CYTOCHROME *c* OXIDASE IS NOT A PROTON PUMP

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Received 23 January 1978

1. Introduction

Mitchell postulated that cytochrome *c* oxidase is plugged through the coupling membrane of mitochondria and bacteria so that the reduction of O_2 to 2 H_2O involves the translocation of 4 e^- from cytochrome *c* at the outer surface of the membrane to 4 H^+ ions that enter the reaction domain from the inner aqueous phase [1]. As evidence for this putative electron-translocating function of cytochrome *c* oxidase, we observed that the reduction of a pulse of oxygen by initially anaerobic antimycin-treated mitochondria in a 150 mM KCl medium containing EGTA caused the inward translocation of about 4 H^+ ions per O_2 only when FCCP was present so that H^+ could be pulled through the membrane by the electric field produced by electron translocation. Further, when valinomycin was present to enable the electric field to be neutralised mainly by K^+ translocation, most of the expected alkalinity of the inner aqueous phase corresponding to the reduction of 4 H^+ + O_2 was not rapidly detectable in the outer aqueous phase unless pH equilibration across the membrane was facilitated by FCCP [2,3]. We also showed that pulsed respiration in mitochondrial suspensions preincubated anaerobically with succinate or β-hydroxybutyrate gave $\Delta H_o^+/2\ e^-$ quotients of 4 and 6 no matter whether O_2 or ferricyanide was used as oxidant [2]. As ferricyanide is impermeant and oxidises cytochrome *c*, bypassing cytochrome *c* oxidase, we concluded that cytochrome *c* oxidase does not act as a proton pump, but catalyses only the net translocation of electrons [3]. Work by Hinkle, Papa, Racker and others, using mitochondria, sonically-prepared mitochondrial vesicles and liposomes inlaid with cytochrome *c* oxidase has greatly strengthened our conclusion that cytochrome *c* oxidase only translocates electrons (see [4–6]).

This comparatively firm conclusion has recently been challenged by Wikström [7,8], who has claimed that the cytochrome *c* oxidase complex is equipped with a proton pump which may be conformationally coupled to the redox reaction. Wikström observed proton translocation associated with ferrocyanide oxidation in aerobic antimycin-treated mitochondria, and attributed this proton-pumping activity to cytochrome *c* oxidase [7,8]. We show here that this proton translocation is not coupled to cytochrome *c* oxidase activity, but is caused by the ferrocyanide oxidation-dependent oxidation of an unidentified hydrogenated reductant.

2. Materials and methods

The method of isolating rat liver mitochondria, and the methods of measuring and recording pH_o and oxygen concentration were as described [9,10]. Digitonin was recrystallised from ethanol before use [11]. Cytochrome *c* was from horse heart (Type III, Sigma, London). It was reduced by treatment with ascorbate followed by dialysis (see [11]) and made

Abbreviations: pH_o, pH of suspension medium; ΔH_o^+, increase of quantity of H^+ in the suspension medium; $\rightarrow H^+$, quantity of H^+ translocated; EGTA, ethyleneglycol-bis(aminoethyl)-tetraacetic acid; NEM, *N*-ethyl maleimide; FCCP, carbonylcyanide trifluoromethoxyphenylhydrazone; Q, ubiquinone; TMPD, *N,N,N',N'*-tetramethylphenylenediamine; $TMPD^+$, Wurster's blue; dig, digitonin; val, valinomycin; [red], concentration of reductant

 Elsevier/North-Holland Biomedical Press

up as a stock 2% solution in 10 mM Tris/HCl buffer, at pH 7.0. Preliminary experiments, using various concentrations of digitonin to facilitate entry of cytochrome *c* through the outer mitochondrial membrane, showed that in the EDTA-containing media used in our experiments, cytochrome *c* equilibrated across the outer membrane and accumulated in the mitochondrial intramembrane space in the usual 20 min preincubation even in the complete absence of digitonin.

We did two kinds kinds of respiratory experiment. In (initially aerobic) reductant-pulse experiments, ferrocyanide was injected into aerobic (rotenone- and antimycin-inhibited) mitochondrial suspensions, and the resulting changes of pH_0 and O_2 consumption were recorded. In (initially anaerobic) O_2-pulse experiments, air-saturated saline of known O_2 content was injected into anaerobic (rotenone- and antimycin-treated) mitochondrial suspensions after preincubation with ferrocyanide or with ferrocytochrome *c*, and the resulting changes of pH_0 were recorded. In either type of experiment, proton translocation was measured in the usual way in the presence of valinomycin [10]. As an essential control, it was also necessary to measure ΔH_0^+ in the presence of FCCP or valinomycin + FCCP to estimate the quantity of oxygen reduced by ferrocyanide or ferrocytochrome *c* and protonated from the inner aqueous phase with a stoicheiometry of 2 H^+ per O reduced. Any excess of oxygen reduced per 2 H^+ consumed should indicate a failure of antimycin to block oxidation of some hydrogenated reductant on the substrate side of the antimycin-inhibited site.

