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FLASH-INDUCED ELECTRON TRANSFER THROUGH MITOCHONDRIAL QH_2: CYTOCHROME *c* OXIDOREDUCTASE IN THE PRESENCE OF BACTERIAL REACTION CENTRES AND CYTOCHROME *c*

ANALYSIS OF SUBSEQUENT PROCESSES AND EFFECT OF INHIBITORS

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In a system containing reaction centres isolated from *Rhodopseudomonas sphaeroides* mutant R26, and variable amounts of horse-heart cytochrome *c* and bovine-heart mitochondrial QH_2: cytochrome *c* oxidoreductase in a medium containing 2 mM ascorbate and 0.1 μM phenazine methosulphate, electron transfer was induced by a single flash. Three distinct phases of electron transfer can be distinguished: the first event is the oxidation of cytochrome *c*, and this is followed by an equilibration between cytochrome *c*, cytochrome c_1 and the Rieske [2Fe-2S] cluster. The actual rates of these processes depend on the concentrations of cytochrome *c* and the reductase. The slower third phase is the oxidation of ubiquinol, which can follow two pathways: one sensitive to antimycin and one sensitive to myxothiazole. The antimycin-sensitive pathway ($t_{1/2} \approx 10$ ms) is an equilibration between the Q/QH_2 couple and cytochrome *b*, but may also include a direct reduction of cytochrome *b* by the Q_B of the reaction centres. The myxothiazole-sensitive pathway is a coupled reduction of cytochrome *b* and the Rieske [2Fe-2S] cluster which rapidly equilibrates with cytochromes c_1 and *c*. Both pathways are sensitive to 7-(*n*-heptadecyl)mercapto-6-hydroxy-5,8-quinoline quinone, but with different affinities. In the absence of inhibitors the initial reduction of cytochrome *b* (via both pathways) is followed by a net oxidation which is the resultant of a continuing reduction (together with the reduction of the Rieske [2Fe-2S] cluster) and an oxidation (via the antimycin-sensitive site) by quinone. The results are discussed in the light of linear and cyclic models proposed to explain electron transfer between cytochromes *b* and *c*. It is concluded that only the Q-cycle model fits the present experimental data.

Introduction

In a previous paper [1] we have shown that in hybrid systems containing bacterial reaction centres and mitochondrial ubiquinol: cytochrome *c* oxidoreductase, photooxidized reaction centres can oxidize the mitochondrial oxidoreductase rapidly when (horse-heart) cytochrome *c* is present to mediate electron transfer between the two sys-

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Abbreviations: (oxido) reductase, ubiquinol: cytochrome *c* oxidoreductase; Q, ubiquinone-10; HMHQQ, 7-(*n*-heptadecyl)mercapto-6-hydroxy-5,8-quinolinequinone; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

tems in agreement with the data presented by Packham et al. [2] and Matsuura et al. [3].

In the absence of cytochrome *c*, the transfer of reduction equivalents from cytochrome c_1 to the reaction centres is very slow. The kinetics of the reaction between cytochrome c_1 and cytochrome *c* and of that between cytochrome *c* and the reaction centres have been described [1] and the conclusion was drawn that reduced cytochrome *c* forms a stoicheiometric complex with the (cytochrome c_1 of the) oxidoreductase, but not with the reaction centres. The rate of reduction of cytochrome *c* by cytochrome c_1 was found to be proportional to the concentration of the reductase. No evidence was obtained for an association between reaction centres and reductase (contrast Ref. 4).

We further described the reduction of cytochrome *b* by the quinol present before the flash or produced by the flash and concluded that, in this system as in substrate-reduced mitochondria, two pathways exist for the reduction of cytochrome *b*; one through a site that is inhibited by antimycin (and HQNO or HMHQQ) (centre i) and one through a site that is inhibited by myxothiazole (or HMHQQ) (centre o).

In the present paper we focus on the pathway of reducing equivalents from quinol or cytochrome *b* to cytochrome c_1, the Rieske [2Fe-2S] cluster and cytochrome *c*. From the analysis of the various steps in the process it will be concluded that the data are in line with a cyclic model of electron transfer and not with a linear model for electron transfer from cytochrome *b* to the Rieske [2Fe-2S] cluster via a QH_2/Q^- couple as proposed by Matsuura and Dutton [5,6]. In particular only the cyclic model is able to explain the kinetic matching between oxidation of cytochrome *b* and reduction of cytochrome $c + c_1$, in the absence of any inhibitor.

