Alternative hypotheses of proton ejection in cytochrome oxidase vesicles

Transmembrane proton pumping or redox-linked deprotonation of phospholipid-cytochrome *c* complex(es)

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A review of published experimental and interpretative knowledge concerning proton ejection associated with cytochrome *c* oxidation by artificial phospholipid vesicles inlaid with cytochrome *c* oxidase indicates that the detailed characteristics of the redox-linked proton ejection cannot be simply explained by proton pumping. We propose an alternative hypothesis according to which proton ejection is due to the redox-linked deprotonation of a complex involving phospholipid and cytochrome *c* at the surface of the vesicles. The postulates upon which this hypothesis depends are explicitly outlined, and some methods of testing the hypothesis are suggested.

Cytochrome oxidase vesicles *Redox-linked proton pump* *Redox-linked deprotonation*
DCCD-sensitive phosphatidyl serine–cytochrome c complex *Redox pulse* *Proton ejection*

Abbreviations: pH_O or pK_O and pH_I or pK_I, pH or pK of outer and inner aqueous media, respectively; H_O^+ or K_O^+ and H_I^+ or K_I^+, H^+ ion or K^+ ion content of outer and inner aqueous media, respectively; $\rightarrow e^-/e^-$ and $\leftarrow H^+/e^-$, number of electrons and protons translocated per electron transferred; $\Delta H_O^+/e^-$ or $\Delta K_O^+/e^-$ and $\Delta H_I^+/e^-$ or $\Delta K_I^+/e^-$, increment of quantity of H^+ or K^+ in the outer and inner media per electron transferred, respectively; $\leftarrow H^+$(acid) and $\leftarrow H^+$(charge), numbers of protons translocated measured by the acidic and electric property of the proton, respectively; Δe^-, quantity of electrons transferred in redox pulse; $\Delta\psi$, electric membrane potential; B_O and B_I, pH buffering powers of outer and inner aqueous media, respectively; terminations obs, tot, pmp, dep and err stand for observed, total, pump, deprotonation and error, respectively; val, valinomycin; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexyl-carbodiimide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

1. INTRODUCTION

It is ten years since Hinkle and colleagues [1,2] introduced suspensions of artificial phospholipid vesicles containing purified cytochrome *c* oxidase plugged through the membrane to study the vectorial characteristics of the oxidation of ferrocytochrome *c* and the electronation and protonation of dioxygen by cytochrome *c* oxidase. In this work, anaerobic cytochrome oxidase vesicle suspensions with externally added ferrocytochrome *c*, were pulsed with oxygen in the presence of valinomycin + K^+, and the changes of pH_O and pK_O were recorded. In the simplest experiments, ferrocytochrome *c* was the only reductant present, but in other experiments, additional artificial reductants, such as benzoquinol or ascorbate, were included to keep the cytochrome *c* reduced. The results were considered to give an important quantitative

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verification of the previously observed [3] vectorial conduction to the catalytic site in cytochrome *c* oxidase of one electron from the outside and one proton from the inside per electron transferred from cytochrome *c* to dioxygen in the cytochrome *c* oxidase action ($\rightarrow e^-/e^- = 1.0$; $\Delta H_I^+/e^- = -1.0$); and no evidence was found for any proton-pumping function ($\leftarrow H^+/e^- = 0.0$). More recently, following Wikström [4], the changes of pH_O and pK_O observed in aerobic cytochrome oxidase vesicle suspensions pulsed with ferrocytochrome *c* in the presence of valinomycin + K^+, or the changes of pH_O in the presence of another lipophilic cationic agent such as tetraphenyl phosphonium, and other related experiments [5–16], have been widely [17–28] but not unanimously [29–33] interpreted as reliable evidence for a proton-pumping as well as a vectorial electron-conducting function of cytochrome *c* oxidase, in which one proton is pumped per electron transferred ($\rightarrow e^-/e^- = 1.0$; $\leftarrow H^+/e^- = 1.0$; $\Delta H_O^+/e^- = 1.0$; $\Delta H_I^+/e^- = -2.0$).

The object of this paper is to comment on relevant experimental observations on the oxidation of ferrocytochrome *c* by cytochrome oxidase vesicle suspensions, and to suggest that the characteristics of the proton ejection observed under certain conditions cannot be simply explained by the activity of a redox-linked proton pump in cytochrome *c* oxidase. We endeavour to show that our earlier suggestion that proton ejection may be attributed to the redox-linked deprotonation of certain cytochrome *c*-binding components at the surface of the membrane [29,30], may provide a relatively simple and acceptable explanation of the observations; and we indicate how this hypothesis may be directly tested.

