Evidence for a mobile semiquinone in the redox cycle of the mammalian cytochrome bc_1 complex

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Experimental evidence is presented to demonstrate that cytochromes *b* of the mammalian cytochrome bc_1 complex may be rapidly oxidised by a pulse of oxidising equivalents which react with cytochrome c_1, even when all cytochrome *b* is fully reduced before the pulse. The oxidation is sensitive both to antimycin and to myxothiazol. Such behaviour is inconsistent with models in which only the fully oxidised ubiquinone may move beween the centres 'o' and 'i' of the complex. It is proposed that the charged semiquinone ($Q^{\cdot-}$) may move between these centres, which may constitute separate reaction domains of a single ubiquinone-binding site. The bearing of this on the mechanism of electron, proton and charge transfer in the complex is discussed.

Cytochrome b *Ubisemiquinone Electron transfer Energy conservation Q cycle Cytochrome* b *cycle*

1. INTRODUCTION

Two general ideas have been central for the present understanding of the mechanism of action of cytochrome *bc*-type complexes. The first was that of 'branching' of electron transfer from ubiquinol, which is oxidised in two discrete one-electron steps by different electron acceptors, and with ubisemiquinone as an intermediate [1]. The second was the further development of this idea into cyclic (Q cycle or b cycle) types of reaction schemes [2–6]. The experimental observations of 'oxidant-induced reduction' of cytochromes *b* [1,7–9], the identification of means of specific inhibition of the two proposed centres of ubiquinone interaction with the complex [4,10–12], as well as of two forms of semiquinone with predicted behaviour by EPR spectroscopy [13,14], have provided sound support for such models.

A central feature of the Q cycle model has been the proposed movement of electrical charge across the membrane by electron transfer from the low- to high-potential cytochrome *b*. Model building of protein structure from the amino acid sequence of the cytochrome *b* apoprotein suggests that the two haem *b* groups may indeed be arranged across the membrane [15,16]. This lends weight to the proposal that such electron transfer would indeed be electrogenic.

The experiments reported here for isolated cytochrome bc_1 complex and for intact mitochondria concern the kinetics of oxidation of cytochrome *b* after a pulse of oxidant that oxidises cytochrome c_1. The results are not consistent with models where ubiquinone generated by oxidation of quinol at centre 'o' would provide the only possible oxidant for cytochrome *b* at centre 'i'. A modification appears necessary which allows for electrogenic movement of semiquinone between these centres, such as that in the recent version of the b cycle model [6].

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2. MATERIALS AND METHODS

2.1. *Cytochrome* bc_1 *complex*

Succinate-cytochrome *c* oxidoreductase and cytochrome bc_1 complex were prepared from beef heart mitochondria by the methods of Hatefi and Rieske [17,18]. Preparations were stored frozen at 77 K until required.

The kinetic measurements were performed in a stirred cuvette in which the sample was kept anaerobic with a flow of O_2-free nitrogen maintained above the surface. Redox changes of cytochromes *b* were monitored at 562–575 nm, and of cytochrome c_1 at 552–542 nm. Reduced minus oxidised extinction coefficients of 20 $mM^{-1} \cdot cm^{-1}$ were used for both components.

Cytochrome bc_1 complex activity was assayed in 50 mM potassium phosphate, 2 mM EDTA and 0.1 mM KCN at pH 7.0 and 25°C. Cytochrome *c* was added to 20 μM final concentration and its reduction was monitored at 550 nm on addition of 100 μM ubiquinol-2. Succinate-cytochrome *c* oxidoreductase activity was monitored in a similar manner, but substituting 10 mM potassium succinate for the ubiquinol.

Succinate-ferricyanide oxidoreductase activity of the complex was measured under similar conditions by substituting an appropriate amount of potassium ferricyanide for the cytochrome *c*, and monitoring the reaction at 420 nm with an extinction coefficient of 1 $mM^{-1} \cdot cm^{-1}$. Other additions were made as indicated in table 1.

