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MECHANISM OF RESPIRATION-DRIVEN PROTON TRANSLOCATION IN THE INNER MITOCHONDRIAL MEMBRANE

ANALYSIS OF PROTON TRANSLOCATION ASSOCIATED WITH OXIDATION OF ENDOGENOUS UBIQUINOL

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SUMMARY

A study is presented of the kinetics and stoichiometry of fast proton translocation associated to aerobic oxidation of components of the mitochondrial respiratory chain.

1. Aerobic oxidation of ubiquinol and *b* cytochromes is accompanied in EDTA particles, obtained by sonication of beef-heart mitochondria, by synchronous proton uptake.

2. The rapid proton uptake associated to oxidation of ubiquinol and *b* cytochromes is greatly stimulated by valinomycin plus K^+, but is unaffected by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

3. 4 gion H^+ are taken up per mol ubiquinol oxidized by oxygen. This $H^+/2e^-$ ratio, measured in the rapid anaerobic-aerobic transition of the particles is unaffected by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

4. In intact mitochondria aerobic oxidation of oxygen-terminal electron carriers is accompanied by antimycin-insensitive synchronous proton release, oxidation of ubiquinol and reduction of *b* cytochromes. The amount of protons released is in excess with respect to the amount of ubiquinol oxidized.

5. It is concluded that electron flow along complex III, from ubiquinol to cytochrome *c*, is directly coupled to vectorial proton translocation. The present data suggest that there exist(s) between ubiquinol and cytochrome *c* one (or two) respiratory carrier(s), whose oxido-reduction is directly linked to effective transmembrane proton translocation.

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

INTRODUCTION

Analysis of the kinetic and stoichiometric relationship between oxido-reduction of individual components of the respiratory chain and proton translocation in the inner-mitochondrial membrane represents a most direct approach to the study of the molecular mechanism of the respiration-linked proton pump in mitochondria [1]. These investigations are necessarily based upon the use of suitable techniques for time resolution of fast reactions.

Kinetics study of the mitochondrial redox proton pump has recently been undertaken in our laboratory [1–3] with the help of flow potentiometric techniques [4, 5] and flow dual wavelength spectrophotometry [6]. Previous work along this line [1–3] has shown that fast aerobic oxidation of the oxygen-terminal electron carriers of the respiratory chain results in synchronous and stoichiometric consumption of protons at the inner side of the mitochondrial membrane. Evidence was also obtained that aerobic oxidation of the terminal electron carriers generates a membrane potential. These results show that cytochrome oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) is oriented transversely across the membrane, reacting with cytochrome *c* at the outer side and with oxygen at the inner side of the membrane (cf. refs 7 and 8).

In this paper a study is presented of the kinetics and stoichiometry of proton translocation in the inner-mitochondrial membrane associated to the oxidation of endogenous ubiquinol and components of complex III of the respiratory chain.

Kinetics [9, 10] as well as extraction-reactivation studies [11] have provided evidence demonstrating that ubiquinone is an obligatory component of the respiratory chain. The quinone appears to act as a hydrogen-transporting pool, which connects NADH and succinate dehydrogenase to the cytochrome chain [9, 10] and is present in the mitochondrial membrane in significant excess with respect to the other components of the chain [12].

The present paper shows that 4 gion H^+ are taken up per mol ubiquinol oxidized in the rapid anaerobic-aerobic transition of sonic submitochondrial particles. This $H^+/2e^-$ ratio is unaffected by uncouplers. These findings indicate the existence of one (or two) respiratory carrier(s) between ubiquinone and cytochrome *c*, whose oxido-reduction is directly linked to effective transmembrane proton translocation

METHODS AND MATERIALS

Mitochondria and submitochondrial particles

Heavy, beef-heart mitochondria were prepared as described by Löw and Vallin [13], stored for 2–7 days at −10 °C and thawed immediately before use. EDTA submitochondrial particles were prepared by exposing mitochondria to ultrasonic energy as described by Lee and Ernster [14] (see also ref. 15).

Kinetic analysis of aerobic proton translocation

The kinetics of proton translocation was analyzed with a Roughton-type, repetitive, continuous-flow pH meter (mixing ratio, 1 : 60) with a resolution time of 2 ms [5]. The electrodes used were: a 50–100 MΩ glass electrode (Ingold KG, Frankfurt/Main, Germany) and a calomel electrode connected to the incubation

mixture in the measuring cell of the apparatus through a saturated KCl bridge. The electrodes were connected to a Vibron Precision Electrometer (Model 62A) set up at a sensitivity at the output of 24 V/pH unit. The input capacitance of the electrometer was 1 pF. The electrometer output was displayed on a Honeywell strip-chart Recorder, model Electronik 194. The sensitivity of the recorder was adjusted to give a 5-cm deflection/V. The circuit used allowed the pH to be measured with a precision of 0.001 pH unit. Potential changes were quantitated as proton equivalents by double titration with standard solution of KOH and HCl. These titrations were performed by following the pH changes which occurred within 30 ms after the addition, to aerobic or KCN-inhibited aerobic mitochondria or sonic particles, of KOH or HCl at a final concentration of 35 μM. The short time interval used avoids titration of the interior of the particles. The syringes of the continuous-flow pH meter, the mixing chamber and the measure-compartment were surrounded by a water bath thermostated at $\pm$0.01 °C. The reaction times at the measuring tip of the glass electrode during the continuous-flow phase (see the pH traces in the figures) could be preselected by changing the speed of the flow and the distance between the mixing chamber and the electrode tip. The shortest time that could be analyzed with the continuous-flow pH meter employed was 10 ms.

