The oxidation of exogenous cytochrome *c* by mitochondria

Resolution of a long-standing controversy

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Several reports in the past have dealt with the oxidation of cytochrome *c* added to suspensions of rat liver mitochondria. Yet, it is generally believed that the cytochrome cannot penetrate the outer membrane. Probably it has been assumed that the permeability of the outer membrane to cytochrome *c* is very low but finite, and that fast oxidation may be observed if time is allowed for sufficient penetration before initiation of electron flow. Here we show that this view is false. The main fraction of rat liver mitochondria, as isolated by conventional procedures, does not catalyse any significant oxidation of added cytochrome *c*, even after prolonged incubation. The observed appreciable oxidation of added cytochrome *c* is catalysed by a very small fraction (5–12%) of the mitochondria that apparently has a damaged outer membrane. Consequently, the turnover of cytochrome oxidase is very high in this fraction during oxidation of added cytochrome *c*. This finding readily explains why Moyle and Mitchell (e.g., FEBS Lett. 88 (1978) 268–272; 90 (1978) 361–365) have failed to observe proton translocation by cytochrome oxidase during oxidation of ferrocytochrome *c* added to rat liver mitochondria, which has been their main reason for rejecting the proton-pumping function of cytochrome oxidase.

Mitochondrial outer membrane *Cytochrome oxidase* *Proton pumping* *NADH-cytochrome* b_5 *reductase*

1. INTRODUCTION

The oxidation of cytochrome *c* added to a suspension of rat liver mitochondria has become something of a paradox. Although it is generally agreed that cytochrome *c* cannot penetrate the outer membrane [1–5], there are several reports showing appreciable rates of oxidation of added cytochrome *c* (e.g., [6–12]). An implicitly adopted explanation for this seems to be that the impermeability of the outer membrane for cytochrome *c* is a relative concept. Thus, the permeability may be very low, but the cytochrome may still penetrate at a finite velocity. According to this, even fast oxidation of added cytochrome *c* may be observed if time is allowed for sufficient penetration before electron flow is initiated.

The functioning of cytochrome oxidase as a proton pump [13] has been supported in a large number of laboratories (reviews, [14,15]). Only two groups have persistently rejected this proposal. The main argument of Moyle and Mitchell [8–10] against the proton-pumping concept has been that they observed no proton translocation during oxidation of the 'natural substrate', cytochrome *c*, added to intact rat liver mitochondria, yet, considerable rates of oxidation of the added cytochrome *c* were measured.

In this laboratory we have found H^+ translocation linked to oxidation of external cytochrome *c* by mitochondria suspended in high salt media [11,12,16]. However, the H^+/e^- ratio has general-

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

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ly been relatively low and, more strikingly, the velocity of backflux of protons across the membrane has been much higher than anticipated for well-coupled mitochondria. In sucrose-based media, at low salt concentrations, no H^+ translocation was observed at all although electron transfer was considerable [9,16].

In this communication we will demonstrate that the outer mitochondrial membrane presents an absolute barrier against penetration of cytochrome *c*. Oxidation of added cytochrome is catalysed by a very small fraction of the mitochondria, which apparently have a damaged outer membrane. This readily explains why proton translocation linked to oxidation of added cytochrome *c* by mitochondria is difficult and sometimes impossible to observe.

2. MATERIALS AND METHODS

Rat liver mitochondria were isolated in a 250 mM sucrose-1 mM EGTA medium by a conventional technique [16,17] and finally suspended in 250 mM sucrose at a concentration corresponding to 10 μM cytochrome aa_3 [16].

The reaction media were either 'KCl-medium', consisting of 120 mM KCl, 1 mM EGTA, 10 mM Hepes (pH 7.4), or 'sucrose medium', consisting of 240 mM sucrose, 10 mM $MgSO_4$, 1 mM EGTA, 10 mM Hepes (pH 7.4, adjusted with KOH; final K^+ concentration about 5 mM). All experiments were done in reaction chambers or cuvettes thermostatted at 25°C.