3. Results and discussion

Table 1 summarises the results of reductant-pulse and O_2-pulse respiratory experiments on mitochondria treated with rotenone and antimycin. These results (lines 1 and 2) confirm our observation [12], reproduced by Wikström [7], that cytochrome *c* oxidase activity is not accompanied by proton translocation in the absence of NEM and added substrate. Under these conditions, net H^+ uptake in the presence of FCCP corresponds to the uptake of 4 H^+ from the inner aqueous phase per O_2 reduced by cytochrome *c* oxidase, indicating that (apart from antimycin-insensitive respiration at a rate of only about 4% of the normal uncoupled rate) the only significant reaction is the oxidation of ferrocyanide via cytochrome *c* and cytochrome *c* oxidase. However, as shown in table 1 (line 3), when NEM was present in the absence of added substrate, or when the medium contained choline, which acted as a reducing substrate in the absence (line 4) or in the presence (line 5) of NEM, there was not only proton translocation, as observed by Wikström [7,8], but there was also excess O reduction. In the O_2-pulse type of experiment with ferrocyanide as reductant (table 1, lines 6–13), both proton translocation and excess O reduction occurred under all the conditions tested. This excess O reduction, other than by ferrocyanide oxidation in the reductant-pulse and O_2-pulse experiments (table 1, lines 3–13), indicates that about one H^+ is produced by oxidation of some unidentified hydrogenated reductant per net oxidation of about 3 ferrocyanide ions, independently of the rate of ferrocyanide oxidation but synchronously with it. The rate of oxidation of the unidentified reductant by the excess O depended on the ferrocyanide concentration and on other conditions (table 1), and ranged from 1–5 times the rate of antimycin-insensitive O reduction in the absence of ferrocyanide. The number of H^+ ions translocated per excess O reduced $[\rightarrow H^+/(\text{excess O})]$ was about 4. In O_2-pulse experiments, not included in table 1, it was shown that 0.1 mM KCN completely abolished the increase of O_2-reduction rate and any proton translocation caused by ferrocyanide.

The reductant-pulse and O_2-pulse experiments with ferrocyanide described in table 1 (lines 1–13), show that proton translocation does not correspond to cytochrome *c* oxidase activity, but invariably corresponds to the activity of a protonmotive system through which the excess O is reduced by the unidentified hydrogenated reductant. It is possible that the unidentified reductant is endogenous NADH, and that the protonmotive system is NADH dehydrogenase (which is still 15% active in the presence of rotenone) connected to an oxidant site at the surface of the membrane by a trans-membrane QH_2/Q couple, bypassing the Q cycle [13]. The oxidant could be ferricyanide produced at high concentration at the membrane surface by the oxidation of ferrocyanide by cytochrome *c*. To test this possibility, we con-

Table 1
Characteristics of oxygen reduction by ferrocytochrome *c* oxidase and by an interfering protonmotive redox system actuated during ferrocyanide oxidation in rat liver mitochondria

Medium	[red] (mM)	ΔO	→H^+ (val)	ΔH_o^+ (FCCP)	excess O	→H^+/(excess O)
Reductant-pulse experiments						
	$K_4Fe(CN)_6$					
A	0.5	4.8	0	− 9.5	0	–
B	0.3	2.9	0	− 6.0	−0.1	–
B + NEM	0.3	3.0	2.5	− 4.8	0.6	4.1
C	0.3	4.0	4.0	− 6.0	1.0	4.0
C + NEM	0.3	4.0	4.2	− 5.8	1.1	3.8
O_2-pulse experiments						
	$K_4Fe(CN)_6$					
B	0.3	5.3 (19.8)	4.3	− 8.3	1.1	3.9
B + NEM	0.3	5.3 (19.8)	4.2	− 8.4	1.1	3.8
C	0.3	5.2 (49.8)	5.7	− 7.4	1.5	3.8
C + NEM	0.3	5.1 (49.8)	5.4	− 7.4	1.4	3.9
D	0.3	10.9 (47.6)	10.8	−16.6	2.6	4.1
D	0.9	28.8 (47.6)	28.3	−44	6.8	4.1
B	0.9	12.1 (24.9)	10.0	−19.4	2.4	4.2
B	0.9	12.1 (49.8)	8.8	−18.4	2.9	3.1
	Ferrocyto-chrome *c*					
B	0.025	23.2 (24.9)	0	−46	0.2	–
B	0.05	27.4 (24.9)	0	−54	0.4	–
B	0.05	27.4 (49.8)	0	−54	0.4	–
B + dig	0.025	28.5 (24.9)	0	−57	0	–
B + NEM	0.025	25.8 (24.9)	0	−52	−0.2	–
B + NEM	0.05	30 (24.9)	0	−61	−0.5	–
E	0.05	24.6 (24.9)	0	−49	0.1	–