Oxido-reduction of respiratory carriers

Oxido-reduction of respiratory carriers was followed with a dual wavelength spectrophotometer, using either a static cuvette or a regenerative stopped-flow apparatus (mixing ratio, 1 : 80) [6]. The flow cell was surrounded by a water bath thermostated at $\pm$0.01 °C. The amplifier output was displayed, in parallel, on a Leeds and Northrup strip-chart recorder and on a storage oscilloscope (Textronik Type 564B). A mercury arc (ultraviolet strahler, Type ST-75 Original, Quarz-Lampen, GmbH, Hanau) was used as ultraviolet light source. Ubiquinone oxido-reduction was monitored at 280/289 nm and a $\Delta\varepsilon$ mM of 8.8 [16] was used to quantitate the redox changes of ubiquinone. This narrow wavelength difference minimized light-scattering and stray-light artifacts. In addition a 10-mm thickness of a saturated nickel-cobalt sulfate solution [16] was used as light filter and placed between the observation chamber and the phototube.

The reaction time at the point of observation in the stopped-flow spectrophotometer (continuous-flow phase) ranged, from experiment to experiment, between 6 and 9 ms, depending upon the speed of the discharge (the flow trace in the figures indicates the duration of the flow phase, this is determined by the N_2 pressure applied to push the pistons as well as by the distance that the pistons travel).

Experimental procedure

The main compartments of the pulsed-flow spectrophotometer and the continuous-flow pH meter were filled with the aerobic suspension of submitochondrial particles or mitochondria. This contained, as respiratory substrate and buffer, potassium succinate. The compartments of the two flow apparatus were then hermetically closed and the oxygen dissolved in the suspension allowed to be consumed by succinate oxidation. Anaerobiosis was revealed by alkalinization of the mitochondrial suspension (acidification in the case of submitochondrial particles) and reduction of respiratory carriers. When indicated malonate was subsequently added. The small

amount of oxygen conveyed by malonate solution caused immediate acidification of the suspension and oxidation of the respiratory carriers. Both the processes reversed when oxygen was exhausted through the slow residual succinate oxidase activity. At 3 min intervals of equilibration of the anaerobic system, 6–8 repetitive oxygen pulses were delivered from the side syringes of the two flow apparatus as oxygen-saturated reaction mixture.

Other experimental conditions are described in the text and in the legends to figures and tables.

Materials

Valinomycin was a generous gift of Dr Godtfredsen of Leo Pharmaceutical Products, Ballerup, Denmark. Antimycin A was obtained from Sigma. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift of Dr C. P. Lee.

RESULTS

Fig. 1 illustrates the flow analysis of proton uptake and oxidation of respiratory

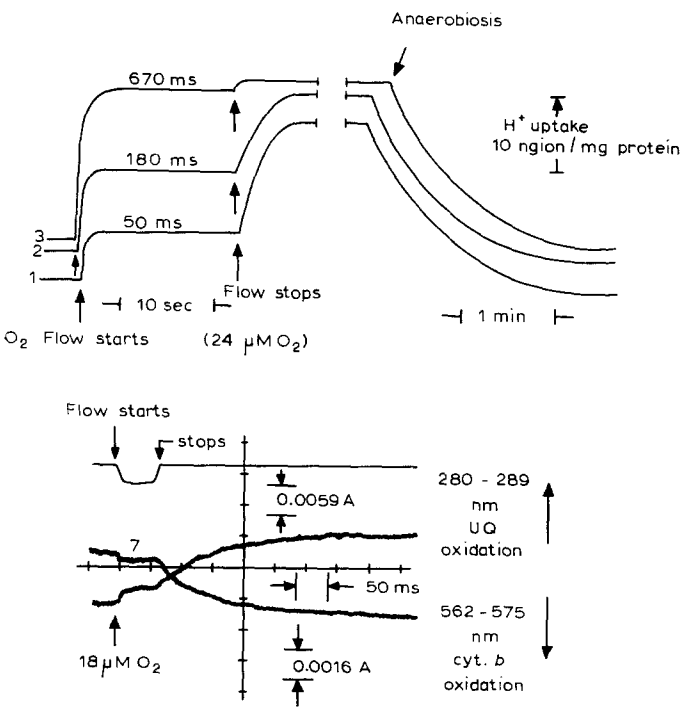

Fig. 1. Flow analysis of proton uptake and oxidation of respiratory carriers on the substrate side of the antimycin site induced by repetitive oxygen pulses of anaerobic submitochondrial particles. The main syringe contained: 250 mM sucrose, 30 mM KCl, 10 mM potassium succinate, 3 μg/mg protein oligomycin, 0.5 μg/mg protein valinomycin, 0.05 mg/ml purified catalase and EDTA submitochondrial particles (1 mg protein/ml). Final pH 7.5. Temperature 20 °C. The smaller syringe contained 0.2% H_2O_2. The figures (ms) given on the pH traces and on the absorbance traces indicate, in this and in the following illustrations, the reaction times during the continuous-flow phase. For other details see under Methods and Materials.

carriers on the substrate side of the antimycin site, induced by repetitive oxygen pulses of anaerobic submitochondrial particles supplemented with succinate as respiratory substrate, valinomycin plus K^+ and oligomycin. Oligomycin served to depress passive proton back-flow across the vesicle membrane [17]. Within 50 ms after oxygenation a very rapid initial phase of proton uptake occurred. This amounted to 6 ngion H^+ per mg particle protein. It has been previously shown [1, 3] that this fast initial phase of proton uptake is in large part (50–70 %) associated to aerobic oxidation of the oxygen-terminal electron carriers. Progression of ubiquinone and *b* cytochromes from the anaerobic reduced state to the aerobic redox steady state was accompanied by a second phase of proton uptake. At an interval of 180 ms, when net oxidation of these components had practically reached completion, proton uptake amounted to a total of 11 ngion H^+ per mg protein. Subsequent steady-state turnover of the respiratory chain was accompanied by further slower proton uptake and a steady-state extent of 22 ngion H^+ taken up per mg protein was reached. With anaerobiosis these protons were released back in the medium.

