The Role of Ubiquinone-10 in Cyclic Electron Transport in *Rhodopseudomonas capsulata* Ala pho$^+$: Effects of Lyophilization and Extraction

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1. The effects of lyophilization and the extraction of ubiquinone-10 on the kinetics of electron transport in *Rhodopseudomonas capsulata* Ala pho$^+$ have been investigated.

2. Lyophilization reduced the amount of ferrocytochrome c_2 photo-oxidized on a microsecond time scale following a single excitation.

3. Lyophilization increased the reactivity of the electron transfer components with redox mediators, particularly N-methyl phenazonium methosulphate (PMS). At a concentration of 1 μM, PMS accelerated reaction center re-reduction, ferricytochrome c_2 re-reduction and ferrocytochrome b_{50} oxidation. The cytochrome c_2 re-reduction stimulated by PMS was antimycin A insensitive but the cytochrome b_{50} oxidation was partially antimycin sensitive.

4. Removal of 25–30 molecules of ubiquinone 10 per reaction center removed a secondary acceptor pool, had very little effect on the kinetics of ferricytochrome b_{50} reduction and ferricytochrome c_2 re-reduction, but markedly inhibited ferrocytochrome b_{50} oxidation. Ubiquinone extraction also caused an increased stimulation of ferrocytochrome b_{50} oxidation by PMS.

5. The involvement of tightly bound ubiquinone in cytochrome b reduction and in the cytochrome b-c_2 oxido-reductase, and the role of semiquinone species is discussed.

Introduction

In the cyclic electron transport system of *Rps. capsulata* and the closely related *Rps. sphaeroides*, there is a possibility that ubiquinone-10 (UQ) acts in several separate sections as an electron or hydrogen carrier [1–5]. Thermodynamic and kinetic evidence [4] suggests that a ubiquinone/ubisemiquinone couple acts between the bound UQFe complex of the reaction center (primary acceptor) and cytochrome b_{50} (Cyt b_{50}) in the presence of antimycin A. UQ depletion experiments also indicate a role for UQ as the secondary acceptor in *Chromatium vinosum* [5].

It has been suggested that UQ is also involved in cytochrome c_2 (Cyt c_2) re-reduction [1]. Several features of the kinetics and thermodynamics of electron transfer between Cyt b_{50} and Cyt c_2 (the donor to the reaction center), indicate the involvement of a hydrogen carrier [1, 3, 6–11]. Prince and Dutton [12, 13] have found that the extent of rapid Cyt c_2 re-reduction following flash activation titrates in like a two-electron, two-proton carrier of $E_{m(7.0)} \sim 155$ mV and, in the absence of any other known components with these properties, have suggested that this component may be a bound ubiquinone. Crofts *et al.* [1] and Crofts and Bowyer [14] have suggested that the Cyt c_2 re-reduction kinetics observed in chromatophores of *Rps. capsulata* show a second order process indicating a pool of reductant, presumed to be a special UQ (called ZH_2) in a 4–5 fold molar excess over Cyt c_2, in which ubiquinol is the reductant for Cyt c_2. The resulting unstable semiquinone ($ZH\cdot$) is proposed to act as the *oxidant* for ferrocyt b_{50} [1, 3]. Dutton *et al.* [15] and Prince *et al.* [16] have also demonstrated the second order nature of the interaction between Cyt c_2 and its rapid reductant in *Rps. sphaeroides*, but they propose that the $ZH\cdot$ produced following Cyt c_2 reduction by ZH_2 is the *reductant* for ferricyt b_{50}.