2.2. *Rat liver mitochondria*

Mitochondria were prepared by a conventional procedure [19], suspended in 0.25 M sucrose, and kept on ice until used. The reaction medium consisted of 1 vol. of 0.25 M sucrose, 20 mM KCl, 20 mM Hepes-KOH buffer, pH 7.2, and 0.2 vol. ethylene glycol. 3 μM rotenone and 1 μM FCCP were further added together with mitochondria to a final concentration expressed as μM cytochrome aa_3 (see legend to fig.2). The cytochrome aa_3 content was determined from the dithionite-reduced minus oxidised difference at 605–630 nm, using an extinction coefficient of 27 $mM^{-1} \cdot cm^{-1}$.

The mitochondrial suspension was supplemented with potassium succinate at room temperature and allowed to become anaerobic, with monitoring of the redox state of cytochrome *b* (see below). When indicated, an aliquot of freshly prepared sodium dithionite (made up in O_2-free sucrose medium) was further added. The DBS-1 dual-wavelength spectrophotometer (Johnson Foundation Workshops, University of Pennsylvania) cell was cooled to an equilibrated temperature of -7 ± 0.1°C in the cuvette. Redox changes in cytochromes *b* were monitored at 566–575 or 565–575 nm, where the relative contributions of cytochromes *b*-566 and *b*-562 are approx. 55 and 45% (see [9]). The reaction was initiated by rapid and efficient stirring with ferricyanide and air (see legend to fig.2 for further details). The response of the instrumentation was limited by the recorder, with 90% response in 320 ms.

3. RESULTS

3.1. *Oxidation of cytochrome* b *in isolated succinate-cytochrome* c *oxidoreductase complex*

These results are illustrated in fig.1. The cytochrome *b* was prereduced in the complex by addition of a small aliquot of freshly prepared sodium dithionite solution. Full reduction was achieved in about 60 s, and was confirmed by the fact that further additions of dithionite had no effect, and by the observation that the absorbance change corresponded to two cytochrome *b* haems per complex.

The reaction was initiated by addition of an aliquot of anaerobic potassium ferricyanide. In the control (fig.1A) this caused a rapid oxidation of the *b* cytochromes. The half-time of oxidation was significantly faster than the mixing half-time of about 200 ms, in agreement with earlier data [20].

The subsequent traces of fig.1 demonstrate that the process was sensitive to either antimycin or to myxothiazol, hence ruling out the possibility that the ferricyanide might be oxidising the *b* cytochromes directly.

3.2. *Maximum rates of removal of semiquinone from site o by* O_2 *and by ferricyanide*

On providing a pulse of oxidant, the initial reaction presumably involves oxidation of cytochrome c_1 and the Rieske Fe-S centre, followed by their re-reduction by the quinol associated with the complex to produce semiquinone at centre o.

