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Limited-Turnover Studies on Proton Translocation in Reconstituted Cytochrome *c* Oxidase-Containing Vesicles

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We have investigated ferrocytochrome *c*-induced proton ejection from reconstituted cytochrome *c* oxidase-containing vesicles using careful control of the number of enzyme turnovers. Ferrocytochrome *c* caused the appearance of protons at the vesicle exterior, and this could be abolished by using a protonophore. In addition, its decay was dependent on the permeability of the vesicle membranes to protons and the number of turnovers of the oxidase. These observations indicate that the ejection of protons was the result of genuine translocation. The possibility of this translocation occurring via a Mitchellian loop as a result of the presence of a reduced hydrogen carrier contaminating the enzyme was considered and excluded. Proton-translocating activity in this reconstituted system depended critically on the ratio of enzyme to lipid used in the reconstitution process and we propose a rationale to account for this. We conclude that our data provide strong support for the proposal that cytochrome *c* oxidase acts as a proton pump and that approx. 0.9 H^+ is excluded per ferrocytochrome *c* molecule oxidized.

Much controversy exists at present concerning the role of cytochrome *c* oxidase (ferrocytochrome *c*–oxygen oxidoreductase, EC 1.9.3.1) in proton translocation by the mitochondrial respiratory chain. According to the chemiosmotic hypothesis of Mitchell, this enzyme, together with cytochrome *c*, forms the electron-carrying arm of the third loop in his scheme of alternating hydrogen and electron carriers (Mitchell, 1966; Mitchell & Moyle, 1970). The passage of electrons from cytochrome *c* oxidase to molecular oxygen results in the consumption of protons from the mitochondrial interior in the formation of water (Mitchell, 1969; Mitchell & Moyle, 1970). Consequently, reduction of oxygen via cytochrome *c* oxidase might produce an electrochemical proton gradient by vectorial electron transport and by removal of protons at only one side of the mitochondrial membrane. Cytochrome *c* oxidase alone, however, could not achieve net proton translocation. There has been considerable support for this mode of action of the enzyme (Hinkle, 1973; Papa *et al.*, 1974; Skulachev, 1974; Wikström, 1974).

According to proposals made by Wikström and his co-workers, cytochrome *c* oxidase itself may act as a proton pump, without the requirement of an associated hydrogen carrier. These workers have observed proton translocation in intact mitochondria (Wikström, 1977; Wikström & Saari, 1977; Wikström & Krab, 1978, 1979*a*,*b*) and submitochondrial particles (Wikström & Saari, 1977) on the addition of electron donors to cytochrome *c*, and their studies have been supported by work in other laboratories (Sigel & Carafoli, 1978; Sorgato & Ferguson, 1978). It has been suggested that the observed proton translocation occurs via a chemiosmotic mechanism and does not reflect proton translocation by cytochrome *c* oxidase (Moyle & Mitchell, 1978*a*), though this criticism has been countered (Wikström & Krab, 1978). Furthermore, stoicheiometries of diaminodurene-induced proton and charge movements in mitochondria have been reported (Moyle & Mitchell, 1978*b*; Mitchell & Moyle, 1979) that indicate that there is no proton translocation via cytochrome *c* oxidase, and that all charge flux can be accounted for by electron influx linked to O_2 reduction. Wikström & Krab (1979*a*,*b*), however, have criticized these measurements and reported that diaminodurene does indeed cause proton extrusion via cytochrome *c* oxidase. It seems clear that the complexities of the intact mitochondrial respiratory chain make it unsuitable for providing unequivocal evidence for cytochrome *c* oxidase having a proton-translocating activity.

A more reliable system for studying functions specific to cytochrome *c* oxidase is provided by

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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artificial lipid vesicles, prepared in the presence of the purified enzyme. The method of preparation of such reconstituted cytochrome *c* oxidase-containing vesicles is well established (Hinkle *et al.*, 1972; Jasaitis *et al.*, 1972; Carroll & Racker, 1977; Eytan & Broza, 1978), and their properties have been studied extensively (Hunter & Capaldi, 1974; Vik & Capaldi, 1977; Hansen *et al.*, 1978; Hansen & Nicholls, 1978). Wikström and co-workers have reported ferrocytochrome *c*-induced proton extrusion from such reconstituted vesicles (Wikström & Saari, 1977; Krab & Wikström, 1978), in contrast with previous findings of Hinkle *et al.* (1972), and this affords the most convincing evidence so far for a proton-translocating activity of cytochrome *c* oxidase. In the studies reported here, we have extended the investigation of proton translocation in reconstituted cytochrome *c* oxidase-containing vesicles, employing controlled turnovers of the enzyme. Our results provide strong support for the proposal that cytochrome *c* oxidase can carry out true proton translocation and indicate that the number of protons translocated per ferrocytochrome *c* molecule oxidized is close to 0.9.