2. METHODOLOGY

2.1. *Possible protonic complexities in the interaction of cytochrome* c *with cytochrome oxidase vesicles*

Ferrocytochrome *c* binds positive ionic groups, whereas ferricytochrome *c* binds negative ionic groups such as carboxyl or phosphate [34–38]. Also, cytochrome *c* interacts strongly with phospholipids [39–43], and phospholipids contain proton-binding positive and negative ionic groups, some of which have effective p*K* values not far from 7 [44]. Consequently, the oxidation of ferrocytochrome *c*, complexed with proton-binding phospholipids at the surface of cytochrome oxidase vesicles in a salt medium containing dilute pH buffers, may result in a net deprotonation of proton-binding groups, and may cause a corresponding ejection of protons into the aqueous medium at the surface of the vesicles. The net acidification that is observed when anaerobic suspensions of mitochondria in certain media with added ferrocytochrome *c* are pulsed with oxygen or ferricyanide appears to be satisfactorily explained in this way [29,30]. Care must therefore be taken to avoid mistaking the proton ejection that results from such redox-linked deprotonation processes for evidence of redox-linked transmembrane proton pumping by cytochrome *c* oxidase molecules in cytochrome oxidase vesicle suspensions.

2.2. *Criteria for recognising and measuring redox-linked transmembrane proton pumping represented quantitatively by* $\leftarrow H^+/e^-$

In redox pulse experiments for measuring $\leftarrow H^+/e^-$ ratios, the extent of electron transfer represented by e^- is given, either by the quantity of oxidant injected in the presence of excess reductant, or by the quantity of reductant injected in the presence of excess oxidant. In most cytochrome oxidase vesicle experiments the oxidant and reductant are injected as standard solutions of O_2 and ferrocytochrome *c* respectively, and the accurate determination of e^- in the $\leftarrow H^+/e^-$ ratio therefore presents little difficulty. However, the estimation of $\leftarrow H^+$ is more problematical. There are two main methods of estimating $\leftarrow H^+$, which depend: (i) on the acidic property; and (ii) on the electric property, of the proton, respectively. The corresponding estimates of $\leftarrow H^+$ are conveniently described as $\leftarrow H^+$(acid) and $\leftarrow H^+$(charge) [31].

In method (i), the electric membrane potential $\Delta\psi$ is collapsed by the presence of a lipophilic cationic agent, such as K–valinomycin$^+$ in a K^+-containing medium, and the total quantity of H^+ ions (ΔH_O^+tot) ejected into the outer medium during the redox pulse is estimated from a continuous recording of pH_Oobs, the observed outer pH, and from appropriate acid calibrations [45]. The acid calibrations under these conditions measure the effective buffering power B_O of the outer medium and enable changes in H_O^+obs, the

quantity of H^+ ions in the outer medium, to be estimated from changes of pH_Oobs, as follows:

$$\Delta H^+_Oobs = -B_O \Delta pH_Oobs \quad (1)$$

As illustrated in fig.1A, the observed ejection of protons per electron transferred ($\Delta H^+_Oobs/e^-$), measured from the initial H^+_Oobs baseline, decays during and after the redox pulse. Usually, the rate of cytochrome *c* oxidation is approximately constant during the redox pulse and the total quantity of protons ejected during the redox pulse, ΔH^+_Otot, may accordingly be equated with the value of

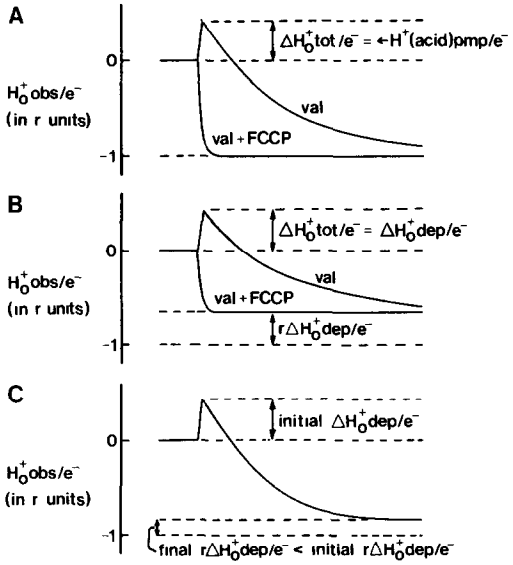

Fig.1. Theoretical time-courses of the observed H^+ ion content of the outer medium (H^+_Oobs) in cytochrome oxidase vesicle suspensions during and after a redox pulse, in the presence of K^+ + valinomycin (val), or K^+ + valinomycin + FCCP (val + FCCP), in cases where proton ejection is due to the following: (A) transmembrane proton pumping, $\leftarrow H^+$(acid)pmp; (B) deprotonation (ΔH^+_Odep) associated with oxidation of a superficial phospholipid-cytochrome *c* complex that is stable; (C) deprotonation (initial ΔH^+_Odep) associated with oxidation of a superficial phospholipid-cytochrome *c* complex that is partially unstable (final ΔH^+_Odep < initial ΔH^+_Odep). In (C) the conditions are similar to those of the curve marked val in (B) but are modified; e.g., by the presence of certain lipophilic cationic or anionic agents. Unit of H^+_Oobs scale $r = B_O/(B_O + B_I)$, as explained in text. The value of *r* was taken as 0.75 in (B) and (C).

