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THE MECHANISM OF ENERGY CONSERVATION AND TRANSDUCTION BY MITOCHONDRIAL CYTOCHROME *c* OXIDASE

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SUMMARY

Oxidation of ferrocytochrome *c* by molecular oxygen catalysed by cytochrome *c* oxidase (cytochrome aa_3) is coupled to translocation of H^+ ions across the mitochondrial membrane. The proton pump is an intrinsic property of the cytochrome *c* oxidase complex as revealed by studies with phospholipid vesicles inlayed with the purified enzyme. As the conformation of cytochrome aa_3 is specifically sensitive to the electrochemical proton gradient across the mitochondrial membrane, it is likely that redox energy is primarily conserved as a conformational "strain" in the cytochrome aa_3 complex, followed by relaxation linked to proton translocation. Similar principles of energy conservation and transduction may apply on other respiratory chain complexes and on mitochondrial ATP synthase.

INTRODUCTION

Cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) is the terminal member of the respiratory chain catalysing transfer of electrons from cytochrome *c* to molecular oxygen. The enzyme is a lipoprotein complex of approx. 200 000–250 000 molecular weight localised in the mitochondrial inner (cristae) membrane of eucaryotic cells, and in the cytoplasmic membrane of some bacteria [1]. The complex contains 6–7 different subunits [2–5] and four one-electron redox centres, two hemes (*a* and a_3) and two protein-bound copper ions [6, 7]. In addition to its function as a terminal oxidase, cytochrome aa_3 must somehow be involved in conservation of the free energy released in the redox reaction at the so-called third coupling site of oxidative phosphorylation. In the present report, the latter function has been elucidated showing that the enzyme complex is equipped with a proton pump which may be conformationally coupled to the redox reaction. A preliminary account of these findings has been recently published [8].

Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonate; NEM, *N*-ethylmaleimide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Tricine, *N*-tris(hydroxymethyl)methylglycine; WB, Wurster's blue; 1799, α,α'-bis(hexafluoracetonyl)acetone.

A central feature of Mitchell's chemiosmotic hypothesis of oxidative phosphorylation [9, 10] is that the respiratory chain complexes are arranged asymmetrically (in so-called redox loops) so that alternating transfer of hydrogen atoms and electrons in opposite directions across the mitochondrial membrane gives rise to an electrochemical proton gradient (protonmotive force) as the primary form of conserved energy. Cytochrome *c* oxidase is thought to form the electron-carrying arm of one such redox loop [11], in which the electrons are transferred from ferrocytochrome *c* on the outside of the membrane across the membrane continuum with consumption of H^+ ions inside upon reduction of dioxygen to water. This model has recently gained much support from studies with phospholipid vesicles inlayed with purified cytochrome *c* oxidase [12, 13] and has been considered the best documented example of transmembrane electron transfer in the respiratory chain [14, 15]. In contrast, the chemical and conformational hypotheses of oxidative phosphorylation [16–22] assume primary energy conservation as a high-energy form or conformational "strain" of the redox carrier complex itself, the relaxation of which couples to ATP synthesis by protein-protein interaction between the redox complex and ATP synthase. In the chemiosmotic hypothesis, ATP synthesis is assumed to occur at the expense of the protonmotive force by reversal of the proton-translocating ATPase of the mitochondrial membrane [9, 23].

The demonstration of energy-dependent shifts in the light absorption spectrum of mitochondrial cytochrome aa_3 that could not be explained as being due to a change in the redox state of the complex [24–28] suggested that the chemistry and conformation of the hemes is dependent on the mitochondrial energy state. Such a dependence would be expected a priori if redox energy were primarily conserved as a conformational "strain" in the cytochrome aa_3 complex (see e.g. refs. 25, 29, 30), since reversal of the partial reactions of oxidative phosphorylation would then be expected to result in accumulation of such a "high-energy" form of the respiratory complex. Since it has been shown that the spectral shift is accompanied by sizeable alterations in the ligand affinities and redox properties of the aa_3 hemes [27, 28, 31, 32] and in the kinetics of the intramolecular electron transfer reactions [31], it is safe to conclude that changes of considerable chemical and physical relevance with respect to the functional properties of the enzyme take place upon mitochondrial energisation.

We have recently demonstrated that the energy-dependent spectral shift in ferric cytochrome aa_3 correlates with the total electrochemical proton gradient across the mitochondrial membrane rather than with either of its component forces, the membrane potential or the pH gradient, alone [8, 33]. This finding is of great interest since it suggests that the conformational changes in hemes aa_3 may be linked to proton translocation across the mitochondrial membrane and further, that cytochrome *c* oxidase may function as a proton pump that is conformationally coupled to the redox reaction (see also ref. 34).