Experimental

Cytochrome *c* oxidase was prepared from bovine heart by the method of Yu *et al.* (1975). It was stored at −70°C in 0.25M-sucrose/0.05M-sodium phosphate buffer, pH7.4.

Reconstituted cytochrome *c* oxidase-containing vesicles were prepared by a modification of the method of Hinkle *et al.* (1972). A suspension of 40mg of soya-bean phospholipids/ml in 24.5mM-potassium cholate/100mM-Hepes, pH7.2, was sonicated at 6μ peak to peak in an MSE sonicator for 1min per ml of suspension. Cytochrome *c* oxidase was added to give a final concentration of 7.5 or 15μM on a haem basis, depending on the preparation used. The suspension was dialysed for 4h against 100 vol. of 100mM-Hepes, pH7.2, then for a further 4h against 200 vol. of 10mM-Hepes/27mM-KCl/73mM-sucrose, pH7.2, and finally for 12h against 200 vol. of 1mM-Hepes/30mM-KCl/79mM-sucrose, pH7.2. All solutions were adjusted to the indicated pH with KOH. The procedure was carried out at about 4°C. The maximal stimulation of cytochrome *c* oxidase activity in the resulting vesicles by valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone was approx. 5.

Ferrocytochrome *c* was prepared by adding a few grains of sodium dithionite to 0.5ml of 40mg of ferricytochrome *c*/ml/100mM-Hepes/40mM-Tris, pH7.4. To remove excess dithionite, the solution was applied to a column (15cm×1cm) of Sephadex G-25 (coarse grade) eluted with 100mM-choline chloride. The eluted ferrocytochrome *c* had a concentration of approx. 1mM, as measured from its change in absorbance at 550–540nm on oxidation, with a millimolar absorption coefficient of 19.6litre·mmol^{-1}·cm^{-1} (Yonetani, 1965*a*). It was adjusted to the pH of the experimental medium before use.

Changes in pH were measured by using a pH electrode or the pH indicator dye Phenol Red. An Ingold LoT 405-M3 combined pH electrode was used in combination with a Radiometer PHM 64 pH-meter and a W+W 600 chart recorder. The change in absorbance of Phenol Red at 556.5–504.5nm was monitored in an Aminco DW-2a spectrophotometer, fitted with a magnetic stirring device and a thermostatically controlled cuvette holder. At the start of each experiment the $A_{556.5-504.5}$ of the experimental system was adjusted to the same level as that of 100mM-Hepes/50μM-Phenol Red, pH as indicated in the Figure legends. Extreme care was taken in choosing the above wavelengths to ensure that changes in the absorbance of cytochrome *c* or of cytochrome *c* oxidase contributed negligibly to the measured absorbance changes. In addition, control experiments were performed in the presence of 20mM-Hepes, pH7.4, to exclude cytochrome *c*-induced absorbance changes not linked to changes in pH. Merck Titrisol-grade HCl was used to calibrate both methods.

Ferrocytochrome *c* oxidation was measured by monitoring either the change in absorbance of cytochrome *c* at 550–540nm in an Aminco DW-2a spectrophotometer or the consumption of oxygen by using a Clark-type oxygen electrode obtained from Yellow Springs Instrument Co.

Soya-bean phospholipids were recrystallized by the method of Kagawa & Racker (1971) and were obtained from Sigma Chemical Co., as were cytochrome *c*, valinomycin, carbonyl cyanide *m*-chlorophenylhydrazone and Hepes. Cholic acid was recrystallized from sodium cholate obtained from Fluka A.G., CH-9470 Buchs, Switzerland. Triton X-100 was obtained from Merck at 'gas-chromatography' grade. All other reagents were of analytical grade.

