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ON THE STOICHIOMETRY AND THERMODYNAMICS OF PROTON-PUMPING CYTOCHROME *c* OXIDASE IN MITOCHONDRIA

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Summary

Different approaches have been used to evaluate the stoichiometry of proton translocation linked to cytochrome *c* oxidase in rat liver mitochondria.

A mathematical model was designed that successfully describes the kinetics of redox-linked proton translocation provided that the rate of electron transfer is not too high.

With ascorbate as reductant, an essentially pH-independent (in the pH range 6—8.5) proton ejection stoichiometry ($\overleftarrow{H}^+/e^-$) is obtained from either initial rates of H^+ ejection (0.86 ± 0.12), or the model (0.87 ± 0.14).

Similar results are obtained with either ferrocyanide, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine or externally added cytochrome *c* mediating between ascorbate and cytochrome *c* in rotenone- and antimycin-inhibited mitochondria.

Oxygen pulse experiments with ferrocytochrome *c* as substrate show fully uncoupler-sensitive redox-linked proton ejection with a stoichiometry of 0.78 ± 0.14.

With murexide to measure Ca^{2+} uptake during oxidation of ferrocyanide, we found a stoichiometry of two positive charges taken up/electron transferred, confirming earlier findings.

These results provide strong evidence that cytochrome *c* oxidase functions as a redox-linked proton pump with a stoichiometry of one H^+ ejected and two charges translocated/electron transferred.

The thermodynamic consequences of the proton pump are discussed and a

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Abbreviations: $\overleftarrow{H}^+/e^-$, protons ejected from mitochondria/electron transferred; $\overleftarrow{q}^+/e^-$, net charge translocation (positive charge, inside to outside)/electron transferred; $\overrightarrow{H}^+$/ATP, protons translocated (outside to inside)/ATP synthesised; EGTA, ethylene glycol bis(β-aminoethylether)-*N,N'*-tetraacetate; FCCP, trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

maximal P/O ratio of $1\frac{1}{3}$ for 'site 3' is predicted in agreement with state 4 redox potentials and phosphate potential.

Introduction

In contrast to the view that cytochrome *c* oxidase exclusively catalyses vectorial electron transfer across the mitochondrial inner membrane [1,2], much evidence has recently been brought forward indicating that the redox reaction is linked to true proton transport across the membrane with a $\overleftarrow{H}^+/e^-$ stoichiometry of 1 and a $\overleftarrow{q}^+/e^-$ stoichiometry of 2. This evidence has been obtained from studies with intact mitochondria [3—6], inverted submitochondrial particles [4,7—9] and liposomes reconstituted with purified cytochrome *c* oxidase [4,10].

As it is now generally understood that redox energy can be stored as an electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) across the mitochondrial inner membrane before it is used (e.g. to drive the phosphorylation of ADP to ATP) [11], it is clear that a study of the mechanism of generation of $\Delta\tilde{\mu}_{H^+}$ is essential for the understanding of the mechanism of oxidative phosphorylation. Therefore it is of great importance to test our proposal of a proton-translocating function of cytochrome *c* oxidase under as many conditions as possible.

Main consequences of the proposed proton-pumping activity of cytochrome *c* oxidase are, that for every electron transferred from cytochrome *c* to oxygen, two protons are taken up from the aqueous matrix space (M-space), one proton is released into the outside (cristae) aqueous space (C-space; the other proton being consumed in the reduction of oxygen to water) and two positive charges are translocated from the M- to the C-side of the membrane [12]. One of these charges originates from the translocated proton, and the other results from the combination of an electron originating in the C-space (where cytochrome *c* reacts) with a proton originating in the M-space, in the reduction of oxygen.

It is obvious that the exact stoichiometries of proton uptake and release and of charge transfer are important not only for the information they contain about the actual mechanism of energy conservation, but also because they have direct consequences for the thermodynamic relationship between redox potential and protonmotive force ($\Delta\tilde{\mu}_{H^+}$) and, indirectly, for that between redox potential and phosphorylation potential.

In this paper we will demonstrate the proton-translocating function of cytochrome *c* oxidase with the oxygen pulse technique [13,14], using externally added ferrocytochrome *c* as substrate. We will also evaluate the stoichiometry of proton ejection when mitochondria catalyse oxidation of ascorbate by oxygen (with several reagents mediating between ascorbate and endogenous cytochrome *c*).

Proton ejection stoichiometry is measured from extents of H^+ ejection, from initial rates, or indirectly (and independently) by using a mathematical model designed to describe the proton-pumping function of oxidase. All methods yield values close to one H^+ ejected/electron transferred. The effects of extramitochondrial pH and of rate of electron transfer on this stoichiometry are studied, and limitations of the model used are discussed.

The stoichiometry of charge translocation is determined with ferrocyanide as reductant and Ca^{2+} as permeant ion. From the influx of Ca^{2+} a stoichiometry of two charges/electron is obtained, confirming earlier findings [5,6].

These results are in contrast to recently published experiments of Moyle and Mitchell, who, using the oxygen pulse technique with ferrocytochrome *c* as substrate, did not observe any H^+ ejection [2,15]. We will discuss some probable reasons for this discrepancy.

Methods and Materials

Rat liver mitochondria were prepared as described previously [16]. Ferrocytochrome *c* was prepared by reducing horse heart cytochrome *c* (Boehringer) with a few grains of solid dithionite, an excess of which was removed by chromatography on a Sephadex G-25 column.

Mitochondrial protein was determined according to Lowry et al. [17] using human serum albumin as a standard.

