

Experimental conditions were as in Fig. 1, except that 7.2 mg of phospholipid vesicles lacking cytochrome *c* oxidase were used instead of COVs (see the text).

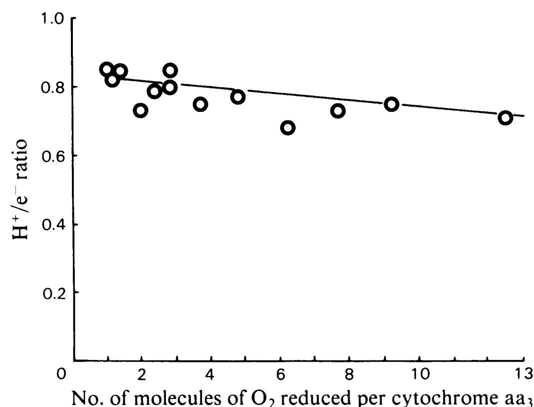

Fig. 4. *Number of protons pumped per molecule of ferrocytochrome c oxidized as function of the number of O_2 molecules reduced per cytochrome aa_3*

The individual H^+/e^--ratios were obtained by experiments similar to those described in Fig. 1 and were calculated as described in Fig. 2.

same amount of reductant and was cyanide-insensitive. During this reaction, 1.0 H^+ was consumed for each molecule of ferrocytochrome *c* oxidized. These results may be interpreted to indicate that a Fenton-type reaction of the ferrocytochrome *c* with lipid peroxides present in the asolectin occurred [for a more detailed description of Fenton reactions, see Svingen *et al.* (1979)]. However, comparing the phospholipid vesicles with the COVs, the non-enzymic oxidation of ferrocytochrome *c* associated with the proton consumption was only responsible for maximally 1% of the overall reaction and thus it may be overlooked.

The introduction of a higher amount of valinomycin (to give single-exponential relaxation kinetics of the pH gradient) and the use of the protonophore CCCP (to facilitate the proton movements across the membrane) made the system ideal for studying the effect of multiple turnovers on the apparent reaction stoichiometry. The number of protons released into the external medium per molecule of ferrocytochrome *c* oxidized, the H^+/e^- ratio, is presented in Fig. 4 as a function of the number of dioxygen molecules reduced. Only a minor decrease of the H^+/e^- ratio occurred with increasing number of O_2 molecules reduced (up to 12.5). These findings differ from the earlier observations of Casey *et al.* (1979) and Proteau *et al.* (1983), in which a much larger decrease of the H^+/e^- ratio was observed with increasing number of dioxygen molecules reduced. Possible explanations of this phenomenon are as follows.

(1) Owing to the use of an insufficient charge-compensation system (Casey *et al.*, 1979), a significant transmembrane potential may have remained (Casey *et al.*, 1984) with increasing amounts of ferrocytochrome *c* added, through which respiratory control on the COVs occurred.

(2) The abstraction of a large number of protons from the intravesicular space of COVs at a low b_{in} created a significant pH gradient across the membrane. The presence of a ΔpH greater than 0.5 pH unit represents a thermodynamic load on the incorporated enzymes which may lead to a decreased oxidation rate (respiratory control).

The equilibration kinetics of the respiration-induced pH gradients in the two cases discussed above may therefore be complicated by the slow oxidation rates, which lead to simultaneous pH equilibration and ferrocytochrome *c* oxidation and by changes in the membrane proton conductance due to the developed membrane potential (O'Shea *et al.*, 1984).