Chromatophores of *Rps. capsulata* contain large amounts of UQ (25–100 molecules per reaction

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Abbreviations: PMS, N-methyl phenazonium methosulphate; PES, N-ethyl phenazonium ethosulphate; FCCP, carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone; MOPS, morpholinopropane sulphonic acid; UQ, ubiquinone-10; BChl, bacteriochlorophyll.

center) [17, 18 and Dr. P. L. Dutton, personal communication]. Baccarini-Melandri and Melandri [18] showed that extraction of a very large proportion of the UQ results in a marked inhibition of photophosphorylation, in the rate of Cyt b_{50} reduction and oxidation and in Cyt c_2 re-reduction observed under continuous illumination. Addition of pure UQ in an amount roughly equal to the amount extracted restored the rates to their values before extraction. Furthermore, the quinone antagonist 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) has been shown to inhibit electron transport between Cyt b_{50} and Cyt c_2 under continuous illumination in *Rhodospirillum rubrum* chromatophores [19, 20] and to inhibit photophosphorylation in *Rps. capsulata* [33].

We have attempted in this work to relate the effects due to UQ extraction observed with continuous illumination to those observed when using saturating light flashes to elicit only one turnover per electron transport chain.

Methods

Chromatophores from the carotenoidless mutant of *Rps. capsulata* strain Ala pho$^+$ were prepared as described previously [21]. The use of a carotenoidless strain precluded measurement of the carotenoid electrochromic response to monitor transmembrane electrogenic electron transport. However, overlapping electrochromic absorption changes of light harvesting bacteriochlorophyll which seriously interfere with the measurement of cytochrome b_{50} kinetics, particularly under coupled conditions, in carotenoid containing strains, are relatively insignificant in carotenoidless strains [24]. The methods used for membrane lyophilization, ubiquinone extraction and reconstitution, and for ubiquinone estimation are described in ref 18. The ubiquinone-10 used for reconstitution was a gift of Hoffman La Roche Co. Ltd. Bacteriochlorophyll was measured in acetone: methanol extracts according to Clayton [22].

Flash-induced absorbance changes were recorded using a rapid single beam spectrophotometer equipped for single averaging and data processing [23]. The averaging computer was based on LSI-11 microcomputer modules (Digital Equipment Corporation). Traces for time resolved spectra were averaged and processed by a PDP 11/10 minicomputer (Digital Equipment Corporation). Chromatophores were suspended in 60 mM glycylglycine, 10 mM $MgCl_2$, 100 mM KCl, 10 μM FCCP, 2 mM sodium fumarate, and 0.2 mM sodium succinate at pH 8.0 in a stirred anaerobic cuvette at room temperature. These conditions poised the redox potential at about 15 mV, as determined following the addition of redox mediators.

When carrying out redox titrations (reviewed in 25) great care was taken to ensure that the mediating dyes did not affect the kinetics of electron transport. Since PMS or PES at concentrations as low as 1 μM markedly modified the kinetics of cytochrome changes, these reagents were omitted, and 5 μM each of 2-hydroxy-1,4-naphthoquinone, 1,4-naphthoquinone and 1,2-naphthoquinone were used.

In order to ensure equilibration of the Pt electrode with the membrane redox components, a rather long equilibration time (15 – 20 min) at each redox potential (E_h) was allowed before measurements were made, and 1 μM PMS was added at the end of redox titrations (at low E_h) to confirm that the Pt electrode was at least equilibrated with the solution phase.

Because of uncertainty in the effectiveness of the mediators which had to be used for redox titrations, we hesitate to draw definite conclusions from redox titrations performed on lyophilized chromatophores.

Results and Discussion

Effects of lyophilization

Lyophilization affected the electron transport system in a number of ways which must be taken into account when considering the effects of UQ extraction. There was some dislocation of cytochrome c_2 from its site of interaction with the reaction center such that, in the presence of antimycin A, only about 15% of the total photo-oxidizable cytochrome c_2 was oxidized on a millisecond time scale following a single flash (Fig. 1) (c. f. 30 – 50% in native chromatophores). Of the "rapidly" oxidized Cyt c_2, about 50% was oxidized with a $t_{1/2}$ of less than 50 μs. Treatment with isooctane increased the amount of Cyt c_2 oxidized on a submillisecond time scale following a single activation, perhaps by providing an environment in which some membrane structural rearrangement could occur (see Fig. 2).

