Biochimica et Biophysica Acta, 357 (1974) 181–192

BBA 46760

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

ANALYSIS OF PROTON TRANSLOCATION ASSOCIATED TO OXIDO-REDUCTIONS OF THE OXYGEN-TERMINAL RESPIRATORY CARRIERS

S. PAPA, F. GUERRIERI and M. LORUSSO

Institute of Biological Chemistry, Faculty of Science, Institute of Biological Chemistry, Faculty of Medicine, Centre for the Study of Mitochondria and Energy Metabolism, CNR, University of Bari, Bari (Italy)

(Received November 26th, 1973)
(Revised manuscript received February 22nd, 1974)

SUMMARY

The kinetics and stoicheiometry of fast proton translocation associated to aerobic oxidation of the oxygen-terminal components of the mitochondrial respiratory chain have been analyzed by means of continuous- and stopped-flow techniques.

1. In intact mitochondria the aerobic oxidation of the respiratory carriers situated on the oxygen side of the antimycin site was accompanied by synchronous release of protons. When the proton conductivity of the membrane was increased by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, oxidation of the terminal respiratory carriers was accompanied by stoicheiometric consumption of protons. This proton disappearance from the medium was, however, much slower than the oxidation of the respiratory carriers.

2. In sonic sub-mitochondrial particles oxidation of the terminal respiratory carriers was accompanied by synchronous and stoicheiometric proton consumption. This proton uptake was practically unaffected by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; its rate was markedly increased by valinomycin plus K^+.

3. The results presented provide functional evidence that cytochrome oxidase is a transmembranous molecule with haeme a_3 reacting with oxygen at the matrix side of the inner mitochondrial membrane and haeme *a* reacting with cytochrome *c* at the outer side.

4. The fast proton release accompanying the oxidation of the terminal respiratory carriers in intact mitochondria appears to be associated to antimycin-insensitive oxidation of a hydrogen carrier.

INTRODUCTION

In previous papers [1–6] evidence was presented showing that respiration-linked proton translocation in mitochondria consists of a single, electrogenic flux

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

mediated by system(s) different from cation carriers in the mitochondrial membrane (cf. refs 7 and 8; contrast refs 9 and 10). Results have also been obtained [4, 6, 11, 12] indicating that the proton pump is directly coupled to electron flow without the intervention of labile, uncoupler-sensitive, chemical intermediates (see also ref. 13).

As already pointed out [4, 6, 14–16], elucidation of the molecular mechanism by which oxido-reductions are coupled to vectorial proton translocation requires full knowledge of: (i) the functional components of the respiratory chain and their sequence, (ii) the physical-chemical properties of the respiratory carriers and (iii) their topological organization in the membrane.

In this paper a study is presented of the kinetics and stoicheiometry of proton translocation associated to oxido-reductions of the oxygen-terminal part of the respiratory chain, which appears to throw light on some of the above-mentioned points.

An account of these investigations has already been communicated [11, 12].

METHODS

Mitochondria and sub-mitochondrial particles

Heavy, beef-heart mitochondria were prepared as described by Löw and Vallin [17], stored for 2–7 days at −10 °C and thawed immediately before use. EDTA sub-mitochondrial particles were prepared as described by Lee and Ernster [18]: heavy, beef-heart mitochondria were suspended at a concentration of 20 mg protein per ml in 0.25 M sucrose containing 4 mM EDTA (final pH 8.5) and exposed to ultrasonic energy for 60 s at 0 °C (Ultrasonic-Branson Sonifier, model W 185; output, 70 W).

Kinetic analysis of aerobic proton translocation

The kinetics of proton translocation was analyzed with a Roughton-type continuous-flow pH meter (mixing ratio 1 : 60) with a resolution time of 10 ms [19]. 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 Electrometer, model 62A, and from this to a Honeywell Recorder, model Electronik 194. Potential changes were quantitated as proton equivalents by double titration with standard solutions 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 of KOH or HCl at a final concentration of 35 μM; the short time interval used avoids titration of the interior of the particles.

Oxido-reduction of respiratory carriers

Oxido-reduction of respiratory carriers was followed with a Johnson Foundation dual-wave-length spectrophotometer using either a static cuvette or a stopped-flow apparatus (mixing ratio 1 : 80) [20].

Incubation procedure

Mitochondria and sub-mitochondrial particles were incubated at 25 °C in 250 mM sucrose. Potassium succinate served as the respiratory substrate and buffer. The specific additions are given in the legends to figures and tables.