In these media the mitochondria exhibited good respiratory control; usually the rate of respiration with succinate as substrate (with rotenone, and in the absence of ADP or P_i) was stimulated 10-fold or more on addition of the uncoupler FCCP.

Oxygen consumption was measured in a closed glass chamber equipped with a conventional Clark electrode.

Spectrophotometric determinations were performed with a DBS-1 dual wavelength spectrophotometer, designed and constructed at the Johnson Foundation Workshops, University of Pennsylvania. The cuvettes had a light path of 1 cm. The molar absorptivities of cytochrome aa_3 and cytochrome *c* (reduced minus oxidised), at 605–630 and 550–540 nm, respectively, were taken to be 27000 and 21000 cm^{-1}.

Horse heart cytochrome *c* was purchased from Sigma (Type VI). The FCCP and myxothiazol were generous gifts from Drs P.G. Heytler and H. Reichenbach, respectively. Other reagents were commercially available products of the highest grade available.

3. RESULTS

The paradoxical oxidation of exogenous cytochrome *c* by mitochondria (see section 1) could be due either to a slow but finite penetration of the cytochrome through the outer membrane, or by fast penetration exclusively into some of the mitochondria that might have a damaged outer membrane. In the former case the system should behave homogeneously, with all cytochrome oxidase on the average turning over at approximately the same velocity. But if the latter were true one would expect that only a fraction of cytochrome oxidase should turn over rapidly, while the rest might not turn over at all. A distinction between these alternatives is made below.

Table 1

Respiration with added cytochrome *c*

Substrate	Reduction of added cytochrome *c* (%)	Respiratory rate (e^-/s per aa_3)
NADH	–	1.1 (0.4)
NADH + cytochrome *c*	≈95	13.8 (9.1)
NADH + cytochrome *c* + TMPD	≈95	13.8
Ascorbate	–	0.7[a]
Ascorbate + cytochrome *c*	≈50	6.0

[a] Only a fraction of this oxygen consumption was sensitive to cyanide

The KCl medium (see section 2) was supplemented with 3 μM rotenone, 0.3 μg/ml antimycin, 1.1 μM myxothiazol, 0.7 μM FCCP and rat liver mitochondria (0.29 μM cytochrome aa_3, or approx. 2.1 mg protein/ml). Further additions (when indicated) were 2.3 mM NADH, 34 μM cytochrome *c*, 1 μM TMPD and 5.7 mM potassium ascorbate. Values in parentheses are for the sucrose medium (see section 2)

Rat liver mitochondria were treated with rotenone, antimycin and myxothiazol to block the respiratory chain, apart from the cytochrome *c* oxidase segment, as tightly as possible. Table 1 shows that such mitochondria are able to oxidise added cytochrome *c* at an appreciable rate (cf. [7]). The reductant of cytochrome *c* was either added NADH or ascorbate. In the former case cytochrome *c* is reduced by the rotenone-insensitive NADH-cytochrome *c* reductase of the outer membrane (see [6,7,18]). This activity is very efficient, keeping added cytochrome *c* highly reduced in the steady state (table 1; cf. [6]). The observed maximal respiratory rate (about 14 e^-/s per aa_3, assuming that all cytochrome oxidase turns over; table 1) corresponds to almost 50% of that during uncoupled respiration with succinate (see [19]). Ascorbate is a less effective reductant and the redox level of cytochrome *c*, as well as the respiratory rate, depended strongly on the concentration of added ascorbate (in the tested range of 0–18 mM; table 1).

Note that oxygen consumption started immediately after addition of cytochrome *c*, and did not require preincubation of the mitochondria with the cytochrome.