Lyophilization also affected electron transport from Cyt b_{50} and to Cyt c_2. Since the proportion of

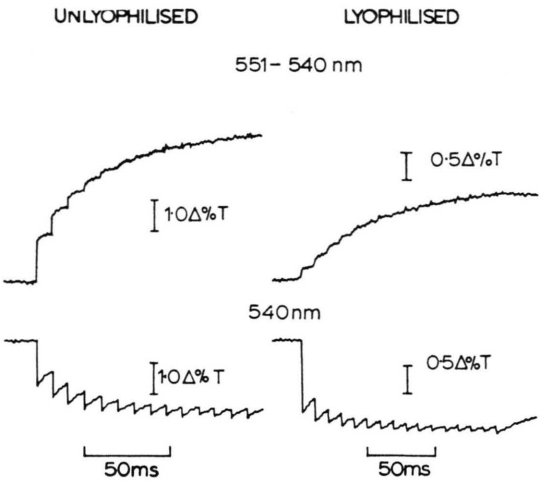

Fig. 1. Effects of lyophilization on the Cyt c_2-reaction center interaction. Unlyophilized: Chromatophores (25 μM BChl, 1 μM reaction centers) were suspended in 2.5 ml of a medium containing 50 mM MOPS, 100 mM KCl, 1 mM potassium cyanide, 1 mM sodium ascorbate and 2 μM valinomycin at pH 7.0 in an open unstirred cuvette. Traces are an average of 4. The instrument response time was 0.2 ms. Lyophilized: Chromatophores (24 μM BChl, 0.6 μM reaction centers) were suspended in the medium as described in Materials and Methods. Traces were not averaged. The instrument response time was 0.2 ms.

Cyt c_2 undergoing rapid photo-oxidation was low, the kinetics of Cyt c_2 reduction were masked by the slow phase of oxidation. By comparing the kinetics in the presence and absence of antimycin A, the rate of reduction could be assayed. A similar procedure was used to assay the rate of Cyt b_{50} oxidation. At E_h 20 mV, pH 8.0, the $t_{1/2}$ for both processes was about 20 ms. However, considerable variations in rate were observed, and in some preparations the two processes did not appear to be kinetically matched, in contrast to the results obtained with unlyophilized chromatophores by Prince and Dutton [28] and Crofts *et al.* [1]. The rates of Cyt b_{50} oxidation and Cyt c_2 reduction were much slower than the rates in native chromatophores, where the half-times of Cyt b reoxidation and of Cyt c_2 re-reduction are both about 2 ms at E_h 20 mV, pH 8.0. This could be due to the lowered level of photo-oxidised Cyt c_2 in lyophilized chromatophores if Cyt c_2 reduction is a second order process [16].

Effects of N-methyl phenazonium methosulphate (PMS)

The disruption of the electron transport system caused by lyophilization also gave rise to an increased reactivity of the electron transfer components with the redox couples used as mediators. This effect was particularly marked with PMS and PES. Addition of PMS at low (1 – 5 μM) concentration dramatically affected the kinetics of Cyt b_{50} oxidation, Cyt c_2 reduction and reaction center reduction.