Ferrocyanide, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate solutions were prepared freshly each day.

pH was measured with an Ingold combination electrode connected to an Instrulab IM 555 pH meter. Traces were calibrated using standard solutions of oxalic acid and HCl.

Oxygen consumption was measured with a Clark electrode (Yellow Springs) equipped with an extra thin Teflon membrane and connected to an oxygen probe amplifier designed and built at the Johnson Research Foundation, Philadelphia. Calibration was done as described in detail previously [18].

Optical measurements were done with a DBS-1 dual wavelength spectrophotometer (Johnson Research Foundation).

FCCP was a gift of Dr. P.G. Heytler; all other chemicals were commercially available products of the highest purity.

Results

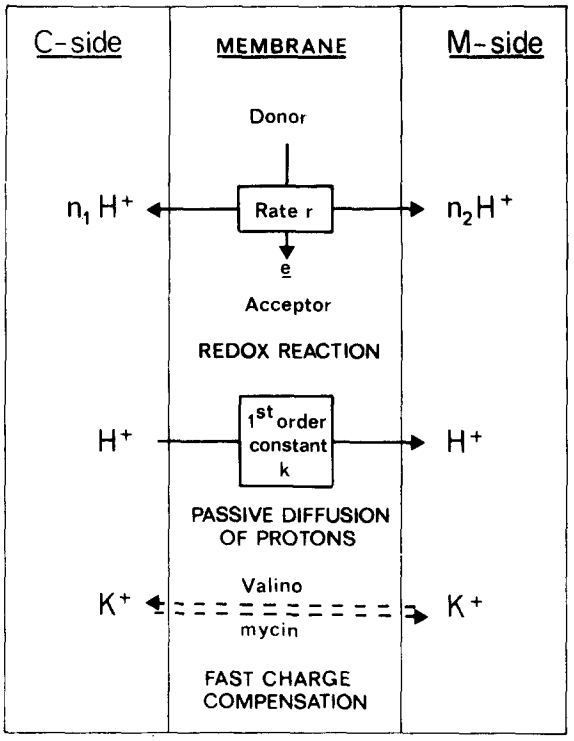
Mathematical model of H^+ translocation

In Fig. 1A a scheme is given which is used to describe the pH changes which may occur when aerobic mitochondria are pulsed with a reductant. It shows the mitochondrial inner membrane together with the two aqueous phases that surround it. The membrane-bound respiratory enzyme catalyses electron transfer from reductant to oxidant with a rate r, which is assumed to be constant during the experiment. n_1 and n_2 indicate the stoichiometry (H^+/e^-) by which protons are released on either side of the membrane, obligatorily linked to electron transfer. These numbers include uptake or release of protons by the reductant or by the oxidant, and may be negative (indicating proton uptake). V_1 and V_2 are factors with the dimension of volume, which relate the total number of protons available in the two water phases (N_1 and N_2) to the free proton concentrations:

$$[H^+]_1 = N_1/V_1 \tag{1a}$$

$$[H^+]_2 = N_2/V_2 \tag{1b}$$

V_1 and V_2 thus contain the buffering properties of the two phases, and are



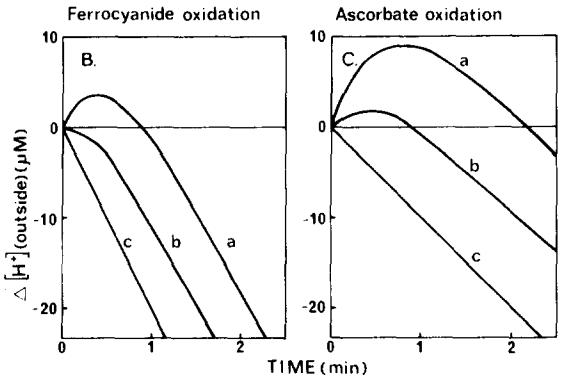


Fig. 1. A theoretical model of redox-linked proton translocation. (A) Scheme illustrating the model. Explanation see text. (B) Simulated proton ejection with ferrocyanide as reductant. Curve a: $n_1 = 1$, $n_2 = -2$, $k/V_2 = 0.3\ s^{-1}$. Curve b: $n_1 = 0$, $n_2 = -1$, $k/V_2 = 0.3\ s^{-1}$. Cuve c: $n_1 + n_2 = -1$, k/V_2 infinitely large. (C) Simulated proton ejection with ascorbate as reductant. Curve a: $n_1 = 1.5$, $n_2 = -2$, $k/V_2 = 0.2\ s^{-1}$. Curve b: $n_1 = 0.5$, $n_2 = -1$, $k/V_2 = 0.3\ s^{-1}$. Curve c: $n_1 + n_2 = -0.5$, k/V_2 infinitely large. In all simulations $r = 20\ \mu M\ e^-$/min.

assumed to be constant during the experiment under consideration.

The single arrow in Fig. 1A indicates unspecific proton diffusion across the membrane with first-order rate constant k. The scheme also indicates that any electric potential difference across the membrane is efficiently nullified by electrophoretic movement of a permeant ion, e.g. K^+ in the presence of valinomycin (double arrow in Fig. 1A). It may therefore be assumed that proton

diffusion is linearly proportional to the concentration difference of H^+ across the membrane. Using the definitions of Eqn. 1, the unspecific diffusion rate of H^+ is therefore given by:

$$V_{diff} = k(N_2/V_2 - N_1/V_1) \tag{2}$$

where k is the diffusion constant.