MATERIALS

Valinomycin was a generous gift of Dr W. O. 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

Oxido-reduction-linked proton translocation in intact mitochondria

Fig. 1 illustrates a typical experiment in which respiration-linked proton release from mitochondria was analyzed with the continuous-flow pH meter. Respiration was activated by repetitive pulses with an oxygen-saturated medium of strictly anaerobic mitochondria supplemented with succinate as the respiratory substrate. In this way proton translocation could be measured at various ms intervals after oxygenation. Oxygenation caused an immediate release of protons amounting, after 15 ms, to 1.5 ng ion H^+ per mg protein. After this fast proton production the rate of proton release sharply decreased. The total extent of the reaction, measured from the pH decrease which ensued as the flow stopped, amounted to 16 ng ion H^+ per mg protein. Similar observations have recently been reported by Penniston [21] who

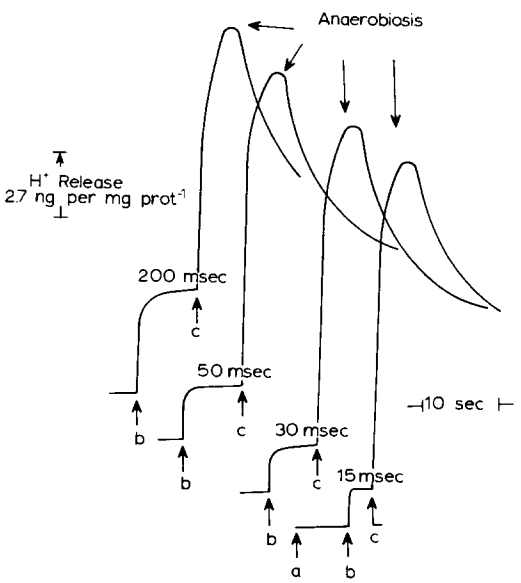

Fig. 1. Continuous-flow measurement of the initial rate of respiration-driven proton translocation in beef-heart mitochondria. The main syringe of the continuous-flow pH meter contained 250 mM sucrose, 10 mM potassium succinate, 30 mM KCl, 0.5 μg/ml rotenone and 3 mg/ml of mitochondrial protein. Final pH, 7.4. Temperature, 25 °C. The smaller syringe contained an oxygen-saturated mixture consisting of 250 mM sucrose and 30 mM KCl. Arrows indicate: (a) start of the flow of the content of the main syringe, (b) start of the flow of the oxygen-saturated medium with oxygenation of the anaerobic mitochondrial suspension and (c) stop of the flow. For other details see under Methods.

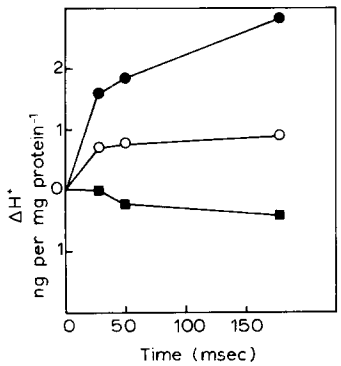

Fig. 2. Effect of antimycin and FCCP on the initial rate of respiration-driven proton translocation in beef-heart mitochondria. For incubation conditions see the legend to Fig. 1: ●, control; ○, plus 1 μg/mg protein antimycin; ■, antimycin plus 1 μM FCCP.

found that oxygen uptake and the accompanying proton ejection from beef-heart mitochondria occurred at a very high rate within the first 500 ms after oxygenation, after which both rates sharply decreased. Evidently respiration and proton ejection reflect, in the first 500-ms interval, rapid oxidation of anaerobically reduced respiratory carriers. This has been directly verified in our laboratory [4, 6, 11, 12].

Fig. 2 shows the effect of antimycin A and FCCP on the respiration-linked proton release from mitochondria. The experimental points were obtained with the continuous-flow pH meter by exposing anaerobic mitochondria to repetitive oxygen pulses. It should be stressed that in these, as well as in the other experiments, anaerobiosis was also monitored by spectrophotometric analysis of the redox state of cytochromes. When antimycin was present sufficient time was allowed, before repeating the oxygen pulse, for cytochromes $c+c_1$ and $a+a_3$, oxidized in the aerobic state, to be maximally reduced through the slow antimycin-resistant succinate oxidase activity. Antimycin suppressed the slow phase of proton release; however, the fast initial phase of the process was largely antimycin-insensitive. Fig. 2 also shows that when 1 μM FCCP was added to the anaerobic mitochondria supplemented with succinate and antimycin, no pH change occurred within 30 ms after oxygenation. However, the continuous-flow signal revealed the disappearance of 0.2 ng ion H^+ per mg protein at 50 ms and 0.4 at 180 ms (cf. ref. 22).