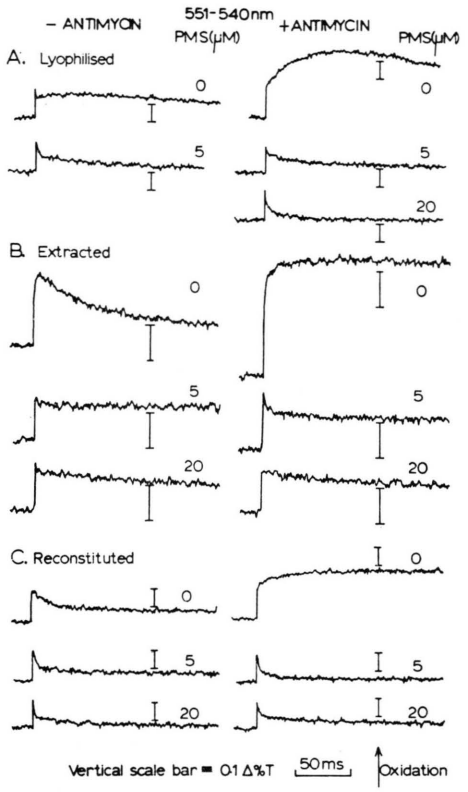

Fig. 2. Effects of PMS and antimycin A on the single turnover kinetics of cytochrome c_2 in lyophilized, isooctane extracted, and UQ reconstituted chromatophores. Antimycin A was added to 8 μM and PMS to the concentrations indicated. Traces are an average of 4. Lyophilized membranes: 24 μM BChl. Instrument response time 0.2 ms. Extracted membranes: 14 μM BChl. Instrument response time 0.5 ms. Reconstituted membranes: 15 μM BChl. Instrument response time 0.2 ms.

1 – 5 μM PMS greatly accelerated the rate of re-reduction of the photo-oxidized reaction centers presumable by direct reduction of the reaction center. However, similar concentrations had very little apparent effect on the Cyt c_2 re-reduction kinetics in the absence of antimycin A, but entirely eliminated any effect of antimycin A on Cyt c_2 re-reduction, implying that PMS was bypassing the inhibition of this reaction by antimycin A. Although the PMS mediated rate appeared to be similar to

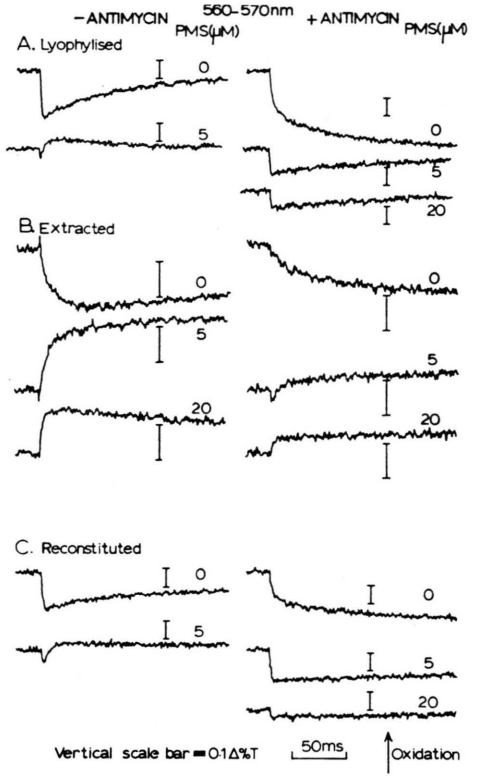

Fig. 3. Effects of PMS and antimycin A on the single turnover kinetics of cytochrome b_{50} in lyophilized, isooctane extracted, and UQ reconstituted chromatophores. Conditions as for Fig. 2.

