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

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Tween 80 substituting for Emasol, and was stored at -75°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 *aa*₃) of $250\text{--}300\text{ s}^{-1}$ in the presence of 0.5% Tween, and 0.05% asolectin, in 65 mM-sodium phosphate buffer, pH 7.4, with ascorbate, *NNN'*-tetramethyl-*p*-phenylenediamine dihydrochloride and horse heart 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_2SO_4 to isolate the reduced cytochrome, which was adjusted to the 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*- α -phosphatidylcholine, Sigma type IV-S) was dispersed in 10 ml of 50 mM-potassium phosphate buffer, 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 $27\,000\text{ g}$ for 10 min, and any pellet was discarded. The supernatant was passed through a Sephadex G-75 column equilibrated with 50 mM- K_2SO_4 to remove external buffer.

Proteoliposomal cytochrome *c* oxidase turnover is stimulated by at least 6-fold by addition of valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone 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 anaerobic with externally added ascorbate and cytochrome *c*. The addition of tetramethyl-*p*-phenylenediamine or deoxycholate (0.4%) resulted in the remaining 50% of the cytochrome *c* oxidase becoming reduced. From these and other results (Wrigglesworth, 1978; Wrigglesworth & Nicholls, 1979; Nicholls *et al.*, 1980) it can be concluded that the preparation comprised vesicles incorporating cytochrome *c* oxidase in a transmembrane orientation with a 50:50 distribution of the cytochrome *c* reaction sites on either side of the membrane.

Changes in H^+ activity were monitored in a thermostatically controlled glass chamber fitted with

a magnetic stirrer, by using a glass pH electrode (type G 2222 B or C) connected to a digital pH-meter (pH M64) and recorder (Rec 61 Servograph) (Radiometer, Copenhagen, Denmark). A calomel reference electrode (K701) was used. Samples were introduced into the chamber by injecting small volumes ($5\text{--}100\ \mu\text{l}$) of solution. Spectra were obtained with an Aminco 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. 1*a*, trace i); this H^+ pulse slowly decays to give a net alkalinity change. In the absence of valinomycin (Fig. 1*a*, trace ii) a small acidification is seen, which decays very slowly. Spectral measurements ($A_{550} - A_{540}$) show that cytochrome *c* is rapidly oxidized in the presence and in the absence of valinomycin during the initial part of the H^+ -production phase (Fig. 1*c*). 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 acidification is dependent on the presence of valinomycin plus K^+ , and does not occur in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Fig. 1*a*, trace iii). Nigericin, an electro-neutral ionophore, also abolishes the initial ferrocytochrome *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. 1*b*). The initial pH change caused by addition of HCl was therefore used to calculate the H^+/e^- ratio for ferrocytochrome *c*-induced acidification; the final net changes were calculated from the eventual pH change induced by the 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 H^+ production to the amount of ferrocytochrome *c* oxidized under various experimental conditions are shown in Table 1. An initial, uncoupler-sensitive, acidification was observed 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*₃ concentration of $1.28\ \mu\text{M}$, the initial acidification was

Fig. 1. Ferrocyanide oxidation and induced pH changes in an aerobic suspension of proteoliposomes containing cytochrome *c* oxidase

(a) Ferrocyanide-induced pH changes in a reaction vessel containing 6 ml of K₂SO₄ (50 mM) and 2 ml of proteoliposomes (1.28 μM-cytochrome *aa*₃) at pH 7.0 and 30°C. pH changes were initiated by the addition of 100 μl of ferrocyanide (Cyt. c²⁺) (1.45 mM) (i) in the presence of valinomycin (1.25 μg/ml), (ii) in the absence of valinomycin, and (iii) in the presence of valinomycin (1.25 μg/ml) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (1.25 μM). (b) pH changes observed after external acid addition to the vesicle suspensions (i) and (iii) of (a). (c) Ferrocyanide oxidation at pH 7.0 and 30°C monitored by spectral changes at A₅₅₀ - A₅₄₀ in a parallel experiment. The reaction mixture, in a final volume of 1 ml, contained (i) 0.6 ml of K₂SO₄ (50 mM) plus 0.4 ml of proteoliposomes (1.34 μM-cytochrome *aa*₃), (ii) 0.6 ml of K₂SO₄ (50 mM) plus 0.4 ml of liposomes without incorporated cytochrome *c* oxidase, and (iii) 1.0 ml of K₂SO₄ (50 mM). The changes were started in each case by the addition of 3 μl of ferrocyanide (1.74 mM).

Table 1. Ferrocyanide-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 *aa*₃ concentrations (vesicular). Conditions of measurement were as described in Fig. 1(a) legend. The results are given as the averages ± range for two experiments.

