Review Letter

An evaluation of the evidence for H^+ pumping by reconstituted cytochrome *c* oxidase in the light of recent criticism

Robert P. Casey and Angelo Azzi

Medizinisch-chemisches Institut, Universität Bern, Bühlstrasse 28, 3000 Bern 9, Switzerland

Received 4 February 1983

We review the evidence for H^+ translocation by reconstituted cytochrome *c* oxidase; attention is paid to those aspects which we feel most open to criticism. Possible alternative hypotheses are assessed, with regard to experiments carried out to test them directly and with regard to published data. We conclude that, whilst certain aspects of this system are worthy of further clarification, the reported observations are all consistent with proton translocation by reconstituted cytochrome *c* oxidase; most of these observations provide a positive indication of such an activity.

Cytochrome c oxidase *Proton pumping* *Reconstituted vesicle* *Chemiosmosis* *Energy coupling*

1. INTRODUCTION

The proposal of an H^+ translocating activity for mitochondrial cytochrome *c* oxidase [1] would do more than simply add this enzyme to the ever-growing list of biological H^+ pumps. The indirect coupling of redox chemical energy to H^+ transfer would be a clear deviation from the principles of ligand conduction [2] which form the theoretical basis of the chemi-osmotic hypothesis [3]. Perhaps as a consequence of this, the association of an H^+-translocating activity with cytochrome *c* oxidase has been vigorously disputed at both conceptual and experimental levels (see [4–6]).

Whilst the first evidence for H^+ pumping by cytochrome *c* oxidase was obtained using mitochondrial systems [1], the criticism that other components of the respiratory chain might contribute artefactually to this activity led to the use of the structurally simpler and experimentally more reliable reconstituted cytochrome *c* oxidase vesicles [7–10]. A large number of studies has now been carried out using this system and these form a major part of the experimental foundation for the cytochrome *c* oxidase H^+ pump.

In a recent review article [11], Mitchell and Moyle have criticised most of the published observations of H^+ translocation by reconstituted cytochrome *c* oxidase as being experimentally unsound or misinterpreted. In the light of this criticism, we re-evaluate here the evidence for H^+ pumping in this system on the basis of published experiments and some new data.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; TPP-Cl, tetraphenylphosphonium chloride; TMPD, *N,N,N',N'*-tetramethyl phenylene diamine

2. MATERIALS AND METHODS

The preparation of bovine cytochrome *c* oxidase and its incorporation into vesicles were as in [9,12,13], as were experimental techniques and materials.

3. THE PHENOMENON OF CYTOCHROME *c*-INDUCED H^+ EXTRUSION FROM CYTOCHROME *c*-OXIDASE VESICLES

When ferrocytochrome *c* is oxidised aerobically

Published by Elsevier Science Publishers
00145793/83/0000–0000/$3.00

by cytochrome *c* oxidase reconstituted into lipid vesicles in the presence of fairly high ionic strength and of a permeant counterion to avoid transmembrane potential build-up, there is an acidification of the external medium with a ratio of up to 1 H^+/e^- passing to oxygen; following the addition of uncoupler, however, this acidification is abolished and instead the oxidation of cytochrome *c* leads to an immediate alkalinisation corresponding to the consumption of 1 H^+/e^- reducing oxygen. This has been observed when the reaction was initiated by either oxygen [8,14] or by reduced cytochrome *c* (e.g., [7–10,15]) and has been interpreted as the translocation of H^+ from the vesicle interior coupled to electron flow from ferrocytochrome *c* to oxygen.

These observations have been corroborated by measurements indicating that the number of monovalent positive ions accumulated by oxidase vesicles per electron passing to oxygen is close to 2 both in steady state [10,16] and limited turnover [14] experiments. As one positive charge per electron (a proton) is required in the internal phase for the reduction of oxygen to water [17], these results would be consistent with the second monovalent cation being accumulated in exchange for an extruded proton.

H^+ ejection from cytochrome *c* oxidase vesicles is strongly inhibited by N,N′-dicyclohexylcarbodiimide (DCCD) whilst the electron-transfer rate is decreased to a lesser extent [12,18]. This is accompanied by specific covalent binding of 1 mol DCCD/mol enzyme to subunit III [12] at glutamic acid residue number 90 [19]. The primary structure of the region of subunit III containing the DCCD-binding residue has >70% similarity to the analogous regions of the H^+-channeling proteolipids from a number of H^+-translocating ATPases [19]. Since subunit III does not contain copper or haem [20] it is unlikely that the inhibition of proton translocation is secondary to a block of the redox reaction. It has been proposed thus that subunit III of cytochrome *c* oxidase may be involved in H^+ channeling through the enzyme.