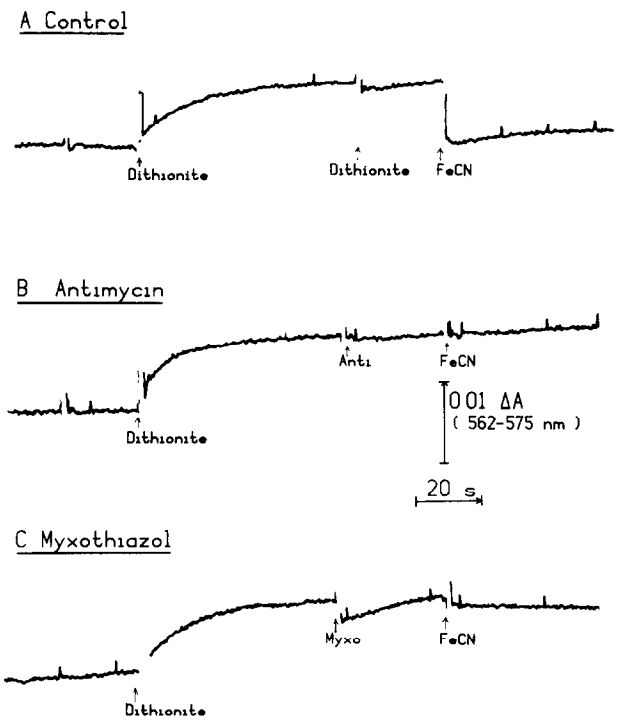

Fig.1. Oxidation of cytochrome *b* in isolated succinate-cytochrome *c* oxidoreductase. Succinate-cytochrome *c* oxidoreductase was added to the reaction mixture (section 2) at a final cytochrome *b* concentration of 0.5 μM, and the whole was kept anaerobic with a flow of oxygen-free nitrogen. Additions (where indicated) were: 20 μM sodium dithionite, 80 μM potassium ferricyanide, 1.2 μM antimycin and 1.2 μM myxothiazol.

It was of interest to determine how fast this semiquinone could be converted to quinone by further oxidation, either by the Rieske centre, by O_2, or by the added ferricyanide. Maximal values for these routes could be determined experimentally on the basis that each would allow an antimycin-insensitive electron transfer pathway from quinol to cytochrome *c*, or to the acceptor which may directly oxidise the generated semiquinone. Such antimycin-insensitive fluxes were determined (table 1). The fastest of these possible routes appears to be that to ferricyanide. Even at a ferricyanide concentration as high as 1 mM, and in the presence of O_2, a maximal rate of semiquinone removal was measured which could support a turnover number of the complex of approx. 1.6 s^{-1}. This process was therefore not rapid enough to support the much faster rates of oxidation of cytochrome *b* which were observed in fig.1, and obtained at much lower concentrations of ferricyanide.

Table 1

Maximum rates of removal of ubisemiquinone from centre o

Acceptors present	Inhibitors present	Turnover number (s^{-1})
12 μM cytochrome *c* 256 μM O_2	antimycin	0.065
12 μM cytochrome *c* anaerobic	antimycin	0.03
1 mM ferricyanide 256 μM O_2	antimycin	1.64
1 mM ferricyanide anaerobic	antimycin	1.5
1 mM ferricyanide 256 μM O_2	myxothiazol	0.4
1 mM ferricyanide 256 μM O_2	myxothiazol + antimycin	0.3

These turnover numbers are expressed in terms of turnovers of bc_1 complex per s. They were determined by measuring the maximum rates of antimycin-insensitive electron transfer catalysed by succinate-cytochrome *c* oxidoreductase, from succinate to the appropriate acceptor. Antimycin or myxothiazol were 2 μM where added. Anaerobiosis was obtained by addition of 10 mM glucose and 0.5 mg glucose oxidase (Sigma, type II from *Aspergillus niger*, 40000 U/g at saturating oxygen) to the buffer which had been bubbled with oxygen-free nitrogen

From the data of table 1 it may also be seen that around 75% of this antimycin-insensitive succinate-ferricyanide oxidoreductase activity is insensitive to O_2 and sensitive to myxothiazol. This fraction then probably arises from direct oxidation of semiquinone by ferricyanide. Most of the remainder of the myxothiazol-insensitive fraction occurs even under anaerobic conditions and is probably therefore caused by oxidation of quinol or succinate dehydrogenase directly by ferricyanide. The rest (which can support a turnover of less than 0.2 s^{-1}) is presumably caused by the direct reduction of O_2 by semiquinone at centre o.