Fig. 3 illustrates the kinetics of the oxidation of cytochromes $c+c_1$ and cytochrome a, a_3 caused by oxygenation of beef-heart mitochondria supplemented with antimycin and succinate and their subsequent anaerobic reduction. These redox changes were analyzed with the stopped-flow dual-wave-length spectrophotometer; the rapid oxidation of cytochromes was simultaneously recorded on a storage oscilloscope and a strip-chart recorder. It can be seen that cytochrome a, a_3 was practically completely oxidized during the flow (this phase corresponded to 6 msec after mixing); cytochromes $c+c_1$ were 55 % oxidized during the flow; their oxidation continued when the flow stopped and reached completion in about 60 ms. The anaerobic reduction of cytochrome oxidase and cytochromes $c+c_1$, caused by the antimycin-resistant succinate oxidase was, as expected, very slow and reached completion in about 3 min.

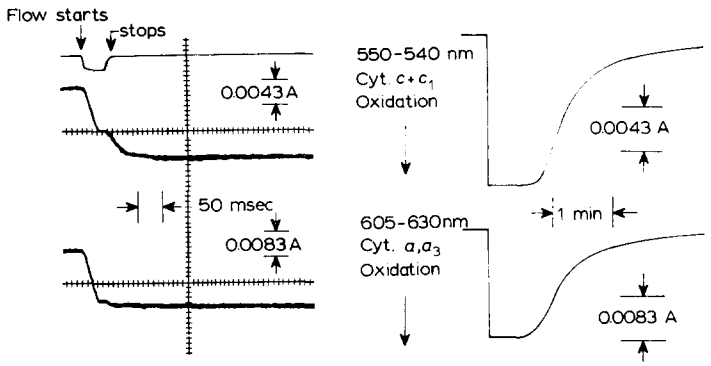

Fig. 3. Kinetics of aerobic oxidation and anaerobic reduction of cytochromes $c+c_1$ and cytochrome oxidase in beef-heart mitochondria in the presence of antimycin. The redox kinetics were analyzed with the stopped-flow spectrophotometer. Mitochondria (2.2 mg protein per ml) were incubated at 25 °C in a reaction mixture containing 200 mM sucrose, 30 mM KCl, 5 mM potassium succinate, 2 μg/mg protein oligomycin, 0.1 μg/mg protein rotenone and 1 μg/mg protein antimycin A. Oxygenation of anaerobic mitochondria was obtained by adding an oxygen-saturated medium containing 200 mM sucrose and 30 mM KCl. Final pH 6.8. For other details see under Methods and Results.

The pH traces presented in Fig. 4 give a picture of the various phases of proton translocation accompanying respiratory pulse of antimycin-treated mitochondria and of the effect on this process of FCCP. In the absence of FCCP 0.8 ng ion H^+ per mg protein were released from the mitochondria at 30 ms after oxygenation of anaerobic mitochondria. When the flow stopped the acidification of the medium continued until anaerobiosis was reached, but this phase of proton release occurred with a rate about 3 orders of magnitude lower than that of the initial burst of acidification. This very slow phase is attributed to the antimycin-resistant succinate oxidase activity. Upon anaerobiosis the acidification of the medium reversed completely. In the presence of FCCP 0.3 ng ion H^+ were taken up per mg protein at 200 ms after oxygenation. This proton uptake continued when the flow stopped and reached a maximum extent of 1.6 ng/mg protein. Upon anaerobiosis an equal amount of protons was slowly released from the mitochondria.

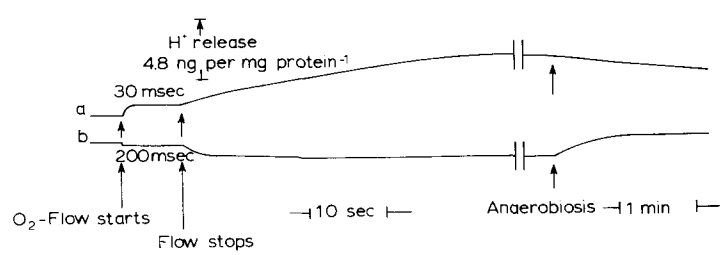

Fig. 4. Continuous-flow traces of respiration-driven proton translocation in beef-heart mitochondria in the presence of antimycin and antimycin plus FCCP. For experimental conditions see legends to Figs 1 and 2 and under Methods. Trace a: 1 μg/mg protein antimycin; trace b: antimycin plus 1 μM FCCP.