Recently [7,8], it has been concluded that a monomeric Q-cycle model is not sufficient to explain the observed pre-steady-state kinetics obtained with duroquinol as substrate for the mitochondrial oxidoreductase. In the experiments reported here, only some of the molecules present undergo oxidation-reduction, since the amounts of flash-produced oxidizing and reducing equivalents are always less than the amount of enzyme. This makes the discrimination between a monomeric and a dimeric cycle difficult or even impossible. For that reason we restrict ourselves to the monomeric model, although we assume that at centre i (cf. Refs. 9 and 10) cytochrome *b* can reduce ubiquinone to ubiquinol which step most likely needs the functioning of the oxidoreductase as a dimer, one monomer reducing Q to Q^- and the other Q^- to QH_2. The variant proposed in Ref. 11, which includes the dismutation equilibrium $QH_2 + Q \rightleftharpoons 2Q^- + 2H^+$, in fact also requires a dimer, since stabilization of the Q^- requires two sites.

Materials and Methods

The system studied contained 1 μM reaction centres, isolated from *Rhodopseudomonas sphaeroides* R26 [12], variable amounts of horse-heart cytochrome *c* and bovine-heart ubiquinol : cytochrome *c* oxidoreductase (isolated according to Refs. 13 and 14), 2 mM ascorbate and 0.1 μM phenazine methosulphate or 10 μM naphthoquinone in 50 mM Tris-HCl buffer (pH 8.0) or 50 mM morpholinepropanesulphonic acid (brought to pH 7.0 with NaOH). The light intensity used was sufficient to oxidize 20% of the bacteriochlorophyll dimer of the reaction centres (0.2 μM). The pathway through the cuvette was 0.5 cm. For the determination of cytochrome *c* and cytochrome c_1, extinction coefficients of 21.1 $mM^{-1} \cdot cm^{-1}$ at 549.6 [15] and 19.2 $mM^{-1} \cdot cm^{-1}$ at 552.5 nm [16], respectively, were used for the difference red-ox. All further methods and materials used have been described in Ref. 1.

Results

Characterization of the system studied

At the moment when the reaction centres were flashed, the [2Fe-2S] cluster and the cytochrome c_1 in the QH_2 : cytochrome *c* oxidoreductase were completely reduced by the ascorbate present in the reaction mixture (with phenazine methosulphate or naphthoquinone as mediator). Added cytochrome *c* was also completely reduced. The ubiquinone and cytochrome *b* present in the reductase were partially reduced.

Immediately after the light flash – which oxidizes the bacteriochlorophyll dimer and reduces the electron acceptor on the reaction centres – the

following reactions take place:

			$\Delta A_{551\ nm}$
(1)	$(BChl)_2^+ + c^{2+} \rightarrow (BChl)_2 + c^{3+}$	fast	–
(2)	$c^{3+} + c_1^{2+} \rightleftharpoons c^{2+} + c_1^{3+}$	fast	0
(3)	$c_1^{3+} + [2Fe\text{-}2S]^+ \rightleftharpoons c_1^{2+} + [2Fe\text{-}2S]^{2+}$	very fast	+
(4)	$QH_2 + [2Fe\text{-}2S]^{2+} + b^{3+} \rightarrow Q + [2Fe\text{-}2S]^{1+} + b^{2+}$	slow	0

In addition, a very slow reduction of c, c_1 and the Fe-S cluster by ascorbate takes place as well as other reactions leading to the reduction or oxidation of Q and cytochrome b that will be discussed later.

In the absence of reductase, only reaction (1) and the slow re-reduction by ascorbate of the cytochrome c oxidized by reaction (1) can take place. This is illustrated by the curves marked 0 in Fig. 1. The amount of cytochrome c rapidly oxidized equals the amount of reaction centres oxidized by the flash (0.2 μM).

In the presence of 3 μM reductase (curves marked 3 in Fig. 1), the cytochrome c oxidized by reaction (1) is re-reduced by cytochrome c_1 (reaction 2). Since at this wavelength the two cytochromes have about the same absorbance coefficient in the difference spectrum, this would not be observed. However, the subsequent reduction of cytochrome c_1 by the Fe-S cluster (reaction 3) leads to an increase of the absorbance at 551 nm. Reactions 2 and 3 are sufficiently rapid to prevent the initial decline in $A_{550\ nm}$, reaching the same value as in the absence of reductase. This effect is enhanced by the fact that the rate of oxidation of cytochrome c itself (reaction 1) is slower than in the absence of reductase since a substantial part of cytochrome c is bound to the reductase [1]. However, the total concentration of reaction centres oxidized by the flash (followed at 604 nm) is not affected by addition of reductase.

Since reaction (4) is much slower than reactions (1)–(3), a quasi-equilibrium is set up after a few milliseconds between the 2Fe-2S cluster and the cytochromes c and c_1. In the experiment shown in Fig. 1, 0.08 μM cytochrome $c + c_1$ had been re-reduced by the Fe-S cluster 5 ms after the flash. Thus, 0.12 μM cytochrome $c + c_1$ remained oxidized. The spectrum of cytochromes $c + c_1$ oxidized at this time, is shown in Fig. 2. From the positions of the absorbance maxima of the two individual cytochromes (549.6 nm and 552.5 nm) and their absorbance coefficients [15,16], we calculate that 0.039 μM cytochrome c and 0.081 μM cytochrome c_1 were oxidized in this experiment.

