Protonmotive functions of cytochrome c oxidase in reconstituted vesicles

Influence of turnover rate on 'proton translocation'

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1. Oxidation of ferrocytochrome c by cytochrome c oxidase incorporated into proteoliposomes induces a transient acidification of the external medium. This change is dependent on the presence of valinomycin and can be abolished by carbonyl cyanide p-trifluoromethoxyphenylhydrazone or by nigericin. The H⁺/e⁻ ratio for the initial acidification varies with the internal buffering capacity of the vesicles, and under suitable conditions approaches +1, the pulse slowly decaying to give a net alkalinity change with H⁺/e⁻ value approaching -1. 2. Inhibition of cytochrome c oxidase turnover by ferricytochrome c or by azide addition results in ferrocytochrome c-dependent H⁺ pulses with decreasing H⁺/e⁻ ratios. The rate of the initial H⁺ production remains higher than the rate of equilibration of the pH gradient, indicating an intrinsic dependence of the H⁺/e⁻ ratio on enzyme turnover. The final net alkalinity changes are relatively unaffected by turnover inhibition.

Respiring mitochondria are able to generate an electrochemical gradient of protons across the inner membrane (Mitchell, 1966; Mitchell & Moyle, 1969; Nicholls, 1974), although the stoichiometry of proton extrusion by different segments of the respiratory chain is still in question (Reynafarje et al., 1976; Mitchell et al., 1978; Brand et al., 1978; Wikström & Krab, 1979; Papa et al., 1980). One such segment, cytochrome c oxidase, has previously been thought to generate an electrochemical gradient by translocating electrons from cytochrome c on the outer surface of the inner membrane to an oxygen and proton reaction site on the inner surface (Mitchell, 1966). More recent experimental evidence, however, had led Wikström (1977, 1981) and others (Brand et al., 1978; Sigel & Carafoli, 1978; Sorgato & Ferguson, 1978) to propose that cytochrome c oxidase can also act as a proton pump to translocate H⁺ across the membrane in the opposite direction. This proposal has been challenged by Moyle & Mitchell (1978), Mitchell (1979) and Papa et al. (1980).

Ferrocytochrome c-induced proton extrusion from reconstituted phospholipid vesicles incorporating purified cytochrome c oxidase has been demonstrated by Krab & Wikström (1978), Casey et al. (1979), Sigel & Carafoli (1980) and Prochaska et al. (1981). The H⁺/e⁻ stoichiometry of the extruded protons is reported to depend on the internal buffering power of the vesicles and (as a consequence) on the number of turnovers of the incorporated cytochrome c oxidase. Nevertheless, extrapolation to zero-turnover conditions still appears to give different stoichiometries of initial proton extrusion for bovine heart and Paracoccus denitrificans enzymes (Solioz et al., 1982).

We now report that the H⁺/e⁻ stoichiometry of proton extrusion from reconstituted proteoliposomes incorporating bovine heart cytochrome c oxidase is dependent on turnover rate rather than on the absolute number of turnovers. The dependence on turnover reported previously could arise from the inhibitory effect of repeated cytochrome c additions on enzyme activity. We suggest that the apparent dependence of stoichiometry on rate could arise as a consequence of a two-state enzyme mechanism or from localized back-diffusion of protons into the vesicle interior.

Materials and methods

Cytochrome c oxidase was prepared from bovine heart by the method of Kuboyama et al. (1972), with
Tween 80 substituting for Emasol, and was stored at 
\(-75^\circ\text{C}\) as described by Nicholls & Hildebrandt 
(1978). The final preparations of the enzyme had a 
haem a/protein ratio between 8 and 10 \(\mu\text{mol/g}\) and 
maximal turnovers (electrons/cytochrome \(a\alpha_2\)) of 
250–300 s\(^{-1}\) in the presence of 0.5% Tween, and 
0.05% asolectin, in 65 mM-sodium phosphate buffer, 
\(\text{pH} 7.4\), with ascorbate, \(\text{NHN}^+\text{N}^-\text{tetramethyl-p-}
\text{phenylenediamine dihydrochloride and horse heart} 
\text{cytochrome c (Sigma type VI) as substrate.} 

Ferrocytochrome c was prepared by incubating 
5 mM-cytochrome c with 7.5 mM-ascorbate for 
30 min at 4°C. The solution was then eluted from a 
Sephadex G-25 column with 50 mM-K\(_2\)SO\(_4\) to isolate 
the reduced cytochrome, which was adjusted to the 
\(\text{pH}\) of the experimental medium before use.