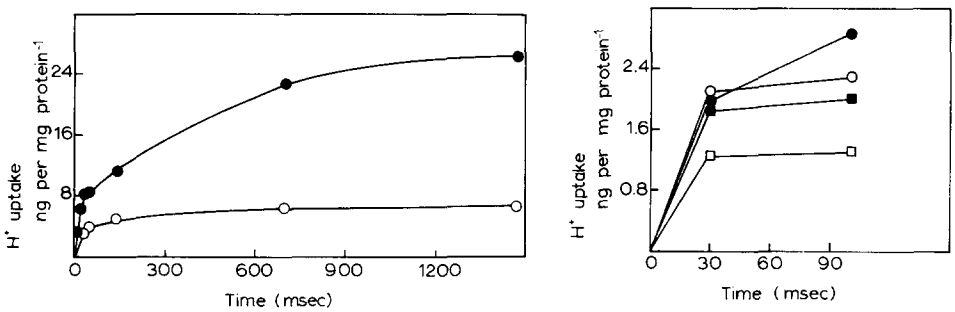

Fig. 5. Continuous-flow analysis of the respiration-linked proton uptake by EDTA sub-mitochondrial particles. The main syringe contained 250 mM sucrose, 0.1 mg/ml catalase, 15 mM potassium succinate, 50 mM KCl, 3 μg/ml oligomycin and 1 mg/ml EDTA-sub-mitochondrial particles. Final pH, 7.7. Temperature, 25 °C. The smaller syringe contained 0.2 % H_2O_2. ○, Control; ●, plus 0.5 μg/mg protein valinomycin.

Fig. 6. Effect of antimycin, valinomycin and FCCP on the kinetics of respiration-linked proton uptake in EDTA sub-mitochondrial particles. The main syringe contained 250 mM sucrose, 5 mM potassium succinate, 30 mM KCl, 2.5 μg/mg protein oligomycin and EDTA sub-mitochondrial particles, 1.5 mg/ml. Final pH, 7.5. Temperature, 25 °C. The smaller syringe contained an oxygen-saturated mixture consisting of 250 mM sucrose and 30 mM KCl. ●, Control; □, plus 0.5 μg/mg protein antimycin; ○, antimycin plus 0.34 μg/mg protein valinomycin; ■, antimycin, valinomycin and 1 μM FCCP. For other details see under Methods.

Proton translocation in sonic sub-mitochondrial particles

Sub-mitochondrial particles obtained by exposure of mitochondria to ultrasonic energy consist of vesicles of the inner membrane turned inside out [17, 23]. These vesicles exhibit an opposite polarity of the proton pump with respect to intact mitochondria [1–3]. The continuous-flow analysis of the respiration-linked proton uptake by sonic particles presented in Fig. 5 shows that the process was biphasic. It can be distinguished a very fast initial phase, which apparently completed itself in about 50 ms, and a much slower phase which took about 5 s to go to completion. The overall proton uptake amounted to 12 ng ion H^+ per mg protein. The biphasicity of the aerobic proton uptake was more evident in the presence of valinomycin plus K^+, which caused a marked stimulation of both the phases of proton uptake. This shows that dissipation by valinomycin-mediated K^+ migration of the membrane potential, set up by aerobic proton translocation, takes place very rapidly in the ms range (cf. ref. 5).

In the presence of the ionophore, about 4 ng ion H^+ were taken up per mg particle protein within 10 ms after oxygenation of anaerobic particles. This very fast proton uptake has to be associated with electron flow along the cytochrome *c* cytochrome oxidase region of the respiratory chain. This is substantiated by the experiment of Fig. 6, which shows that antimycin suppressed the slow phase of proton uptake but caused only a 26 % decrease of the fast initial part of the process. Fig. 6 shows, in addition, that the antimycin-insensitive proton uptake, induced by oxygenation of anaerobic particles, was also markedly stimulated by valinomycin. FCCP had, on the other hand, no significant effect on proton uptake (see Fig. 6).