Freeze–fracture analysis of the COVs used in the present study (M. Müller, M. Thelen & A. Azzi, unpublished work) indicate that most of the cytochrome *c* oxidase vesicles have a single protein particle incorporated. Most of the vesicles were of 35–45 nm diameter. From these data the average vesicular volume was calculated to be 1.8×10^{-20} litre/vesicle. The intravesicular compartment corresponded to an average number of 2185 molecules of Hepes per vesicle. Thus from the Henderson–Hasselbalch equation it was calculated that the uptake of the first 125 protons changed the time-average pH of the intravesicular medium only by 0.1 unit. The experimental observation presented in Fig. 4 supports the proposal of a proton-pumping activity of cytochrome *c* oxidase and therefore it makes unlikely the hypothesis of

Papa *et al.* (1983) that the transient acidification of the extravesicular space after a ferrocytochrome *c* addition is the result of a 'membrane-Bohr' effect. In fact it is unlikely that the oxidation of up to 50 molecules of ferrocytochrome *c* per enzyme molecule results in almost an equivalent number of protons released from the membrane or the enzyme surface. It is known that a high concentration of cytochrome *c* severely disrupts the membraneous structure of phospholipid vesicles (De Kruiff & Cullis, 1980; Tiessie, 1981). This therefore limits the number of turnovers that may be initiated during the course of a single experiment.

To conclude, the results presented above provide further evidence in favour of the hypothesis that cytochrome *c* oxidase is equipped with a redox-linked proton pump. Some experimental inconsistencies reported previously (Casey & Azzi, 1983) with the requirements for a redox-linked proton pump (Mitchell & Moyle, 1983) have been re-evaluated and found to be a consequence of the inappropriate conditions of the earlier experiments.

We thank Dr. M. Müller for help with the freeze-fracture analysis of the COVs. This work was supported by the Swiss National Science Foundation 3.501.083 and the Emil-Barrel Stiftung.

References

Arents, J. C., v. Dekken, H., Hellingwerf, K. J. & Westerhoff, H. V. (1981) *Biochemistry* **20**, 5114–5123

Casey, R. P., Chappell, J. B. & Azzi, A. (1979) *Biochem. J.* **182**, 149–156

Casey, R. P. & Azzi, A. (1983) *FEBS Lett.* **154**, 237–242

Casey, R. P., O'Shea, P. S., Chappell, J. B. & Azzi, A. (1984) *Biochim. Biophys. Acta* **765**, 30–37

Coin, J. T. & Hinkle, P. C. (1979) in *Membrane Bioenergetics* (Lee, C. P., Schatz, G. & Ernster, L., eds.), pp. 405–412, Addison–Wesley Publishing Co., Reading, MA

De Kruiff, B. & Cullis, P. R. (1980) *Biochim. Biophys. Acta* **602**, 477–490

Hinkle, P. C., Kim, J. J. & Racker, E. (1972) *J. Biol. Chem.* **247**, 1338–1339

Krab, K. & Wikström, M. (1978) *Biochim. Biophys. Acta* **504**, 200–214

Mitchell, P. & Moyle, J. (1967) *Biochem. J.* **104**, 588–600

Mitchell, P. D. & Moyle, J. (1983) *FEBS Lett.* **151**, 167–178

O'Shea, P. S., Petrone, G., Casey, R. P. & Azzi, A. (1984) *Biochem. J.* **219**, 719–726

Papa, S., Lorusso, M., Capitanio, N. & De Nitto, E. (1983) *FEBS Lett.* **157**, 7–14

Prochaska, L. J., Bisson, R., Capaldi, R. A., Steffens, G. C. M. & Buse, G. (1981) *Biochim. Biophys. Acta* **637**, 360–373

Proteau, G., Wrigglesworth, J. M. & Nicholls, P. (1983) *Biochem. J.* **210**, 199–205

Svingen, B. A., Buege, J. A., O'Neal, F. O. & Aust, S. D. (1979) *J. Biol. Chem.* **254**, 5892–5899

Thelen, M., O'Shea, P. S., Petrone, G. & Azzi, A. (1985) *J. Biol. Chem.* in the press

Tiessie, J. (1981) *Biochemistry* **20**, 1554–1560

Wikström, M. K. F. & Saari, H. T. (1977) *Biochim. Biophys. Acta* **462**, 347–361

Yu, C., Yu, L. & King, T. E. (1975) *J. Biol. Chem.* **250**, 1383–1392