4. DO THESE OBSERVATIONS PROVIDE A RELIABLE INDICATION OF H^+ PUMPING?

The above summary, whilst by no means comprehensive (see [6,21] for more detailed treatments), presents the essential experimental basis for the proposal that reconstituted cytochrome *c* oxidase pumps protons. Despite the clear consensus of various lines of evidence, however, certain aspects of the experimental system are still unclear and some inconsistencies remain. In particular, Mitchell and Moyle [11] have recently criticised a number of the experimental observations cited above as being anomalous, erroneous or misinterpreted. In this section, we will examine these points individually and consider whether they simply provide a stimulus to further studies or are sufficient to jeopardise the hypothesis of proton pumping by reconstituted cytochrome *c* oxidase at its basis.

4.1. *The backflux of extruded protons*

A noteworthy feature of many published measurements of H^+ translocation by oxidase vesicles, where K^+ and valinomycin form the counterion system (e.g., [8,9,15]), is the very slow

Table 1

The rates of decay of acidifications resulting from cytochrome *c* oxidation or from addition of acid in suspensions of cytochrome *c* oxidase vesicles [half-time of H^+-pulse decay (s)]

Addition	K^+/valinomycin	TPP-Cl
Cytochrome *c*	129.0 ± 11.9	14.2 ± 1.9
Oxalic acid	19.2 ± 0.8	12.3 ± 0.4

Cytochrome *c*-induced H^+ translocation: 2 nmol ferrocytochrome *c* were added to 1.4 ml 75 mM choline chloride, 25 mM KCl, 50 μM phenol red (pH 7.8) containing cytochrome *c* oxidase vesicles (0.5 nmol haem) and either 0.5 nmol valinomycin or 1 mM tetraphenylphosphonium chloride (TPP-Cl) at 20°C. The subsequent acidification and its decay were recorded spectrophotometrically as the change in phenol red absorbance [9]

Acid pulses: Measurements were as for H^+ translocation except that 5 μl of a freshly prepared solution of 0.5 mM oxalic acid was added instead of cytochrome *c*

In both cases, the measurements were then repeated in the presence of 3.5 μM CCCP. The extent of the pH change obtained with CCCP present was taken as a baseline in order to calculate the half-times of the decays shown below. The means and standard deviations of values obtained using 3 vesicle preparations are shown

decay of the cytochrome *c*-induced proton extrusion. Mitchell and Moyle have claimed this slow decay to be a serious anomaly, incompatible with genuine H^+ translocation [11]. According to our measurements, the return of the pH change caused by cytochrome *c* oxidase activity to the predicted equilibrium level has a half-time of ~130 s (table 1) whilst the decay of the acidification caused by a pulse of added acid is more rapid (~19 s, table 1).

Using the lipophilic tetraphenylphosphonium ion as counterion, however, the acidification caused by cytochrome *c* oxidase activity decays to the predicted level with the same rate (within experimental error) as the decay of an added pulse of acid (table 1). Thus the discrepancy observed with K^+ and valinomycin is not intrinsic to the system but may be the consequence of a hindered diffusion of the K^+/valinomycin complex in the membrane during or following the oxidase turnover. In this regard, it is noteworthy that the decay of the cytochrome *c*-induced acidification with K^+/valinomycin as counterion is accelerated by addition of very low concentrations of uncouplers such as CCCP [9,15]. At these concentrations, such uncouplers have been shown to complex with K^+/valinomycin [22] and thus to facilitate its transmembrane diffusion.

We conclude that, whilst the slow decay of the cytochrome *c*-induced H^+ pulse in the presence of K^+ and valinomycin is not immediately explicable, it does not argue against true H^+ translocation by the oxidase.

4.2. *The value of charges translocated by cytochrome* c *oxidase per electron*

Sigel and Carafoli [10,16] have measured the steady state rates of K^+ uptake and oxygen consumption in suspensions of oxidase vesicles supplemented with valinomycin and from these they found close to 2 K^+/e^- being taken up. In these experiments, oxidase turnover was initiated by adding to the vesicle suspension a mixture of ascorbate and TMPD. The $[K^+]$ in this solution was slightly different to that in the experimental medium, thus causing a small deflection of the K^+ trace in the presence of CCCP. Mitchell and Moyle [11] have argued that when the K^+-uptake trace in fig.2 of [16] is corrected for this artefact the K^+/e^- ratio ceases to be significantly greater than 1. This step change, however, clearly should have no considerable effect on the rate measurement. In fact, according to our evaluation, correction of the K^+ traces in [16] for this and an uncoupler-insensitive drift yields a K^+/e^- ratio of 1.7 for the initial enzyme turnovers, consistent with 0.7 H^+/e^- being extruded.