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

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

Turnover effects on ferrocyanide-induced acidifications

Ferrocyanide inhibits the ferrocyanide *c*-induced acidifications catalysed by cytochrome oxidase vesicles (Fig. 2a). The accumulation of oxidized product after successive injections of ferrocyanide *c* or the addition of extra ferrocyanide 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). Ferrocyanide *c* is known to inhibit the oxidation of ferrocyanide *c* by cytochrome *c* oxidase (Smith & Conrad, 1956). If the oxidation time courses are plotted by the

Fig. 2. Inhibitory effect of ferricytochrome *c* on the ferrocyanochrome *c*-induced acidification in an aerobic suspension of cytochrome *c* oxidase proteoliposomes

(a) Ferrocyanochrome *c*-induced pH changes in a mixture of 6 ml of K_2SO_4 (50 mM), 2 ml of proteoliposomes ($1.28 \mu M$ -cytochrome *aa*₃) and valinomycin ($1.25 \mu g/ml$) at pH 7.0 and 30°C. Repetitive injections of ferrocyanochrome *c* (Cyt. c^{2+}) were made (i) before the addition of ferricytochrome *c*, (ii) after the addition of 0.2 ml of ferricytochrome *c* (1 mM), and (iii) after a further addition of 0.2 ml of ferrocyanochrome *c* (1 mM). (b) Variation of H^+/e^- ratios with total cytochrome *c* concentration. Conditions of measurement were as for (a).

Guggenheim method (Fig. 3a), it can be seen that the turnover is progressively decreased by successive repetitive pulses of ferrocyanochrome *c*, because the oxidized cytochrome *c* concentration increases. The magnitude of the ferrocyanochrome *c*-induced acidification is apparently dependent on the rate of cytochrome *c* oxidase turnover.

This conclusion was further tested by use of the cytochrome *c* oxidase inhibitor azide (Fig. 4a). When the enzyme is inhibited by azide, the H^+/e^- ratio falls in parallel with the decline in catalytic activity (Fig. 4b). Under certain conditions, azide can act as a weak uncoupling agent; it increases the proton-leakage rate. Although such an effect may interfere with the measurements of initial proton stoichiometries, it can be seen to be absent or negligible in the present experiments. The equilibra-

tion of the ferrocyanochrome *c*-dependent proton gradient in the presence of azide was always much slower than the rate of proton production, and was about 5 times slower than the equilibration rate in the presence of nigericin (Fig. 4c).

Discussion

The present results support the view that turnover of proteoliposomal cytochrome oxidase is associated with an initial phase of proton release into the bulk suspending medium when ferrocyanochrome *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).

Fig. 3. Inhibitory effect of ferricytochrome *c* on ferrocyanochrome *c* oxidation in an aerobic suspension of cytochrome *c* oxidase proteoliposomes

The oxidation of successive ferrocyanochrome *c* pulses was monitored at $A_{550}-A_{540}$ in a mixture containing 1 ml of K_2SO_4 (50 mM), 75 μl of proteoliposomes (1.28 μM -cytochrome aa_3) and valinomycin (1 $\mu g/ml$) at pH 7.4 and 30°C. (a) Guggenheim plot of $n_i + \ln([Cyt. c^{3+}]_{t+\Delta t} - [Cyt. c^{3+}]_t)$ versus time (s), where $[Cyt. c^{3+}]_t$ is the concentration of ferricytochrome *c* at time *t*, the interval Δt 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_1 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 pre-

viously 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 ferrocyanochrome *c* pulses are used because of the inhibitory effect of the product, ferricytochrome *c*, on turnover. Negatively charged (asolectin) 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 ferrocyanochrome *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 ferrocyanochrome *c* or azide, ferrocyanochrome *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

Fig. 4. Inhibitory effect of azide on the ferrocyanide-induced pH changes in an aerobic suspension of cytochrome *c* oxidase proteoliposomes

The reaction mixture contained 6 ml of K_2SO_4 (50 mM), 2 ml of proteoliposomes ($1.28 \mu\text{M}$ -cytochrome *aa*₃) and valinomycin ($1.25 \mu\text{g/ml}$) at pH 7.0 and 30°C . (a) Ferrocyanide (Cyt. c^{2+}) induced pH changes in the presence of various concentrations of NaN_3 . The broken line shows the trace in the presence of 100 μM - NaN_3 plus nigericin ($1.25 \mu\text{g/ml}$). (b) Variation of the magnitude of (i) the initial acid pulse, expressed as H^+/e^- ratio, with increasing azide concentrations (O), and (ii) turnover of cytochrome *c* oxidase (●) measured in a parallel experiment with an oxygen electrode. (c) Semi-logarithmic plot of decay rates of ferrocyanide-induced acidifications from (a).

an intrinsic dependence of stoichiometry 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 stoichiometry. Intramembranous protons could be able to diffuse back into the liposome interior without ever becoming detectable in the external medium.

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