Results

Ferrocytochrome c-induced acidification in suspensions of cytochrome c oxidase-containing vesicles

When ferrocytochrome *c* was added to a suspension of reconstituted cytochrome *c* oxidase-containing vesicles in the presence of valinomycin, there was an acidification of the medium, as shown in Fig. 1. Parallel experiments indicated that ferrocytochrome *c* was oxidized and O_2 was consumed concomitantly with the acidification (results not shown). The ratio of protons appearing to molecules of ferrocyto-

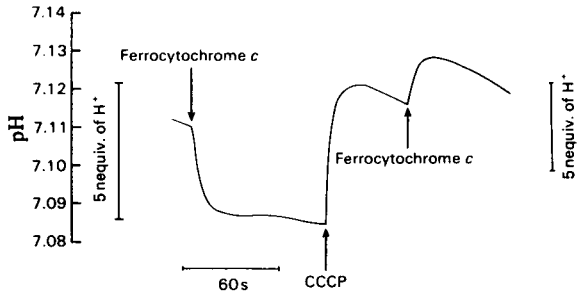

Fig. 1. *Ferrocytochrome c-induced proton ejection from reconstituted cytochrome c oxidase-containing vesicles*
A suspension of cytochrome *c* oxidase-containing vesicles (0.2ml containing 0.86nmol of the enzyme) was added to 2ml of 55mM-KCl/45mM-choline chloride at 15°C, followed by 10μl of 0.2mM-valinomycin. The pH of the suspension was adjusted to that shown and was then monitored with a combined pH electrode. Ferrocytochrome *c* (10μl, 0.35mM) was added as indicated. After approx. 80s, 10μl of 1mM-carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added, followed by a further 10μl of 0.35mM-ferrocytochrome *c* when the resulting pH change was complete. The bars to the left and right of the pH trace indicate the change in pH caused by 5μl of 1mM-HCl in the absence and presence respectively of 10μl of 1mM-carbonyl cyanide *m*-chlorophenylhydrazone.

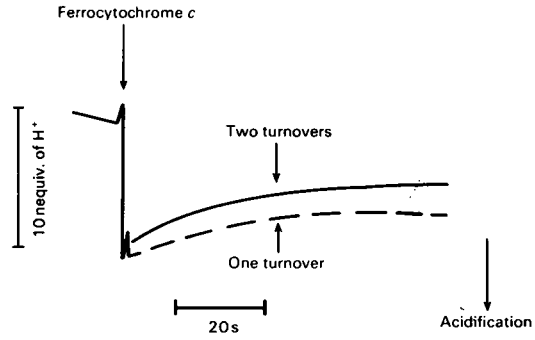

Fig. 2. *Proton ejection from cytochrome c oxidase-containing vesicles induced by one and two turnovers of the oxidase*
A suspension of cytochrome *c* oxidase-containing vesicles (0.2ml containing 2.6nmol of the enzyme) was added to 2.3ml of 30mM-KCl/70mM-choline chloride/50μM-Phenol Red at 15°C, followed by 10μl of 0.2mM-valinomycin. The pH of the suspension was adjusted to 7.2 and its absorbance at 556.5–504.5nm was monitored as described in the Experimental section. At the time indicated 10μl (broken trace) or 20μl (solid trace) of 1.08mM-ferrocytochrome *c* was added. The absorbance trace after the latter addition of ferrocytochrome *c* has been drawn on half-scale to facilitate its comparison with the other trace.

chrome *c* oxidized was 0.9±0.03 (mean±S.D. for six determinations). When carbonyl cyanide *m*-chlorophenylhydrazone was present (Fig. 1) and when valinomycin was omitted (results not shown) ferrocytochrome *c* addition caused no acidification of the medium, but an alkalinization with a stoicheiometry of 1 H^+/mol of ferrocytochrome *c* oxidized. When the oxidative activity of the incorporated cytochrome *c* oxidase was inhibited by approx. 98% with NaN_3, addition of ferrocytochrome *c* under conditions similar to those of Fig. 1 caused no acidification of the extravesicular medium, and oxidation of the ferrocytochrome *c* by 30μM-potassium ferricyanide, thus by-passing cytochrome *c* oxidase, also caused no pH changes (results not shown). The pH changes described here and below were calibrated by using pulses of 1mM-HCl added in the presence or absence of carbonyl cyanide *m*-chlorophenylhydrazone. Much larger pH changes always occurred when no protonophore was present, indicating that the bulk of the buffering capacity lies at the vesicular interior as expected.

The response of the pH electrode used here was relatively slow. Consequently, under conditions where there was an appreciable decay of the ferrocytochrome *c*-induced acidification, changes in the absorbance of Phenol Red were used to give a more exact measure of the pH changes.