4.3. *The interaction of DCCD with reconstituted cytochrome* c *oxidase*

In contrast to the proposals mentioned above, Mitchell and Moyle [11] have claimed that DCCD does not inhibit H^+ pumping by reconstituted cytochrome *c* oxidase but instead blocks a transient, cytochrome *c*-induced net acidification which has been mistaken for H^+ pumping. As evidence, they cite fig.1 of [23] which shows cytochrome *c*-induced acidifications with DCCD-inhibited and control oxidase vesicles which decay with approximately similar initial rates. They argue that if H^+ pumping were decreased in the DCCD-inhibited sample, the transmembrane pH gradient caused by cytochrome *c* oxidation should be proportionally less and the decay slower. The initial back-flux rates measured in these experiments are shown, however, in table 1 of [23] and from these it is clear that the decay rate in the inhibited sample is only 65% of that in the control. The inhibition of H^+ pumping observed there (76%) would predict that the pH gradient in the DCCD sample should be 62% of that in the control in good agreement with the measured decay rates. We consider, thus, the objection of Mitchell and Moyle [11] to be unfounded.

Despite the clear correlation of DCCD binding to cytochrome *c* oxidase with its inhibitory effects [12,19], Mitchell and Moyle [11] have proposed that inhibition of H^+ ejection is due instead to binding of DCCD to carboxyl groups of phosphatidylserine in the vesicle membranes. As evidence for such an interaction, they cite experiments [24,25] which show that certain lipids reverse the inhibitory effect of DCCD on the mitochondrial ATPase. These workers, however, show no evidence for covalent binding of DCCD to phosphatidylserine.

As a test of their proposal, Mitchell and Moyle suggest [11] that the extent of inhibition caused by DCCD in oxidase vesicles should vary according to the phosphatidylserine concentration in the vesicle membranes. In the experiment of fig.1, vesicles

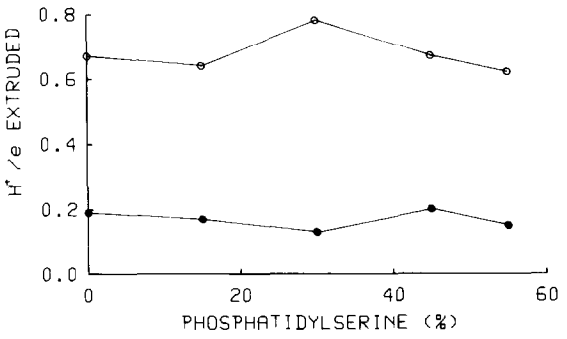

Fig.1. H^+ translocation and its inhibition by DCCD in cytochrome *c* oxidase vesicles having different contents of phosphatidylserine. Cytochrome *c* oxidase vesicles were prepared as in [9] except that, instead of asolectin, pure phospholipids were used. Vesicles containing phosphatidylserine also contained phosphatidylethanolamine and phosphatidylcholine at a molar ratio of 4:1; for the vesicles containing no phosphatidylserine, this ratio was 1:3 and cardiolipin was added (10% of total lipid). These compositions afforded maximal coupling [30]. H^+ translocation was measured in samples of the vesicles as described for table 1, after incubation for 20 h at 4°C with DCCD (200 mol/mol haem) added as an ethanolic solution (●) or with an equal amount of ethanol (○).