Reconstitution of the cytochrome c oxidase in 
phospholipid vesicles was performed essentially as 
described by Racker (1973). A 0.5 g portion of 
dry asolectin (L-\(\alpha\)-phosphatidylycholine, Sigma type 
IV-S) was dispersed in 10 ml of 50 mM-potassium 
phosphate buffer, \(\text{pH} 7.4\), by rapid shaking on a 
mechanical mixer. Cytochrome c oxidase was then 
added to give a final phospholipid/protein weight 
ratio of approx. 20:1. The mixture was gently 
hand-shaken, placed in a container surrounded 
by ice and sonicated to clarity (15 min) with a 
Heats–Systems Ultrasonics model W375 sonicator 
set on the pulsed mode at 30% duty cycle. 
The solution was then centrifuged at 27000 \(\text{g}\) for 10 min, 
and any pellet was discarded. The supernatant 
was passed through a Sephadex G-75 column equi-
librated with 50 mM-K\(_2\)SO\(_4\) to remove external 
buffer.

Proteoliposomal cytochrome c oxidase turnover 
is stimulated by at least 6-fold by addition of valino-
mycin and carbonyl cyanide \(p\)-trifluoromethoxy-
phenylhydrazone to a system respiring in the 
presence of oxygen, ascorbate, tetramethyl-p-
phenylenediamine and cytochrome c. A further 
2-fold stimulation can be obtained by addition of 
8 mM-deoxycholate (final concentration).

Only 50% of the cytochrome c oxidase molecules 
were fully reduced when the sample became 
aerobic with externally added ascorbate and 
cytochrome c. The addition of tetramethyl-p-phenyl-
enediamine or deoxycholate (0.4%) resulted in the 
remaining 50% of the cytochrome c oxidase becom-
ing reduced. From these and other results 
(Wrigglesworth, 1978; Wrigglesworth & Nicholls, 
1979; Nicholls et al., 1980) it can be concluded 
that the preparation comprised vesicles incorpor-
ating cytochrome c oxidase in a transmembrane 
orientation with a 50:50 distribution of the cyto-
ochrome c reaction sites on either side of the mem-
brane.

Changes in \(\text{H}^+\) activity were monitored in a 
thermostatically controlled glass chamber fitted with 
a magnetic stirrer, by using a glass \(\text{pH}\) electrode 
(type G 2222 B or C) connected to a digital 
\(\text{pH}\)-meter (\(\text{pH}\) M64) and recorder (Rec 61 Servo-
graph) (Radiometer, Copenhagen, Denmark). A 
calomel reference electrode (K701) was used. 
Samples were introduced into the chamber by 
injecting small volumes (5–100 \(\mu\text{l}\)) of solution. 
Spectra were obtained with an Amino DW-2 
spectrophotometer as previously described (Nicholls 
et al., 1980).

All other reagents were of analytical grade.

**Results**

**Ferrocytochrome c addition to aerobic cytochrome oxidase vesicles**

Addition of ferrocytochrome c to an aerobic 
suspension of vesicles plus valinomycin induces a 
transient acidification of the external medium (Fig. 
1a, trace i); this \(\text{H}^+\) pulse slowly decays to give a 
net alkalinity change. In the absence of valinomycin 
(Fig. 1a, trace ii) a small acidification is seen, which 
decays very slowly. Spectral measurements (\(A_{550}–
A_{450}\)) show that cytochrome c is rapidly oxidized in 
the presence and in the absence of valinomycin 
during the initial part of the \(\text{H}^+\)-production phase 
(Fig. 1c). In the absence of cytochrome c oxidase, 
vesicles prepared from asolectin alone catalyse a slow 
autoxidation of the ferrocytochrome c, but this rate 
is too low to affect the enzyme-catalysed reaction.
The initial ferrocytochrome c-dependent acidific-
ation is dependent on the presence of valino-
mycin plus \(\text{K}^+\), and does not occur in the presence 
of carbonyl cyanide \(p\)-trifluoromethoxyphenyl-
hydrazone (Fig. 1a, trace iii). Nigericin, an electro-
nutral ionophore, also abolishes the initial ferro-

cytochrome c-dependent acidification. Addition of a 
small amount of external acid indicates that buffer 
internal to the vesicles is not titrated immediately 
unless uncoupler is present (Fig. 1b). The initial \(\text{pH}\) 
change caused by addition of \(\text{HCl}\) was therefore 
used to calculate the \(\text{H}^+/\text{e}^-\) ratio for ferrocyto-

chrome c-induced acidification; the final net 
changes were calculated from the eventual \(\text{pH}\) change 
induced by the \(\text{HCl}\) pulse. As originally pointed out 
by Mitchell & Moyle (1967), considerable errors in 
calculated stoichiometries can occur if this 
approach is not taken.