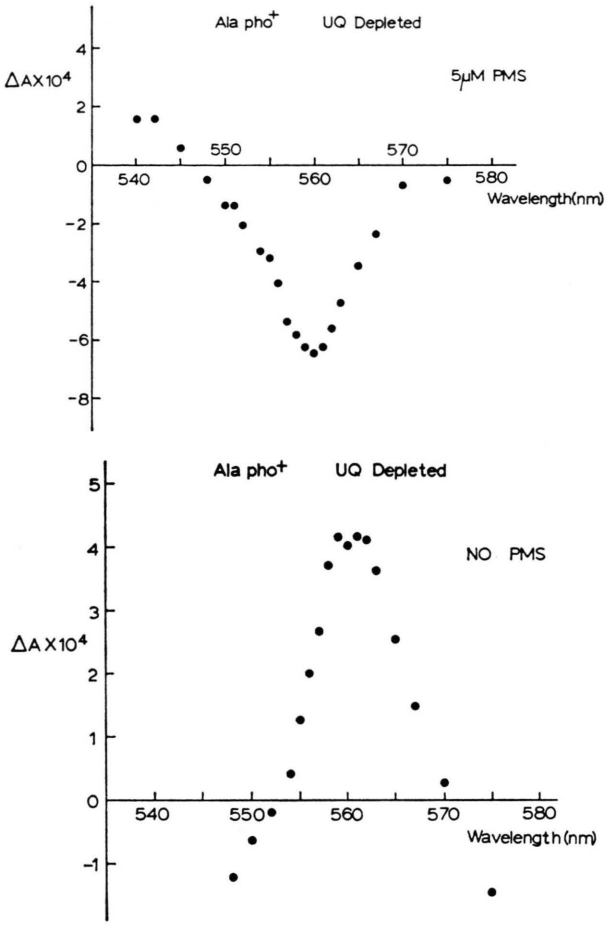

Fig. 4. A. Spectrum of the absorption charge, 100 ms after the flash in extracted chromatophores in the presence of 5 μM PMS. The spectrum is corrected for the reaction center change, assuming that the change at 605 nm represents only a reaction center change. Each point is derived from a non-averaged trace of the flash induced absorption charge recorded at that wavelength. The instrument response time was 10 ms. Chromatophores were at 14 μM BChl in the medium described in Materials and Methods.
B. Spectrum of the absorption charge 140 ms after the flash in extracted chromatophores. Conditions were as for Fig. 4 A except that no PMS was present, and the change due to Cyt c_2 photo-oxidation has also been substrated out.

the control rate, it is also possible that the step bypassed by PMS was not the rate limiting step in Cyt c_2 re-reduction (Fig. 2). Although PMS stimulated rapid *complete* re-reduction of the photo-oxidized reaction centers, re-reduction of Cyt c_2 in the presence of PMS was not aways complete even after 200 ms (Fig. 2).

Low concentrations of PMS also stimulated Cyt b_{50} photo-oxidation (Fig. 3). A spectrum of the change confirms that Cyt b_{50} was being oxidized (Fig. 4 A). The oxidation was partially antimycin sensitive, and became less sensitive as the PMS concentration was raised (Fig. 3). These effects are interpreted as being due to oxidation of Cyt b_{50} by an oxidized species of PMS (possibly the semiquinone PMS·) resulting from oxidation of reduced PMS (PMSH) by the photo-oxidized reaction centers. The different sensitivities of the PMS stimulated Cyt c_2 and Cyt b_{50} kinetics to antimycin imply that, under these conditions, the redox changes of the two cytochromes were not obligatorily linked. These results explain the observation of Baccarini-Melandri and Melandri [18] on the effects of PMS and antimycin on photophosphorylation and the steady-state redox poises of the cytochromes under continuous illumination in lyophilized chromatophores. The results also help to reconcile their results with those of Gromet-Elhanan and Gest, who found that PMS could not bypass the antimycin block on photophosphorylation in *unlyophilized* chromatophores.

The relative insensitivity of the PMS mediated effects to antimycin, particularly in the case of Cyt c_2 reduction, suggests that PMS was interacting directly with the electron transport components on the time scale of cyclic electron transport rather than simply increasing the concentration of "ZH_2", the physiological component responsible for mediating rapid electron transport through the Cyt b-c_2 oxido-reductase [2, 13]. The antimycin concentration used was about eight times greater than that of the reaction center, more than enough to inhibit cyclic electron transport at low redox potentials in native chromatophores. However, it is also possible that some components of the electron transport system do not come to complete equilibrium with the succinate/fumarate couple even after several hours, and that PMS catalyzes a rapid equilibration. Such an effect has been observed with the b-type cytochrome in mitochondria [26]. It is noteworthy that even in some preparations of native chromatophores, low concentrations of PMS (in the range normally used as a redox mediator when performing redox titrations of Cyt c_2 reduction rates) accelerate the rate of reaction center re-reduction and modify the kinetics of the cytochrome changes and their sensitivity to antimycin. Thus, in some preparations of unlyophilized chromatophores of *Rps. capsulata* Ala pho$^+$, PMS at 10 μM overcomes the inhibition of Cyt c_2 re-reduction by antimycin A (present at 2 μM, double the reaction center concentration) but does not significantly affect the inhibition by antimycin of Cyt b_{50} oxidation. The effects of PMS in unlyophilized chromatophores will be reported in more detail in forthcoming papers. The oxidation of reduced PMS (at 70 μM) by chromatophores of *Rps. sphaeroides* and *Rhodospirillum rubrum* in continuous illumination has also been followed spectrophotometrically by Zaugg *et al.* [27].