Fig. 7 illustrates the kinetics of oxidation of cytochromes $c+c_1$ and cytochrome a, a_3 and of the accompanying proton uptake induced by oxygenation of

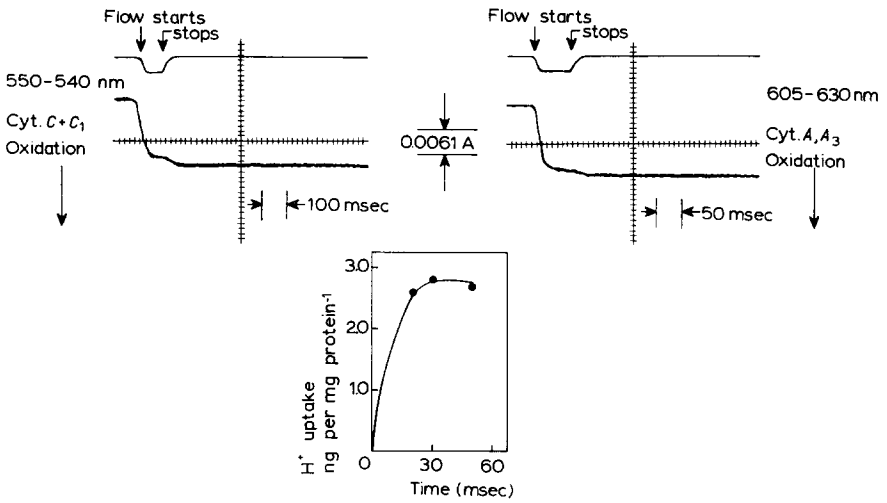

Fig. 7. Kinetics of cytochrome oxidation and proton uptake induced by oxygen pulses of sonic sub-mitochondrial particles in the presence of antimycin. Sub-mitochondrial particles (2.0 mg protein per ml) were incubated at 25 °C in a reaction mixture containing 250 mM sucrose, 5 mM potassium succinate, 30 mM KCl, 2.5 μg/mg protein oligomycin, 0.5 μg/mg protein valinomycin and 1 μg/mg protein antimycin. Oxygenation of anaerobic particles was obtained by adding an oxygen-saturated medium containing 250 mM sucrose and 30 mM KCl. Final pH, 6.85. Proton uptake was measured with the continuous-flow pH meter and cytochrome oxidation with the dual-wave-length spectrophotometer and stopped-flow apparatus.

anaerobic particles in the presence of antimycin and valinomycin. The oxidation of cytochrome a, a_3 was, as in the case of intact mitochondria, completed during the flow; this phase corresponded to an interval of 6 ms after mixing with oxygen. The oxidation of cytochromes $c+c_1$ was, under these conditions, considerably faster than in intact coupled mitochondria (see Fig. 3); it had in fact occurred to an extent of 87 % during the flow and reached completion, after the flow stopped, in about 30 ms. Proton consumption completed itself in 20–30 ms. Thus, the antimycin-insensitive aerobic proton uptake is synchronized to oxidation of the oxygen-terminal respiratory carriers, i.e. in sonic sub-mitochondrial particles there is no measurable lag between completion of oxidation of the electron carriers, on the oxygen-side of the antimycin site, and proton consumption.

Further kinetic resolution of the individual steps of electron transfer in this span of the respiratory chain and of the oxygen protonation is in progress in our laboratory.

From the extent of cytochromes $c+c_1$ oxidation during the flow (6 ms after mixing) a turnover of 206 moles cytochromes $c+c_1$ oxidized per s per mole of cytochrome oxidase ($\Delta\varepsilon = 28$) [25] could be calculated. The extent of proton uptake measured at 20 ms gave a turnover number of 260 gion H^+ per s per mole of cytochrome oxidase. This latter number is higher since it includes proton consumption due to oxidation of the reduced cytochrome oxidase. These measurements probably underestimate the turnover of the cytochrome oxidase reaction; however, the present turnover numbers are very close to those measured by other authors in intact mitochondria and in the purified oxidase (see ref. 25 for review), where at pH 7, 25 °C

and in phosphate buffer, which activates the reaction, a turnover of 300 moles of cytochrome *c* oxidized per s per mole of cytochrome oxidase was calculated [25].

Table I compares the extent of proton uptake which occurred upon oxygenation of anaerobic particles pre-treated with succinate, valinomycin plus K^+ and antimycin, to the extent of oxidation of cytochromes $c+c_1$ and haemes $a+a_3$.

TABLE I

FAST H^+ UPTAKE ASSOCIATED TO THE AEROBIC OXIDATION OF THE TERMINAL SPAN OF THE RESPIRATORY CHAIN IN EDTA SUB-MITOCHONDRIAL PARTICLES

Sub-mitochondrial particles (1.6–2.0 mg protein per ml). The experimental conditions and procedure are those given in the legend to Fig. 7. In the presence of antimycin cytochrome oxidation and proton uptake were completed at 30 ms after oxygenation (see Fig. 7); the figures given in the table refer to this interval. The extent of cytochrome oxidation was calculated by using a $\Delta\varepsilon_{mM}$ at 550–540 nm of 19.1 for cytochromes $c+c_1$ [24] and a $\Delta\varepsilon_{mM}$ at 605–630 nm of 14 for haemes $a+a_3$ [25]. For other details see under Methods.