From these data the relative E_m values of the [2Fe-2S] cluster, cytochrome c_1 and cytochrome c can be determined.

Since all these components are present at a

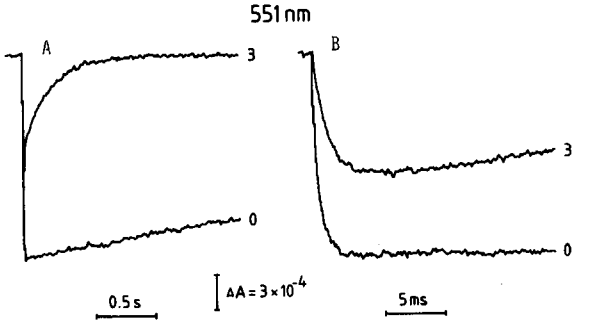

Fig. 1. The redox change of cytochromes $c + c_1$ after a single flash in the presence and absence of the reductase on slow (A) and fast (B) time scales. The system contained 1 μM reaction centres, 3 μM horse-heart cytochrome c, no reductase or 3 μM of the reductase as indicated by the numbers, with 2 mM ascorbate and 0.1 μM phenazine methosulphate in 50 mM Tris-HCl buffer (pH 8.0).

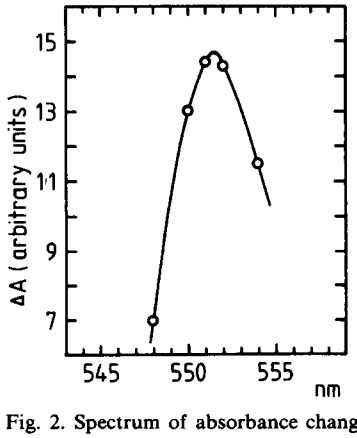

Fig. 2. Spectrum of absorbance change around α-band region of cytochromes $c + c_1$ optical absorption 5 ms after a flash. The experimental conditions were the same as in Fig. 1, but with 3 μM of the reductase. At the moment this spectrum was taken, all photooxidized reaction centres had been re-reduced.

concentration of 3 μM, we obtain:

for the Fe-S cluster	red/ox	= 2.92/0.08	= 36.5
for the cytochrome c_1	red/ox	= 2.919/0.081	= 36.0
for the cytochrome c	red/ox	= 2.961/0.039	= 76.0

Thus, at pH 8.0, the E_m of the Fe-S cluster is about equal to that of cytochrome c_1 but the E_m of cytochrome c is about 20 mV higher than that of cytochrome c_1.

Since the amounts of the oxidized components can be determined quite accurately, the ratio red/ox and the difference in E_m between the different components are quite accurate. The ΔE_m of 20 mV fits exactly with the result of Fig. 3, where very different concentrations of reductase and cytochrome c are used. For Fig. 4, the calculations are more complicated, since these measurements have been done at 550 nm where cytochrome c absorbs much more strongly than cytochrome c_1.

The slow reduction of cytochrome $c + c_1$ shown in Fig. 1A ($t_{1/2} = 50$–60 ms) is due to reaction (4) followed by reequilibration between the [2Fe-2S] cluster, cytochrome c and cytochrome c_1. This can be inhibited by myxothiazole or HMHQQ, so that only the slow reduction by ascorbate remains after quasi-equilibrium has been reached (Fig. 3).

The level of flash-induced oxidation of cytochrome c ($+c_1$) as measured by the absorbance at 550 or 551 nm varies with the concentrations of

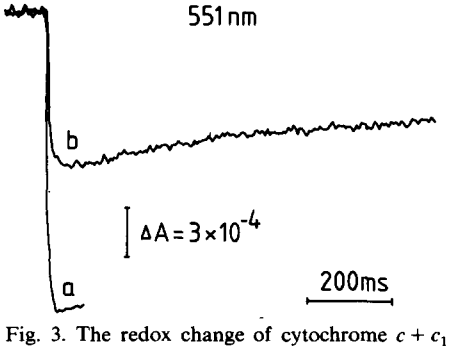

Fig. 3. The redox change of cytochrome $c + c_1$ after a single flash in the presence of reductase (7 μM) and HMHQQ (80 μM) is shown in b. A control showing the oxidation of cytochrome c in the absence of reductase (and HMHQQ) is shown in a. Other experimental conditions were the same as in Fig. 1, except that 0.1 μM phenazine methosulphate was replaced by 10 μM naphthoquinone.