With the three assumptions made (constant r, constant V_1 and V_2 and linear diffusion) the changes in amounts of protons in the two aqueous phases with respect to time can be described by:

$$\frac{dN_1}{dt} = n_1 r + k(N_2/V_2 - N_1/V_1) \tag{3a}$$

$$\frac{dN_2}{dt} = n_2 r - k(N_2/V_2 - N_1/V_1) \tag{3b}$$

Solution of these equations yields:

$$\Delta N_1 = \frac{V_1 V_2}{V_1 + V_2}\left[\frac{(n_1 + n_2)r}{V_2} \cdot t - \frac{(V_2 n_1 - V_1 n_2)r}{(V_1 + V_2)k}\left(\exp\left\{-kt\left(\frac{1}{V_1} + \frac{1}{V_2}\right)\right\} - 1\right)\right] \tag{4a}$$

$$\Delta N_2 = \frac{V_1 V_2}{V_1 + V_2}\left[\frac{(n_1 + n_2)r}{V_1} \cdot t + \frac{(V_2 n_1 - V_1 n_2)r}{(V_1 + V_2)k}\left(\exp\left\{-kt\left(\frac{1}{V_1} + \frac{1}{V_2}\right)\right\} - 1\right)\right] \tag{4b}$$

Since V_1 and V_2 reflect to a large extent the actual volumes of the outer and inner aqueous phase, respectively, V_2 is much smaller than V_1. Eqn. 4a may therefore be approximated by:

$$\Delta N_1 = (n_1 + n_2)rt + (n_2 r V_2/k)[\exp\{-kt/V_2\} - 1] \tag{5}$$

In Fig. 1B and C some theoretical curves derived from this expression are shown. In Fig. 1B curves are given for the oxidation of ferrocyanide by oxygen. This is shown for a pure electron-translocating oxidase, as proposed by Mitchell and Moyle [1,2] (b), for a proton-translocating oxidase as proposed by Wikström [3] (a), and for both cases in the presence of uncoupler (k is very large, c). In Fig. 1C similar curves are given with ascorbate as reductant, which itself produces one proton/two electrons on oxidation at pH 7.

Oxidation of ascorbate

Fig. 2 depicts an experiment using ascorbate plus ferrocyanide as reducing system. As shown in Fig. 2B this results in a reasonably constant rate of electron transfer, one of the necessary conditions for using Eqn. 5. The medium contains Mg^{2+} to promote the reaction between ferrocyanide and the respiratory chain [19], so that a relatively low concentration of ferrocyanide can be used to mediate between ascorbate and endogenous cytochrome *c*.

It may be derived from Eqn. 5 that the trace shown in Fig. 2A (as the traces in Fig. 1) has an asymptote with slope $(n_1 + n_2)r$, and that the vertical distance d from the curve to this asymptote (see Fig. 2A) depends exponentially on time.

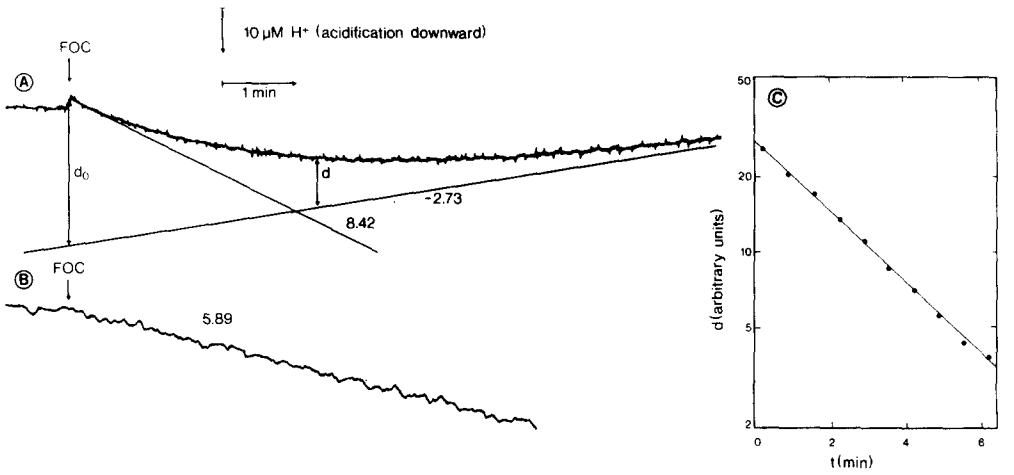


Fig. 2. Proton ejection and uptake coupled to oxidation of ascorbate catalysed by rat liver mitochondria plus ferrocyanide. The medium contained 150 mM sucrose, 30 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM Hepes, 1.4 mM ascorbate, 5.6 μM rotenone, 0.12 μg/ml antimycin, 1.87 μg/ml oligomycin, 187 μM *N*-ethylmaleimide, 0.19 μg/ml valinomycin and 1.51 mg/ml mitochondrial protein, at pH 7.1 and 21°C. This medium was divided between a pH meter vessel (2 ml) and an oxygraph (1.75 ml), both connected to the same thermostate, and the reaction was started by addition of 1 mM potassium ferrocyanide (FOC) to both vessles. (A) Proton uptake and release. ———, initial rate of proton release, and asymptotic rate of proton uptake. *d*, the distance between the curve and the asymptote (d_0 at $t = 0$). (B) Parallel measurement of oxygen uptake. (C) Semilogarithmic plot of *d* against time.