Expt No.	H^+ uptake (ngion/mg protein)	Cytochrome oxidation (nmoles/mg protein)	
		$c+c_1$ 550–540 nm	$a+a_3$ 605–630 nm
1	4.38	0.57	0.98
2	3.17	0.57	1.10
3	2.47	0.68	1.04
Mean	3.34	0.61	1.04

DISCUSSION

The present continuous-flow potentiometric analysis of the respiration-linked proton pump in the mitochondrial membrane affords kinetic resolution of protonic reactions accompanying fast electron transfer along the oxygen-terminal span of the respiratory chain. The respiration-linked proton ejection from mitochondria showed a very rapid initial phase in the 15–30-ms range (Figs 1 and 2). Within this interval a smaller, but significant, proton release could also be detected when electron flow to oxygen was limited by antimycin to the terminal electron carriers of the respiratory chain (Figs 2 and 4). Since rapid electron flow from these carriers to oxygen requires consumption of one proton per electron transferred, two explanations can in principle be offered for the present findings: (i) the reaction of cytochrome oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) with oxygen takes place at a site of the membrane which is not directly exposed to the electrode; (ii) the rapid protonation of oxygen by cytochrome oxidase occurs at the external side of the membrane but is compensated by an equally fast proton-releasing reaction promoted by electron flow along the terminal carriers.

As regards the topography of the cytochrome *c*–cytochrome oxidase region various observations show that cytochrome *c* is located at the external side of the inner mitochondrial membrane (see for reviews refs 26–28). Investigations with

antibodies [26] and selective surface reagents [29] indicate that cytochrome oxidase is a transmembranous molecule. There are also results on the reactivity of cytochrome oxidase with azide [30] and cross-linked poly-L-lysine [29] which have been taken as evidence for the location of haeme a_3 at the inner side of the membrane.

Internal location of haeme a_3 has been inferred by Mitchell and Moyle [22] from the observation that in intact mitochondria the oxidation of respiratory carriers on the oxygen side of the antimycin site was accompanied by proton uptake provided that a high proton permeability was induced in the membrane by FCCP. However their results, obtained with conventional potentiometric techniques, did not rule out the alternative possibility that the uncoupler unmasks protonation of oxygen at the external surface of the membrane by inhibiting a proton-ejecting $\sim$ driven pump (see ref. 14). The present investigation appears to provide discrimination between these two mechanisms.

In the presence of antimycin and FCCP the extent of proton uptake induced by oxygenation of anaerobic mitochondria amounted to 1.6 ngion H^+ per mg protein. Upon anaerobiosis an equal amount of protons was slowly released from mitochondria (see Fig. 4). These protonic reactions, which are exactly opposite in direction to those observed in the absence of FCCP are, evidently due to aerobic oxidation and subsequent anaerobic reduction by hydrogen carrier(s) of the terminal electron carriers. In fact their extent roughly corresponds to the estimated content of the terminal electron carriers (see below and ref. 31). Thus, the uncoupler, by abolishing effective transmembrane proton translocation, allows the scalar protonic reactions associated to redox processes to be detected directly by the electrode. It can be noted that in the presence of FCCP the proton uptake associated to the aerobic oxidation of the terminal electron carriers manifested itself to the electrode much more slowly than the proton release observed in the absence of the uncoupler. The proton uptake had occurred only by 23 % at 200 ms after oxygenation and took more than 5 s to reach completion. Since the oxidation of the terminal electron carriers was practically completed within 60 ms (see Fig. 3 and ref. 32), it can be concluded that the site where oxygen reacts with haeme a_3 and is protonated is located on the internal side of the membrane, the delay for detecting the latter process being due to the inward diffusion time of protons. On the other hand it seems possible to exclude that the uncoupler acted by inhibiting a $\sim$ driven proton pump. Since FCCP took seconds to completely unmask the protonation of oxygen it could not act by inhibiting a protonreleasing process which is shown to be completed within a ms range (see Fig. 2). These conclusions are further reinforced by the results obtained with sonic submitochondrial particles.