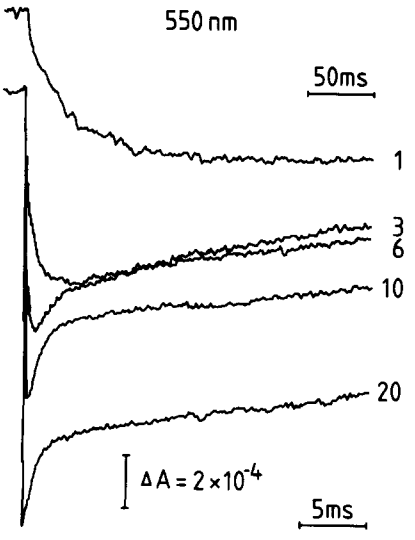

Fig. 4. The redox change of cytochrome c after a single flash in the presence of 3 μM of the reductase and at different concentrations of cytochrome c as indicated by the numbers (in μM). Other experimental conditions were the same as in Fig. 1.

both cytochrome c and oxidoreductase. Increasing concentrations of cytochrome c cause an increase in the rate of oxidation of cytochrome c. Thus, at high concentrations of cytochrome c, oxidation is virtually completed before a substantial re-reduction by cytochrome c_1 and the Rieske Fe-S cluster occurs (Fig. 4). The maximum level of oxidation of cytochrome c, therefore, increases with increasing concentrations of cytochrome c, but it decreases with increasing concentrations of reductase, since this causes both an enhancement of the rate of reduction of cytochrome c and a slowing down of its oxidation (see, for example, Fig. 6C).

When cytochrome c is not in large excess, the amount of the photo-oxidized cytochrome $c + c_1$ that remains oxidized is little dependent on the concentration of the reductase. This is in agreement with the conclusion that it is mainly the [2Fe-2S] cluster and cytochrome c_1 that become partly oxidized (their ratio does not change at variable concentrations of reductase) and that both components share the oxidation on a fifty-fifty basis, again indicating that their E_m values are about equal at the pH used (pH 8).

At substantially higher concentrations of cytochrome c, however, more cytochrome c remains

oxidized and less cytochrome c_1 and [2Fe-2S] cluster. This effect can be seen in Fig. 4 where the level of oxidation at 550 nm after the initial equilibration between cytochrome c, cytochrome c_1 and the [2Fe-2S] cluster increases with increasing concentrations of cytochrome c (cytochrome c is in excess of the reductase).

In Fig. 3. the absorbance change at 551 nm in the presence of HMHQQ is shown. We have reported earlier [17] that HMHQQ binds in the vicinity of the [2Fe-2S] cluster and that it binds 100-times more strongly to reductase in which this cluster is reduced than when it is oxidized. It would be expected, then, that HMHQQ would increase the E_m of the [2Fe-2S] cluster. However, according to Fig. 3 the distribution of the oxidation between [2Fe-2S] cluster and cytochrome c_1 is not significantly changed by HMHQQ: also here about 50% of the oxidized cytochrome $c + c_1$ is re-reduced by the [2Fe-2S] cluster, although the concentration of the reductase is higher than in Fig. 1 (7 μM). It appears that the presence of HMHQQ in concentrations high enough to inhibit the reduction of the [2Fe-2S] cluster does not affect the equilibrium between the [2Fe-2S] cluster and cytochrome c_1. Moreover, it does not seem to affect the rate of oxidation of the Fe-S cluster by cytochrome c_1, contrary to what is concluded for UHDBT [18,19], a compound very similar in behaviour to HMHQQ. In the Discussion we will return to this contradiction. At pH 7 (experiments not shown) less [2Fe-2S] cluster is oxidized and more cytochrome c_1 (the re-reduction at 551 nm is much smaller), indicating that at pH 7.0 the E_m of the Fe-S cluster is higher than that of cytochrome c_1 (cf. Refs. 1, 8 and 20).

The primary reduction of cytochrome b

In the absence of inhibitors a rapid initial reduction of cytochrome *b* can be seen, within a few milliseconds, followed almost immediately by an oxidation (see later). Since, however, only the initial phase of the reduction can be seen, it is not possible to calculate the $t_{1/2}$. This reduction can occur via two pathways, one sensitive to antimycin and one to myxothiazol. The antimycin-sensitive reduction (in the presence of myxothiazole) is biphasic (see Fig. 5), in agreement with the finding of De Vries et al. [8] on mixing QH_2 : cytochrome

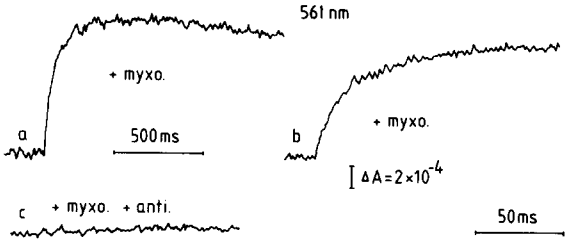

Fig. 5. The reduction of cytochrome *b* through centre i. The experimental conditions were the same as in Fig. 1 (3 μM reductase) but with 2 μM cytochrome *c* and 10 μM myxothiazole (myxo.) added. The concentration of antimycin, when added, was 6 μM.