EXPERIMENTAL

The possibility that the cytochrome *c* oxidase reaction is coupled to the function of a proton pump was tested by pulsing isolated rat-liver mitochondria with ferrocyanide. The substrate end of the respiratory chain was appropriately inhibited by rotenone and antimycin to minimise oxidation of endogenous substrates. Ferro-

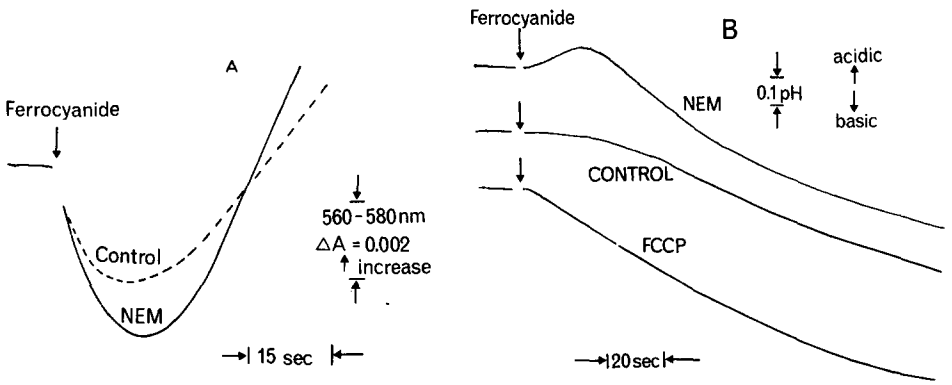

Fig. 1. Proton ejection from mitochondria coupled to oxidation of ferrocyanide. A, Rat liver mitochondria (4.9 mg protein/ml) were suspended in 0.12 M KCl/1 mM HEPES buffer, pH 7.2, containing 5 μM rotenone, 0.23 μg/ml antimycin, 0.07 μg/ml valinomycin, 7.1 μg/ml oligomycin, 0.71 mM EDTA and 25 μM phenol red. The suspension was preincubated for 5 min at room temperature in the presence or absence of 0.36 mM NEM before addition of 1.4 mM potassium ferrocyanide (shown in the figure). Dual wavelength spectrophotometry at the indicated wavelength couple. A downward deflection of the trace corresponds to a decreased absorption difference and acidification of the suspension. B, Rat liver mitochondria (1.8 mg protein/ml) were suspended in 0.15 M KCl/1 mM HEPES, pH 7.2, in the presence of the same constituents as described under A except phenol red. 0.18 mM NEM and 1 μM FCCP were present when indicated. The reaction was started by addition of 1.8 mM potassium ferrocyanide. Direct pH electrode measurements as described in Materials and Methods.

cyanide donates electrons to cytochrome *c* under such conditions [35], the latter being reoxidised by oxygen as catalysed by cytochrome aa_3. Oligomycin was further added to abolish re-entry of protons coupled to phosphorylation of endogenous ADP. The experiments were performed in KCl medium (see Materials and Methods) in the presence of valinomycin to ensure that possible electrogenic efflux of H^+ ions would be electrically balanced by influx of potassium ions.

Fig. 1 shows that protons are indeed ejected into the reaction mixture upon addition of ferrocyanide, as measured both with a pH indicator (Fig. 1A) and a pH electrode (Fig. 1B). Proton ejection is not observed if ferricyanide is added instead of ferrocyanide [8], nor in the presence of cyanide, indicating that proton ejection is coupled to oxidation of the added ferrocyanide by oxygen as catalysed by cytochrome oxidase. The initial acid burst following addition of ferrocyanide is soon followed by monotonous alkalinisation as expected from the reduction of O_2 by an electron donor (Fig. 1). After rendering the mitochondrial membrane permeable to protons with the uncoupling agent FCCP (Fig. 1B), only the latter alkalinisation is observed. This suggests that the initial appearance of protons may indeed be due to proton transport, and is supported by the finding that nigericin, which catalyses electroneutral H^+/K^+ exchange [36], has the same effect (see also Fig. 5). Leaving out the valinomycin from the reaction mixture considerably decreased or abolished net proton appearance suggesting that it may be due to electrogenic proton transport. In the absence of a charge-compensating counterflow of K^+ ions, electrogenic H^+ transport would be expected to come quickly to a halt due to build-up of a membrane potential. Leaving out the oligomycin had, in general, a much less pronounced inhibitory effect.