It has to be pointed out that whilst stopped-flow spectrophotometry allows, when the flow stops, a continuous read-out of fast absorbance changes during one single discharge, with continuous-flow potentiometry rapid pH changes can be satisfactorily analyzed only from the flow phase. In this case no more than three time intervals can be analyzed per discharge, during the continuous-flow phase. Thus in order to analyze the overall process of proton transport the oxygen pulses are

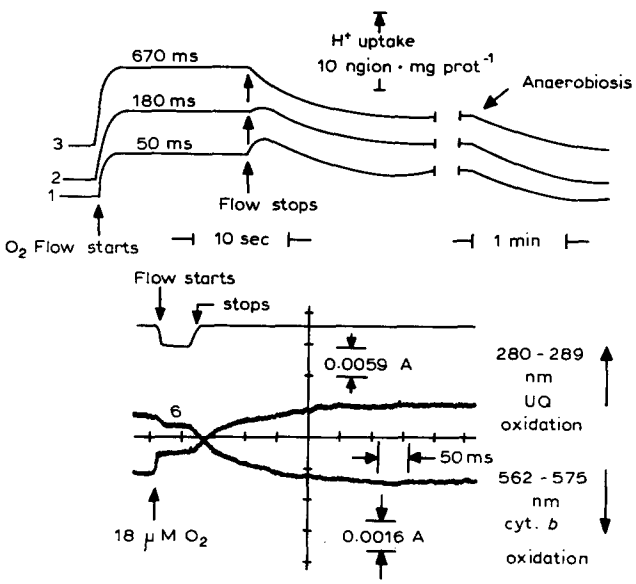

Fig. 2. Kinetics of proton uptake and oxidation of respiratory carriers on the substrate side of the antimycin site in submitochondrial particles supplemented with succinate plus malonate. The main syringe contained: 250 mM sucrose, 30 mM KCl, 10 mM potassium succinate, 5 mM potassium malonate, 3 μg/mg protein oligomycin, 0.5 μg/mg protein valinomycin and EDTA submitochondrial particles (1 mg protein/ml). Final pH 7.5. Temperature 20 °C. The smaller syringe contained an oxygen-saturated mixture consisting of 250 mM sucrose and 30 mM KCl. For other details see under Methods and Materials.

repeated more times depending upon the number of data required [1, 5] (see Figs 5 and 6).

Addition to the system of an excess of malonate suppresses succinate oxidation and hence limits oxygen-induced electron flow to net oxidation of anaerobically reduced respiratory carriers. In the experiment of Fig. 2 malonate was, thus, added to the system and, once respiratory carriers were maximally reduced in anaerobiosis through the residual succinate dehydrogenase activity, electron flow was activated by oxygen pulses. Comparison of Figs 1 and 2 shows that malonate had practically no effect on the phase of proton uptake which accompanied the oxidation of ubiquinol and *b* cytochromes. However, once the oxidation of these components was completed (compare traces 2 and 3 of Fig. 2) net proton uptake ceased. It can be observed that as the system passed into the aerobic steady state and the fast rate of electron flow associated to ubiquinol oxidation subsided to the much slower rate supported by the residual succinate dehydrogenase activity, the alkalinization of the medium partly reversed. This lower level maintained itself constant untill, upon anaerobiosis, the external pH returned to the original anaerobic value.

Table I summarizes the parameters of respiration-linked proton translocation and oxidation of respiratory carriers in sonic submitochondrial particles. Proton

TABLE I

CHARACTERISTICS OF H^+ TRANSLOCATION AND OXIDATION OF RESPIRATORY CARRIERS IN THE ANAEROBIC-AEROBIC TRANSITION OF SUCCINATE-REDUCED SONIC PARTICLES FROM BEEF-HEART MITOCHONDRIA

The conditions of Expt a were those described in the legend to Fig. 1. The aerobic external pH rise showed discontinuities around 20 and 200 ms (see also Fig. 5 of ref. 3). The extent of phase I proton uptake was obtained from the observed proton uptake at 20 ms after oxygenation. The extent of phase II was obtained by subtracting from the observed proton uptake at 200 ms that accounted for by phase I and by succinate oxidase (4 ngion H^+ per natom oxygen taken up). The extent of phase III was obtained by subtracting from the overall aerobic proton uptake that accounted by phase I and II. Expt b: 250 mM sucrose, 30 mM KCl, 5 mM potassium succinate, 2.5 μg/mg protein oligomycin, 0.5 μg/mg protein valinomycin and EDTA submitochondrial particles (2 mg protein/ml), pH 6.8. Temperature 25 °C. For the details of Expt c see Fig. 5. The extent of proton uptake was obtained by subtracting from the observed value at 200 ms the antimycin-insensitive proton uptake.