ΔH^+_Oobs, extrapolated to the centre of the redox pulse, or:

$$\Delta H^+_Otot = \Delta H^+_Oobs(extrapolated) \quad (2)$$

It is generally agreed that the reduction of O_2 by cytochrome *c* oxidase involves the uptake of one proton from inside the vesicles per electron transferred inwards from cytochrome *c*, and this must be manifested as a downward shift of H^+_O, the H^+ ion content of the outer medium, by the fraction *r* of a proton per electron transferred, where $r = B_O/(B_O + B_I)$, because, at protonic equilibrium, the net uptake of protons must be supplied in the ratio B_O/B_I, or $r/(1-r)$, by the outer and inner media, respectively – as seen in control experiments in which both the $\Delta\psi$-collapsing agent and a proton-conducting agent, such as FCCP, are present. Thus, if the redox-linked proton ejection ΔH^+_Otot were exclusively attributable to proton pumping across the membrane, or:

$$\leftarrow H^+(acid)pmp = \Delta H^+_Otot \quad (3)$$

the time-course of H^+_Oobs should resemble curve A in fig.1.

Equation (3) has generally been considered to apply in cytochrome oxidase vesicle experiments. However, as shown in section 2.1, proton ejection could be caused by deprotonation processes associated with cytochrome *c* oxidation as well as proton pumping. Therefore, we suggest that a correction for the redox-linked deprotonation (ΔH^+_Odep) is required, as follows:

$$\Delta H^+_Otot = \Delta H^+_Opmp + \Delta H^+_Odep \quad (4)$$

or

$$\leftarrow H^+(acid)pmp = \Delta H^+_Otot - \Delta H^+_Odep \quad (5)$$

If ΔH^+_Opmp were zero, and ΔH^+_Odep were finite and time-invariant after the oxidation of the cytochrome *c*, the time-course of H^+_Oobs should resemble curve B in fig.1. The expected alkalinisation corresponding to the uptake of one H^+ ion from the inner medium should show a shortfall corresponding to $r\Delta H^+_Odep$, because after transmembrane equilibration, only the fraction *r* of the protons ΔH^+_Odep initially released at the surface of the vesicles would remain in the outer medium. However, if ΔH^+_Opmp were zero, and ΔH^+_Odep were finite and decayed towards a lower but still finite value after oxidation of the

cytochrome *c*, the time course of H^+_oobs should resemble curve C in fig.1, showing a shortfall in the alkalinisation that is less than $r\Delta H^+_o$dep. The reason for considering these special cases will become clear later.

In method (ii), the membrane is made specifically permeable to K^+ by valinomycin, and $\leftarrow H^+$(charge) attributable to redox-linked proton pumping is customarily estimated from changes in pK_oobs, the observed pK_o, on the basis that:

$$\leftarrow H^+(\text{charge})\text{pmp} = -\Delta K^+_o\text{tot} - \Delta e^- \quad (6)$$

where $-\Delta K^+_o$tot represents the total K^+ ion uptake, measured by extrapolation of calibrated pK_oobs recordings (as in the pH_o and H^+_o measurements), and Δe^- represents the extent of inward electron translocation corresponding to electron transfer from cytochrome *c* to oxygen in the redox pulse [45].

The K^+ ion is much less likely to be subject to specific redox-linked binding or release in the outer medium by components at the surface of the cytochrome oxidase vesicles than is the proton, especially when other univalent ions such as sodium and choline are present at relatively high concentration. However, in view of the known redox-linked cation binding by cytochrome *c* [34–38], it is possible that significant binding changes of limited extent could contribute to ΔK^+_otot, and that a correction term should be included in eq. (6). Unlike the estimation of ΔH^+_otot from ΔpH_o, the estimation of ΔK^+_otot from ΔpK_o is very susceptible to small dilution errors that shift the pK_o baseline on addition of the pulse of oxidant or reductant solution, because there is virtually no pK_o buffering, and it is prudent to allow for such dilution errors. Accordingly, we suggest that eq. (6) should be amended as follows:

$$\leftarrow H^+(\text{charge})\text{pmp} = -\Delta K^+_o\text{tot} + \Delta K^+_o\text{err} - \Delta e^- \quad (7)$$

where ΔK^+_oerr (which could, of course, be positive or negative) stands for the increase in K^+_o attributable to redox-linked K^+ desorption or dilution error in the outer medium.