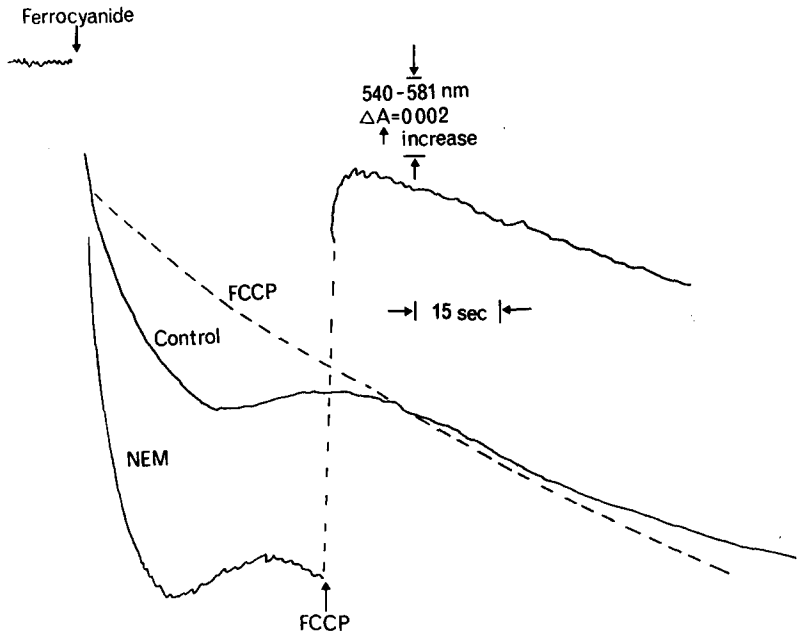

Fig. 2. Intramitochondrial alkalinisation coupled to oxidation of ferrocyanide. Experimental conditions as described in the legend to Fig. 1A, except that the medium consisted of 0.12 M KCl/20 mM HEPES, pH 7.2, and 71 μM neutral red was added instead of phenol red. When indicated, 0.36 mM NEM and 1 μM FCCP were also present. Dual wavelength spectrophotometry at the indicated wavelength couple. Downward deflection indicates intramitochondrial alkalinization.

Inhibition of the H^+/P_i symporter of the mitochondrial membrane by *N*-ethylmaleimide (NEM, see ref. 37) was initially found to be necessary for observation of net proton ejection under the above conditions (ref. 8 and Fig. 1B). However, we have more recently observed proton ejection, though less extensive, also in the absence of NEM (Fig. 1A). Hence, it is likely that variations in the amount of inorganic phosphate that leaks out of the mitochondria during preincubation and is subsequently taken up with H^+ ions account for this variation in the effect of NEM. The absence of valinomycin, oligomycin and NEM in the ferrocyanide pulse experiments of Mitchell and Moyle [38] may be the reason why these authors did not observe net proton ejection.

Intramitochondrial pH may be monitored spectrophotometrically using the permeable pH indicator neutral red under conditions of a well-buffered suspending medium [8, 39]. As shown in Fig. 2, definite alkalinisation of the intramitochondrial space follows the pulse of ferrocyanide. This effect is strongly counteracted by making the membrane permeable to protons by FCCP or by leaving out the valinomycin. The alkalinisation is strongly enhanced by NEM, which is in line with the effect of this compound upon extramitochondrial pH (cf. Fig. 1). This data is consistent with electrogenic transport of protons across the mitochondrial membrane driven by the cytochrome oxidase reaction, but is also expected from mere H^+ consumption in O_2 reduction in the matrix space [11]. True H^+ transport is supported by the fact that following large pulses of ferrocyanide, the number of protons ejected extramitochon-

TABLE I

H^+ EJECTION COUPLED TO FERROCYANIDE OXIDATION BY RAT LIVER MITOCHONDRIA

Experimental conditions were as described in the legend to Fig. 1A, but using varying concentrations of ferrocyanide. Parallel experiments were performed at the wavelength couple 420 minus 500 nm in the absence of phenol red for measurement of the rates of production of ferricyanide (see also ref. 8). The initial rates of H^+ ejection and ferricyanide production were calculated from initial slopes (mixing time 1 s or less) using calibration of respective signals with HCl and ferricyanide standard solutions respectively. 0.36 mM *N*-ethylmaleimide was present in all experiments.

Ferrocyanide (mM)	H^+ ejection (nmol/min per mg protein)	Ferricyanide production	H^+/e^-
0.13	4.1	3.6	1.14
0.34	8.5	8.8	0.97
0.67	19.4	18.3	1.06
1.35	31.3	38.1	0.82

drially exceeds the concentration of respiratory chains (calculated as the concentration of cytochrome c_1) by a factor larger than 25. It is therefore unlikely that the protons are released from membrane components on initiation of respiration. However, this does not of course imply that the protons may not be transiently bound to membrane components (a "proton pump") during their passage across the membrane.

Direct comparison of the initial rates of proton ejection with the rate of electron transfer (conventiently measured spectrophotometrically as the rate of appearance of ferricyanide, see Table I) yields data suggesting a stoichiometry of 1 ejected H^+ ion per transferred electron ($2H^+/2e^-$) over a wide range of electron transport rates (Table I). As expected from the overall reaction: $2e^- + 1/2\ O_2 + 2H^+ \rightarrow H_2O$, the stoichiometry of protons consumed per electron in the presence of an uncoupling agent was found to be 1.0. The latter result serves as an internal control of appropriate calibration of the phenol red and ferricyanide signals. In addition, care was taken to control the validity of the absorption change in phenol red as a measure of H^+ ions. Thus experiments were performed at various wavelength pairs to test for possible interference from light scattering or absorption changes in cytochromes. It was found that such changes did not significantly affect the data reported in Table I. The observed H^+/e^- ratio of proton ejection was apparently lowered upon increasing the the rate of respiration even further than shown in Table I, by employing higher concentrations of ferrocyanide (at 7.2 mM ferrocyanide the respiratory rate was 84 nmol ferricyanide produced per min per mg protein; observed $H^+/e^- = 0.52$). This is most likely an artifact due to very fast development of a large pH gradient (see Fig. 2) due to the rapid turn-over. This is expected to cause significant rates of proton re-entry at the time of measuring the "initial rate" of proton appearance extramitochondrially, and therefore to an underestimated H^+/e^- ratio. At the lower rates of electron transport (Table I), intramitochondrial pH had only shifted very slightly (as revealed by the neutral red signal) at the time of the extramitochondrial rate determination.