Experiments	Additions	H^+ uptake		Ubiquinone oxidation 280–289 nm	Cyto-chromes *b* oxidation 562–575 nm	Cyto-chromes $c+c_1$ oxidation 550–540 nm
		$t_{\frac{1}{2}}$ (ms)	Extent ngion H^+ / mg protein	$t_{\frac{1}{2}}$ (ms)	$t_{\frac{1}{2}}$ (ms)	$t_{\frac{1}{2}}$ (ms)
a	None	Phase I < 10	≈ 3			
		Phase II ≈ 100	≈ 7	50	75	4
		Phase III ≈ 350	≈ 14			
b	+0.5 μg antimycin/ mg protein	< 10	3	—	—	4
c	+3.5 mM malonate	110	7	100	90	4

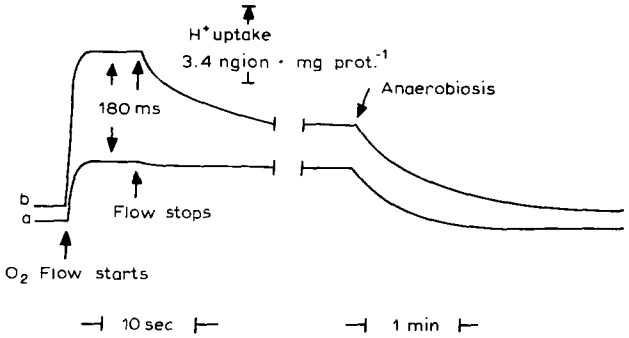

Fig. 3. Effect of valinomycin plus K^+ on proton uptake associated to oxidation of reduced respiratory carriers in malonate supplemented EDTA submitochondrial particles. The main syringe contained: 200 mM sucrose, 30 mM KCl, 3.5 mM potasssium succinate, 3.5 mM potassium malonate and EDTA submitochondrial particles (1.9 mg protein/ml). Final pH 7.5. Temperature 20 °C. The smaller syringe contained an oxygen-saturated mixture consisting of 250 mM sucrose and 30 mM KCl. Trace a refer to the control; trace b plus 0.5 μg/mg protein valinomycin. For other details see under Methods and Materials.

uptake induced by oxygenation of reduced particles presented three phases. A very fast phase I, which was practically insensitive to antimycin and was synchronous with the oxidation of the terminal electron carriers (see also refs 1–3). Phase II of proton uptake was synchronous to oxidation of ubiquinone and *b* cytochromes and was unaffected by suppressing succinate oxidation with malonate. The third slow phase of net proton uptake was supported by steady-state turnover of the respiratory chain.

The experiment of Fig. 3 shows the effect of valinomycin plus K^+ on proton uptake associated to oxidation of reduced respiratory carriers in malonate supplemented submitochondrial particles. The ionophore caused a 3-fold enhancement of proton uptake measured at an interval of 180 ms after oxygenation. Papa et al. [18] have shown that valinomycin plus K^+ causes a marked enhancement of the rate of oxidation of *b* cytochromes and ubiquinone in the anaerobic-aerobic transition of sonic particles but leaves the steady-state respiration unaffected. Thus proton translocation associated to oxidation of endogenous ubiquinol, like proton translocation supported by oxidation of respiratory substrates [15, 19–21] and proton consumption associated to oxidation of the oxygen-terminal carriers [1, 3] is of electrogenic nature.

Fig. 4 shows that 1 μM FCCP, added during the anaerobic equilibration phase to the particle suspension supplemented with valinomycin plus K^+ and oligomycin, had, at an interval of 200 ms after oxygenation, no effect on the rapid proton uptake associated to the oxidation of endogenous ubiquinol neither did it affect the rate of oxidation of *b* cytochromes and ubiquinol. Thus there is no uncoupler-sensitive intermediary step in the proton translocation associated to ubiquinol oxidation. During the subsequent aerobic steady state FCCP slowly reversed the oxygen-induced alkalinization of the medium. This delayed collapse of the aerobic pH change (see also refs 1 and 3), brought about by an uncoupling agent, which enhances the proton conductivity of the membrane [22], verifies that the rapid alkalinization of the particle suspension associated to ubiquinol oxidation represents an effective transmembrane proton translocation.