Fig. 2C shows that this prediction from the model is fulfilled experimentally. From an experiment such as that of Fig. 2 the stoichiometric numbers n_1 and n_2 can be calculated in two ways.

(1) From the initial rate of H^+ ejection. From the slope of the asymptote of the trace in Fig. 2A ($(n_1 + n_2) \cdot r = -2.73$ μM H^+/min) and the slope of the trace in Fig. 2B ($r = 5.89$ μM e^-/min) it can be calculated that $(n_1 + n_2) = -0.46$ H^+/e^- (which corresponds to a proton release by ascorbate of 1.08 $H^+/2e^-$ at this pH). From Eqn. 5 it can be derived that the initial slope of the trace in Fig. 2A (after an initial alkalinisation artefact upon addition of ferrocyanide that is discussed elsewhere [18]) should be equal to $n_1 \cdot r$ (=8.42 μM H^+/min). Hence it can be calculated that $n_1 = 1.43$ and $n_2 = -1.89$ H^+/e^-. Subtracting the H^+ released on oxidation of ascorbate (0.54 H^+/e^-, see above) the cytochrome *c* oxidase reaction is found to be linked to ejection of 0.89 H^+/e^- into the C-space, and to uptake of 1.89 H^+/e^- (n_2) from the M-space. This agrees well with our previous findings (see Introduction).

(2) From the exponential parameter by use of the model. Measurements of initial rates are usually difficult and prone to error. Therefore a second method to calculate n_2 may be used. From Eqn. 5 it can be seen that the initial distance to the asymptote (d_0, see Fig. 2A) equals $n_2 \cdot rV_2/k$ (=34.15 μM H^+). When this is multiplied by the apparent diffusion constant obtained from Fig. 2C ($k/V_2 = 0.325$ mM^{-1}), and divided by r (=5.89 μM e^-/min), the result yields directly $n_2 = -1.88$ H^+/e^-, which corresponds to 0.88 H^+/e^- translocated across the membrane, and is in excellent agreement with the result obtained independently above.

In Fig. 3 the results of a treatment like this are shown for experiments performed over a range of different pH values. The number of protons pumped across the membrane/electron transferred (a) is plotted, as calculated either from the initial slope (A) or from the parameters of the exponential (B). It is clear that the stoichiometry of H^+ ejection is essentially independent of extramitochondrial pH in the range between pH 6 and pH 8.5, and that the stoichiometries obtained with the two methods agree closely.

Variation of mediator in the ascorbate system

Fig. 4 shows the comparison of several reducing systems for the oxidase. In this case the slopes of traces like that of Fig. 2A are divided by r and then plotted, so that a curve is obtained described by:

$$\text{slope} = (n_1 + n_2) - n_2 \cdot \exp\{-kt/V_2\} \qquad (6)$$

For clarity the results which are obtained in the presence of uncoupler are also included. From these plots an impression can be easily obtained of n_1 (from the initial value), $(n_1 + n_2)$, and of the kinetics of back diffusion of protons.

It is clear that TMPD can substitute for ferrocyanide (see also [6]) without changing either n_1 (which is about 1.5) or the diffusion kinetics (compare Fig. 4A and B). However, with externally added ferrocytochrome c as mediator between ascorbate and oxidase, n_1 appears to be 1 and the backflow of protons is much faster (Fig. 4C).

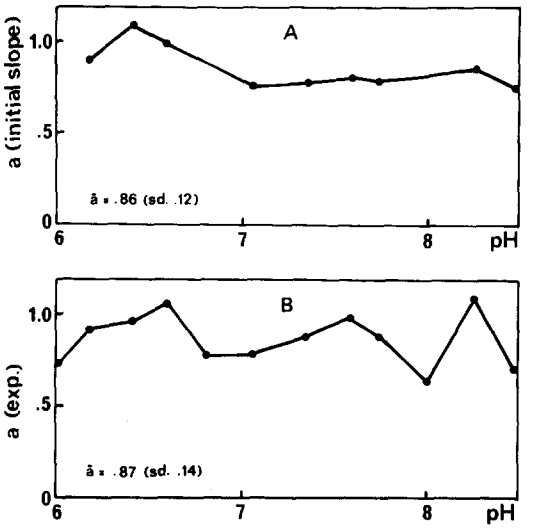


Fig. 3. Calculated stoichiometry of proton translocation as a function of pH. The medium contained 150 mM sucrose, 20 mM KCl, 10 mM $MgCl_2$, 5 mM EDTA, 1 mM Hepes, 9,6 μM rotenone, 0.06 μg/ml antimycin, 160 μM *N*-ethylmaleimide, 0.06 μg/ml oligomycin, 0.03 μg/ml valinomycin, 1.6 mM ascorbate and 1.05 mg/ml mitochondrial protein at 22°C. The reaction was started by addition of 0.4 mM potassium ferrocyanide. Oxygen consumption and changes in pH were recorded simultaneously as described in the legend to Fig. 2. (A) The number of protons (a) transferred across the membrane as calculated from the initial slope. (B) The number of protons as calculated from the exponential parameters (see text). The numbers given refer to the means of the determinations (when it is assumed that there is no effect of pH) together with the standard deviation.