c oxidoreductase with duroquinol. These authors propose that first the low-potential *b*-562 is reduced (60% of the total absorbance change at 561 nm) followed by the high-potential *b*-562. However, this cannot be the explanation for the two phases observed in our system, since high-potential *b*-562 is completely reduced before the flash. The most simple explanation is that the fast phase corresponds to reduction of residual oxidized low-potential *b*-562, while the slower phase corresponds to a partial reduction of *b*-566. Considering, however, the fact that in the absence of oxidant-induced reduction of cytochrome *b*, *b*-566 is scarcely reducible by substrate, it is possible that the rapid phase is due to direct reduction of cytochrome *b* by Q_B of the reaction centres, and the slow phase is due to reduction by Q_B via the Q-10 present in the reductase. It should be noted, however, that both phases are fully sensitive to antimycin as shown in Fig. 5C, suggesting that, if indeed cytochrome *b* is directly reducible by Q_B, this reduction proceeds via the site for Q_i.

The secondary reduction of cytochrome b and cytochrome c

To study the reactions at centre o, without involvement of the reaction of centre i, it is necessary to add antimycin which blocks the rapid reduction of cytochrome *b* via centre i (and also its oxidation), but has no effect on the rapid equilibration between the [2Fe-2S] cluster, cytochrome c_1 and cytochrome *c*, described above.

Fig. 6 shows traces at 561, 566 and 551 nm at varying concentrations of reductase, in the presence of antimycin. At 551 nm it can be seen that,

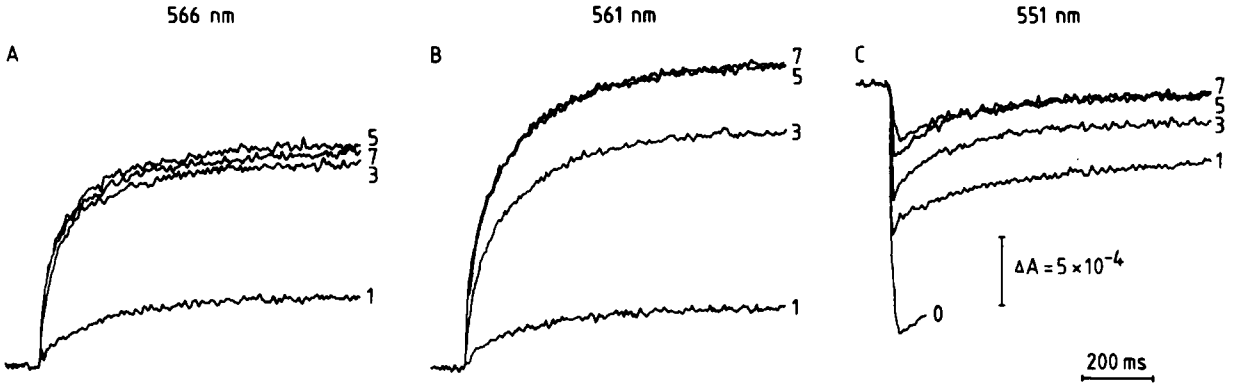

Fig. 6. The flash-induced reduction of cytochrome *b* followed at 561 nm (A) and 566 nm (B) as well as the re-reduction of cytochromes $c + c_1$ (C), at different concentrations of the reductase (indicated by numbers) and in the presence of saturating amounts of antimycin. Other experimental conditions were the same as in Fig. 1, except that 0.1 μM phenazine methosulphate was replaced by 10 μM naphthoquinone.

in contrast to Fig. 3. where HMHQQ was used as inhibitor, more than 50% of the total oxidized cytochrome $c + c_1$ finally becomes re-reduced. The extra re-reduction is due to electrons from QH_2 via centre o and at 5 μM reductase almost all cytochrome c_1 and [2Fe-2S] clusters become re-reduced. At the same time also cytochrome *b* is reduced and at full re-reduction of cytochrome *c*, c_1 and Fe/S cluster (in fact a full re-reduction of the oxidized cytochrome *c* via the [2Fe-2S] cluster and cytochrome c_1 as catalysts) the concentrations of reduced cytochrome *b* and re-reduced cytochrome *c* are about equal. To compare the amount of reduced cytochrome *b* with that of re-reduced cytochrome *c*, it should be noted that the absorbance coefficient of cytochrome *b* at its maximum wavelength is 1.3 that of cytochrome *c* at 551 nm and that both *b*-562 and *b*-566 contribute about 50% to the absorbance at the wavelength maximum of the other *b* component (see also Ref. 21). Thus, 0.5 s after the flash, the absorbance change due to cytochrome $c + c_1$ followed at 551 nm and that due to cytochrome *b*-561 and *b*-566 followed at 561 and 566 nm are, respectively, 11.0, 13.5 and 19.5 arbitrary units. From the extent of the mutual contribution of cytochrome *b*-561 and *b*-566 to the spectra it can be calculated that the absorbance changes due to cytochrome $c + c_1$, *b*-561 and *b*-566 at their respective maxima, are 11.0, 11.7 and 3.7 arbitrary units. The total amount of cytochrome *b* reduced relative to cytochrome *c* re-reduced is then:

$$\frac{11.7+3.7}{1.3} : 11.0 = 11.8 : 11.0 = 1.07 : 1$$

At intermediate stages of re-reduction of cytochrome $c + c_1$ it has to be taken into account that also some Fe-S cluster remains oxidized. At low concentrations of reductase no full re-reduction occurs, since the equilibrium does not allow full reduction. At these low concentrations of reductase (relative to cytochrome *c*) relatively more cytochrome c_1 and [2Fe-2S] become oxidized by cytochrome *c* and cannot be fully re-reduced by the QH_2 present.

The kinetics of the reduction of cytochromes *b* and c_1 by QH_2 cannot be determined from the traces at 551 nm in Fig. 6, since part of this reaction, together with the rapid equilibration of cytochrome c_1, cytochrome *c* and Fe-S cluster, cannot be seen, and the part that can be seen reflects re-reduction of cytochrome c_1 and Fe-S cluster together. The traces at 561 or 566 nm, however, do correspond to the actual rate of the reaction and from these traces the $t_{1/2}$ can be determined to be 10–15 ms. This value is in good agreement with the value determined by De Vries et al. [5] for the turnover at centre o (15–20 ms) with duroquinol as substrate.

In our previous paper [1] we have shown that in

the absence of cytochrome *c* equal amounts of cytochromes *b* and c_1 are reduced. In that case the rate of re-reduction of cytochrome c_1 is limited by its oxidation by the reaction centres and the rate of *b* reduction is correspondingly slow. This is consistent with the postulate of the Q cycle that oxidation of QH_2 via centre o occurs only when both cytochrome *b* and the Fe-S cluster (the electron donor to cytochrome c_1 [22]) can be reduced together. The scheme depicted by Matsuura et al. [6] according to which QH_2 is oxidized by the Fe-S cluster to Q^-, which is then re-reduced to QH_2 by cytochrome *b* is, however, not consistent with our finding that, in the presence of antimycin, equal amounts of cytochromes *b* and c_1 are reduced at centre o, which is unaffected by antimycin.

The re-reduction of cytochrome c, accompanied oxidation of cytochrome b

In the previous paragraph it was shown that, under the conditions of our experiments, cytochrome c_1 and the [2Fe-2S] cluster could not be fully re-reduced in the presence of antimycin when the concentration of reductase is lower than 5 μM. In the absence of antimycin, however, complete reduction is obtained, but at a slower rate (Fig. 7). The reason for this complete reduction is clear: since antimycin is absent, cytochrome *b* can be oxidized again via centre i and therefore the equilibrium of the reaction

$$QH_2 + b^{3+} + c_1^{3+}(\text{Fe-S})_{ox} \rightleftharpoons Q + b^{2+}H^+ + c_1^{2+}(\text{Fe-S})_{red} + H^+$$

shifts to further reduction of cytochrome c_1 and the Fe-S cluster because of the additional reaction $2b^{2+}H^+ + Q \rightleftharpoons 2b^{3+} + QH_2$.

In Fig. 7 it can be seen also that cytochrome *b* after an initial rapid reduction becomes oxidized, while cytochromes $c + c_1$ are reduced with the same half-time as that with which cytochrome *b* is oxidized (cf. Refs. 5 and 6). The initial re-reduction of cytochrome *c* as the result of equilibration with the [2Fe-2S] cluster and cytochrome c_1 is invisible, since under the conditions of the experiment this reduction is as fast as the oxidation by the reaction centres. In agreement with Fig. 1, the 'missing' cytochrome *c* is about one-half the total oxidized cytochrome *c*. The half-time of reduction of cytochrome $c + c_1$ (and the Fe-S cluster) is now larger than in Fig. 6 with the same concentration of reductase (and QH_2), since more cytochrome $c + c_1$ can be reduced and the quinol has to be re-formed via oxidation of cytochrome *b* at centre i.