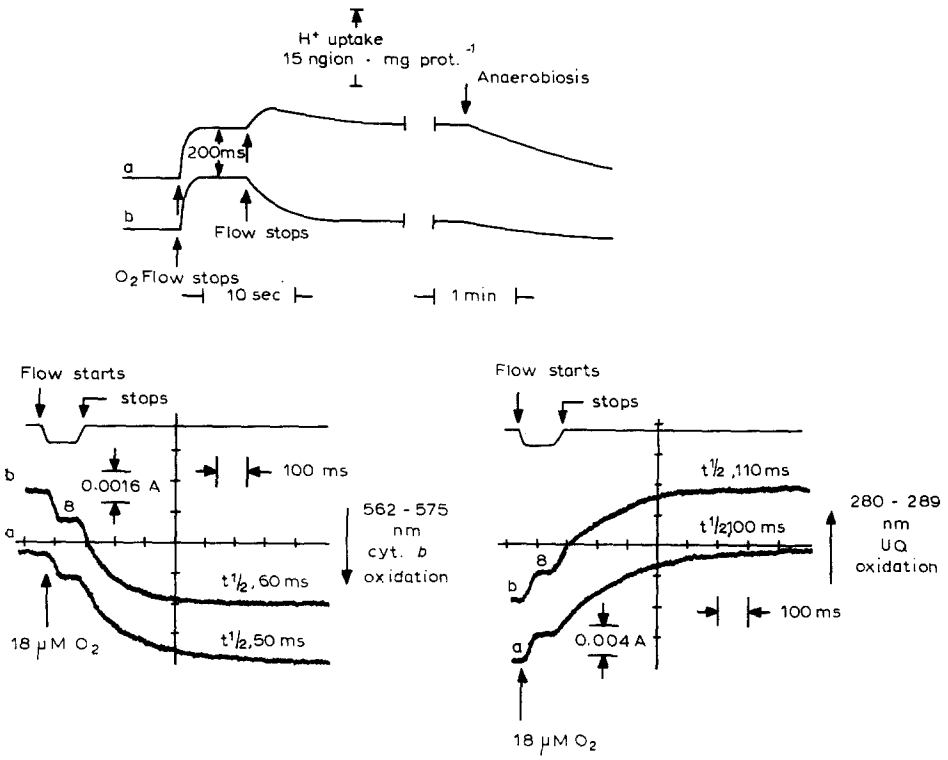

Fig. 4. Effect of FCCP on proton uptake associated to oxidation of reduced respiratory carriers in malonate supplemented EDTA submitochondrial particles. The main syringe contained: 200 mM sucrose, 30 mM KCl, 5 mM potassium succinate, 3.5 mM potassium malonate, 2 µg/mg protein oligomycin, 0.5 µg/mg protein valinomycin and EDTA submitochondrial particles (1.9 mg protein/ml). Final pH 6.8. Temperature 20 °C. The smaller syringe contained an oxygen-saturated mixture consisting of 200 mM sucrose and 30 mM KCl. Trace a refer to the control; trace b plus 1 µM FCCP. For other details see under Methods and Materials.

Fig. 5 illustrates one of a series of experiments which were utilized to calculate the kinetics parameters of the oxidation of respiratory carriers and of the accompanying proton uptake (see Table I) and the $H^+/2e^-$ ratio for oxidation of endogenous ubiquinol in submitochondrial particles. Valinomycin plus K^+ and oligomycin were added to the system; the concentrations of succinate and malonate were 3.5 mM. The pH of the suspension was 6.8. At this pH the rate of ubiquinol oxidation was slower than at 7.5. The kinetics of the aerobic proton uptake was biphasic; the initial part of the process was apparently completed in 36 ms. This phase corresponded to the antimycin-insensitive proton uptake associated to oxidation of the terminal electron carriers, which in fact completed itself within 36 ms (see also refs 1 and 3). The second slower phase of proton uptake was synchronous to the oxidation of *b* cytochromes and ubiquinol.

The mean values of the $H^+/2e^-$ ratio for the oxidation of endogenous ubiquinol in submitochondrial particles are given in Table II. The $H^+/2e^-$ ratios of the first row were calculated from the ngions H^+ taken up and the nmol ubiquinol oxidized

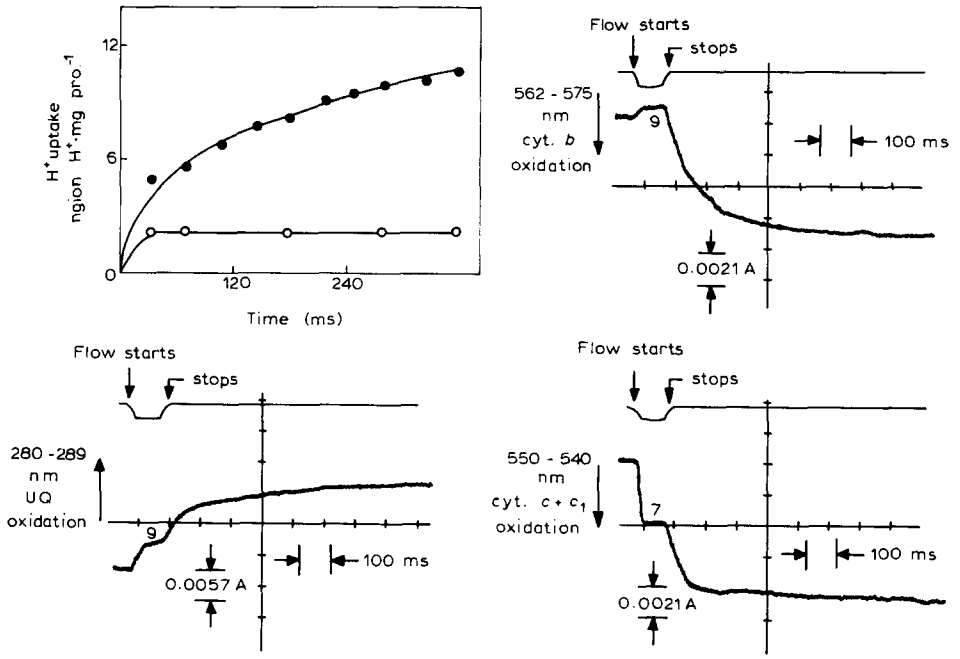

Fig. 5. Kinetics of proton uptake and oxidation of respiratory carriers in EDTA submitochondrial particles. The main syringe contained: 200 mM sucrose, 3.5 mM potassium succinate, 3.5 mM potassium malonate, 30 mM KCl, 2 μg/mg protein oligomycin, 0.5 μg/mg protein valinomycin and EDTA submitochondrial particles (2 mg protein/ml). Final pH 6.8. Temperature 20 °C. The smaller syringe contained an oxygen-saturated mixture consisting of 200 mM sucrose and 30 mM KCl. ●—●, control; ○—○, plus 1 μg/mg protein antimycin. For other details see under Methods and Materials.

TABLE II

$H^+/2e^-$ RATIOS FOR THE AEROBIC OXIDATION OF ENDOGENOUS UBIQUINOL IN SONIC SUBMITOCHONDRIAL PARTICLES

H^+ uptake in the first 72 ms was corrected for the antimycin-insensitive aerobic proton uptake. The experimental conditions and procedure are those given in the legend to Fig. 5. The rate of succinate ubiquinol reductase v_{red} was calculated as described by Kröger et al. [9]. For other details see under Methods and Materials and in the text. UQ, ubiquinol. The results are expressed as means ± S.E.