The ratios of the initial \(\text{H}^+\) production to the 
amount of ferrocytochrome c oxidized under various 
experimental conditions are shown in Table 1. An 
initial, uncoupler-sensitive, acidification was ob-
erved under all conditions. However, the final (net) 
alkalization varied with different types of vesicle. 
With 50 mM-potassium phosphate as internal buffer 
and a final proteoliposomal cytochrome \(aa_2\) con-
centration of 1.28 \(\mu\text{M}\), the initial acidification was
Table 1. Ferrocytochrome c-induced pH changes in an aerobic suspension of proteoliposomes containing cytochrome c oxidase

Proteoliposomes were prepared in the indicated potassium phosphate buffers to give the internal buffer concentrations shown. Cytochrome c concentrations are given as the effective final cytochrome aa₃ concentrations (vesicular). Conditions of measurement were as described in Fig. 1(a) legend. The results are given as the averages ± range for two experiments.

<table>
<thead>
<tr>
<th>Conc. of internal potassium phosphate buffer (mM)</th>
<th>Conc. of cytochrome c oxidase (µM)</th>
<th>H⁺/e⁻ (initial)</th>
<th>H⁺/e⁻ (final)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1.06</td>
<td>+0.71 ± 0.02</td>
<td>-0.66 ± 0.09</td>
</tr>
<tr>
<td>20</td>
<td>0.35</td>
<td>+0.67 ± 0.03</td>
<td>-0.56 ± 0.02</td>
</tr>
<tr>
<td>50</td>
<td>1.28</td>
<td>+0.78 ± 0.15</td>
<td>-0.99 ± 0.05</td>
</tr>
<tr>
<td>50</td>
<td>0.32</td>
<td>+0.64 ± 0.02</td>
<td>-0.86 ± 0.04</td>
</tr>
</tbody>
</table>

found to decay to give a net alkalinity approaching that predicted from the scalar reaction:

\[
4\text{Cyt. c}^{2+} + \text{O}_2 + 4\text{H}^+ \rightarrow 4\text{Cyt. c}^{3+} + 2\text{H}_2\text{O} \quad (1)
\]

Turnover effects on ferrocytochrome c-induced acidifications

Ferricytochrome c inhibits the ferrocytochrome c-induced acidifications catalysed by cytochrome oxidase vesicles (Fig. 2a). The accumulation of oxidized product after successive injections of ferrocytochrome c or the addition of extra ferricytochrome c results in acidification pulses with decreasing H⁺/e⁻ ratios (Fig. 2b, upper curve). The final net alkalinity change remains relatively unaffected (Fig. 2b, lower curve). Ferricytochrome c is known to inhibit the oxidation of ferrocytochrome c by cytochrome c oxidase (Smith & Conrad, 1956). If the oxidation time courses are plotted by the
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Fig. 2. Inhibitory effect of ferricytochrome c on the ferrocytochrome c-induced acidification in an aerobic suspension of cytochrome c oxidase proteoliposomes

(a) Ferrocytochrome c-induced pH changes in a mixture of 6ml of K$_2$SO$_4$ (50mM), 2ml of proteoliposomes (1.28 μM-cytochrome aa$_3$) and valinomycin (1.25 μg/ml) at pH 7.0 and 30°C. Repetitive injections of ferrocytochrome c (Cyt. c$^{2+}$) were made (i) before the addition of ferricytochrome c, (ii) after the addition of 0.2ml of ferricytochrome c (1mM), and (iii) after a further addition of 0.2ml of ferrocytochrome c (1mM). (b) Variation of H$^+$/e$^-$ ratios with total cytochrome c concentration. Conditions of measurement were as for (a).

Discussion

The present results support the view that turnover of proteoliposomal cytochrome c oxidase is associated with an initial phase of proton release into the bulk suspending medium when ferrocytochrome c is added to the aerobic enzyme. Similar acidifications have been observed by Wikström & Saari (1977), Wikström (1981), Casey et al. (1979), Coin & Hinkle (1979), Sigel & Carafoli (1980), Prochaska et al. (1981) and Solioz et al. (1982).
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Fig. 3. Inhibitory effect of ferricytochrome $c$ on ferrocytochrome $c$ oxidation in an aerobic suspension of cytochrome $c$ oxidase proteoliposomes