In fig.1 the redox level of cytochrome aa_3 was followed spectrophotometrically. Trace A shows the well-known behaviour during respiration with TMPD, but without added cytochrome *c*. NADH was the ultimate reductant here via the NADH-cytochrome *c* reductase system of the outer membrane, which also catalyses effective reduction of $TMPD^+$ (Wurster's blue; see [7]). TMPD, being membrane permeable, shuttles electrons to cytochrome oxidase via endogenous cytochrome *c* [20]. The point to note in trace A is that on anaerobiosis all of cytochrome aa_3 is reduced rapidly (within 10 s), as expected.

Fig.1, trace B, shows the analogous experiment with exogenous cytochrome *c* replacing the TMPD. The overall respiratory rate is here more than twice that in trace A, as determined separately. Yet, only a very small fraction (about 8–10%) of cytochrome oxidase is rapidly reduced upon anaerobiosis. The majority (90–92%) is reduced extremely slowly over about 8 min. Notice the interesting biphasic slow reduction of the haem system of cytochrome oxidase, which resembles that observed during an anaerobic potentiometric titration [21,22].

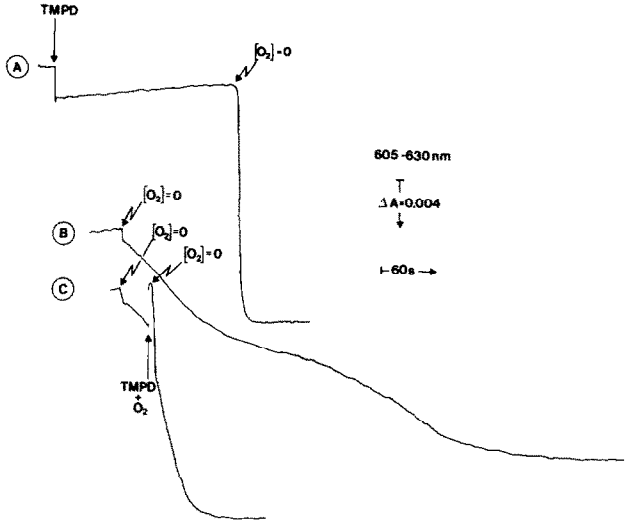

Fig.1. Redox change in cytochrome oxidase upon anaerobiosis. The KCl medium (see section 2) was supplemented with 3 μM rotenone, 0.4 μg/ml antimycin, 3 μM myxothiazol, 1 μM FCCP and rat liver mitochondria (0.76 μM cytochrome aa_3, i.e., about 5.4 mg protein/ml). The experiment in traces B and C further contained 25 μM cytochrome *c*. After 5 min preincubation 3.1 mM NADH was added. In trace A, this was followed by addition of 15 μM TMPD, as shown. In all traces the points where anaerobiosis occurred are indicated. In trace C, which is a continuation of the experiment in trace B, some oxygen was first added by stirring (not shown); an addition of 1 μM TMPD together with some O_2 is also shown. Downward deflections reflect an increase of absorption at 605 vs 630 nm, i.e., reduction of cytochrome oxidase (see section 2).

Trace C is a direct continuation of trace B. First some oxygen was added by stirring (not shown), which yielded the aerobic steady state redox level of cytochrome oxidase. This was soon again followed by anaerobiosis (fig.1; trace C), showing the beginning of the same unique pattern of reduction as in trace B. Then 1 μM TMPD was added together with some oxygen. This amount of TMPD had no effect on the overall velocity of respiration (table 1), but upon subsequent anaerobiosis cytochrome oxidase was now fully reduced very much faster than without the TMPD (within about 1 min; trace C).

Under the conditions used for the experiment of fig.1 (trace B) we also measured the corresponding redox changes in cytochrome *c* at 550–540 nm (not

shown). Fast reduction of an amount corresponding to approx. 5% of added cytochrome *c* occurred on anaerobiosis. This was followed by very slow reduction of the rest of cytochrome *c*, which corresponded to 1.3–1.4-times the total concentration of cytochrome aa_3. This is likely to be reduction of endogenous cytochrome *c*, which took place with kinetics comparable to the slow phase of cytochrome aa_3 reduction; it occurred later than the primary, but slightly prior to or simultaneously with the secondary slow phase of reduction of cytochrome oxidase (see fig.1; trace B).