Number of experiments	Time (ms)	$\frac{\Delta \text{ngion } H^+}{\Delta \text{nmol UQ}}$	$\frac{\Delta H^+ - (v_{red}\ UQ \cdot t \cdot 4)}{\Delta UQ + (v_{red}\ UQ \cdot t)}$	$\frac{\Delta H^+ - (\Delta b^{3+} + v_{red}\ UQ \cdot t \cdot 4)}{\Delta UQ + (v_{red}\ UQ \cdot t)}$
9	0–72	4.3±0.5	4.1±0.5	3.8±0.5
9	36–180	5.6±0.7	4.7±0.6	4.4±0.5
			Control	
10	0–72	4.3	4.0	3.9
			+1 μM FCCP	
		5.0	4.8	4.6

in the first 72 ms and from 36 to 180 ms after oxygenation. To calculate the $H^+/2e^-$ ratio associated to ubiquinol oxidation in the first 72-ms interval the observed proton uptake had to be corrected for the antimycin-insensitive proton consumption which is associated to the oxidation of reduced oxygen-terminal electron carriers. Since this process is completed within 36 ms such a correction had not to be applied to calculate the $H^+/2e^-$ ratio in the 36–180-ms interval.

The $H^+/2e^-$ ratios were 4.3 in the 0–72-ms interval and 5.6 in the 36–180-ms interval. The increase of the ratio in the 36–180-ms interval is likely due to the fact that, as net oxidation of reduced ubiquinol approaches completion, the influence of the residual succinate dehydrogenase activity becomes more significant. Correction for this activity was done by subtracting from the proton uptake that accounted for by the residual succinate oxidation and by adding to the amount of ubiquinone oxidized that re-reduced by succinate. The $H^+/2e^-$ ratio for succinate oxidation was taken equal to 4. The $H^+/2e^-$ ratios so obtained (see the second row of Table II) were lowered to 4.1 and 4.7 in the 0–72- and 36–180-ms interval, respectively. The fact that the $H^+/2e^-$ ratio for ubiquinol oxidation is higher than two implies that there exist(s) one (or two) respiratory carrier(s) in the respiratory chain between ubiquinone and cytochrome *c*, whose oxido-reduction is directly coupled to proton translocation. Thus the $H^+/2e^-$ ratios of the second row of Table II were further corrected for proton translocation associated to net oxidation of *b* cytochromes. The $H^+/2e^-$ ratios so corrected (see the last row) were 3.8 and 4.4 in the 0–72- and 36–180-ms interval, respectively. It should be pointed out that these values were obtained on the basis of 1 gion H^+ translocated per mol of cytochrome *b* oxidized; however, the quotients did not change significantly if a ratio of 2 gion H^+ per mol of cytochrome *b* oxidized was used in the correction. Expt 10 in Table II shows that FCCP practically did not influence the $H^+/2e^-$ ratio for ubiquinol oxidation measured during the initial interval of the oxygen pulse. At longer intervals, however, the uncoupler caused a decrease of the $H^+/2e^-$, (not shown), which was evidently due to passive proton back-flow induced by the uncoupler.

Papa et al. [1, 3] have previously found that aerobic oxidation of the oxygen-terminal respiratory carriers in intact beef-heart mitochondria is accompanied by synchronous, antimycin-insensitive proton release in the suspending medium. This process has to be the expression of the antimycin-insensitive oxidation of a hydrogen carrier and might be directly related to the rapid proton uptake associated to ubiquinol oxidation in submitochondrial particles.

It has, on the other hand, been shown that oxygenation of the antimycin-inhibited reduced respiratory chain results in the rapid reduction of *b* cytochromes and oxidation of ubiquinol [18, 23–25]. In the experiment of Fig. 6 the kinetics relationship between proton release and the various redox events elicited by oxygenation of antimycin-inhibited anaerobic mitochondria is shown. The oxygen pulse caused immediate oxidation of cytochrome oxidase (not shown); this process was almost completed during the continuous-flow phase (6 ms after oxygenation). The oxidation of cytochrome *c* proceeded with a $t_{\frac{1}{2}}$ of 9 ms and was accompanied by synchronous oxidation of ubiquinone and by a somewhat slower reduction of cytochromes b_{562} and b_{566}. Proton release was synchronous to the oxidation of cytochrome $c+c_1$ and ubiquinone. The kinetics and stoichiometric parameters of the events described in Fig. 6 are summarized in Table III. It can be seen that the amount of ubiquinol oxi-