The H^+/e^- ratios were found not to change significantly upon lowering the temperature from 23 to 5 °C. Variation in the pH of the mitochondrial suspension between 6.5 and 7.8 also had little effect, with slightly lower stoichiometries at the pH extremes probably attributable to partial uncoupling.

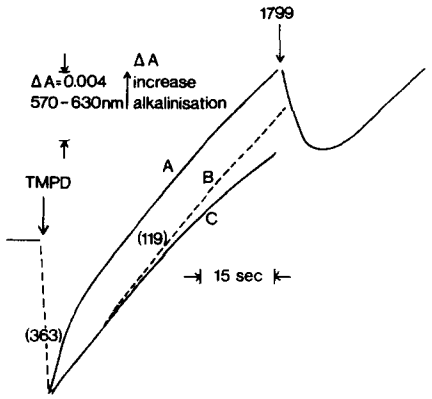

Fig. 3. Proton uptake into sonicated submitochondrial particles. The reaction medium consisted of 150 mM KCl/1 mM HEPES (pH 7.4) and was supplemented with 0.07 μg/ml valinomycin (traces A and C), 0.36 mM *N*-ethylmaleimide, 2.5 μg/ml oligomycin, 58 μM phenol red, 4.2 mM potassium ascorbate and submitochondrial particles (2.85 mg protein per ml; final concentration of cytochrome aa_3 = 1.4 μM). In trace C, a further addition of 0.96 μM of the uncoupling agent 1799 (α,α'-bis (hexafluoracetonyl)acetone) was made initially. Respiration was initiated by addition of 12 μM TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) as indicated. Dual wavelength spectrophotometry at the indicated wavelength couple where medium alkalinisation causes an upward deflection of the trace. The absorption changes were calibrated in the measured range by additions of standard solutions of HCl (not shown). The numbers adjacent to the traces refer to rates of proton consumption in nanomol H^+/min. Final volume, 1.34 ml. Temperature, 23 °C. The rate of oxygen consumption under identical conditions was 108 nanomol O/min as measured polarographically, and was unaffected by the presence or absence of 1799 and/or valinomycin.

Fig. 3 presents experiments performed with sonicated submitochondrial particles from beef-heart mitochondria. These vesicles of the inner mitochondrial membrane are oriented "inside out" in comparison to the parent mitochondrion [40]. It is therefore possible to test whether the protons seen ejected extramitochondrially linked to cytochrome oxidase do indeed originate in the matrix space as should be the case for a true transport process. Ascorbate plus the redox couple *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)/Wurster's Blue was used as electron-donating system to cytochrome *c*, which is located on the inside of the membrane in these "inverted" vesicles (see ref. 41). The TMPD/Wurster's Blue couple is a pure electron carrier ($n = 1$) at physiological pH. Oxidation of ascorbate to dehydroascorbate ($n = 2$) is associated with release of one H^+ ion at pH 7 followed by slower hydrolysis of the dehydroascorbate with release of another equivalent of H^+ ions. As shown in Fig. 3, the addition of TMPD (ascorbate is present) initiates alkalinisation of the vesicle suspension. In the presence of an uncoupling agent (trace C) or in the absence of valinomycin (trace B) the rate of alkalinisation for the first 30 s matches closely the rate of respiration on a two-electron basis as expected from the overall reaction: ascorbate$+1/2\,O_2+H^+ \rightarrow$ dehydroascorbate$+H_2O$. At later stages the rate of alkalinisation declines though the rate of oxygen consumption remains constant, apparently due to the production of acid linked to hydrolysis of dehydroascorbate. In contrast, as shown in trace A, under conditions optimal for electrogenic proton transport (cf. Fig. 1 and Table I) H^+ ions are consumed at a rate three times faster for the first 5 s after TMPD addition before the steady state is reached. Thus a total of

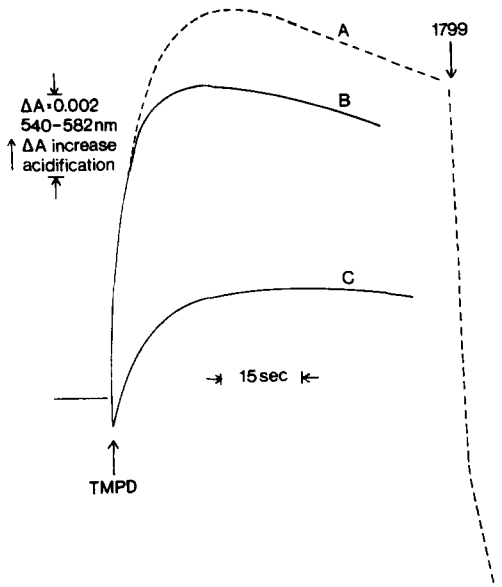

Fig. 4. Intravesicular acidification in sonicated submitochondrial particles. Reaction medium: 200 mM sucrose/20 mM KCl/20 mM HEPES (pH 7.2). Valinomycin, NEM, oligomycin, ascorbate and submitochondrial particles were added as described in the legend to Fig. 3. Neutral red was present at a concentration of 77 μM. Trace A: the uncoupler 1799 was added (0.96 μM) as shown in the figure. Trace B: in the absence of oligomycin and NEM. Trace C: in the absence of valinomycin. Dual wavelength spectrophotometry at the indicated wavelength couple where intravesicular acidification corresponds to an upward deflection of the trace. The conditions were in all other respects identical to those described in the legend to Fig. 3, respiration being initiated by addition of 12 μM TMPD as indicated. Neutral red is sensitive to intravesicular pH changes only due to penetration of the dye through the membrane and efficient extravesicular buffering (cf. Fig. 2 and ref. 39).