In the experiment of Fig. 2, addition of sufficient ferrocytochrome *c* for one turnover of the oxidase resulted in an acidification of the medium, which had only a slight tendency to decay. The extent of acidification was 0.9H^+/mol of ferrocytochrome *c* oxidized, as in Fig. 1. Addition of sufficient ferrocytochrome *c* for two turnovers of the oxidase caused a burst of acidification with a slightly lower ratio of H^+/ferrocytochrome *c* oxidized than with one turnover, and having a more rapid decay.

Effect of low concentrations of uncoupler on the cytochrome c-induced pH change

When carbonyl cyanide *m*-chlorophenylhydrazone was added to cytochrome *c* oxidase-containing vesicles at very low concentrations, the ferrocytochrome *c*-induced acidification was retained with only slightly diminished magnitude, though the subsequent alkalinization was accelerated (Fig. 3*a*). Increasing the concentration of the protonophore further caused the rate of alkalinization to be increased, such that the burst of acidity was decreased in magnitude until finally it was abolished and ferrocytochrome *c* addition led only to alkalinization of the medium. Use of the carbonyl cyanide *m*-chlorophenylhydrazone concentration that caused

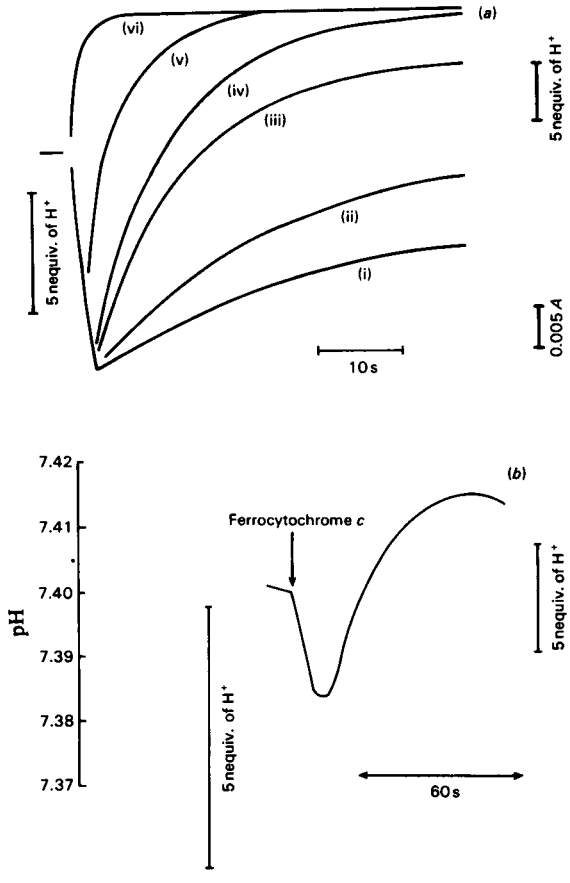

Fig. 3. *Effects of carbonyl cyanide* m-*chlorophenylhydrazone at various concentrations on the ferrocytochrome* c*-induced proton ejection from cytochrome* c *oxidase-containing vesicles*

(*a*) The procedure was as for the experiment of Fig. 2 with the following modifications. The vesicles contained 1.36nmol of cytochrome *c* oxidase, and at the point indicated 10μl of 1.17mM-ferrocytochrome *c* was added. For trace (i) there were no other additions. In all other cases carbonyl cyanide *m*-chlorophenylhydrazone was added immediately after the valinomycin to give concentrations of 4nM (ii), 12nM (iii), 16nM (iv), 20nM (v) or 4μM (vi). Before addition of ferrocytochrome *c*, the suspension was always adjusted to pH7.4 as described in the Experimental section. The bars to the left and right of the absorbance traces indicate the change in absorbance caused by 5μl of 1mM-HCl in the absence and presence respectively of 10μl of 1mM-carbonyl cyanide *m*-chlorophenylhydrazone. (*b*) The procedure was essentially as described for the experiment of Fig. 1. Before the addition of ferrocytochrome *c* (20μl of 0.35mM), however, 35μl of 1μM-carbonyl cyanide *m*-chlorophenylhydrazone was added to the suspension and the pH re-adjusted to that indicated.

the greatest acceleration of the alkalinization phase while only slightly affecting the size of the acid burst made it possible to measure in a single experiment the protons appearing extravesicularly and those consumed in reduction of O_2. In the experiment of Fig. 3(*a*), these were 0.7 and 1 respectively per molecule of ferrocytochrome *c* oxidized. As shown in Fig. 3(*b*), these effects could be demonstrated in a qualitatively similar fashion by using a pH electrode. Although a large proportion of the acid burst was not detected by the electrode, owing to its relatively slow response, the net stoicheiometry of protons consumed/mol of cytochrome *c* was again 1. When ferrocytochrome *c* was added to the vesicles in the presence of uncoupler at any of the above concentrations but in the absence of valinomycin, there was no acidification of the medium.