3.3. *Oxidation of cytochrome* b *in mitochondria at low temperature*

Fig.2 shows the results in different conditions

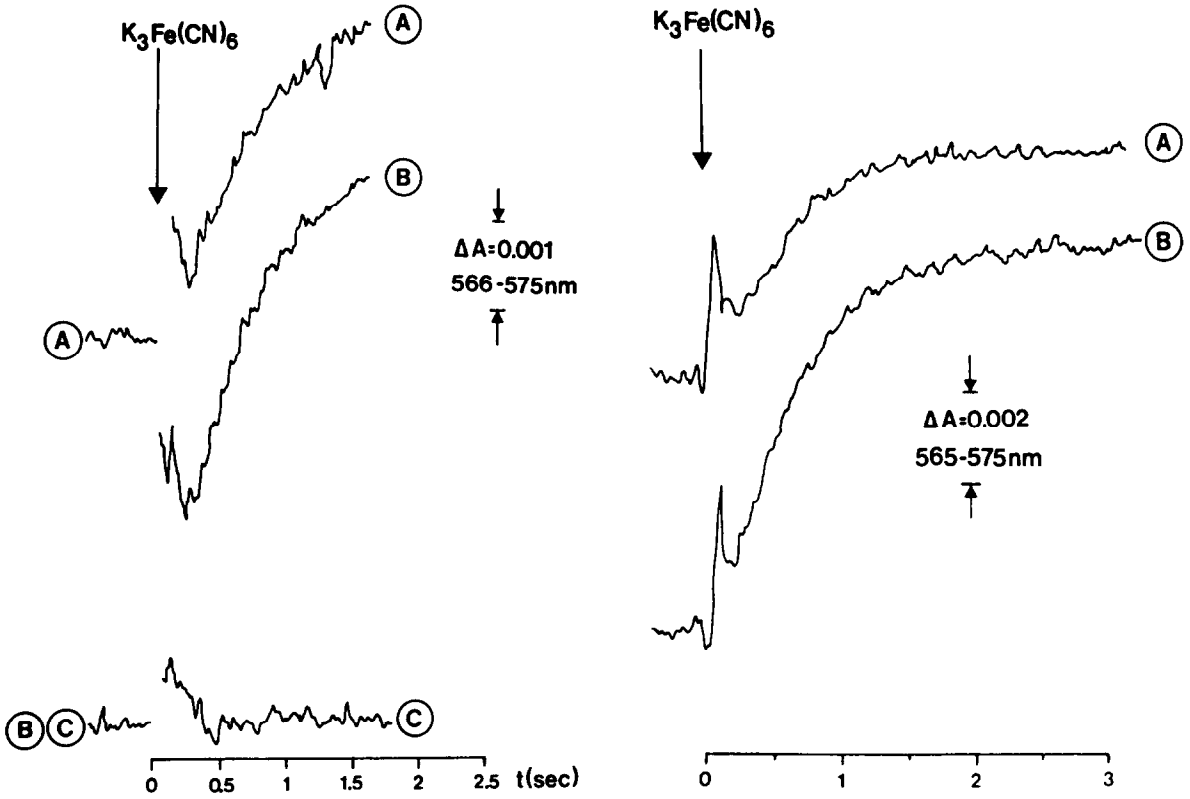

Fig.2. Kinetics of cytochrome *b* oxidation in rat liver mitochondria at low temperature. General conditions are described in section 2. Oxidation of cytochrome *b* corresponds to an upward deflection of the traces. In traces marked A succinate was the only added reductant (6.7 mM in the left and 3.3 mM in the right panel). In traces B, 1.9 mM (left panel) or 0.67 mM (right panel) sodium dithionite was further added, which caused complete reduction of cytochrome *b*. In trace C (left panel), 1 μM antimycin was also present. The reaction was started (arrow) by rapid and efficient stirring with 4.6 mM (left panel) or 3.3 mM (right panel) potassium ferricyanide (together with air) when the system had equilibrated at -7°C. The concentration of mitochondria corresponded to 0.5 (left panel) and 0.67 (right panel) μM cytochrome aa_3.

where, in each, the reaction was initiated at -7°C by addition of ferricyanide plus air to oxidise rapidly cytochromes $c + c_1$. In anaerobic succinate-supplemented mitochondria (traces labelled A) cytochrome *b*-562 is initially highly reduced while *b*-566 is highly oxidised (cf. [9]). When an aliquot of freshly prepared dithionite is added as well (traces B), all cytochrome *b* is fully reduced before the oxidant pulse. This was ascertained by the finding that further addition of dithionite caused no further reduction. In both cases (traces A and B) the pulse of oxidant yielded oxidation of cytochrome(s) *b* with similar kinetics, the apparent first-order rate constant being approx. 1.1 s^{-1}.