Experiments otherwise identical to fig.1 (traces B and C) were also conducted in conditions where the mitochondria were incubated anaerobically with cytochrome *c* for up to 30 min, with no significant difference in the results. Likewise, when the mitochondria were incubated anaerobically with cytochrome *c* in conditions typical for performing O_2 pulse experiments (see, e.g., [16]), i.e., in the presence of valinomycin in place of the FCCP, the results were again the same as those shown in fig.1.

When these experiments were performed in a medium where the KCl was replaced by sucrose (section 2), it was found that the respiratory rate was somewhat less (table 1) and the quickly-reduced proportion of the oxidase slightly smaller (not shown; see below) than in the KCl medium, all other parameters being kept constant. Qualitatively, however, the findings were the same.

With ascorbate in place of NADH the kinetic difference between the fast and slow phases of reduction of cytochrome oxidase on anaerobiosis was much less distinct, though still observable (see section 4). In addition, the measurements at 605–630 nm were less easily interpretable at most ascorbate concentrations because added cytochrome *c* was not usually very highly reduced in the aerobic steady state (see table 1). This caused spectral interference at 605–630 nm from the large redox change in exogenous cytochrome *c* upon anaerobiosis. With NADH as the reductant this was no problem, since the degree of reduction of added cytochrome *c* was very high already aerobically (table 1).

We also tested the effect of inhibiting cytochrome oxidase by azide [23] on the aerobic redox state of cytochrome oxidase at 605–630 nm, during respiration with TMPD + ascorbate or with NADH + exogenous cytochrome *c* at similar respiratory velocities. Cytochrome aa_3 rapidly became successively more reduced on adding azide in the former system, as is well known [23]. However, azide had a very small effect on the highly-oxidised, aerobic steady-state redox level of cytochrome aa_3 with NADH and added cytochrome *c* as substrate, although respiration became effectively inhibited (not shown).

4. DISCUSSION

Our results show that the relatively high overall respiratory activity of rat liver mitochondria with added cytochrome *c* as electron donor is due solely to the activity of a very small fraction of the mitochondria. The great majority of the mitochondria do not oxidise added cytochrome *c* at all. This is most probably due to an intact outer membrane [1–5]. With exogenous cytochrome *c* as electron donor a blockade of cytochrome oxidase by anaerobiosis, or by azide, led to fast reduction only of those relatively few oxidase molecules that turned over prior to inhibition, having access to the added cytochrome *c*. Anaerobically, the remainder of the oxidase is reduced extremely slowly, probably via very slow leakage of reducing equivalents from endogenous substrates through the inhibited respiratory chain. This explains why the slow reduction pattern is similar to that during anaerobic potentiometric titrations. The slow anaerobic reduction could be dramatically accelerated by catalytic amounts of the redox mediator TMPD (fig.1, trace C), which delivers reducing equivalents equally to all cytochrome oxidase molecules.

When ascorbate replaced NADH as the reductant the distinction between slowly and rapidly reacting cytochrome oxidase was much less clear-cut. This is probably because endogenous cytochrome *c* in mitochondria with an intact outer membrane can be reduced by ascorbate (but not by added NADH). Although ascorbate is oxidised only very slowly by the respiratory chain via endogenous cytochrome *c*, this effect is apparently sufficient to speed up reduction of cytochrome aa_3 anaerobically to an extent well beyond that with added NADH.

This difference between ascorbate and NADH suggests that reduction of cytochrome *c* by the

NADH-cytochrome b_5 reductase system occurs exclusively on the outer side of the outer mitochondrial membrane [24]. Our results do not support the possibility of a physiological intermembranous cytochrome *c* shuttle of reducing equivalents from extramitochondrial NADH to the respiratory chain, as proposed in [25].