In the experiment of Fig. 4, ferrocytochrome *c* was again added to a suspension of cytochrome *c* oxidase-containing vesicles in the presence of a low concentration of carbonyl cyanide *m*-chlorophenylhydrazone. Here, however, when the ferrocytochrome *c*-induced changes were complete, the pH of the suspension was adjusted to its initial value. Subsequent ferrocytochrome *c* addition then induced the same acidification and re-alkalinization, and the cycle was repeated several times. This repetitive effect has important consequences for the validity of the cytochrome *c* oxidase proton pump as discussed below.

Effect of varying the number of allowed turnovers of the cytochrome c oxidase on the ratio of protons appearing per cytochrome c molecule

An experiment was carried out similar to that of Fig. 4, except that the quantity of ferrocytochrome *c* was varied in successive additions to allow one, two, three or four turnovers of the oxidase. As shown in Fig. 5, the number of protons appearing in the external medium decreased linearly with the number of allowed turnovers. The net stoicheiometry of protons consumed/mol of ferrocytochrome *c* oxidized, however, was always 1 ± 0.02.

Effect of varying the cytochrome c oxidase/lipid ratio of the reconstituted vesicles on the ferrocytochrome c-induced pH changes

The phenomenon of ferrocytochrome *c*-induced acidification depended critically on the ratio of cytochrome *c* oxidase/lipid employed in the reconstitution process. In addition, the optimal ratio of these substances varied from one enzyme preparation to another. This is illustrated in Fig. 6, which shows the ferrocytochrome *c*-induced pH changes in suspensions of vesicles prepared with the same cytochrome *c* oxidase preparation but having three different oxidase/lipid ratios. With a ratio of

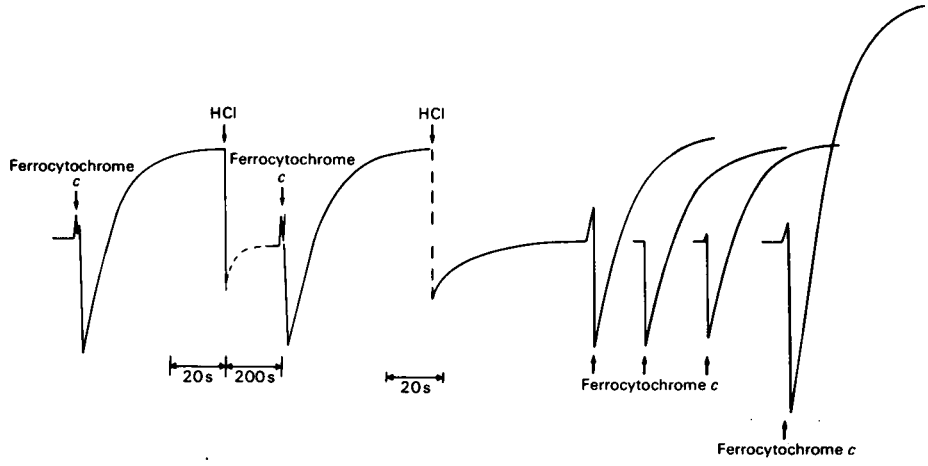

Fig. 4. *Repetitive ferrocytochrome c-induced proton ejection from cytochrome c oxidase-containing vesicles in the presence of a low concentration of carbonyl cyanide m-chlorophenylhydrazone*

A suspension of cytochrome *c* oxidase-containing vesicles (0.2ml containing 1.36nmol of the enzyme) was added to 2.3ml of 66.7mM-choline chloride/25.3mM-KCl/50μM-Phenol Red at 15°C, followed by 10μl of 0.2mM-valinomycin and 14μl of 1μM-carbonyl cyanide *m*-chlorophenylhydrazone. The pH of the suspension was adjusted to 7.4 and its absorbance at 556.5–504.5nm was monitored as described in the Experimental section. At the points indicated, samples of 10μl (and finally of 25μl) of 1.17mM-ferrocytochrome *c* were added. When the pH changes induced by each addition of ferrocytochrome *c* were complete, the absorbance at 556.5–504.5nm was adjusted to its initial value with HCl.