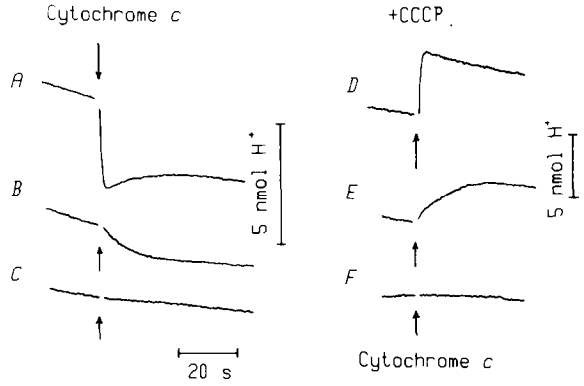

Fig.2. Changes in pH in suspensions of oxidase vesicles linked to the oxidation of ferrocytochrome *c*. Changes in pH were measured in the absence (A–C) and presence (D–F) of 3.5 μM CCCP as described for table 1, except that 5.7 nmol ferrocytochrome *c* were added and that valinomycin was used in all cases: (A,D) no further additions. The other samples had been incubated for 10 min with 2 mM NaN_3 and, in (C,F) 30 μM $K_3(Fe(CN)_6)$ was added immediately before the cytochrome *c*. The square-ended lines indicate the change in phenol red absorbance caused by the addition of 5 μl 0.5 mM oxalic acid. All absorbance traces are corrected for small step artefacts. A downward deflection of the trace indicates acidification.

were prepared with phosphatidylserine contents varying from 0–55%. The effect of DCCD on H^+ ejection was the same, within experimental error, regardless of phosphatidylserine content. On this basis, we consider that the mechanism proposed by Mitchell and Moyle [11] is improbable and maintain that DCCD inhibits H^+ pumping in cytochrome *c* oxidase by covalent interaction with subunit III.

4.4. *The cytochrome* c*-induced H^+ ejection occurs vectorially via cytochrome* c *oxidase and is not due to scalar release from the membrane lipid*

It seems clear from the above that, although our understanding of this system is not fully complete, there are no experimental inconsistencies so far which seriously contradict the large body of evidence indicating that reconstituted cytochrome *c* oxidase pumps protons. However, it is still worth considering whether H^+ ejection from oxidase vesicles occurs via some other mechanism than translocation through the enzyme. In particular, it has been proposed [11] that cytochrome *c* interacts with the head groups of membrane phospholipids, especially phosphatidylserine, and that changes in these interactions, resulting from oxidation of cytochrome *c*, cause H^+ ejection. Two essential predictions of this model, which were stressed in [11] are:

(*i*) That oxidation of cytochrome *c* otherwise than via cytochrome *c* oxidase but in the presence of oxidase vesicles should result in H^+ ejection;

(*ii*) That the H^+ ejection should depend on the lipid composition of the vesicles, in particular their phosphatidylserine content.

Fig.2 shows that when cytochrome *c* was oxidised by ferricyanide in the presence of vesicles containing azide-inhibited oxidase no H^+ ejection was detected. This would tend to exclude prediction (*i*) as would our observation of no cytochrome *c*-induced H^+ ejection with vesicles containing oxidase from *Rhodopseudomonas sphaeroides* even though these were prepared under identical conditions to proton-pumping vesicles of bovine oxidase [26].

Although oxidase proton pumping has been

usually observed in asolectin vesicles, mitochondrial lipids have also been used succesfully [7]. Furthermore, in the experiment of fig.1, vesicles with a range of lipid compositions showed very similar H^+ pumping activities; in particular, the phosphatidylserine content had no significant effect. We consider thus that the model proposed in [11] is very improbable and maintain that the H^+ ejection from oxidase vesicles occurs via the enzyme.

5. PROSPECTS FOR FURTHER STUDIES ON H^+ PUMPING IN CYTOCHROME *c* OXIDASE VESICLES

The major strength of reconstituted oxidase vesicles is that they enable the purified enzyme, free from other redox components, to express its osmotic function as well as its electron-transfer activity. The experimental simplicity of this system compared to the enzyme in situ is particularly advantageous in studying energy coupling by bacterial oxidases. The 3-subunit oxidase from the thermophilic bacterium PS3 carries out the DCCD-sensitive translocation of close to 1 H^+/cytochrome *c* oxidised [27]. *Paracoccus denitrificans* [28] and *Rps. sphaeroides* [26] possess 2-subunit oxidases, the latter not pumping H^+ and the former doing so with a ratio of ~0.6 H^+/e^- [29].

There is little information so far on the involvement of the various subunits of mitochondrial cytochrome *c* oxidase in H^+ pumping. A further advantage of the vesicle system is that it allows energy coupling to be studied in subunit-depleted forms of the enzyme. In this way, it was demonstrated that the bovine oxidase, after removal of subunit III, loses its H^+-pumping activity [14] in agreement with the inhibitory effects of DCCD; similar studies using other subunit-depleted effects of DCCD; similar studies using other subunit-depleted forms of the enzyme are under way in our laboratory. The reconstituted system should also prove suitable for the study of H^+ pumping in oxidase altered in other ways; e.g., by chemical modification.