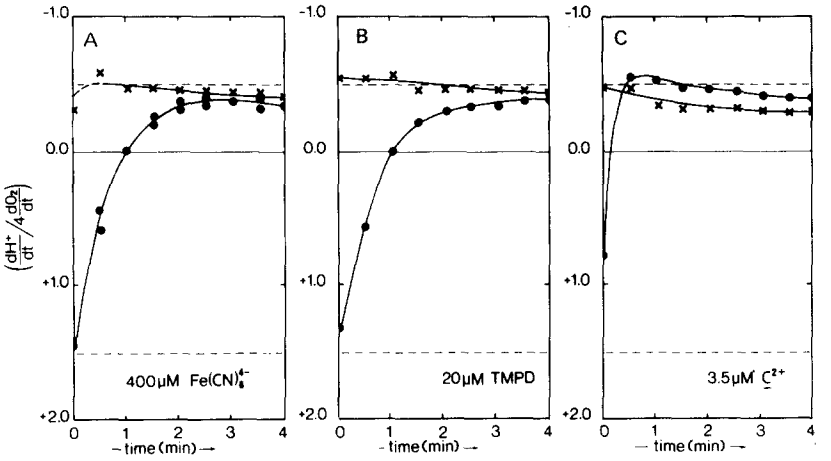


Fig. 4. The effect of different mediators between ascorbate and (endogenous) cytochrome *c*. The medium contained 150 mM sucrose, 30 mM KCl, 10 mM $MgCl_2$, 5 mM EDTA, 1 mM Hepes, 6 mM ascorbate, 11 μM rotenone, 0.23 μg/ml antimycin, 0.07 μg/ml mitochondrial protein, at pH 7.1 and 19.5°C. The reaction was started with 0.4 mM ferrocyanide (A), 20 μM TMPD (B), or 3.5 μM ferrocytochrome *c* (C). Oxygen uptake and pH changes were recorded as described for Fig. 2, and the ratio of the slopes of the pH trace to the O_2 uptake trace is plotted as a function of time. •, in the absence of uncoupler; X, plus 0.9 μM FCCP. - - - - - -, theoretical initial (+1.5) and final (—0.5) values of the ratio of slopes.

The lower value of n_1 is caused by the fact that reduced cytochrome *c* (which is oxidised very rapidly) was used to initiate the reaction, and that the reduction rate of oxidised cytochrome *c* by ascorbate is slow under the prevailing conditions (not shown). So during the initial stages of the reaction cytochrome *c* (an electron donor at this pH) is oxidised, and all the proton production seen is due to the proton pump (with the expected stoichiometry of one H^+/e^-). In later stages also the protons produced by ascorbate oxidation become significant, so that the final value for $(n_1 + n_2)$ in Fig. 4C is similar to those in Fig. 4A and B.

The fast backflow of H^+ seen in Fig. 4C may be explained by the very high oxidation rate of ferrocytochrome *c*, which leads to a fast exhaustion of the proton buffer inside the mitochondria. Especially under these conditions the assumption of a constant V_2 (see above) might be longer hold. The observation that slow backflow kinetics are seen, when oxidised cytochrome *c* is used to start the reaction in the presence of ascorbate (not shown) agrees with this explanation.

It is possible to test the effect of an exhausted inner proton buffer also with ascorbate plus ferrocyanide as reducing system. From the traces shown in Fig. 5A it can be seen that when *r* is increased by raising the concentration of ferrocyanide, the proton backflow rate becomes faster (which can be seen from a decrease of the time at which the trace 'turns', τ). Moreover, the proton uptake phase no longer follows the simple kinetics predicted by the model. Hence the H^+ transport can be described adequately by Eqn. 5 only at relatively low respiratory rates (*r*). Fig. 5B shows that there is a linear relationship between *r* and the inverse of τ, the latter of which can be shown to be proportinal to k/V_2 in cases where Eqn. 5 applies.