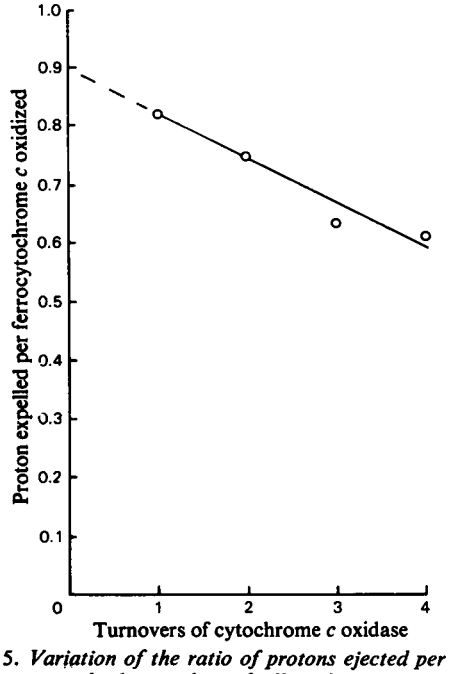

Fig. 5. *Variation of the ratio of protons ejected per cytochrome c with the number of allowed turnovers of the oxidase*

The experimental procedure was as described in the legend to Fig. 4, except that the quantities of 0.08nmol of enzyme/mg of lipid, ferrocytochrome *c* induced a small acidification, but this was made unsuitable for quantitative study by the rapid alkalinization that followed. When the ratio was raised to 0.19 (the optimal ratio for this preparation of enzyme), a stable acidification resulted from ferrocytochrome *c* addition. When the ratio was increased further to 0.38, however, ferrocytochrome *c* caused an alkalinization of the medium with no acidification phase.

Orientation of cytochrome c oxidase in the vesicle membranes

Carroll & Racker (1977) reported incorporation of cytochrome *c* oxidase into lipid vesicles with all the catalytic sites of the enzyme facing outwards and thus accessible to cytochrome *c*. As the reconstitution procedure used here differed slightly from theirs, it was decided to investigate the orientation of cytochrome *c* oxidase in our reconstituted vesicles. The procedure that we employed made use of the fact that dithionite is a slowly permeant anion (Salhany & Swanson, 1978), whereas cytochrome *c* is impermeant through biological membranes. Sodium ascorbate,

1.17mM-ferrocytochrome *c* added were 5, 10, 15 and 20μl, allowing respectively one, two, three and four turnovers of the oxidase.

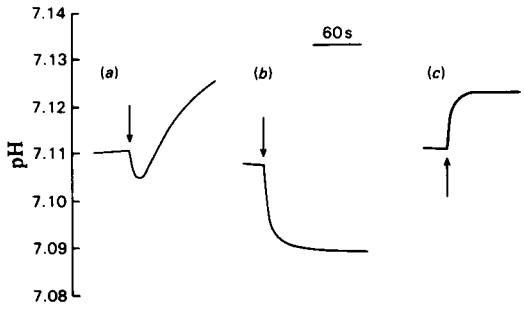

Fig. 6. *Ferrocytochrome c-induced pH changes in suspensions of cytochrome c oxidase-containing vesicles having different cytochrome c oxidase/lipid ratios*
The experimental procedure was as described in the legend to Fig. 1, except that ferrocytochrome *c* was always added in the absence of carbonyl cyanide *m*-chlorophenylhydrazone. The final concentrations of cytochrome *c* oxidase were 0.20 μM (*a*), 0.43 μM (*b*) or 0.93 μM (*c*), giving enzyme/lipid ratios of 0.08, 0.19 and 0.38 nmol/mg respectively.

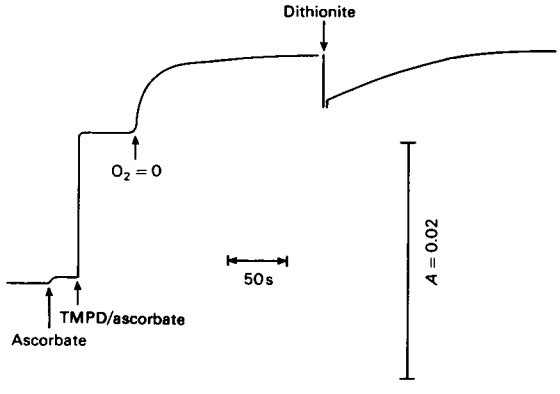

Fig. 7. *Reduction of cytochrome c oxidase by cytochrome c, sodium ascorbate/tetramethyl-p-phenylenediamine and by sodium dithionite*
A suspension of cytochrome *c* oxidase-containing vesicles (0.2 ml containing 3.4 nmol of the enzyme) was added to 2.3 ml of 0.1 M-KCl/10 mM-Hepes/2.3 μM-ferricytochrome *c*, pH 7.07 at 15°C, and the absorbance of the suspension at 603–630 nm was monitored. Then 25 μl of 200 mM-sodium ascorbate, 25 μl of 20 mM-sodium ascorbate/20 mM-tetramethyl-*p*-phenylenediamine (TMPD) and a few grains of dithionite were added at the times indicated.