We conclude that the case for reconstituted cytochrome *c* oxidase vesicles providing a reliable system for the measurement and analysis of cytochrome *c*-induced H^+ pumping is a good one and that studies employing these should continue to prove informative in further investigations into the nature and mechanism of energy coupling in this enzyme.

ACKNOWLEDGEMENTS

This article was made possible through the grant 3.739-080 from Schweizerischer Nationalfonds and by the Sandoz Stiftung. We thank Professor M. Wikström and Dr P. O'Shea for helpful discussion.

REFERENCES

[1] Wikström, M.K.F. (1977) Nature 266, 271–273.
[2] Mitchell, P. (1979) Eur. J. Biochem. 95, 1–20.
[3] Mitchell, P. (1966) Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research Ltd., Bodmin.
[4] Moyle, J. and Mitchell, P. (1978) FEBS Lett. 88, 268–272.
[5] Mitchell, P. (1980) Ann. NY Acad. Sci. 341, 564–584.
[6] Wikström, M.K.F. and Krab, K. (1979) Biochim. Biophys. Acta 549, 177–222.
[7] Wikström, M.K.F. and Saari, H.T. (1977) Biochim. Biophys. Acta 462, 347–361.
[8] Krab, K. and Wikström, M.K.F. (1978) Biochim. Biophys. Acta 504, 200–214.
[9] Casey, R.P., Chappell, J.B. and Azzi, A. (1979) Biochem. J. 182, 149–156.
[10] Sigel, E. and Carafoli, E. (1979) J. Biol. Chem. 254, 10572–10574.
[11] Mitchell, P. and Moyle, J. (1983) FEBS Lett. 151, 167–178.
[12] Casey, R.P., Thelen, M. and Azzi, A. (1980) J. Biol. Chem. 255, 3994–4000.
[13] Casey, R.P., Ariano, B.H. and Azzi, A. (1982) Eur. J. Biochem. 122, 313–318.
[14] Pentillä, T. and Wikström, M.K.F. (1981) in: Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F. et al. eds) Dev. Bioenerg. Biomemb. vol.8, pp.71–80, Elsevier Biomedical, Amsterdam, New York.
[15] Coin, J.T. and Hinkle, P.C. (1979) in: Membrane Bioenergetics (Lee, C.P. et al. eds) pp.405–412, Addison-Wesley, Reading MA.
[16] Sigel, E. and Carafoli, E. (1980) Eur. J. Biochem. 111, 299–306.
[17] Mitchell, P. and Moyle, J. (1967) in: Biochemistry of Mitochondria (Slater, E.C. et al. eds) pp.53–74, Academic Press, London, New York.

[18] Casey, R.P., Thelen, M. and Azzi, A. (1979) Biochem. Biophys. Res. Commun. 87, 1044–1051.
[19] Prochaska, L.J., Bisson, R., Capaldi, R.A., Steffens, G.C.M. and Buse, G. (1981) Biochim. Biophys. Acta 637, 360–373.
[20] Winter, D.B., Bruyninckz, W.J., Foulke, F.G., Grinich, N.P. and Mason, H.W. (1980) J. Biol. Chem. 255, 11408–11414.
[21] Azzi, A. and Casey, R.P. (1979) Mol. Cell. Biochem. 28, 169–184.
[22] O'Brien, T.A., Nieva-Gomez, D. and Gennis, R.B. (1978) J. Biol. Chem. 253, 1749–1751.
[23] Casey, R.P., Broger, C., Thelen, M. and Azzi, A. (1981) J. Bioenerg. Biomemb. 13, 219–228.
[24] Bruni, A., Pitotti, A., Contessa, A.R. and Palatini, P. (1971) Biochem. Biophys. Res. Commun. 44, 268–274.
[25] Contessa, A.R. and Bruni, A. (1971) Biochim. Biophys. Acta 241, 334–340.
[26] Gennis, R.B., Casey, R.P., Azzi, A. and Ludwig, B. (1982) Eur. J. Biochem. 125, 189–195.
[27] Sone, N. and Hinkle, P.C. (1982) J. Biol. Chem. 257, 12600–12604.
[28] Ludwig, B. and Schatz, G. (1980) Proc. Natl. Acad. Sci. USA 77, 196–200.
[29] Solioz, M., Carafoli, E. and Ludwig, B. (1982) J. Biol. Chem. 257, 1579–1582.
[30] Eytan, G.D., Matheson, M.J. and Racker, E. (1976) J. Biol. Chem. 251, 6831–6837.