3. INTERPRETATIVE REVIEW

3.1. *Oxygen pulse experiments*

Hinkle's original O_2 pulse experiments [2] with cytochrome oxidase vesicles preincubated anaerobically with ferrocytochrome *c* in a KCl medium showed an initial valinomycin-dependent acidification (ΔH^+_otot) and an equal shortfall in the expected CCCP-dependent net alkalinisation attributable to the uptake of one H^+ per e^- transferred from ferrocytochrome *c* to O_2. From his experimental data [2], it seems reasonable to infer, as Hinkle did at first [2,7], that the initial acidification (ΔH^+_otot), and the subsequent equal shortfall in the expected net alkalinisation, which corresponded closely to curve B of fig.1, may be caused by a redox-linked deprotonation that is dependent on and stable after ferrocytochrome *c* oxidation, and corresponds to ΔH^+_odep in eq. (5), while redox-linked proton pumping ($\leftarrow H^+$(acid)pmp) was zero. When 1,4-naphthoquinol-2-sulphonate or benzoquinol were present to re-reduce the cytochrome *c* during the redox pulse in O_2 pulse experiments with cytochrome oxidase vesicles at pH_o near 7, Hinkle observed ΔH^+_otot values very close to the expected ΔH^+_odep associated with oxidation and deprotonation of the added reductant, and there was no significant shortfall in the expected ΔH^+_o baseline (given by ΔH^+_otot − ΔH^+_odep) after the redox pulse [2]. The redox-linked deprotonation ΔH^+_odep in his experiments with ferrocytochrome *c* as the only reductant corresponded to about 0.2 H^+ per e^- transferred. Therefore, if it had occurred in the experiments with added reductants of cytochrome *c*, it should have been easy to identify, especially as a shortfall in the CCCP-dependent net alkalinisation control experiments. Presumably, the redox-linked deprotonation observed with ferrocytochrome *c* as the only reductant is prevented when the cytochrome *c* is maintained in the reduced state by the added reductant, because it involves ferricytochrome *c*.

We find it hard to escape the conclusion that the proton ejection observed in Hinkle's O_2 pulse experiments with ferrocytochrome *c* as the only reductant was not due to proton pumping, but was due to the deprotonation of some complex involving cytochrome *c*, anionic phospholipid, and possibly other components. As Hinkle pointed out [8], the proton ejection at pH_o near 6 or 7 could not be due to a classical cytochrome *c* Bohr effect, the apparent p*K* of which is near 9 [46]. It could, however, occur by interaction of cytochrome *c* with proton-binding phospholipids, such as car-

diolipin (as suggested by Hinkle [8]), or phosphatidyl serine.

3.2. *Ferrocytochrome* c *pulse experiments*

Wikström and Saari [5] and Hinkle [8] observed that, when pulsed with ferrocytochrome *c*, aerobic suspensions of cytochrome oxidase vesicles in a KCl medium showed an initial valinomycin-dependent acidification ($\Delta H^+_{\bar{o}}$tot) very much like that seen in Hinkle's O_2 pulse experiments [2]; and this observation has been confirmed by several research groups [6,8–10,13,16]. Proteau, Wrigglesworth and Nicholls [16] recently described ferrocytochrome *c* pulse experiments done in a sodium sulphate and choline chloride medium of low K^+ content, so that both $\leftarrow H^+$(acid)pmp and $\leftarrow H^+$(charge)pmp could be estimated from the observed pH_O and pK_O changes. We corrected the results shown in their fig.1 for a slow baseline drift, and replotted them for comparison with Hinkle's O_2 pulse experiments, discussed above. We found that the initial $\Delta H^+_{\bar{o}}$tot pulse, that may be identified with $\Delta H^+_{\bar{o}}$dep, was practically equal to $1/r$ times the shortfall in the FCCP-dependent alkalinisation, as in fig.1B, and in Hinkle's O_2 pulse experiments, although $\Delta H^+_{\bar{o}}$dep was greater in the ferrocytochrome *c* pulse experiment of Proteau and colleagues than in Hinkle's O_2 pulse experiments – a fact that may be attributed to differences in the cytochrome oxidase vesicle preparations, in the suspension media and in other experimental conditions, rather than to any intrinsic difference of behaviour of the cytochrome oxidase vesicle system. According to our analysis of the data of Proteau, Wrigglesworth and Nicholls [16] along the lines described in section 2.2, the observed values of $\leftarrow H^+$(acid)pmp/e^- and $\leftarrow H^+$(charge)pmp/e^- were not greater than 0.1, and certainly did not approach the value of 1.0 mentioned in their report [16]. They have kindly informed us that their re-analysis of the data of their fig.2 in [16] gives values of less than 0.25 for $\leftarrow H^+$(acid)pmp/e^- and $\leftarrow H^+$(charge)pmp/e^-, but that the average values obtained in the course of this work were 0.75, and that they intend to publish the supporting experimental evidence in due course. Meanwhile, it would seem to be unwise to discount the fact that the data published so far [16] are more consistent with a superficial redox-linked deprotonation mechanism of proton ejection than with a redox-linked transmembrane proton pumping mechanism.

Estimates by Sigel and Carafoli [10] of $\leftarrow H^+$(charge)pmp/e^- from measurements of changes of $K^+_{\bar{o}}$obs and of oxygen consumption during redox activity initiated by ascorbate + TMPD in cytochrome oxidase vesicle suspensions in the presence of cytochrome *c* and valinomycin + K^+, appeared to indicate significant proton pumping at low cytochrome *c* oxidase turnover. But, as the reader may readily verify from their fig.10, the values of $\leftarrow H^+$(charge)pmp/e^- estimated from their data cease to be significant when allowance is made for the small step-change in $K^+_{\bar{o}}$, corresponding to $\Delta K^+_{\bar{o}}$err in eq. (7), which they observed in control experiments done in the presence of CCCP [10]. Coin and Hinkle's finding [8] of values of $-\Delta K^+_{\bar{o}}/e^-$, which were apparently significantly greater than 1 only in ferrocytochrome *c* pulse experiments involving less than 2 turnovers of cytochrome *c* oxidase, may likewise have been due to a small $\Delta K^+_{\bar{o}}$err that was not taken into account. They themselves remarked [8]:

> *'the fact that high ratios were observed only under conditions of a few enzyme turnovers may be viewed as disturbing.'*

Ferrocytochrome *c* pulse experiments done by Papa and colleagues [33] gave $H^+_{\bar{o}}$obs time-courses that were similar to those described in fig.1B. They considered that the initial acidification in the ferrocytochrome *c* pulse type of experiment was not due to proton pumping but might depend on the initial oxidised resting state of the oxidase, and could:

> *'represent some artifactual scalar acidification process resulting from the interaction of ferro-ferricytochrome* c *with the oxidase in its particular state of insertion in the phospholipid vesicles.'*

While agreeing with the view of Papa and colleagues [33] that the proton ejection observed in ferrocytochrome *c* pulse experiments is most simply explained by a redox-linked deprotonation involving ferro-ferricytochrome *c* at the surface of the cytochrome oxidase vesicles, we do not think that it depends on the initial oxidised resting state of cytochrome *c* oxidase, because Hinkle [2] observed significant proton ejection in oxygen pulse experiments, in which the cytochrome *c* oxidase was initially in a reduced, and presumably active, state.

In the ferrocytochrome *c* pulse experiments

described by Coin and Hinkle [8], the time-course of H^+_oobs in the presence of valinomycin + K^+ resembled curve B of fig.1. But the H^+_oobs baseline after the redox pulse was moved downward to give a H^+_oobs time-course resembling curve C when a lipophilic anionic uncoupler was present, as though the extent and rate of decay of ΔH^+_odep, as well as the rate of proton conduction inwards through the membrane, were dependent on the concentration of lipophilic anion in the membrane of the vesicles. This effect of lipophilic anion has been observed in the majority of ferrocytochrome *c* pulse experiments [6,8,9,13]. It is particularly nicely illustrated in work by Casey, Chappell and Azzi [9], who used 4 nM to 4 μM CCCP. They showed (fig.3 of [9]) that, in the presence of valinomycin + K^+, but without added CCCP, there was a shortfall in the downward shift of H^+_oobs which was even greater than that represented in fig.1B, suggesting that there may have been a redox-linked deprotonation at the surface of the vesicles that was initiated by ferrocytochrome *c* oxidation, but continued to take effect for some time after the end of the redox pulse. With 4 μM CCCP present, there was no shortfall in the downward shift of H^+_oobs, suggesting that the deprotonation process corresponding to ΔH^+_odep may have been completely suppressed by this relatively high concentration of CCCP. But, with 4 nM and 12 nM CCCP, the results suggest that the initial deprotonation ΔH^+_odep decayed to lower values that were stable, as illustrated by fig.1C. It is possible that, in the absence of CCCP, the valinomycin + K^+ did not enable K^+ to pass through the membrane fast enough to prevent a considerable electric membrane potential from building up as a result of electron translocation through cytochrome *c* oxidase during the redox pulse, and that part of the shortfall in the downward shift of H^+_oobs in the absence of CCCP might be attributable to an ionic punch-through effect. This could not, however, account for the observations at intermediate CCCP concentrations, where the shortfall of the downward shift of H^+_oobs was abolished, but the initial proton ejection was not.

Wikström and Krab [17] have expressed the view that:

> *'proton ejection cannot be ascribed to a scalar* net *production of acid by some artefactual side-reaction, because the overall consumption of H^+ is very closely equal to one per electron both in the presence and absence of uncoupler.... This specifically excludes a* net *acid/base shift induced by oxidation of complexes of ferrocytochrome* c *with other membrane components as suggested by Moyle and Mitchell....'*

However, the experimental observations discussed here do not appear to be consistent with this view. Wikström and Krab [17] cited as evidence for their view, ferrocytochrome *c* pulse experiments [6] in which relatively low concentrations of cytochrome oxidase vesicles were used, and the observed proton ejection was small compared with the alkalinisation following the redox pulse. When the proton ejection is small compared with the subsequent alkalinisation, it is relatively difficult to estimate the baseline shift corresponding to ΔH^+_odep in curve B of fig.1, as the reader can verify from the traces shown in fig.12 of the review by Wikström and Krab [17]. However, when Krab and Wikström [6] maximised the extent of proton ejection by appropriate means, the time-courses of H^+_oobs in ferrocytochrome *c* pulse experiments in the presence of valinomycin + K^+ resembled curve B and not curve A in fig.1, as in ferrocytochrome *c* pulse experiments by other workers [8,9,13]. They remarked that the 'stability' of the acidification phase may be enhanced by increasing the concentration of cytochrome oxidase vesicles, keeping all other parameters constant [6].