$3H^+/2e^-$ are consumed initially followed by the steady state at 1 $H^+/2e^-$, the initial burst being abolished by uncoupling agents or by leaving out the valinomycin. The latter reagents did not affect the rate of oxygen consumption in these experimental conditions. This strongly suggests that the cytochrome *c* oxidase reaction is indeed linked to electrogenic transport of $2H^+/2e^-$ across the mitochondrial membrane in excellent agreement with the data from intact mitochondria (Fig. 1, Table I and ref. 8).

As shown in Fig. 4, the redox activity is moreover accompanied by acidification of the intravesicular space as revealed by the neutral red technique (cf. Fig. 2), the acidification being sensitive to uncoupling agents and valinomycin in the same fashion as the alkaline burst observed extravesicularly.

Taken together, the evidence presented in Figs. 1–4 and Table I strongly indicates that the cytochrome *c* oxidase reaction is linked to electrogenic proton transport across the mitochondrial membrane at a stoichiometry of 2 transported H^+ ions per pair of electrons. It should be pointed out that this activity occurs in addition to the consumption of $2H^+/2e^-$ in oxygen reduction (see Discussion).

To distinguish whether the proton pump linked to cytochrome *c* oxidase redox activity is an intrinsic function of the aa_3 complex itself or, alternatively, due to a separate molecular entity which may be only functionally coupled to cytochrome oxidase, we tested phospholipid vesicles inlayed with the purified enzyme [12, 13] for

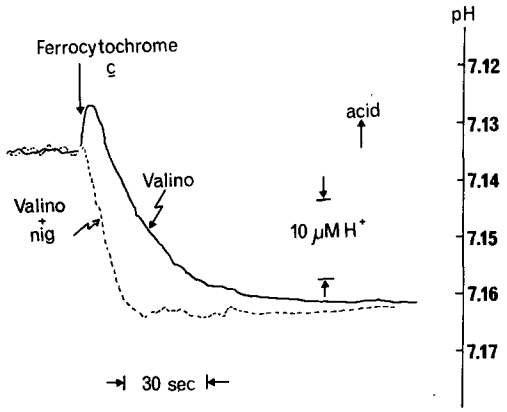

Fig. 5. Proton ejection from cytochrome *c* oxidase vesicles. Cytochrome oxidase vesicles (made from mitochondrial phospholipids, see Materials and Methods) were suspended in 0.1 M KCl (unbuffered) to a final concentration of 0.06 μM cytochrome aa_3 in the presence of 0.96 μg/ml valinomycin, with or without a further addition of 0.2 μg/ml nigericin. After 5 min preincubation 10 μl of ferrocytochrome *c* suspension was added, the pH of which was previously adjusted to be the same as that of the cytochrome oxidase vesicle suspension, to a final concentration of 20 μM. The pH electrode traces are shown for experiments in the presence and absence of nigericin. The changes in hydrogen ion concentration in the measured pH range were calibrated by additions of small aliquots of standard HCl and KOH solutions.

"proton pump" activity. As shown in Fig. 5, a pulse of ferrocytochrome *c* to the suspension of cytochrome oxidase vesicles led first to ejection of protons into the reaction medium, followed by medium alkalinisation (cf. Fig. 1). Net ejection of protons was again counteracted by uncoupling agents or nigericin. It may be noted from Fig. 5 that the total change in pH on addition of 20 μM ferrocytochrome *c* corresponded closely to the consumption of 20 μM of H^+ ions both in the presence and absence of nigericin. This agrees well with the stoichiometry of the overall reaction of oxidation of cytochrome *c* by dioxygen with respect to the consumption of protons. In the absence of nigericin, at least 5 μM of H^+ ions are released initially into the medium, and are subsequently consumed in addition to the 20 μM H^+ consumed in O_2 reduction. Thus release of protons from cytochrome *c* upon oxidation is probably not the explanation for the initial acid burst. Moreover, the redox change in cytochrome *c* is known to occur without dissociation of protons at neutral pH [42]. The amount of protons ejected initially (Fig. 5) exceeds the concentration of cytochrome aa_3 by almost two orders of magnitude, indicating that the protons ejected are indeed derived from the inner aqueous space of the vesicles. Moreover, efficient intravesicular buffering at high pH (accomplished by Tricine, see Materials and Methods) was found necessary in order to observe proton ejection. We therefore conclude that the proton transport function is most probably an intrinsic property of the cytochrome *c* oxidase complex as isolated (see Materials and Methods) and properly embedded in its natural milieu, a phospholipid membrane.