In sonic particles, due to the inverted orientation of the inner mitochondrial membrane, haeme a_3 should be directly exposed to the medium. In fact oxidation of the respiratory carriers on the oxygen side of the antimycin site was accompanied, in this case, by a rapid disappearance of protons from the medium. This fast proton uptake was markedly stimulated by valinomycin plus K^+. In the presence of the ionophore the rapid, antimycin-insensitive proton uptake amounted in 30 ms to 3.3 ng ion H^+ per mg protein; the extent of oxidation of cytochromes $c+c_1$ and haemes $a+a_3$ amounted in the same interval to 0.6 and 1.0 nmole/mg protein, respectively (see Table I). If we add to this 1.0 natom of copper (see refs 31 and 33) and 0.4 natom of non-haeme iron [34] we reach a total of 3.0 oxidized metal natoms. Thus there is,

apparently, a 1 to 1 stoicheiometry between proton consumption and electron flow from the terminal electron carriers to oxygen.

The possibility of the occurrence of an uncoupler-sensitive proton pump activated by a $\sim$ intermediate generated by electron flow at the third coupling site is definitively ruled out by the fact that the antimycin-insensitive, fast proton uptake, induced in sonic particles by oxygenation, was practically unaffected by FCCP (see Fig. 6).

The finding that valinomycin enhanced the rate of oxygen protonation associated to the oxidation of the respiratory carriers on the oxygen side of the antimycin site, is taken as evidence that cytochrome oxidase is a transmembranous molecule, with haeme a_3 reacting with oxygen on the inner side of the membrane and haeme *a* reacting with cytochrome *c* on the opposite side. The fast protonation of oxygen on the inner side of the membrane generates a negative membrane potential on this side. This exerts a back pressure on the oxidation of the electron carriers situated on or towards the opposite side. Dissipation of the potential by valinomycin speeds up oxidation of these carriers.

The spatial arrangement of the cytochrome *c* oxidase region suggested from the present data corresponds to the electron-carrying arm of the third loop of Mitchell's model of the redox proton pump [35, 36]. However this loop could cause effective proton translocation across the membrane only if ubiquinone were oxidized by cytochrome c_1 on the outer side of the membrane (cf. ref. 37).

Another aspect of the process is revealed by the initial fast phase of proton release observed in the anaerobic-aerobic transition of intact mitochondria. This appears to be related to a hydrogen carrier whose oxidation is insensitive to antimycin and synchronized with cytochrome *c* oxidation. The organic hydrogen carriers of the respiratory chain are situated on the substrate side of the antimycin site. However, recent data [38] would indicate that the rapid aerobic oxidation of cytochromes c_1 and *c* is accompanied, in the presence of antimycin, by an equally fast oxidation of ubiquinone and reduction of *b* cytochromes. The oxidation of ubiquinone might be responsible for the rapid antimycin-insensitive proton release provided that it takes place on the outer side of the membrane. Alternatively, one has to look for a hydrogen carrier on the oxygen-side of the antimycin site. The cytochromes of this region act as electron carriers. However, there remains the Rieske's Fe-S-protein [34] which might act as a hydrogen carrier if electron transfer by the metal were linked to proton transfer at the level of acidic groups in the apoprotein. We have in fact proposed [4, 6, 11] that the redox proton pump could represent the result of linkage phenomena [39] between the redox state of the metal of membrane-bound electron carriers and the protonic equilibrium of acidic groups in their apoproteins. Possible candidates for this were considered to be the *b* cytochromes, whose mid-point redox potential decreases by 60 mV per pH unit increase [40–42], and non-haeme iron proteins, provided that the mid-point potential of these components had a similar pH-dependence.

ACKNOWLEDGMENT

The skilful technical assistance of D. Boffoli is gratefully acknowledged.