It is a particularly noteworthy fact that, in ferrocytochrome *c* pulse experiments done in the presence of valinomycin + K^+, the redox-linked proton ejection, shown by the time-course of H^+_oobs, is 'stable' in the sense that it generally fails to fall to the baseline expected of a proton-pump mechanism (as in curve A of fig.1), but persists as though it were due to a stable deprotonation ΔH^+_odep (as in curve B of fig.1). However, since it was discovered, as mentioned above, that the persistence or 'stability' of the initial proton ejection could be abolished by certain lipophilic anionic agents (such as 3,5-ditertiary butyl-4-hydroxybenzylidene malononitrile [8], nigericin [6] or CCCP [9]), it has generally come to be regarded as an anomalous but suppressible, characteristic of the proton-pumping experimental system. This view has been strengthened by Casey's privately communicated observation that the persistence of the initial proton ejection could be abolished by the lipophilic cationic agent tetraphenyl phosphonium.

It has been suggested that the persistence of the initial proton ejection, observed as a failure of pH_o to return to the expected baseline, may be attributable to a very low proton conductance of the vesicles, which can be appropriately raised by lipophilic proton-conducting agents. But this explanation is not supported by estimates of the times of half equilibration of the pH difference across the vesicle membrane in certain ferrocytochrome *c* pulse experiments, as indicated in section 3.3 below.

Perhaps the 'stability' of the initial proton ejection may be most simply explained on the basis that the proton ejection is not due to proton pumping across the membrane, but is caused by the redox-linked deprotonation of a partially salt-linked complex involving cytochrome *c* and anionic phospholipid (and possibly other components) at the surface of the membrane, and that the anionic and cationic components of this lipophilic complex are dissociated or otherwise caused to reprotonate by certain species of lipophilic anion or cation, under certain conditions. It is especially interesting in this context that Krab and Wikström [6] found that proton ejection observed in ferrocytochrome *c* pulse experiments at low ionic strength, as described by Hinkle [7], was completely insensitive to uncoupling agents. They considered it:

> *'possible that the ionic interaction of cytochrome* c *with the phospholipid bilayer surface leads to a* net *production of* H^+ *ions at low ionic strength'*

but that this *net* deprotonation could be excluded in their experiments at relatively high ionic strength [6]. However, the reversal of proton ejection by lipophilic anionic agents such as CCCP or nigericin at relatively high ionic strength under some conditions [6,9] (but not under others [16]) is not acceptable as evidence that uncoupler-insensitive *net* deprotonation is ruled out, and that uncoupler-sensitive transmembrane proton pumping must be occurring under these conditions. It could equally well be that, given certain conditions that are held constant, the putative complex of cytochrome *c* and phospholipid involved in redox-linked deprotonation at the surface of the vesicles is generally dissociated more readily, and its proton-ejecting action is more readily inhibited or reversed, by lipophilic anionic (or cationic) agents at relatively high ionic strength [6,9]; but that under different conditions, such as at the higher temperature (30°C in place of 15°C), or in the different salt medium (50 mM sodium sulphate + 50 mM choline chloride in place of 100 mM uni-univalent electrolyte) used by Proteau and colleagues [16], the putative phospholipid–cytochrome *c* complex responsible for redox-linked deprotonation is sufficiently stable not to be appreciably dissociated by FCCP, despite the relatively high ionic strength of the medium.

The observation that proton ejection by cytochrome oxidase vesicles does not occur significantly in the absence of valinomycin + K^+ (or of some other $\Delta\psi$-collapsing ionophore) has been taken to provide strong support for the interpretation that proton ejection from cytochrome oxidase vesicles must be due to electrogenic transmembrane proton pumping, because it would be inhibited unless the electric membrane potential $\Delta\psi$ that it would create were collapsed [6,9,17]. However, this argument overlooks the fact that the cytochrome *c* oxidase action is electrogenic in any case, and that, unless a $\Delta\psi$-collapsing agent is present, inward electron transfer from ferrocytochrome *c* is strongly inhibited in the well-coupled cytochrome oxidase vesicles that show good proton ejection [6,8,9,47–49]. In the absence of a $\Delta\psi$-collapsing agent, such as valinomycin + K^+, the proton is probably the predominant carrier of electric charge through the membrane in most cytochrome oxidase vesicle experiments. Thus, if proton ejection were due to the dissociation of not more than one proton at the surface of the vesicles per electron transferred from ferrocytochrome *c*, and per proton taken up by oxygen at the cytochrome *c* oxidase catalytic site from the inner medium, the electric membrane potential would be expected to pull protons in through the membrane at least as fast as they were dissociated at the outer surface, and no *net* proton ejection would be visible. Even if significant electrophoretic import of ions other than protons were to prevent electrophoretic proton import from keeping pace with proton dissociation at the surface of the vesicles, owing to the strong inhibition of the rate of electron transfer observed in the absence of a $\Delta\psi$-collapsing agent [6,8,9,47–49], there would be a corresponding inhibition of proton dissociation at the surface of the vesicles, so that the time-course of pH_oobs would show only an apparent change of

baseline drift in place of the proton ejection observed in the presence of valinomycin + K^+.