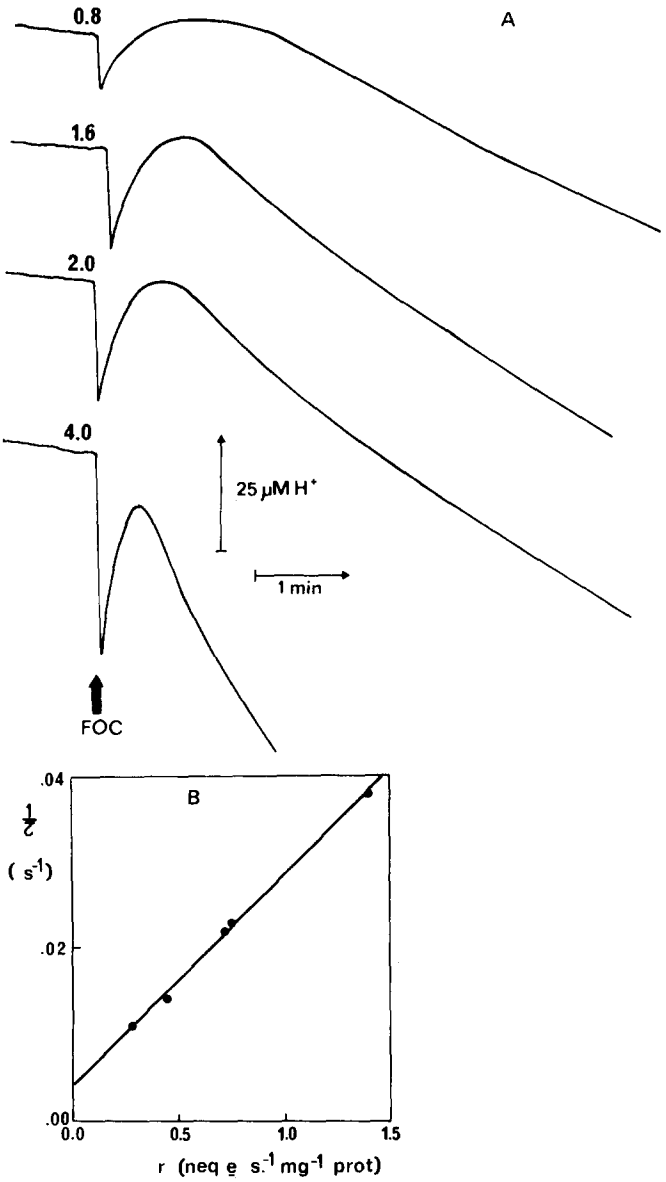


Fig. 5. The effect of the variation of the rate of the redox reaction on the kinetics of proton movement. The medium contained 150 mM sucrose, 30 mM KCl, 10 mM $MgCl_2$, 5 mM EDTA, 1 mM Hepes, 1.6 mM ascorbate, 9.6 μM rotenone, 0.21 μg/ml antimycin, 320 μM *N*-ethylmaleimide, 0.08 μg/ml valinomycin and 0.8 mg/ml mitochondrial protein at pH 7.12 and 22°C. The reaction was started by addition of various amounts of ferrocyanide (FOC, concentrations in mM are given in the figure). (A) Proton ejection and uptake. (B) Plot of $1/\tau$ against r. τ is the time at which the traces shown in (A) turn from net ejection of protons to net uptake.

Oxygen pulse with ferrocytochrome c as substrate

Fig. 6 shows an example of oxygen pulse measurements (for technique, see [14]) with rat liver mitochondria oxidising added cytochrome *c*. Even though cytochrome *c* is a pure electron donor at pH 7, the pulse of oxygen-containing

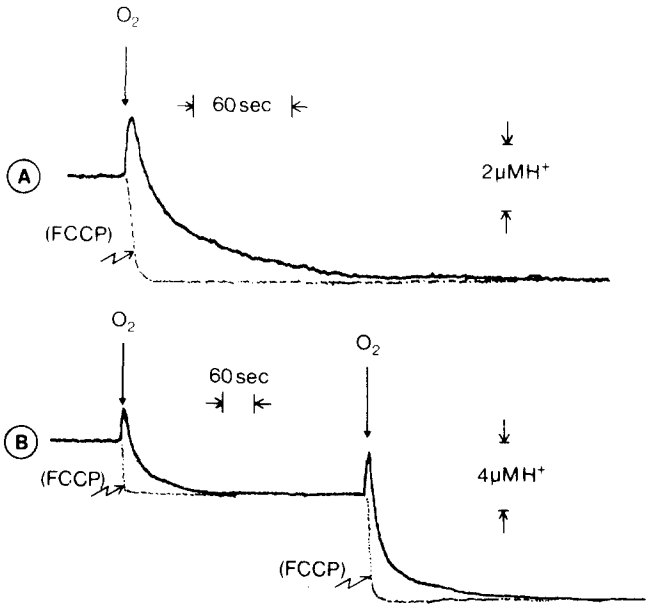


Fig. 6. Proton translocation linked to oxidation of cytochrome *c* by rat liver mitochondria. The medium contained 120 mM KCl, 1 mM EGTA, 1 mM Hepes, 10 mM $MgSO_4$, 15 μM rotenone, 0.53 μg/ml valinomycin, 5.3 μg/ml oligomycin and 263 μM *N*-ethylmaleimide, saturated with N_2 gas. Rat liver mitochondria were added to a final concentration of 0.46 μM cytochrome aa_3 (approx. 3.3 mg/ml mitochondrial protein). After a further incubation for 5 min ferrocytochrome *c* was added to a concentration of 118 μM, and after a further 5 min, 0.084 nmol/mg protein antimycin was added. Final volume 3.8 ml, pH 7.04, temperature 24°C. After a 15—20 min incubation oxygen was added as aliquots of $KCl/MgSO_4$/EGTA/Hepes-containing medium, the oxygen concentration of which was simultaneously measured polarographically in a separate cuvette, connected to the same thermostate. The oxygen addition in (A), and the first addition in (B) correspond each to 1.57 μM O, while the second addition in (B) is 3.13 μM O. · · · · · ·, otherwise identical experiments in the presence of 0.33 μM FCCP. The starting level (before O_2 addition) of the FCCP experiments has been adjusted (with HCl) to be the same as the corresponding trace without uncoupler.

medium unequivocally results in ejection of H^+ from the mitochondria. The net H^+ ejection is completely abolished in the presence of the uncoupler FCCP (dotted traces). In contrast to analogous experiments with hydrogen donors as substrate, the reduction of oxygen by ferrocytochrome *c* is linked to an overall uptake of one H^+/e^-. This is seen as an alkalinisation of the medium after each pulse of oxygen (Fig. 6). It is essential to note that the extent of overall alkalinisation is the same (within 5% experimental error) whether FCCP is present or not, and equivalent to consumption of exactly four H^+/O_2 added. As pointed out previously [18], this finding excludes the possibility that the H^+ ejection is due to a scalar production of acid, coupled to oxidation of cytochrome *c*. The properties of the H^+ ejection are fully consistent with true proton transport across the membrane (see Discussion).