The oxidation of successive ferrocytochrome $c$ pulses was monitored at $A_{550} - A_{540}$ in a mixture containing $1$ ml of K$_2$SO$_4$ (50 mm), $75$ μl of proteoliposomes (1.28 μM-cytochrome aa$_3$) and valinomycin (1 μg/ml) at pH 7.4 and $30^\circ$C. (a) Guggenheim plot of $n_i + \ln ([\text{Cyt. c}^{3+}]_{t = 0} - [\text{Cyt. c}^{3+}]_t)$ versus time (s), where $[\text{Cyt. c}^{3+}]_t$ is the concentration of ferricytochrome at time $t$, the interval Δ$t$ is $5$ s (the time interval between successive determinations of ferricytochrome concentration) and $n_i$ is an increment of 1 for each curve, $n_1 = 1$, $n_2 = 2$ etc. The lines are displaced for clarity. (b) Plot of the $t_i$ values calculated from the slopes of the Guggenheim plots in (a) versus the total concentration of cytochrome $c$. A $K_m$ of $4$ μM is found by extrapolation.

However, it has often been difficult to assess the decay of the initial acidification to the net alkalinity change expected from the scalar reaction shown in eqn. (1). This difficulty also occurred in the present experiments when the vesicles were prepared in a medium with low buffering capacity (see Table 1), and may reflect the degree to which the internal buffering power of the vesicles can respond to the pH changes (Krab & Wikström, 1978; Sigel & Carafoli, 1980). Nevertheless, under conditions of low turnover, it is still possible that some net acidification is taking place. Casey et al. (1979) found that the ratio of protons consumed/electron equivalents only approached 1 (eqn. 1) in the presence of high concentrations of carbonyl cyanide $m$-chlorophenylhydrazone. In the present experiments, the initial acidification in the absence of carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone does decay and reverse to the expected net alkalinity change (eqn. 1) when vesicles with higher internal buffering capacity are used (Table 1). This is consistent with a process of respiration-dependent proton translocation across the vesicle membrane that is followed by a decay of the resulting pH gradient to the pH changes associated with the scalar reaction.

The inhibitory effects of ferricytochrome $c$ and azide on the proton pulse indicate that the initial stoichiometry may depend on the rate of cytochrome $c$ oxidase turnover, rather than, as previously suggested (Casey et al., 1979; Solioz et al., 1982), on the number of times each enzyme molecule has turned over. The initial $H^+/e^-$ ratios may thus decline when repetitive ferrocytochrome $c$ pulses are used because of the inhibitory effect of the product, ferricytochrome $c$, on turnover. Negatively charged (asolvent) vesicles show a higher cytochrome $c$ affinity than do other enzyme preparations (Nicholls et al., 1980), but Fig. 3(a) shows that first-order kinetics still prevail (Smith & Conrad, 1956). Successively smaller proton pulses occur with repeated additions of ferrocytochrome $c$, and suitable corrections have to be made to assess an intrinsic $H^+/e^-$ ratio. A stoichiometry approaching 1 for the initial acidification change is indicated, in agreement with previous work. In the presence of either ferrocytochrome $c$ or azide, ferrocytochrome $c$ oxidation and proton appearance still occurred faster than the rate of equilibration of the proton gradient. The turnover-dependence of the initial acidification appears to be an intrinsic property of the enzyme and not an artifact resulting from fast equilibration of proton gradients under conditions of low turnover.

The present and previous results show that the stoichiometry of proton transfer in reconstituted cytochrome oxidase vesicles is variable. Some of this variability may arise from experimental conditions such as differences in the internal buffering capacity of the vesicles, but the present results also indicate
an intrinsic dependence of stoicheiometry on the rate of turnover of the enzyme. Cytochrome c oxidase is known to exist in (at least) two functionally distinct forms, which have mainly been distinguished on the basis of catalytic activity (Wilson et al., 1977) but may also differ in conformation (Kornblatt et al., 1975) and spin state (Nicholls & Hildebrandt, 1978). The relative proportions of these two forms during steady-state turnover in reconstituted vesicles are dependent on turnover rate and proton gradient (Wrigglesworth & Nicholls, 1978). One explanation for the present findings is that only one of these enzyme forms is capable of proton translocation. Thus, depending on the turnover conditions, the electrogenic transfer of electrons across the membrane catalysed by cytochrome c oxidase could be augmented by proton-pump activity, as proposed by Wikström (1981). Alternatively, the proton-translocation mechanism may involve more than one step within the membrane, each associated with a diffusional back-reaction. In such a system, despite tight 'coupling', a decrease in turnover with no change in diffusion rate could result in an apparent decrease in stoicheiometry. Intramembranous protons could be able to diffuse back into the liposome interior without ever becoming detectable in the external medium.

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References

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