3.3. *Possible mechanisms of proton ejection*

Casey, Thelen and Azzi [11] showed that the redox-linked proton ejection observed in ferrocytochrome *c* pulse experiments is inhibited by prior treatment of the cytochrome oxidase vesicles with DCCD, and that the DCCD reacts specifically with subunit III of cytochrome *c* oxidase under the conditions of these experiments. Interpreting the normal redox-linked proton ejection as a manifestation of transmembrane proton pumping by cytochrome *c* oxidase, Casey and colleagues suggested that subunit III has an important role in H^+ translocation by the enzyme [11,12]. These experimental findings have been confirmed [13], and their interpretation has been supported by ferrocytochrome *c* pulse experiments using cytochrome oxidase vesicles prepared with an ox heart cytochrome *c* oxidase preparation lacking subunit III, which showed relatively little redox-linked proton ejection [14]. It should be realised, however, that the manipulations required to remove subunit III from the normal cytochrome *c* oxidase preparation, and the further treatment needed to remove the Triton used to dissociate the subunit III, may well remove, not only subunit III, but also other components, such as specific phospholipids, from the cytochrome *c* oxidase preparation normally used to produce the cytochrome oxidase vesicles. Moreover, it has been found that certain preparations of cytochrome oxidase vesicles containing the cytochrome *c* oxidase of *Paracoccus denitrificans*, which lacks subunit III, can exhibit redox-linked proton ejection [15].

It seems to us that the inhibitory action of DCCD on redox-linked proton ejection in cytochrome oxidase vesicles may not be due to the reaction of DCCD with cytochrome *c* oxidase, but may be explained otherwise. It has been shown that DCCD reacts with the carboxyl group of phosphatidyl serine in cristae membrane vesicles from mitochondria [50,51]. Therefore, we suggest that phosphatidyl serine is a specific anionic component of a partially salt-linked complex between cytochrome *c* and phospholipid that is involved in redox-linked deprotonation at the surface of the cytochrome oxidase vesicles. This phosphatidyl serine-dependent redox-linked deprotonation, which we suggest corresponds to part or all of the proton ejection $\Delta H^+_{\delta}dep$ of eq. (4), would be expected to be abolished by reaction of the phosphatidyl serine carboxyl group with DCCD.

In their description of the partial abolition of the initial proton ejection by DCCD in ferrocytochrome *c* pulse experiments, Casey and colleagues [12] remarked that the initial rate of H^+ influx after the redox pulse was practically the same in the presence and absence of DCCD. In other words, the initial rate of H^+ influx after the redox pulse was only slightly greater in the absence of DCCD, when there was an initial redox-linked proton ejection ($\Delta H^+_{\delta}tot$), than it was in the presence of DCCD, when there was relatively little redox-linked proton ejection. If the proton conductance were unaffected by DCCD, this observation would imply that the pH difference across the membrane (which determines the rate of H^+ influx at a given proton conductance) must have increased only by a small amount, presumably corresponding to ΔpH_Odep, and the internal pH (pH_I) cannot have been driven appreciably higher when redox-linked proton ejection occurred than when it did not.

The ratio of the outer and inner buffering powers (B_O/B_I) in these experiments was about 3/1, so that the pH_I changes accompanying any proton pumping would have contributed 3-times as much to the pH difference across the membrane as the corresponding pH_O changes. The transmembrane pH difference would have been more than doubled if one proton had been pumped per electron transferred, and the initial rate of H^+ influx should have been decreased by more than a factor of 2 if DCCD had abolished proton pumping.

Casey and colleagues [11] showed by a fluorescent indicator method that the proton conductance of the cytochrome oxidase vesicles was not significantly changed by DCCD in their experiments; and this can be confirmed by measurements of the half-time of pH equilibration after the injection of acid in their acid calibrations [12], which was close to 20 sec both in the presence and absence of DCCD. It follows that redox-linked proton ejection in the experiments of Casey and colleagues [12] in the absence of DCCD was not accompanied by significant withdrawal of H^+ ions from the internal medium, other than the H^+ ions required to protonate the oxygen reduced during the redox

pulse. The implication of these excellently self-consistent and well-documented ferrocytochrome *c* pulse experiments [11,12] is that proton ejection is not due to the outward pumping of H^+ ions from the inner aqueous medium of the cytochrome oxidase vesicles, at least under these experimental conditions. Incidentally, since the proton conductance of the vesicles corresponded to a time for half-equilibration of the transmembrane pH difference of near 20 s in the ferrocytochrome *c* pulse experiments of Casey and colleagues [12], about 99% pH equilibration should have been achieved in $7 \times 20 = 140$ s. The so-called 'stability' of the initial proton ejection, discussed above, involves times at least as great as 140 s, and this 'stability' (which we interpret as the stability of $\Delta H^{\ddagger}_{\delta}$dep) cannot therefore be ascribed to a very low proton conductance of the vesicles in the absence of CCCP.

4. CONCLUSIONS

Definitive experimental evidence has not yet been obtained, either for the extra translocation of electric charge, or for the extra internal uptake of protons, that should be diagnostic of activity of the proposed proton pump of cytochrome *c* oxidase in cytochrome oxidase vesicle experiments. Recent experimental work such as that published by Proteau, Wrigglesworth and Nicholls [16], and from the laboratories of Azzi [9,12] and of Papa [33], does not only fail to provide hard evidence in favour of proton pumping by cytochrome *c* oxidase in cytochrome oxidase vesicles. This work also provides positive evidence against significant proton pumping under the experimental conditions so far employed to measure it. One may, of course, argue that the right experimental conditions have not yet been found for observing the activity of the proposed proton pump. But meanwhile, the interesting phenomenon of redox-linked proton ejection by cytochrome oxidase vesicles requires some explanation.