Effects of ubiquinone-10 extraction and reconstitution

Isooctane extraction of 25 – 30 molecules of UQ per reaction center, enough to cause 95% inhibition of the phosphorylation rate (as compared to non-extracted lyophilized membranes) [18] removed from the system a major component of the redox buffering and this complicates interpretations based on comparison between depleted and undepleted preparations, especially in experiments using antimycin A.

The pattern of reaction center oxidation on multiple flash excitation in the presence of 5 μM PMS to re-reduce the reaction center in the time between flashes (10 ms), was altered by UQ extraction (Fig. 5). By the fourth excitation, the extent of reaction center oxidation was only 8% of the extent following the first excitation, as compared to almost 100% in the unextracted chromatophores, and 30% in the reconstituted chromatophores. It seems likely that this is caused by a loss of the secondary acceptor pool in the depleted particles, so that electrons not reaching Cyt b_{50} remain on the primary acceptor instead of moving on to the UQ pool.

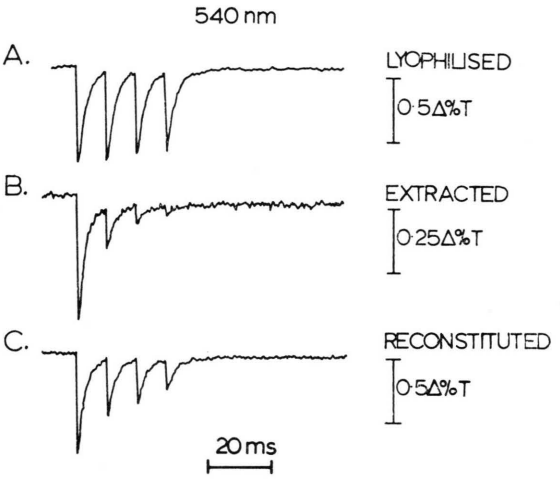

Fig. 5. Reaction center change on excitation with multiple flashes in lyophilized, isooctane extracted and reconstituted chromatophores. Flashes were 10 ms apart. Instrument response time 0.2 ms. Traces are average of 2. PMS was added to 5 μM. Lyophilized membranes added to 18 μM BChl. Extracted membranes added to 14 μM BChl. Reconstituted membranes added to 15 μM BChl. The chromatophores were suspended in the medium described in Materials and Methods.

UQ extraction had very little effect on the initial rate of Cyt b_{50} reduction in the absence of antimycin A (Fig. 3). Time resolved spectra were used to demonstrate that the Cyt b going reduced was Cyt b_{50} (Fig. 4 B). In all the variously treated chromatophores, the apparent $t_{1/2}$ for Cyt b_{50} reduction decreased as the redox potential was lowered (from $\sim$ 10 ms at E_h $\sim$ 200 mV to about 1 ms at E_h $\sim$ 15 mV pH 8.0). This rate of reduction was not kinetically linked to Cyt c_2 re-reduction in either

depleted or non-extracted chromatophores. Thus, in lyophilized UQ containing chromatophores, the rate of Cyt b_{50} reduction at low E_h and in the *absence* of antimycin A was very much more rapid than the rate of Cyt c_2 re-reduction in the same preparation. Dutton and Prince [29] have proposed that the reduction of Cyt b_{50} by ZH·, kinetically linked to Cyt c_2 reduction, operates at low E_h because it is faster than the rate at which Cyt b_{50} may be reduced by electrons delivered form the primary acceptor. This seems unlikely in view of the above observation. However, our observations are compatible with a simple linear scheme in which the reductant for Cyt b_{50} is independent of the redox state of Z/ZH_2 [1], although this redox state may indirectly affect the kinetics of Cyt b_{50} reduction [34].