NNN'N'-tetramethyl-*p*-phenylenediamine and ferricytochrome *c* were added to a suspension of cytochrome *c* oxidase-containing vesicles, causing an increase in the $A_{603-630}$ (Fig. 7), reflecting increased reduction of the enzyme (Yonetani, 1965*b*). After approx. 50s, reduction increased further, indicating exhaustion of O_2 in the cuvette. When a steady absorbance value had been reached, dithionite was added to the suspension, inducing an initial decrease in the $A_{603-630}$, followed by a slow drift back to the value preceding dithionite addition. When this procedure was repeated with the addition of 1% Triton X-100 replacing that of dithionite, there was again no change in the extent of reduction of the enzyme (results not shown). This behaviour was observed with all vesicle preparations used here, including those with high and low enzyme/lipid ratios used in the experiment of Fig. 6, and is consistent with all the incorporated cytochrome *c* oxidase molecules being oriented with their active sites facing the external medium.

Discussion

Cytochrome *c* oxidase from bovine heart spans the inner mitochondrial membrane asymmetrically (Eytan *et al.*, 1975; Henderson *et al.*, 1977). It can thus catalyse the vectorial transfer of electrons from ferrocytochrome *c* to O_2 to form water, the protons being taken from the mitochondrial interior (Mitchell & Moyle, 1970). If this redox process alone were occurring, addition of ferrocytochrome *c* to reconstituted cytochrome *c* oxidase-containing vesicles should result in the consumption of 1 H^+/mol of ferrocytochrome *c* oxidized, as required by the equation:

$$4 \text{ Ferrocytochrome } c + 4\,H^+ + O_2 \rightarrow 4 \text{ ferricytochrome } c + 2\,H_2O$$

In fact, ferrocytochrome *c* caused an acidification of the extravesicular medium, concomitantly with ferrocytochrome *c* oxidation, with a maximal stoicheiometry of 0.9 H^+/electron equivalent. The appearance of protons did not occur in the absence of valinomycin and could be abolished by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone. The possibility that the observed proton appearance was due to a net acidification of the medium on addition or oxidation of ferrocytochrome *c* is ruled out by two observations. First, in the presence of higher concentrations of carbonyl cyanide *m*-chlorophenylhydrazone, the ratio of protons consumed/electron equivalent was 1 in all cases (see Figs. 1, 3*a*, 3*b*, and the experiment of Fig. 5), in agreement with the predictions of the above equation. Any net proton release would have caused this ratio to be less than 1. Second, when vesicles containing azide-inhibited cytochrome *c* oxidase were used, where ferrocytochrome *c* oxidation was carried out with potassium ferricyanide in the absence of significant oxidase activity, there were no changes in the pH of the medium. We believe therefore that

our observations are consistent with the proton-translocating activity of cytochrome *c* oxidase proposed by Wikström and co-workers (Wikström, 1977; Wikström & Saari, 1977; Krab & Wikström, 1978), whereby the oxidation of ferrocytochrome *c* leads to the expulsion of protons from the vesicular interior. The rapid production of a large transmembrane electrical potential, inside negative, through electron influx and proton extrusion would explain the requirement of the acidification for valinomycin. This ionophore, in the presence of external K^+, would allow collapse of the charge differential and, consequently, more extensive and detectable proton translocation. The effect of carbonyl cyanide *m*-chlorophenylhydrazone in allowing equilibration of the proton gradient by increasing the permeability of the vesicles to protons will be looked at in more detail below.

When the number of turnovers of the oxidase was increased, thus enlarging the proposed proton gradient, the decay of the proton pulse was accelerated (Fig. 2). Increasing protonophore concentrations also increased the rate of decay (Fig. 3). This dependence of the decay on the size of the proton gradient and the permeability of the vesicle membranes to protons indicates that it indeed reflected a back-flux of extruded protons.