As shown in Fig. 6, the energy-dependent spectral shift in ferric cytochrome aa_3 previously characterised in intact mitochondria [8, 24–28, 33, 43], may also be demonstrated in the reconstituted cytochrome oxidase vesicles. Imposing a pH gradient across the vesicle membrane (acid outside) results in development of a red shift in the

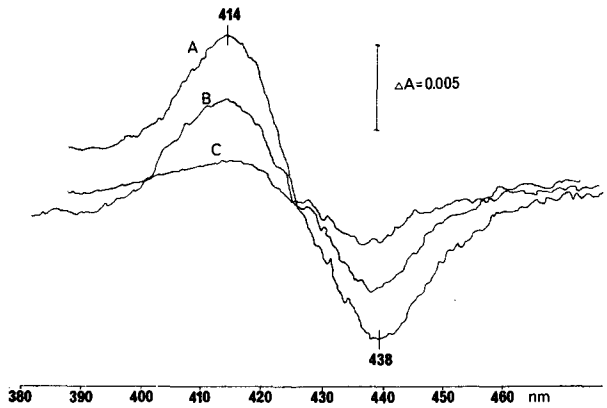

Fig. 6. ΔpH-induced spectral shift in ferric cytochrome aa_3 of cytochrome oxidase vesicles. Cytochrome oxidase vesicles were prepared as described by Hinkle et al. [12] and concentrated (see Materials and Methods). 0.3 ml of the concentrated vesicle suspension (pH 8.5) was then mixed with 0.5 ml of 50 mM HEPES buffer, the pH of which was preadjusted to various values below 8.5 with HCl. Immediately after mixing, which gave a final concentration of 2 μM cytochrome aa_3, a baseline spectrum (380–480 nm) was scanned with reference to the absorption at 380 nm into the spectrophotometer memory unit. After 5 min, when complete decay of the spectral shift had occurred, the spectral interval was scanned again with reference to the memorised "baseline". The difference spectra shown (the absorption difference between the second spectrum and the "baseline" increases upwards) thus represents the relaxation of the spectral shift that was induced by the pH gradient. The spectra marked A, B and C represent different experiments in which the final pH after mixing was 6.78, 7.73 and 8.12 respectively (corresponding to pH gradients of 1.7, 0.8 and 0.4 units).

Soret band which is identical within experimental error to that found in intact mitochondria. Fig. 6 shows the difference spectrum of relaxation of this shift after induction by pH gradients of different magnitude. As in mitochondria, the shift is totally abolished by uncoupling agents, and may be enhanced in extent by an induced electrical diffusion potential (positive polarity outside, cf. ref. 43). It is thus evident that the heme aa_3 structure responds directly to a transmembrane H^+ gradient by a conformational change. This indicates linkage of the conformation of the hemes and their immediate vicinity to proton translocation across the membrane. Since, as we have shown above, cytochrome *c* oxidase activity is coupled to proton transport, the present findings suggest that the linkage between the redox reactions and proton transport takes place through conformational transitions in the heme structure which might be reflected to the aproprotein (see Discussion).

DISCUSSION

It is essential that the present data are not confused with the proton production demonstrated by Hinkle et al. [12] during oxidation of reduced 1,4-naphtoquinone-2-sulfonate or other hydrogen-donating systems [44] by oxygen, catalysed by cytochrome oxidase vesicles. The protons produced in these experiments (often erroneously referred to as translocated protons) have been specifically explained by the reduction of ferricytochrome *c* by the hydrogen donor on the outside of the vesicle, and vectorial electron transfer across the membrane [11, 12, 44]. This explanation

cannot, however, be applied on our results since proton ejection is observed with an electron donor to cytochrome *c* oxidase. This and the accompanying data presented here are strong evidence that cytochrome *c* oxidase catalyses electrogenic proton transport across the mitochondrial membrane with a $2H^+/2e^-$ stoichiometry. It may be argued that if this is the case, then H^+/O ratios higher than 2 should be observed using hydrogen donors (ideally a H^+/O ratio of 4 may be expected on the basis of our findings). The H^+/O ratios of exactly 2.0 found by Hinkle [42] with cytochrome oxidase vesicles supplemented with hydrogen donor and pulsed with low concentrations of oxygen may not, however, contradict our findings since the initial rates of electron transport and proton production were not measured in these experiments.

Since our data show that a net of $2H^+/2e^-$ are ejected extramitochondrially and $2H^+/2e^-$ must be consumed simultaneously in reduction of dioxygen, the transfer of two electrons to 1/2 O_2 must be coupled to separation of a total of four electrical charges across the mitochondrial membrane. This is most easily demonstrated by a scheme such as Fig. 7. This figure is drawn for illustrative purposes only to aid discussion and should not be interpreted mechanistically. To account for our findings (Fig. 1, Table I) the four separated charges (per electron pair) may consist of two electrons and two protons (Fig. 7A) in which case Mitchell's vectorial electron transfer in cytochrome oxidase is retained, but with an additional proton pump of $2H^+/2e^-$ stoichiometry. Alternatively, the four separated charges may consist of protons only (Fig. 7B) in which case electron transport is not transmembraneous and the proton pump stoichiometry is 4 $H^+/2e^-$. In the latter case the $2H^+$ consumed in O_2 reduction are taken from the outside of the membrane.