Trace C (fig.2, left panel) shows that the oxidation of cytochrome *b* is completely blocked by antimycin; the same result was obtained with 2.5 μM myxothiazol in place of the antimycin, or in the presence of both inhibitors.

The dithionite was used in order to ensure conditions (fig.2, traces B and C) where all cytochrome *b* would be reduced before the pulse of oxidant. Due to the dithionite it was found necessary to use an excess of ferricyanide as oxidant (together with air) rather than oxygen alone. However, oxidation of cytochrome(s) *b* is also in these conditions the result of primary oxidation of cytochromes $c + c_1$, as it is after a pulse of O_2. Several findings support this contention. Firstly, oxidation was blocked both by antimycin and by myxothiazol showing that it was not due to direct oxidation of the *b* cytochromes by the oxidant (cf. section 3.1), but

required primary oxidation of ubiquinol by the Rieske centre as well as an intact site i. Secondly, the velocity of oxidation was found to be only about 5-times slower at −7°C ($t_{1/2}$ approx. 600 ms) than at room temperature (see [20]), indicating an energy of activation well below 10 kcal/mol. Experiments with oxygen as the only oxidant in conditions similar to those of fig.2 (traces A) gave similar oxidation velocities (not shown). Thirdly, ferricyanide in the concentration range employed here is known to oxidise specifically cytochromes $c + c_1$ in intact mitochondria [21].

We conclude that the velocity of oxidation of cytochrome *b*, following oxidation of cytochrome c_1 and the Rieske centre, is similar for the cases where part or all of cytochrome *b* is reduced prior to the pulse of oxidant. More specifically, the velocity is still high when the reaction is initiated from the fully reduced state. Within the limits of detection (the 'dead time' due to stirring was approx. 0.3–0.4 s at −7°C; fig.2) we also did not observe any lag in the oxidation of cytochrome *b* when initiated from the fully reduced state.

4. DISCUSSION

The results presented here show that the velocity of oxidation of cytochrome(s) *b*, in response to oxidation of cytochrome c_1 and the Rieske centre, is still high when all cytochrome *b* is reduced prior to the pulse of oxidant, and that the rate constant is not significantly different from that when cytochrome *b* is initially only partially reduced. Although this phenomenon has been alluded to previously [22–25] there have actually been no pertinent experimental data recorded in the literature on this effect. In most cited reports there was no assurance that all cytochrome *b* was reduced prior to the oxidant pulse; in fact the cytochrome *b*-566 component was almost certainly oxidised in most of these studies. The only reported experimental attempt at this question in the past is that of De Vries [25], who showed a slight (20–30 ms) lag in the oxidation kinetics when initiated from the fully reduced state. However, the extent of oxidation was similar for the dithionite- and succinate-reduced cases (cf. fig.2). It is therefore probable that pulsing with O_2 alone in the presence of an excess of dithionite (as in [25]) is an inappropriate procedure that may lead to incomplete oxidation.

The results reported here point out the need to consider further the possible fate(s) of the semiquinone which is produced at centre o after the quinol has donated a single electron to the Rieske centre.

$$QH_2 + FeS \longrightarrow Q^{\cdot -} + FeS^- + 2H^+ \quad (1)$$

Normally, the cytochromes *b* (*b*-566 in particular) are fairly oxidised, and are able to provide the generally accepted route for the semiquinone electron:

$$Q^{\cdot -} + b \longrightarrow Q + b^- \quad (2)$$

Subsequently, electron transfer across the membrane to the second cytochrome *b* haem would then provide the electrogenic reaction, or at least part of it [26].