Main components of the suspension media were: A, 150 mM KCl; B, 150 mM KCl, 1 mM EDTA (K salt); C, 150 mM choline chloride, 1 mM EDTA (K salt); D, 230 mM sucrose, 10 mM KCl, 10 mM $MgCl_2$; E, 150 mM KCl, 1 mM EGTA, 5 mM $MgCl_2$. In addition, all media (3.3 ml) contained 3.3 mM glycylglycine, carbonic anhydrase (30 μg/ml), rat liver mitochondria (about 6 mg protein/ml), 0.4 μM rotenone and antimycin (36 μg/g mitochondrial protein). Where indicated, 0.2 mM NEM and digitonin (0.05 mg/mg mitochondrial protein) were added 5 min before the reductant or O_2 pulses. Experiments were done at 25°C at pH_o 7.0–7.1, and either valinomycin was present (10 μg valinomycin/g mitochondrial protein in media A, B and E or 100 μg valinomycin/g mitochondrial protein in media C and D), or 1 μM FCCP. In each set of experiments, O_2 reduction occurred at the same rate in the presence of valinomycin as in the presence of FCCP.

In reductant-pulse experiments, reductant was injected into the aerobic suspension to give the concentration indicated.

In O_2-pulse experiments, the anaerobic suspension containing the reductant was preincubated for 20 min before injection of air-saturated saline (150 mM KCl, 150 mM choline chloride or 10 mM KCl in 230 mM sucrose, as appropriate). The rate of the antimycin-insensitive O_2 reduction in the absence of added reductant was measured in separate experiments.

The values for ΔO, →H^+ (val), ΔH_o^+ (FCCP) and excess O are expressed as μg atoms O or μg ions H^+ min^{-1} per g mitochondrial protein, corrected for the antimycin-insensitive respiration. The values in brackets in the ΔO column represent the quantity of O_2 (ng atoms O) injected. Values for excess O that are less than 2% of the corresponding ΔO value are not experimentally significant. We express the data as quantities per unit time in the O_2-pulse experiments for comparison with the reductant-pulse experiments. This is done simply by dividing the measured total changes by the times taken for oxygen reduction, because the oxygen reduction rates are virtually constant during these experiments firmed [14] that ferricyanide was reduced by a KCN-insensitive system on the substrate side of the antimycin-sensitive site at a rate that was proportional to the ferricyanide concentration . This antimycin-insensitive reduction of ferricyanide was accompanied by proton translocation. At 15 mM ferricyanide, in a 150 mM KCl, 3.3 mM glycylglycine, 1 mM EDTA medium containing 1 μM FCCP, the rate of electron transfer to ferricyanide was 12 μg equiv. e^- min^{-1} per g mitochondrial protein, corresponding to about 15% of the uncoupled rate of electron transfer to O_2 in the absence of antimycin, and about 4 times the normal uncoupled rate of electron transfer to O_2 in the presence of antimycin. This would be sufficient to account for the observed rates of excess O reduction in the experiments of table 1, lines 3–13. But we would have to assume that the concentration of ferricyanide produced in the neighbourhood of the antimycin-insensitive ferricyanide-reactive component (QH_2 or Q· H?) at the surface of the membrane would be much higher during ferrocyanide oxidation by cytochrome *c* than the equilibrium concentration of ferricyanide in the outer aqueous phase. It is relevant that, unlike the TMPD/TMPD$^+$ couple, the ferrocyanide/ferricyanide couple was found not to provide a continuous bypass of the antimycin-sensitive site. The reduction of the excess O required the net oxidation of ferrocyanide. Ferricyanide was a very potent inhibitor of ferrocyanide oxidation by cytochrome *c*.