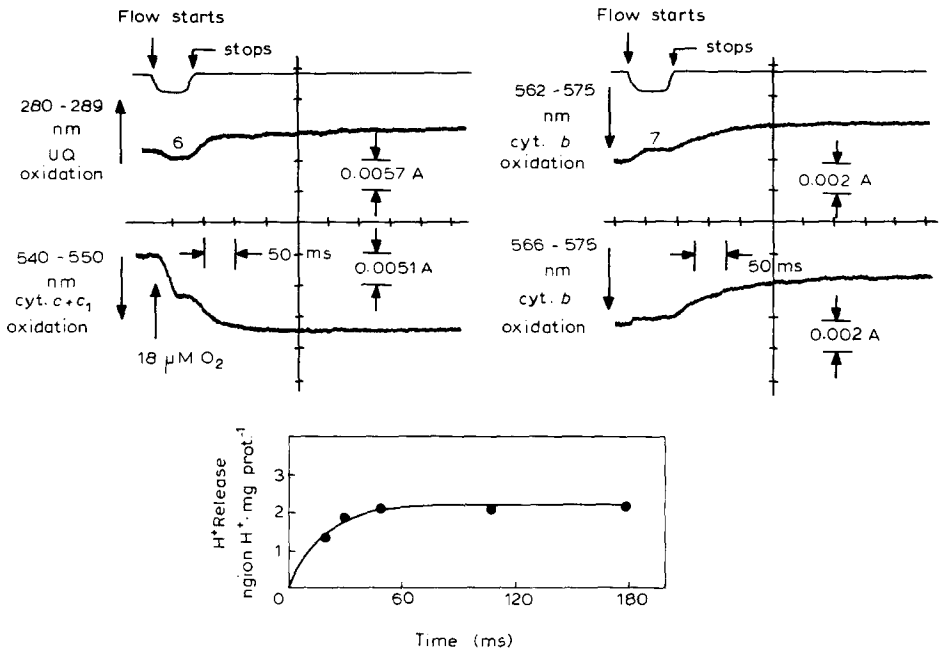

Fig. 6. Kinetic relationship between proton release and the various redox events elicited by oxygenation of antimycin-inhibited anaerobic mitochondria. The main syringe contained 200 mM sucrose, 30 mM KCl, 5 mM potassium succinate, 2 μg/mg protein oligomycin, 0.5 μg/mg protein valinomycin, 1 μg/mg protein antimycin and beef-heart mitochondria (2 mg protein/ml). Final pH 6.8. Temperature 20 °C. The smaller syringe contained an oxygen-saturated medium consisting of 200 mM sucrose and 30 mM KCl. It can be noted from the ubiquinol (UQ) trace that the dilution artifacts in the relatively turbid suspension of mitochondria obscured the small antimycin-insensitive oxidation of ubiquinone occurring during the flow phase. For other details see under Methods and Materials.

TABLE III

CHARACTERISTICS OF PROTON TRANSLOCATION AND OXIDO-REDUCTION OF RESPIRATORY CARRIERS CAUSED BY OXYGEN PULSES OF ANAEROBIC ANTIMYCIN-INHIBITED BEEF-HEART MITOCHONDRIA

The experimental conditions and procedure are those given in the legend to Fig. 6. The extents given in the table refer to 200 ms after oxygenation. The data presented are the mean of three experiments. For other details see under Methods and Materials.

	H^+ release	Ubiquinol oxidation 280–289 nm	Cytochrome *b* reduction 562–575 nm	Cytochrome *b* reduction 566–575 nm	Cytochrome $c+c_1$ oxidation 550–540 nm
$t_{\frac{1}{2}}$ (ms)	<20	11	23	33	14
Extent ngion or nmol/mg protein	2.1	0.2	0.1	0.1	0.5

dized exceeded, on an equivalence basis, the amount of *b* cytochromes oxidized. Thus some of the reducing equivalents which leave ubiquinol must have taken a different path (cf. refs 24 and 25). About 10 gion H^+ were released per mol ubiquinol oxidized. Evidently, besides ubiquinone, other effective hydrogen carriers are involved in the antimycin-insensitive proton release.

DISCUSSION

A number of independent observations (see refs 1, 26–30 and 33 for review), show that active proton translocation is an inherent property of electron transfer chains in the mitochondrial and other energy-transducing membranes. Various theoretical models have been put forward, explaining a direct coupling between scalar redox processes and vectorial proton translocation in coupling membranes [31–33]. These models consider proton translocation as a partial reaction of redox processes between a hydrogen and an electron carrier. Thus they require direct kinetics correspondence between proton translocation and redox reactions.

Flow analysis of oxygen consumption in beef-heart mitochondria [34] and of the aerobic proton ejection in the external medium [1, 3] have revealed for both processes a relatively rapid initial phase which completes itself in about 200 ms. These rapid phases can be ascribed to oxidation of reduced respiratory carriers. In fact it has been found in our laboratory that the addition of malonate leaves the initial rapid phase of aerobic proton release from mitochondria unaffected. In malonate- and rotenone-treated mitochondria the $t_{\frac{1}{2}}$ of oxygen-induced proton release (100 ms) is practically identical to the $t_{\frac{1}{2}}$ of ubiquinol (110 ms) and cytochrome *b* (90 ms) oxidation (Papa, S., Guerrieri, F. and Lorusso, M., unpublished). These observations together with the kinetic data on aerobic proton uptake in sonic particles (Table I), which practically consist of functional vesicles of the inner-mitochondrial membrane turned inside out [13, 35–37], allow to conclude that oxidation of ubiquinone and *b* cytochromes of the mitochondrial respiratory chain is accompanied by synchronous proton uptake at the inner side and proton release at the outer side of the inner-mitochondrial membrane.

According to Mitchell [31, 38] direct coupling between scalar redox processes and vectorial proton translocation is a purely physical consequence of the supramolecular arrangement of the respiratory chain in the membrane. This author has proposed that the respiratory chain is bent upon itself from one side to the other of the membrane at the coupling sites so as to form redox loops each composed of a hydrogen and an electron carrier and translocating $2H^+/2e^-$.

Evidence is available [1–3, 7, 8] showing that the cytochrome *c* oxidase region of the respiratory chain is oriented transversely across the mitochondrial membrane with cytochrome *c* exposed at the outer side and cytochrome oxidase reacting with oxygen at the inner side of the mitochondrial membrane. This configuration accounts for vectorial electron flow across the membrane and corresponds to the electron carrying arm of the third redox loop of Mitchell's model. According to Mitchell [31] the hydrogen arm of this loop is represented by ubiquinone. Thus aerobic oxidation of ubiquinol should result in the translocation of $2H^+/2e^-$.