The stoichiometry of H^+ ejection (the $\overleftarrow{H}^+/e^-$ quotient) may be evaluated in this type of experiment by extrapolation of the decay phase to a point halfway between addition of oxygen and anaerobiosis (see [14]). Application of this procedure to data such as those of Fig. 6 yields $\overleftarrow{H}^+/e^-$ stoichiometries close

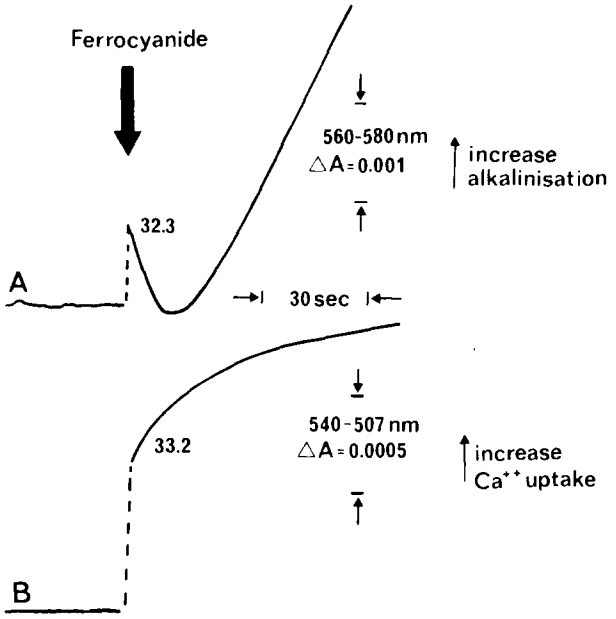


Fig. 7. Calcium uptake induced by oxidation of ferrocyanide by rat liver mitochondria. The medium contained 200 mM sucrose, 20 mM KCl, 1 mM Hepes, 19 μM rotenone, 0.2 μg/ml antimycin, 62.5 μM $CaCl_2$ and approx. 2 mg/ml mitochondrial protein. (A) 62.5 μM phenol red present, pH changes measured at 560—580 nm. (B) 62.5 μM murexide present, changes in Ca^{2+} concentration measured at 540—507 nm.

to unity. In a typical series of eight separate oxygen pulses of different magnitude the quotient was 0.78 ± 0.14 (mean ±S.D.).

Charge transfer stoichiometry

In the absence of valinomycin, Ca^{2+} can take the place of K^+ in compensating for the charge separated caused by the oxidase [5]. In Fig. 7 proton and calcium movements are shown which can be measured when mitochondria are oxidising ferrocyanide. From the initial rates it can be calculated that 1.03 Ca^{2+} are taken up/proton extruded. This confirms our earlier findings, using Arsenazo III as indicator of Ca^{2+} concentration, instead of murexide. Because Ca^{2+} is taken up with two electrical charges [20,21] this means that two charges are translocated/electron transferred, which fits with the expectation based on a proton pump that transports one H^+/e^-. This finding also agrees with the results of Sigel and Carafoli [6], who used a potassium-sensitive electrode to measure K^+ movements in the presence of valinomycin.

Discussion

The model of Fig. 1A, together with the assumptions made, gives an adequate description of proton transport coupled to activity of cytochrome *c* oxidase, and the subsequent back-diffusion of protons, provided that the rate of the redox reaction is not too high, and that the proton buffer in the aqueous M-space is not exhausted. It seems that the critical approximation made in the derivation of Eqn. 5 is considering the factor V_2 a constant, related to the

diffusion of H^+ as indicated by Eqn. 2 *. A better description of the diffusion process might be given by assuming the H^+ diffusion to be proportional to $\Delta\tilde{\mu}_{H^+}$ (in this case, with KCl and valinomycin present: ΔpH, see also [22]), but this would still require a quantitative treatment of the buffering properties of the two water phases. Such an extension would impede the explicit solution of the equivalent of Eqn. 3.

In cases of low electron transfer rates, where the model fits the experimental data, the stoichiometry obtained from the exponential parameters agrees very well with the values obtained from initial rates with ferrocyanide as single reductant (see also [3—5]. This stoichiometry of H^+ ejection falls in the range 0.7—1.0 (Fig. 3).

The same stoichiometry is found whether ferrocyanide, TMPD or (taking into account the fast oxidation of ferrocytochrome *c* and its slow re-reduction by ascorbate) externally added cytochrome *c* is used as a mediator between ascorbate and endogenous cytochrome *c*. It is unlikely that a shunt around the antimycin block [2] is responsible for the observed proton ejection. If this were the case, TMPD, ferrocyanide and cytochrome *c* would be expected to give rise to different stoichiometries, according to their respective tendencies to shortcircuit site 2. Moreover, we have previously shown directly [18] that ferri-/ferrocyanide does not shunt electron across the antimycin block under these conditions, and Sigel and Carafoli [6] have shown that in the case where TMPD mediates between ascorbate and endogenous cytochrome *c*, the TMPD-catalysed shunt around the antimycin block is much to slow to account for the rate of proton ejection observed in this system.

A proton ejection stoichiometry of 0.78 $\overleftarrow{H^+}/e^-$ is obtained from the extents of proton ejection in a series of oxygen-pulse experiments with ferrocytochrome *c* as substrate in excellent agreement with the previous type of experiment. This result moreover agrees with reductant-pulse experiments using the same substrate [18]. In contrast with this, however, Moyle and Mitchell [2,15, 23] have reported that no H^+ extrusion is linked to cytochrome *c* oxidase activity during oxygen-pulse experiments with ferrocytochrome *c* as substrate.