The most likely explanation of our results is that the outer membrane is damaged in a small proportion of the mitochondria during the conventional isolation procedure. Thus, the notion that the outer membrane is completely impermeable to cytochrome *c* is supported by our data, and the apparently contradictory high velocities of oxidation of added cytochrome *c* find a simple solution.

From the spectrophotometric data we estimated that about 5–12% of total cytochrome aa_3 was turning over rapidly with added cytochrome *c*, which would infer that this is the proportion of mitochondria with a damaged outer membrane. We found that this proportion was somewhat lower (close to 5–6%) with mitochondria in the sucrose medium than with the same mitochondria in the KCl medium (close to 8–12%). It is possible, therefore, that some of the isolated mitochondria have a fragile but unbroken outer membrane, which breaks in a high salt medium but not in an approximately equiosmolar sucrose medium.

When it is taken into account that respiration with added cytochrome *c* is due only to a small fraction of cytochrome oxidase, the turnover of this fraction translates to an impressive number of near 150 e^-/s per aa_3. This is, however, a feasible turnover number for this enzyme, for which the maximum may reach values of 400 [22].

In typical oxidant-pulse experiments where proton translocation by cytochrome oxidase has been assessed [9,12,16], a brief burst of respiration is initiated by addition of a limited amount of O_2, and the extramitochondrial pH is recorded with a sensitive pH-meter. In experiments of this kind it is essential that the buffering capacity of the mitochondrial matrix space is not exceeded, otherwise there is a dramatic lowering of the observed H^+/e^- ratio of proton translocation (see, e.g., [26]). In the case of oxidation of added cytochrome *c*, the number of mitochondria that are respiring is only some 1/8–1/20 of the total. This means that the oxygen pulse is 'oversized' by a factor of 8–20; this would be expected to cause a rapid exhaustion of the matrix buffering capacity, subsequent fast back-leakage of protons across the membrane, and a dramatic lowering of the observed H^+/e^- ratio. This explains why the back-decay of proton ejection observed in such experiments is anomalously fast although the corresponding decays of pulses of HCl (with decay of extramitochondrial H^+ into all of the mitochondria) were practically unaffected by added cytochrome *c* (unpublished). In agreement with this, added cytochrome *c* does not cause any considerable general uncoupling of mitochondria (State 4 rates of respiration with succinate were typically accelerated by about 30% only).

The finding of proton translocation in some [11,12,16] but not in other experiments [8–10,16] with cytochrome *c* added to rat liver mitochondria could, in part, be due to variations of the proportion of mitochondria with a broken outer membrane. As discussed above, the present results explain why proton translocation should always be difficult to observe with added *c* as substrate. However, the somewhat lower fraction of mitochondria with a damaged outer membrane in a sucrose as compared with a KCl medium can hardly by itself explain why proton translocation was observed in the latter but not in the former case [16]. But at low K^+ concentrations the electrophoretic counter-movement of potassium ions (+ valinomycin) may easily become limiting for net proton translocation [15] due to the extremely high turnover of the oxidase molecules in the mitochondria with a damaged outer membrane. In any case, it is clear that these previous observations no longer require assumptions related to different proton pumping at high- and low-affinity binding sites of cytochrome *c* [16].

5. CONCLUSIONS

An intact outer mitochondrial membrane provides a complete barrier against penetration of cytochrome *c*. A small fraction of rat liver mitochondria, isolated in sucrose-containing media by conventional techniques, have a damaged outer membrane which permits access of added cytochrome *c* to cytochrome oxidase in the inner membrane. Respiration with exogenous cytochrome *c* is entirely due to the activity of this frac-

tion of the mitochondria, in which cytochrome oxidase may turn over very rapidly.

These results explain why proton translocation has been difficult or in some conditions impossible to observe with rat liver mitochondria oxidising added cytochrome *c*.

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