REFERENCES

1 Papa, S., Guerrieri, F., Lorusso, M. and Quagliariello, E. (1970) FEBS Lett. 10, 295–298
2 Papa, S. (1971) in Energy Transduction in Respiration and Photosynthesis (Quagliariello, E., Papa, S. and Rossi, C. S., eds), pp. 173–204, Adriatica Editrice, Bari
3 Papa, S., Guerrieri, F., Simone, S., Lorusso, M. and Larosa, D. (1973) Biochim. Biophys. Acta 292, 20–38
4 Papa, S., Guerrieri, F., Simone, S. and Lorusso, M. (1973) in Mechanisms in Bioenergetics (Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E. and Siliprandi, N., eds), pp. 451–472, Academic Press, New York and London
5 Papa, S., Scarpa, A., Lee, C. P. and Chance, B. (1972) Biochemistry 11, 3091–3098
6 Papa, S., Guerrieri, F., Lorusso, M. and Simone, S. (1973) Biochimie 55, 703–716
7 Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 7, 471–484
8 Skulachev, V. P. (1971) in Current Topics in Bioenergetics (Sanadi, D. R., ed.), Vol. 4, pp. 127–190, Academic Press, New York and London
9 Harris, E. J. and Pressman, B. C. (1969) Biochim. Biophys. Acta 172, 66–70
10 Massari, S. and Azzone, G. F. (1970) Eur. J. Biochem. 12, 310–318
11 Papa, S. (1973) I.U.B. 9th Internat. Congr. Biochem., Abstract Book 4Sd2, Stockholm
12 Papa, S., Guerrieri, F. and Lorusso, M. in Dynamics of Energy Transducing Membranes (Ernster, L., Estabrook, R. W. and Slater, E. C., eds), Elsevier Publishing Co., Amsterdam, in the press
13 Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147–1162
14 Slater, E. C. (1967) Eur. J. Biochem. 1, 317–326
15 Greville, G. D. (1969) in Current Topics in Bioenergetics (Sanadi, D. R., ed.), Vol. 3, pp. 1–72, Academic Press, New York
16 Mitchell, P. (1971) in Energy Transduction in Respiration and Photosynthesis (Quagliariello, E., Papa, S. and Rossi, C. S., eds), pp. 123–149, Adriatica Editrice, Bari
17 Löw, H. and Vallin, I. (1963) Biochim. Biophys. Acta 69, 361–374
18 Lee, C. P. and Ernster, L. (1968) Eur. J. Biochem. 3, 391–400
19 Rossi Bernardi, L. and Berger, R. L. (1968) J. Biol. Chem. 243, 1297–1302
20 Chance, B., De Vault, D., Legallais, V., Mela, L. and Yonetani, T. (1967) in Fast Reactions and Primary Processes in Chemical Kinetics, Nobel Symposium V (Claesson, S., ed.), pp. 437–464, Interscience, New York
21 Penniston, J. T. (1973) Biochemistry 12, 650–655
22 Mitchell, P. and Moyle, J. (1970) in Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), pp. 575–587, Adriatica Editrice, Bari
23 Lee, C. P. and Ernster, L. (1966) in Regulation of Metabolic Processes in Mitochondria (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), pp. 218–234, Elsevier, Amsterdam
24 Chance, B. (1957) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), pp. 273–329, Vol. IV, Academic Press, New York
25 Nicholls, P. and Kimelberg, H. K. (1972) in Biochemistry and Biophysics of Mitochondrial Membranes (Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E. and Siliprandi, N., eds), pp. 17–32, Academic Press, New York
26 Racker, E., Burstein, C., Loyter, A. and Christiansen, O. (1970) in Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), pp. 235–252, Adriatica Editrice, Bari
27 Klingenberg, M. and Von Jagow, G. (1970) in Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), pp. 281–290, Adriatica Editrice, Bari
28 Lee, C. P. (1966) in Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), pp. 291–300, Adriatica Editrice, Bari
29 Schneider, D. L., Kagawa, Y. and Racker, E. (1972) J. Biol. Chem. 247, 4074–4079
30 Palmieri, F. and Klingenberg, M. (1967) Eur. J. Biochem. 1, 439–446
31 Klingenberg, M. (1968) in Biological Oxidations (Singer, T. P., ed.), pp. 3–54, Interscience Publishers, New York
32 Chance, B., Azzi, A., Lee, I. Y., Lee, C. P. and Mela, L. (1969) in Mitochondria: Structure and Function (Ernster, L. and Drahota, Z., eds), pp. 233–273, Academic Press, London and New York

33 Lemberg, M. R. (1969) Physiol. Rev. 49, 48–121
34 Rieske, J. S., Hansen, R. E. and Zaugg, W. S. (1964) J. Biol. Chem. 239, 3017–3022
35 Mitchell, P. (1966) Biol. Rev. 445
36 Mitchell, P. (1966) in Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research Ltd., Bodmin
37 Hinkle, P. C. and Kim, J. J. (1973) in Mechanisms in Bioenergetics (Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E. and Siliprandi, N., eds), pp. 53–60, Academic Press, New York and London
38 Erecinska, M., Chance, B., Wilson, D. F. and Dutton, P. L. (1972) Proc. Natl. Acad. Sci. U.S. 69, 50–54
39 Wyman, J. (1968) Quart. Rev. Biophys. 1, 35–81
40 Straub, J. P. and Colpa Boonstra, J. P. (1962) Biochim. Biophys. Acta 60, 650–652
41 Urban, P. F. and Klingenberg, M. (1969) Eur. J. Biochem. 9, 519–525
42 Wilson, D. F., Erecinska, M., Leigh, G. S. and Koppelman, M. (1972) Arch. Biochem. Biophys. 151, 112–121