From experiments similar to that of Fig. 7, Matsuura and Dutton [5,6] have concluded that cytochrome *b* reduces the [2Fe-2S] cluster via a QH_2/Q^- couple, without involvement of the Q-pool. According to their explanation, the Q^- is formed via reduction of the [2Fe-2S] cluster by QH_2 and, after a conformational change at the site of Q^- which is needed to make the Q^- an oxidant of cytochrome *b* instead of a reductant, this Q^- oxidizes cytochrome *b*. This explanation would require this conformational change to be extremely fast, since *b* oxidation and [2Fe-2S] reduction occur simultaneously and the Q^- formed by the reduction of the [2Fe-2S] cluster is very unstable and not detectable unless antimycin is added to prevent oxidation of cytochrome *b* and an oxidant is present to keep the [2Fe-2S] cluster highly oxidized [10]. Furthermore, there is a discrepancy between the amounts of *b* oxidized and *c* reduced. According to Fig. 7 (and the traces in Refs. 5 and 6) the absorbance change at 561 nm is smaller than that at 551 nm. Since the absorbance coeffi-

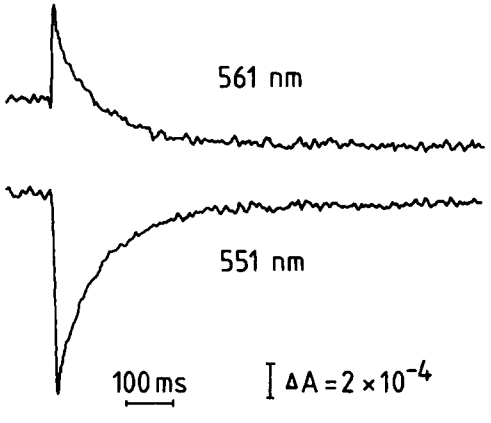

Fig. 7. Flash-induced redox change of cytochrome *b* (561 nm) and cytochrome $c + c_1$ (551 nm) in the absence of inhibitors. The experimental conditions were the same as in Fig. 1 with 3 μM of the reductase present.

cient of cytochrome *b* is higher than that of cytochrome c_1, less than one molecule of cytochrome *b* is oxidized for each cytochrome c_1 that becomes reduced, despite the fact that also electrons are required to reduce the [2Fe-2S] cluster as well as the cytochrome $c + c_1$. It follows that electrons are entering cytochrome c_1 and the Fe-S cluster from a source other than cytochrome *b* and the only possible source of these electrons is QH_2. Since oxidation of QH_2 by the Fe-S cluster is coupled to reduction of cytochrome *b* (see above), it must be concluded that in this reaction a Q cycle is involved in which QH_2 reduces the Fe-S cluster and cytochrome *b*, and cytochrome *b* rapidly equilibrates with the Q-pool via centre i, thereby becoming oxidized (equilibrium with the Q-pool) with kinetics similar to the reduction of cytochrome c_1 and the [2Fe-2S] cluster. In fact both Q and cytochrome *b* become temporarily over-oxidized, since the electrons from the bacteriochlorophyll dimer apparently stay largely on Q_B (see Ref. 1).

The conclusion that the oxidation of cytochrome *b* and the reduction of cytochrome c_1 and Fe-S cluster are coupled to each other via both centre i and centre o is further demonstrated by the effect of increasing concentrations of HMHQQ (Fig. 8). Already at low concentrations of the inhibitor the similarity in the kinetics of cytochrome *b* oxidation and cytochrome $c + c_1$ reduction disappears, the former being inhibited more than the latter. At 7 μM HMHQQ, the net oxidation of cytochrome *b* is no longer visible, whereas cytochrome c_1 and the [2Fe-2S] cluster are still becoming re-reduced. If electron transfer from *b* to *c* would occur via a linear pathway [5,6], the inhibition should affect oxidation and reduction in the same way. Only if we assume that the trace at 561 nm represents a summation of reduction of cytochrome *b* and an oxidation and the two processes have a different sensitivity for HMHQQ (cf. Ref. 17) do the data become explicable. At 7 μM HMHQQ the re-reduction of cytochrome *c* is slow due to the inhibition of centre o. But centre i is not yet inhibited at this concentration [17] and cytochrome *b* equilibrates via this site with the Q-pool, which is more reduced after the flash than before, At very high concentrations of HMHQQ this site also becomes inhibited, blocking all reduction of cytochrome *b*.

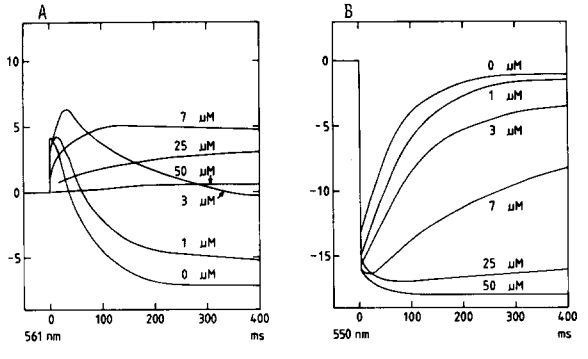

Fig. 8. The redox change of cytochrome *b* (A) and cytochrome *c* (B) after a flash in the presence of different concentrations of HMHQQ. The experimental conditions were similar to those in Fig. 1, with 3 μM of the reductase and 30 μM of cytochrome *c* present. Traces were corrected according to Ref. 1, but by hand. The ordinate indicates the absorbance changes, expressed in arbitrary units.