Instead these authors found an artefactual acidification linked to the reaction, which was insensitive to uncouplers (contrast Fig. 6), and which they assumed could explain our previous experimental findings. Moyle and Mitchell also reported that this artefact was abolished by Mg^{2+} [2,23], and that no H^+ ejection was observed under such conditions. In view of this controversy we must re-emphasize that artefacts of this kind were excluded in all our published cytochrome *c* pulse experiments both with cytochrome *c* oxidase vesicles [4,10] and with mitochondria [18], by the demonstration that the overall consumption of H^+ is independent of uncoupler and, within experimental error, equal to one H^+/e^- (see also p. 10).

Our oxygen-pulse experiments (Fig. 6) were performed under conditions similar to those described by Moyle and Mitchell [2,23], except that we used a

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* Were the assumption not valid that V_2 is much smaller than V_1, comparison of Eqns. 4a and 5 shows that the latter can still be used to fit the data with apparent values for n_1, n_2 and k/V_2. In such a case n_1 (apparent) can be shown to be equal to n_1 so that the correct stoichiometry of H^+ ejection will nevertheless be obtained.

KCl medium instead of sucrose. The absence of the acidification artefact is ascertained by 10 mM $MgSO_4$ (see [23]) and is also directly verified from the extent of overall H^+ uptake/oxygen added. The unequivocal H^+ ejection shown in Fig. 6 is hence in clear contrast to the absence of such an effect in Moyle and Mitchell's experiments. A detailed study of their published experiments reveals, however, that under all conditions where the acidification artefact was suppressed, the experiment was either done in the absence of added K^+ [15,23] or *N*-ethylmaleimide [2].

In the former case the necessary charge compensation for H^+ translocation, viz. by influx of K^+ catalysed by valinomycin, is insufficient as we have shown previously [24], lowering considerably extramitochondrial detection of translocated H^+. In the absence of *N*-ethylmaleimide there is very fast re-uptake of H^+ with phosphate, driven by the pH gradient [24,25], which also largely prevents detection of H^+.

In oxygen-pulse experiments with added ferrocytochrome *c* as substrate, an appreciable antimycin-insensitive re-reduction of the cytochrome by endogenous hydrogenated substrates may occur (see [15]). This side reaction is, however, much slower than oxidation of the cytochrome and H^+ ejection which follows the oxygen pulse. This artefact, when appreciable, nevertheless makes quantitative analysis of the experiment very difficult due to acidic drifts in the pH traces after anaerobiosis. Fortunately this obstacle can be overcome (as shown in Fig. 6) by using relatively low concentrations of mitochondria, but more important, by including *N*-ethylmaleimide. It can be shown directly (unpublished results) that *N*-ethylmaleimide effectively blocks antimycin-insensitive re-reduction of cytochrome *c*. Hence there are two good reasons to include *N*-ethylmaleimide in this type of experiment.

Recently Lehninger and coworkers have claimed that transfer of electrons through cytochrome *c* oxidase is coupled to release of two H^+/e^- [26,27]. According to these authors this result is only obtainable with high ferrocyanide concentration (or with TMPD + ascorbate), hence at high rates of electron transfer. We have not been able to reproduce these results either under identical (unpublished results) of slightly different conditions [4,24].

From a thermodynamic point of view the stoichiometries of proton uptake, proton ejection and charge translocation are compatible with the state 4 redox potential difference between cytochrome *c* and oxygen (see e.g. [28]) and the $\Delta\tilde{\mu}_{H^+}$ in state 4 [29]. When it is taken into account that under most conditions the pH change in the matrix is much larger than that extramitochondrially upon transport of protons from inside to outside, it is clear that ΔpH is determined mainly by the rise of pH in the M-space. Therefore, uptake of two protons at the inside, transfer of two positive charges from the inside to the outside and release of one proton at the outside in the cytochrome *c* oxidase reaction is energetically closely equivalent to electrogenic transport of two protons from the inside to the outside.

In state 4 a maximal $\Delta\tilde{\mu}_{H^+}$ of 228 mV has been measured [29], and the redox potential difference between cytochrome *c* and oxygen amounts to about 500 mV. The transfer of one electron from cytochrome *c* to oxygen hence provides enough energy to transport two protons across the membrane at near-equilibrium conditions. Since it appears most likely, both from thermo-

dynamic considerations [29—32] and from direct measurement of $\ddot{H}^+/ATP$ [33], that three H^+ are translocated back into the M-space for each ATP produced extramitochondrially, we would expect that the maximal P/O quotient of phosphorylation linked to the cytochrome *c* oxidase reaction is $2 \times (2\ddot{H}^+/e^-)/(3\ddot{H}^+/ATP) = 1\frac{1}{3}$. This does not disagree with reported P/O quotients [34], but a more thorough study of 'site 3' phosphorylation seems to be indicated. Recently Brand et al. [35] have found that the stoichiometry of charge translocation of site 3 is about $\frac{2}{3}$ of that of sites 2 + 3, which fits excellently with our expectation [12].

The finding that the H^+/electron stoichiometry hardly varies between pH 6 and pH 8.5 (see also [4]) is a property one would expect from an efficient energy-conserving translocation of protons. As already pointed out before [12] a change of stoichiometry upon changing the pH would imply a loss of energy in the coupling process between redox reaction and phosphorylation of ADP in that pH range.

We conclude from this and earlier [3—5,7,12,10,18] studies that with a variety of substrates, techniques and preparations it can be unequivocally shown that redox activity of cytochrome *c* oxidase is coupled to the transport of one H^+/e^- across the inner mitochondrial membrane (inside to outside), with translocation of two positive charges/e^- in the same direction. The proton-pumping activity is in accordance with the thermodynamic properties of this part of the respiratory chain.

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