However, it is possible and even likely, that the true arrangement of cytochrome oxidase is intermediate to the extremes A and B of Fig. 7. Since we have in the present paper demonstrated that the cytochrome *c* oxidase reaction is coupled to electrogenic proton transport across the mitochondrial membrane, vectorial electron transfer as envisaged in a redox loop (and see Fig. 7A) seems superfluous. We consider it more likely that cytochrome oxidase generates a difference in electrical potential across the membrane by one principal mechanism (proton translocation) rather than by a hybrid between electron and proton translocation as in Fig. 7A. However, this

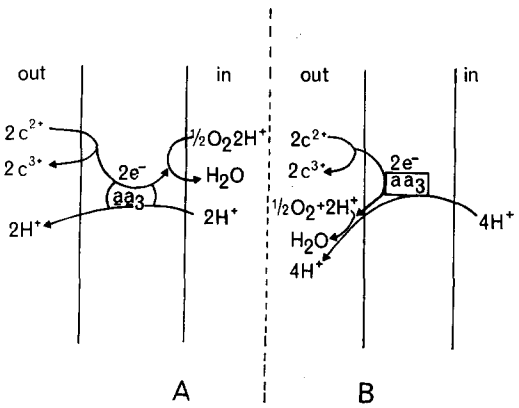

Fig. 7. Two extreme arrangements of the proton pump coupled to cytochrome *c* oxidase. For details, see the text.

does by no means necessitate the extreme solution shown in Fig. 7B, which has two major drawbacks. First, it suggests that the protons consumed in water formation are withdrawn from the outside (cytoplasmic, or C-side) of the membrane. Our results with sonic submitochondrial particles contradict this idea (Fig. 3), since no lag in the proton consumption (measured on the matrix or M-side) was observed on initiation of respiration with TMPD in the absence of valinomycin. A lag may have been expected due to the relative proton impermeability of the membrane if the protons used in water formation were taken from the C-side. Secondly, since the cytochrome *c* oxidase complex catalyses both proton transport (where the protons are clearly extracted from the M-side) and water formation, it appears more likely that all protons (whether consumed or translocated) enter the enzyme by the same route, viz. from the M-side of the membrane.

With the above considerations in mind, a model formally equivalent to Fig. 7B may be constructed where $4H^+$ ions (per $2e^-$) move into the enzyme complex from the M-side of the membrane, perhaps through a channel or molecular "well". Two of the protons are consumed in protonation of reduced oxygen with formation of water, and the two remaining protons are transported all the way across the membrane probably linked to relaxation of a redox-linked conformational change (see below). It is possible that one of the latter transported protons is released as H_3O^+, together with the water formed by reduction of oxygen. It may be added that this model can incorporate any proportion of charge separation due to vectorial electron transfer, depending entirely on the relative location of the hemes of cytochromes *c*, *a* and a_3 with respect to the membrane dielectric.

The correlation between electrochemical proton gradient and the extent of spectral shift in cytochrome aa_3 both in mitochondria [8, 33] and in cytochrome oxidase vesicles (Fig. 6), suggests that the proton pump may be linked to the redox reaction through a conformational change in the hemes and their immediate vicinity. The spectral shift in ferric aa_3 (Fig. 6) is consistent with a spin state transition [27]. Hence the shift from high to low spin ferric heme iron seems to be linked to proton translocation from the C-side to the M-side of the membrane, and vice versa. A spin state transition of heme iron in hemoproteins has quite well understood consequences upon the stereochemistry of the heme itself and may lead to considerable conformational changes in the tertiary and quaternary structure of the apoprotein (cf. hemoglobin, ref. 45, and also refs. 18, 29 and 46). However, so far, the energy-dependent spin state change in cytochrome aa_3 has been observed only in the fully oxidised enzyme, and not under conditions of turnover. Therefore, it must at the present time be strictly regarded only as a model of how proton transport may be linked to the redox reaction, awaiting information on which partial electron transfer reactions of cytochrome *c* oxidase are linked to the proton translocation step. We nevertheless feel that the data already available are very suggestive of a mechanism involving conformational coupling between electron transfer and proton transport.

It is interesting that the energy-dependent spectral shift in ferrous aa_3 may be mimicked in purified cytochrome oxidase by either calcium or proton binding to the complex [43, 47–49]. The binding site may therefore be identical to a H^+ ion-binding site of the proton pump. The spectral shift shows an extraordinary specificity to Ca^{2+} and H^+ [48, 49] which may be consistent with a molecular channel leading to the site. Linkage of this site to the heme structure is not only indicated by the spectral shift, but

also by a change in apparent pK by 0.5 units upon binding of cyanide to cytochrome a_3 (H. T. Saari, unpublished). Linkage of proton dissociation with electron transport is also shown by the dependence of the midpoint redox potential of the hemes upon pH [28, 50].