We do not know why there was no excess O reduction in the reductant-pulse experiments in the absence of choline (reductant substrate) and/or NEM, whereas excess O reduction occurred under all conditions tested in the (initially anaerobic) O_2-pulse experiments with ferrocyanide as reductant. It is possible, however, that the QH_2/Q couple was in a relatively oxidised state, and so failed to reduce ferricyanide at the surface of the membrane, under the conditions where there was no excess O reduction. At all events, as discussed in a broader context [15], the effect of NEM cannot be due to inhibition of the phosphoric acid uniporter, as assumed by Wikström [7,8].

The O_2-pulse experiments of table 1 (lines 14–20) show that, under a variety of conditions, interference by the protonmotive system involved in the excess O reduction was avoided (i.e., there was no excess O reduction) when horse heart ferrocytochrome *c* was used as reductant for the cytochrome *c* oxidase reaction. In these experiments, there was no proton translocation, and the addition of either oligomycin (1 mg/g mitochondrial protein) or 0.2 mM NEM had no significant effect. It is evident, therefore, that the cytochrome *c* oxidase complex has no proton-pumping function. We also observed, in experiments corresponding to those of table 1, lines 6–20, that the net alkalinisation given by the observed value of ΔH_0^+ in the presence of FCCP was not changed when valinomycin was also present to enable the electric field to be neutralised by trans-membrane K^+ equilibration. Thus, it was confirmed that cytochrome *c* oxidase translocates 2 e^- inwards from cytochrome *c* per O reduced and protonated by 2 H^+ from the inner aqueous phase.

The O_2-pulse experiments with ferrocytochrome *c* as reductant (table 1, lines 14–20) showed an interesting acidification artefact which may be relevant to the transient acidification seen by Hinkle [16] and by Wikström and Saari [8] in suspensions of liposomes inlaid with cytochrome *c* oxidase. On injecting the O_2 pulses in our experiments in the KCl media containing EDTA (table 1, lines 14–19), there was a step fall in pH_0, both in the mitochondrial suspensions containing FCCP, and in those containing valinomycin. This fall in pH_0 depended on the presence of added cytochrome *c*. It was proportional to the mitochondrial concentration and was increased by the presence of digitonin; but it was independent of the amounts of O_2 used in table 1. It was virtually complete within 1 s, whereas O_2 reduction by cytochrome *c*, catalysed by cytochrome oxidase, took several seconds and depended on the quantity of O_2 injected. Exactly equivalent falls in pH_0 were produced in control experiments (not included in table 1) by injecting pulses of ferricyanide in place of O_2, either in the absence, or in the presence of 0.1 mM KCN when there could be no cytochrome *c* oxidase activity. The rapid appearance and subsequent persistence of this pH_0 fall indicated that it was synchronous with the transition of part of the added cytochrome *c* from the reduced to the oxidised state. The maximum extent of the acidification artefact seen in this work corresponded to 1 μg ion H^+ per g mitochondrial protein. Moreover, this acidification artefact was completely abolished by 5 mM $MgCl_2$ in the KCl medium containing 1 mM EGTA but no digitonin (table 1, line 20).

The transient acidification artefact is probably explained by the fact that ferricytochrome *c* binds anions, whereas ferrocytochrome *c* binds cations [17]. Presumably the shift of ionic affinity of cytochrome *c*, as it becomes oxidised by the O_2 pulse (or by the control ferricyanide pulse), gives rise to a corresponding shift of protonic dissociation of acid/base groups that interact with the cytochrome *c* in the cristae membrane surface or in the outer membrane or intramembrane space of the mitochondria. This phenomenon sheds serious doubt on the conclusion of Wikström and Saari [8] that transient acidification seen in aerobic suspensions of liposomes inlaid with cytochrome *c* oxidase and pulsed with ferrocytochrome *c* can be taken as evidence for a proton-pumping function of cytochrome *c* oxidase.

4. Conclusion

We conclude that the reduction of O_2 to 2 H_2O by cytochrome *c* oxidase of rat liver mitochondria involves the translocation of 4 e^- from cytochrome *c* at the outer surface of the cristae membrane per O_2 reduced and protonated by 4 H^+ ions that enter the reaction domain from the inner aqueous phase. This net electron-translocating function of cytochrome *c* oxidase plugged through the mitochondrial cristae membrane is not linked to a proton-pumping function, such as that proposed by Wikström [7,8].

Acknowledgements

We thank Mr Robert Harper and Mrs Stephanie Key for expert technical assistance and help in preparing the manuscript. We gratefully acknowledge the financial support of Glynn Research Ltd.

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