In some of the experiments reported here both *b* haems are reduced at the outset and so cannot provide a pathway for the oxidation of semiquinone (reaction 2). Hence there can be no normal formation of quinone at centre o, which according to the Q cycle moves to centre i to provide the antimycin-sensitive oxidant to the *b* cytochromes. Nor can there be any kinetically significant generation of Q by 'abnormal' oxidation of semiquinone (e.g. by FeS, O_2, or ferricyanide) since that would be insensitive to antimycin (see section 3). For the same reasons, release of semiquinone from site o followed by formation of Q by dismutation cannot explain these results.

The fully reduced *b* cytochromes could, instead, be oxidised directly by the semiquinone generated in reaction 1. Such behaviour would not be inconsistent with the Q cycle if this reaction happened at centre o. However, again the sensitivity towards antimycin (a specific centre i inhibitor) and towards myxothiazol (a specific centre o inhibitor) shows that the reaction requires the operation of both centres.

A possible explanation for the phenomenon is provided by a key feature of the b-cycle model [5,6], viz. that the semiquinone produced at centre o can move to centre i in order to act as an oxidant of cytochrome *b*. This idea may then be combined with the key feature of the Q-cycle of a transmembranous arrangement of the centres o and i (as seems likely; see section 1) to suggest that the semiquinone movement between these domains could itself be electrogenic [6], provided that the semi-

quinone moves in its deprotonated charged form. Such a reaction could constantly provide the electrogenicity during turnover, together with electron transfer across the *b* cytochromes, as suggested in the recent version of the b cycle [6]. This version may more appropriately be called the semiquinone, or the SQ cycle [27], to distinguish it clearly both from the b and the Q cycle schemes.

Mitchell [28] has recently pointed out that any combination of the two possible electrogenic events might occur. In this case the actual reaction would be rather 'soft-wired'. However, this presupposes that the oxidised ubiquinone is able to oxidise cytochrome *b* at centre i. In contrast, the SQ cycle is more 'hard-wired' allowing only the quinol/semiquinone couple to interact with cytochrome *b* at centre i. This leads to a fixed 1 : 1 relationship between transmembranous electron transfer catalysed by the *b* haems and transmembranous transfer of the semiquinone anion during turnover. This difference may provide a means of experimentally distinguishing between the modified Q cycle and SQ cycle schemes outlined in this report (see also [27]).

In any case, our data seem to make it necessary to modify current models to include the feature of electrogenic semiquinone transfer from centre o to centre i. Clearly, such transfer must be 'localised' not to allow equilibration of semiquinone with the Q pool. Hence, it could for instance be envisaged as a rotation of the ubisemiquinone molecule in a single active site or 'pocket', to make contact alternatively with o or i reaction domains of that pocket, which may lie at different depths along a normal to the membrane [6,27].

Energetically, the electrogenic movement of the semiquinone anion from the o to the i domain would be made feasible by its stronger binding to the latter. Or stated differently, the i orientation of the semiquinone anion would be energetically favoured over the o orientation [27].

Our results may have bearing on the recent reports [29] that the electrogenic reaction associated with the chloroplast cytochrome *bf* complex, and generally observed as the slow phase of the carotenoid bandshift [30], is relatively unaffected by the redox poise of cytochrome *b*. This has led to models of the electrogenic step which are far removed from the more conventional postulates. An electrogenic semiquinone movement might reconcile these data with the type of SQ cycle or modified Q cycle model which we are suggesting.

The report by Glaser and Crofts [26] that electron transfer between the cytochrome *b* haems accounts only for part of the overall electrogenicity in bacterial chromatophores, and that the oxidation of cytochrome *b*-562 accounts for the rest, could also be explained by an electrogenic semiquinone movement, which would be expected to be kinetically synchronised with the oxidation phase of cytochrome *b*-562.

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