Although the structural counterpart of the proton pump in cytochrome *c* oxidase is obviously not yet known, the studies by Komai and Capaldi [51] and by Phan and Mahler [52] may provide a clue. These authors found that stripping the heavy subunits with hydrophobic characteristics off the enzyme left it with considerable enzymatic activity and apparently unchanged redox centres. By analogy with the mitochondrial ATPase complex [53], in which the hydrophobic proteins (CF_0) are catalytically inactive, but provide the ATPase with an oligomycin-sensitive proton channel or "pump" [54, 55], it may not be unreasonable to suggest as a working hypothesis that the heavy subunits of cytochrome oxidase may constitute the proton-translocating device of this redox complex. This suggestion is presently being tested in our laboratory.

In summary, cytochrome *c* oxidase may conserve the free energy of oxidation primarily by a conformational change involving the hemes and their immediate environment and possibly the apoprotein as well. Relaxation of this conformational change may be directly linked to electrogenic proton transport across the mitochondrial membrane catalysed by a "proton pump", which is inherent in the cytochrome aa_3 complex. Thus cytochrome *c* oxidase transduces the free energy secondarily into a delocalised electrochemical proton gradient. Though the translocation of protons occurs with a stoichiometry of $2H^+/2e^-$, the transfer of a pair of electrons from cytochrome *c* to oxygen is linked to separation of four electrical charges across the mitochondrial membrane. This is in line with the recently reported high H^+/O ratios of respiratory chain-linked proton transport [56]. Though fundamentally different from the redox-loop mechanism of proton translocation [9—11], the proposed mechanism retains several essential features of Mitchell's chemiosmotic hypothesis. These include a necessary asymmetric and transmembrane organisation of the redox complex, the necessity of a membrane structure per se in energy transduction, and finally, the transduction of redox energy into the energy of a protonmotive force. Whether the latter transduction, which clearly occurs in the presence of charge-compensating counterflux of K^+ ions, is also the main route of energy transfer in oxidative phosphorylation is not established with certainty. The alternative is more localised proton circuits between the redox complex and ATP synthase within or at the surfaces of the membrane (ref. 57; see ref. 58 for review). In contrast to the chemiosmotic hypothesis, we suggest that redox energy is primarily conserved as a localised conformational "strain" of the respiratory complex itself in accordance with one of the characteristic postulates of chemical and conformational hypotheses of oxidative phosphorylation.

By analogy to the function of cytochrome *c* oxidase it appears very likely that H^+ translocation occurs by similar principles at the coupling regions 1 and 2 of the respiratory chain as suggested by Papa et al. [59, 60] and by Skulachev [12]. Analogous principles of energy transduction may furthermore apply on mitochondrial ATP synthase, which may utilise the electrochemical proton gradient in ATP synthesis via proton transport linked to conformational changes of the enzyme as considered by Boyer et al. [61, 62].

MATERIALS AND METHODS

Rat liver mitochondria were isolated as described previously [48]*. Cytochrome *c* oxidase was isolated and purified from beef heart mitochondria essentially as described by Kuboyama et al. [64]. The purified enzyme had a heme/protein ratio between 10 and 11 nmol per mg and appeared pure upon electrophoresis in sodium dodecylsulfate-polyacrylamide gels. Cytochrome *c* oxidase vesicles were prepared either exactly as described by Hinkle et al. [12] or by using mitochondrial phospholipids and dialysis at room temperature. Phospholipids were isolated from rat liver mitochondria [65] and dispersed into a medium containing 35 mM KH_2PO_4/15 mM Tricine/1.5 % (w/v) cholate, pH 7.5, by sonication for 10 min, to a final phospholipid concentration of 50 mg (dry weight) per ml. 10 μl cytochrome aa_3 preparation (30 mg protein per ml) was then added to 1 ml of phospholipid suspension and dialysed for 1 h at room temperature against 35 mM KH_2PO_4/15 mM Tricine, pH 7.5, after which the medium was changed into 10 mM KH_2PO_4/40 mM KCl, pH 7.5, and dialysis continued for another 3 h. The resulting vesicle preparations had "respiratory control" indexes above 3.0 (see ref. 12). For spectrophotometry, cytochrome oxidase vesicles [12] were concentrated by centrifugation at 120 000 $\times g$ for 90 min, and the pelleted vesicles eluted through a Sephadex G-25 column with 5 mM Tricine (pH 8.5).

Ferrocytochrome *c* was prepared from concentrated solutions of ferric cytochrome by reduction with an excess of dithionite and subsequent elution through a Sephadex G-25 column. For this purpose crystallised horse heart cytochrome *c* (Boehringer) was used.

Protein was determined by the Folin procedure as described by Lowry et al. [66] with human serum albumin as standard. pH measurements were made using an Ingold combination electrode (W. Ingold, Zürich) in conjunction with an Electronic Instruments Ltd pH meter model C-33-B (Richmond, U.K.) and a strip chart recorder. Spectrophotometry was performed with a scanning dual wavelength spectrophotometer (DBS-1, Johnson Research Foundation Workshops, Philadelphia, Pa.) at indicated wavelength couples (as a function of time) or in indicated wavelength intervals (as a function of wavelength). Glass cuvettes with a 1.0 cm light path were used throughout, and the reaction temperature was 25 °C in all reported experiments.

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* Beef-heart mitochondria and sonicated submitochondrial particles (Mg^{2+}-ATP particles) were isolated as described by Löw and Vallin [63].

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