The present flow analysis shows that in sonic particles $4H^+$ are taken up per

$2e^-$ flowing from endogenous ubiquinol to oxygen. The $H^+/2e^-$ stoichiometry for this span of the respiratory chain has also been analyzed by Lawford and Garland [39], by pulsing intact mitochondria with small amounts of exogenous quinols. The $H^+/2e^-$ ratio for proton release, induced by oxidation of added quinols, amounted, when corrected for proton back-flow, to 4. The identity of these and our measurements is indeed significant if one considers that they were obtained with independent methods and under different experimental conditions.

The observation that the $H^+/2e^-$ ratio measured in sonic particles is with an accuracy of ± 0.5 (see Table II) equal to that of intact mitochondria, shows that the EDTA sonic submitochondrial particles, used in the present investigation, consist by more than 90 % of functional, inverted vesicles of the inner-mitochondrial membrane. In fact it can be calculated that 10 % contamination by functional "right-side out" particles should lower the $H^+/2e^-$ ratio from 4 to 3.2.

The fact that 4 H^+ are translocated per $2e^-$ flowing from ubiquinol to oxygen is inconsistent with the sequence and spatial arrangement proposed by Mitchell [31] for this span of the respiratory chain. The present findings in fact imply that one (or two) more hydrogen carriers should exist on the oxygen side of ubiquinone. No classical hydrogen carrier is known to act in this region of the respiratory chain. However, as we recently proposed [1, 33, 40], metal electron carriers might act as effective hydrogen carriers and might be directly involved in transmembrane proton translocation if oxido-reduction of the metal were linked to protonic equilibria of acidic groups in the apoprotein. These linkage phenomena would be of the same type as those described for soluble hemo-proteins [32, 41]; however the specific attachment of the carriers to the membrane would confer vectoriality to the protonic reactions. Possible candidates for this mechanism are *b* cytochromes, whose midpoint potential decreases by 60 mV per pH unit increase [40, 42–44], and iron-sulphur-proteins provided that the midpoint potential of these components had a similar pH dependence.

Fig. 7 presents two tentative schemes to explain redox-linked proton translocation at the level of complex III of the respiratory chain. According to mechanism (a), ubiquinol, reduced by complex II at the M side of the membrane, diffuses to the outer side where it is oxidized by cytochrome *b* of complex III and releases 2 $H^+/2e^-$; electron transfer along complex III is now associated to release of another 2 $H^+/2e^-$. It might be possible that either cytochrome *b* or the iron-sulphur-protein transfer hydrogen atoms, accepting, upon reduction of the iron, protons at the inner side of the membrane and releasing these at the outer side upon oxidation.

Aerobic oxidation of the terminal electron carriers is accompanied in intact mitochondria by synchronous antimycin-insensitive ubiquinol oxidation and proton release (see Fig. 6 and refs 2, 18 and 24). The fact that this proton production is accompanied in the presence of antimycin by synchronous reduction of both cytochromes b_{562} and b_{566} (Fig. 6, refs 18, 24 and 25), and its extent is in excess with respect to the amount of ubiquinol oxidized, indicates that a component of complex III, different from the *b* cytochromes and situated on the oxygen side of the antimycin site is directly involved in the redox-linked proton pump in this span of the respiratory chain. Since at physiological pH values cytochrome c_1 acts as an electron carrier the only known component of the complex which might be directly involved in proton translocation is the Rieske's iron-sulphur protein [45].

Whilst there is some evidence that ubiquinone is reduced by complex I at the

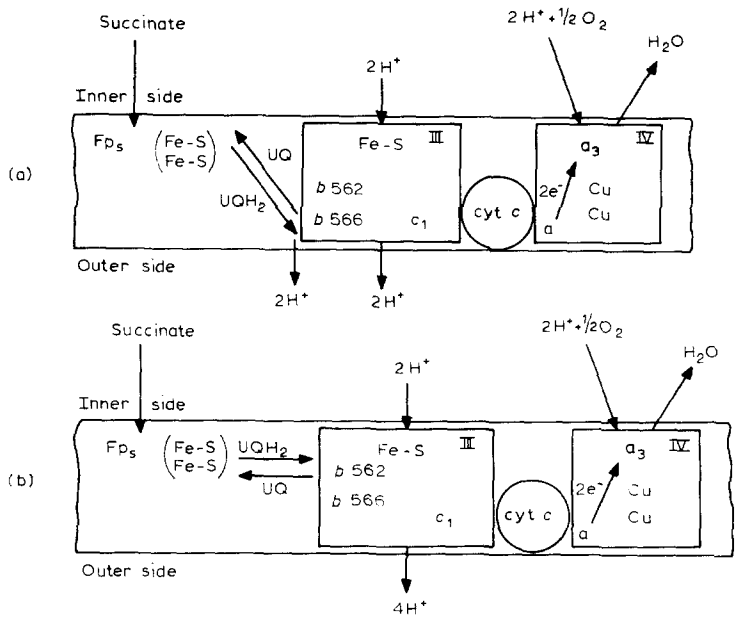

Fig. 7

inner side of the membrane [46] no experimental evidence is available to support the hypothesis that ubiquinol is oxidized by complex III at the outer side of the membrane. In fact the well-known observation that ferricyanide, which does not permeate the cristae membrane [47], interacts with respiratory carriers on the oxygen side of antimycin site but not with those on the substrate side, would suggest that the redox groups of the respiratory carriers on the substrate side of the antimycin site do not become directly exposed to the outer aqueous phase during their catalytic activity. In this case (see mechanism b) it might be possible that ubiquinone shuttles between complexes I, II and III at the inner side or in the hydrophobic domain of the membrane, without getting directly in equilibrium with protons in the external aqueous phase. Therefore the components of complex III should account for the release of 4 H^+ at the outer side of the membrane per $2e^-$ electrons flowing from ubiquinol to cytochrome *c*.

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