By using very low concentrations of carbonyl cyanide *m*-chlorophenylhydrazone it was possible to retain the ferrocytochrome *c*-induced proton extrusion with only slightly diminished magnitude, while accelerating its decay considerably (Fig. 3). When a high concentration of protonophore was employed, ferrocytochrome *c* induced only an alkalinization of the medium with the expected stoicheiometry of $1\,H^+$/mol of ferrocytochrome *c* oxidized. With an appropriate protonophore concentration, however, ferrocytochrome *c* caused the extrusion of 0.7 H^+/electron equivalent, followed by an alkalinization to a final level indicating a net consumption of $1\,H^+$/mol of ferrocytochrome *c* oxidized. This observation of the quantitative removal of the protons appearing in the external medium indicates that they were indeed translocated across the vesicle membranes and forced back into the vesicles by the electrochemical proton gradient formed.

The variation of the number of protons ejected/electron equivalent with the amount of added ferrocytochrome *c* (Fig. 3) is again consistent with the protons being actively expelled. More oxidase activity led to a higher transmembrane proton gradient and, consequently, an accelerated proton back-flux. As a result, the extent of proton efflux (and thus the proton/electron ratio) was more underestimated with increasing turnovers of the oxidase. It is noteworthy that extrapolation of the ratio to zero turnovers (and therefore zero transmembrane proton gradient) indicates a ratio of 0.9, as obtained under conditions where there was very slow decay of the proton extrusion (see Figs. 1 and 2).

The experiment of Fig. 7 indicates that all the incorporated cytochrome *c* oxidase molecules were oriented with their active sites facing outwards. This excludes any underestimations of the true ratio of protons expelled/electron equivalent, owing to some of the enzyme being inaccessible to cytochrome *c*. It is thus reasonable to conclude that the true stoicheiometry is close to 0.9. This agrees quite well with the findings of Krab & Wikström (1978), who found a proton/electron stoicheiometry of 1 on comparing the rates of proton efflux and ferrocytochrome *c* oxidation in cytochrome *c* oxidase-containing vesicles. Their maximal proton pulses, however, indicated a stoicheiometry of 0.71, which is slightly lower than that found here. This small discrepancy may have been due to their use of a non-optimal cytochrome *c* oxidase/lipid ratio, as discussed below.

In the experiment of Fig. 4, the presence of carbonyl cyanide *m*-chlorophenylhydrazone at a low concentration allowed the protons extruded on ferrocytochrome *c* oxidation to pass back into the vesicles, giving a net consumption of $1\,H^+$/electron equivalent. After back-titration and the relaxation of any proton gradient (as indicated by the pH trace), further addition of ferrocytochrome *c* caused proton expulsion of only slightly diminished magnitude. In the absence of the protonophore, repeated additions of ferrocytochrome *c* induced successively much smaller proton pulses (results not shown), presumably owing to the build-up of a restraining electrochemical proton gradient.

It is conceivable that the observed proton extrusion occurred via a Mitchellian loop, which was allowed to operate by the presence of a reduced hydrogen carrier contaminating the cytochrome *c* oxidase. This possibility is excluded by the experiment of Fig. 4, where the proton extrusion was repeated six times with the induction of a total of 15 turnovers of the oxidase. As no external hydrogen donor was added, the enzyme would have had to have been contaminated with 60 times its own amount of a reduced hydrogen carrier for a Mitchellian loop to account for the proton translocation. We consider this highly unlikely. The critical dependence of the ferrocytochrome *c*-induced proton extrusion on the enzyme/lipid ratio (Fig. 6) is a particularly important technical point. We feel that this probably reflects the distribution of cytochrome *c* oxidase among the lipid vesicles. The optimal ratio of enzyme/lipid yielded a stable acidification of the medium on ferrocytochrome *c* addition. When this optimum was exceeded the system behaved as though uncoupler were present. This suggests that the capacity of the vesicles for oxidase had been exceeded and that consequently they were disrupted or there was a large amount of unincorporated enzyme. With

suboptimal ratios of enzyme/lipid, the proton pulse decayed very rapidly. This indicates the formation of a very large proton gradient on ferrocytochrome *c* oxidation. This would occur on induction of several turnovers of oxidase incorporated into relatively few vesicles. The determination of the same orientation of vesicular cytochrome *c* oxidase regardless of enzyme/lipid ratio indicates that, in all three kinds of vesicle, cytochrome *c* oxidase functioned normally.

Our results provide strong support for the proposal that cytochrome *c* oxidase is a proton pump. The fact that the protons appearing in the external medium passed back quantitatively into the vesicles indicates that they were the result of true translocation.

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