Discussion

The effects of variation of the concentrations of cytochrome *c* and the oxidoreductase on the equilibration between the [2Fe-2S] cluster, cytochrome c_1 and cytochrome *c* confirm our previous conclusion that at pH 8 the E_m of the [2Fe-2S] cluster equals that of cytochrome c_1, but is higher at pH 7.0, becoming closer to that of cytochrome *c*.

Unexpected is the finding that HMHQQ, unlike UHDBT with chromatophores [18], does not significantly affect the equilibration between the [2Fe-2S] cluster and cytochrome c_1. We have shown previously [17] that HMHQQ binds more strongly to its site at centre o when the high-potential components of the respiratory chain are reduced. The fact that HMHQQ affects the EPR spectrum of the reduced [2Fe-2S] cluster suggested to us that the reduction of the [2Fe-2S] cluster was responsible for the difference in binding, in analogy with the results obtained with UHDBT. A possible explanation is that it is not the redox state of the [2Fe-2S] cluster or cytochrome c_1 that is relevant for the binding constants of HMHQQ, but that of another component in the reductase, reducible with ascorbate, possibly Q bound to a Q-binding subunit (see Ref. 23). This could fit with the finding [17] that the K_i for the inhibition of the steady-state

oxidation of succinate is low, although the [2Fe-2S] cluster and cytochrome c_1 remain oxidized.

The main conclusion from our data is that they are inconsistent with a linear electron transfer from reduced cytochrome *b* to Q^- and from QH_2 to the [2Fe-2S] cluster, which has been suggested by Matsuura et al. [4] on the basis of the kinetic similarity of the reduction of cytochrome $c_1 + c$ with the oxidation of cytochrome *b* to occur under the conditions of flashing in the absence of any inhibitor. Apart from the reasons already given (lack of stoichiometry, instability of Q^-, differential effect of HMHQQ), additional arguments for rejecting this proposal can be given. First, the proposed effect of antimycin on centre o: in the presence of antimycin, QH_2 reduces both the [2Fe-2S] cluster and cytochrome *b*; when cytochrome *b* is already reduced, the Fe-S cluster cannot be reduced any more, resulting in inhibition of electron transport; should Q^- be only a reductant of cytochrome *b* in the presence of antimycin and an oxidant in its absence? Such an effect of antimycin should be possible if antimycin raises the E_m of cytochrome *b*, but in fact antimycin lowers the E_m of (part of) cytochrome *b* [24].

A second argument can be derived from the experiment shown in Fig. 2 of Ref. 6. While the rates of both reduction of cytochrome *c* and oxidation of cytochrome *b* first increase with decreasing redox potential, these rates become lower again at potentials below 0 mV, where the low-potential *b* becomes reduced. This phenomenon suggests that the reduction of cytochrome *c* becomes inhibited when all cytochrome *b* is reduced, i.e., reduction of cytochrome *c* can proceed only when also cytochrome *b* can be reduced.

For the mitochondrial oxidoreductase it is quite well established that cytochrome *b* is reducible not only via centre o (sensitive to myxothiazol) but also via centre i (sensitive to antimycin). In chromatophores and chloroplasts, however, only the myxothiazol-sensitive pathway has been established, not the antimycin-sensitive pathway. Several authors have reported [25,26] that in chromatophores, in the presence of myxothiazol, cytochrome *b* is not reduced after a flash. On the other hand, the oxidation of cytochrome *b* is inhibited by antimycin, resulting in an oxidant-induced increased reduction of cytochrome *b*. Apparently antimycin inhibits the oxidation of cytochrome *b* in chromatophores just as in mitochondria, but, unlike in mitochondria, the reduced form of the oxidant of cytochrome *b* in this antimycin-sensitive pathway is unable to reduce cytochrome *b*, unless the lack of reduction of cytochrome *b* is due to the fact that the redox state of the Q pool is only marginally increased by light flashes.

Another difference between our system and intact chromatophores is the rate of reduction of cytochrome *b*, which is relatively slow in our system. The size of the Q-pool may be the reason for this difference, although it is possible that the turnover number of the mitochondrial enzyme especially at centre o, is lower, per se.

In Fig. 6 it was seen that at concentrations of reductase of 5 μM or higher, all cytochrome $c + c_1$ become re-reduced by QH_2 in the presence of antimycin. According to the dimeric model of the Q-cycle proposed in Refs. 7 and 8, antimycin blocks the re-reduction of cytochrome c_1 and the [2Fe-2S] cluster in one of the two protomers. We do not observe such an inhibition, but it could be obscured by the cytochrome *c*, equilibrating between the cytochromes c_1 of the different protomers

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