The work reviewed in this paper suggests a hypothetical redox-linked deprotonation mechanism of proton ejection by cytochrome oxidase vesicles, based on the following postulates:

(1) Redox-linked proton ejection is not attributable primarily to cytochrome *c* oxidase, but it is a consequence of the oxidation of ferrocytochrome *c* to ferricytochrome *c* at the surface of cytochrome oxidase vesicles.

(2) The oxidation of ferrocytochrome *c*, complexed with proton-binding phospholipids at the surface of cytochrome oxidase vesicles in salt media containing dilute pH buffers, results in a *net* deprotonation of proton-binding groups, which is observed as proton ejection.

(3) The phospholipid-cytochrome *c* complex or complexes involved in the redox-linked *net* deprotonation process are partially salt linked. Proton ejection may therefore be reversed or prevented by certain lipophilic anions or cations (or other agents or conditions) that dissociate or modify the salt linkages in the phospholipid–cytochrome *c* complex(es).

(4) Phosphatidyl serine is a specific anionic component of the phospholipid–cytochrome *c* complex(es) involved in the redox-linked proton ejection that is sensitive to DCCD.

Cytochrome oxidase vesicles, as prepared so far, do not provide nearly such a simple and well-defined experimental material for elucidating the function(s) of cytochrome *c* oxidase as might have been supposed or desired. Significant differences of lipid composition, enzyme integrity, vesicle size distribution, and general physical topology and homogeneity are obviously to be expected between one preparation and another, depending on the origins and histories of the starting materials, and on the details of the method of incorporation of the enzyme in the phospholipid vesicles [6,8,9,13,15,33,52–57]. Relatively small changes in the ratio of cytochrome *c* oxidase to phospholipid in the incorporation procedure have a profound and complex effect on the behaviour of the cytochrome oxidase vesicles in redox-pulse experiments [9], and considerable variations in the behaviour of preparations of cytochrome oxidase vesicles may thus occur, even when the method of preparation is basically the same. As discussed earlier, the 'stability' of proton ejection evidently depends on a number of complex factors that may be influenced by lipophilic anionic and cationic agents, the detailed ionic composition of the suspension

medium, its pH and ionic strength, and the temperature at which the experiments are conducted. Moreover, it is well known to those who work with cytochrome oxidase vesicles that the quantitative behaviour of the experimental system in ferrocytochrome *c* pulse experiments may depend critically on its recent past history, especially with respect to interaction with ferrocytochrome *c*, as in the induction of larger and larger stable proton ejections with successive ferrocytochrome *c* pulses in Hinkle's experiments at low ionic strength [7]. Such complexities of behaviour might reflect complex structural rearrangements in the cytochrome oxidase vesicles, which could include transmembrane phospholipid head-group transport or flip-flop [58,59] and even penetration of cytochrome *c* into the phospholipid bilayer [42,60].

In view of the scope for structural and behavioural complexity and variability in cytochrome oxidase vesicle preparations, we are not suggesting that all the experimental observations on redox-linked proton ejection by cytochrome oxidase vesicle suspensions can be explained on the basis of the four postulates listed above. In some experiments, the results may, for example, be complicated by field-driven ion punch-through effects, by field-driven phospholipid flip-flop, or by penetration of cytochrome *c* into the lipid membrane. However, we suggest that the above four postulates may provide a sufficient basis for explaining redox-linked proton ejection by topologically stable cytochrome oxidase vesicles in the presence of sufficient valinomycin + K^+, or other suitable ionophoric agent, to collapse the electric membrane potential effectively, under conditions that otherwise enable the artificial lipid membrane to act as a model of the mitochondrial cristae membrane.

5. PROSPECT

The hypothetical redox-linked deprotonation mechanism suggested above is susceptible to a number of simple experimental tests.

If postulate 1 were true, it should be possible to observe proton ejection during oxidation of cytochrome *c* at the surface of cytochrome oxidase vesicles, or even at the surface of phospholipid vesicles not containing cytochrome *c* oxidase, by artificial electron acceptors such as ferricyanide.

If postulate 2 were true, the extent of proton ejection should depend on the lipid composition of the cytochrome oxidase vesicles.

If postulate 3 were true, it should be possible to change the extent and 'stability' of proton ejection with cationic or anionic agents that interact coulombically with cytochrome *c* and/or phospholipid but do not change the proton conductance of the cytochrome oxidase vesicle membrane.

If postulate 4 were true, it should be possible to vary the extent of DCCD-sensitive proton ejection by varying the phosphatidyl serine content of the cytochrome oxidase vesicles.

These and other experimental approaches to the question of the mechanism of redox-linked proton ejection in cytochrome oxidase vesicles are now being pursued in our laboratories. We hope that the suggestions made in this commentary may help to stimulate further experimental initiatives aimed at resolving the present conflict of opinion, and of apparent experimental information, concerning the putative proton-pumping function of cytochrome *c* oxidase.

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