Antimycin A decreased both the initial rate and total extent of Cyt b_{50} reduction in the UQ depleted chromatophores (Fig. 3). This may be due to a direct effect of antimycin on the interaction between Cyt b_{50} and its rapid reductant but the decrease in extent probably reflects the fact that a proportion of the Cyt b_{50} was pre-reduced by endogenous reductant or by photo-reduction by the measuring light under these conditions. Because of these effects, the rate of re-oxidation of Cyt b_{50} could not be determined by subtracting the change in the presence of antimycin from that in its absence. However, inspection of the traces (Fig. 3) does suggest that Cyt b_{50} oxidation was inhibited by UQ extraction and could be partially restored on reconstitution with UQ. Redox titrations indicated that in unextracted and reconstituted chromatophores, the rate of Cyt b_{50} oxidation increased as the potential was lowered below 200 mV, but very little change in rate was seen in extracted chromatophores.

The effects of UQ extraction and reconstitution on Cyt c_2 re-reduction were equivocal because of the disruptive effects of lyophilization. UQ extraction apparently had very little effect on the rate of Cyt c_2 re-reduction after allowing for the acceleration of Cyt c_2 oxidation by isooctane treatment.

Effects of PMS in ubiquinone-extracted chromatophores

The stimulation of Cyt b_{50} oxidation was most marked in UQ-depleted chromatophores. In these membranes, the rate, but not the extent, of Cyt b_{50} oxidation increased as the PMS concentration was raised from above 5 μM (Fig. 3), and even at fairly low concentrations, the PMS mediated Cyt b_{50} oxidation was faster than that mediated by the endogenous oxidant. In terms of the mechanism suggested by Crofts et al. [1] we envisage that the component ZH_2 (ubiquinol in a special environment), when oxidized by ferricytochrome c_2, produces the semiquinones species ZH· which is the endogenous oxidant for ferrocyt b_{50}. Results from unlyophilized chromatophores suggest that the half-time for oxidation of ferrocyt b_{50} bz ZH· may be at least as low as 2 ms, so that the rate constant for oxidation of ferrocyt b_{50} by ZH· must be high. The slow rate of oxidation of Cyt b_{50} in lyophilized unextracted chromatophores is thought to reflect the slow rate of generation of ZH· in these chromatophores. The PMS stimulated rate of oxidation of Cyt b_{50} also appears to proceed via a reaction with a high rate constant as observed after ubiquinone depletion.

In both unextracted and extracted chromatophores, when PMS is present, a substantial part of the re-reduction of Cyt c_2 and reaction center occurs through PMSH, presumably producing PMS·, so that extraction does not significantly affect the concentration of PMS· generated following a flash excitation. Furthermore, removal of a large pool of ubiquinone did not appear to significantly alter the rate of Cyt c_2 reduction. This implies that the level of Z/ZH_2 was not affected, but because of the drastic effects of lyophilization we cannot regard this as a definite conclusion. If correct, however, it would seem that ubiquinone depletion not only results in inhibition of oxidation of ferrocyt b_{50} by the endogenous oxidant ZH· but also enables PMS· to interact more readily with an oxidation site for ferrocyt b_{50}.

UQ extraction did not affect the PMS mediated bypass of the antimycin A block on Cyt c_2 reduction (Fig. 3).

Thus loss of a large UQ pool from lyophilized chromatophores has very little apparent effect on Cyt c_2 reduction over that caused by lyophilization, but has a fairly marked effect on Cyt b_{50} re-oxidation, implying that although the two processes appear to be kinetically linked in normal and in lyophilized particles, they are not obligatorily linked in UQ depleted chromatophores. This is also suggested by the differential sensitivity of the

two processes to antimycin in the presence of PMS. However, because of the modifying effects of lyophilization, we would hesitate on the basis of these results to reach any firm conclusions about the mechanism operating in native unlyophilized chromatophores. The results obtained appear to be somewhat inconsistent with those of Baccarini-Melandri and Melandri [18]. It is possible that isooctane extractable bulk UQ plays a role in Cyt c_2 reduction, but that this role only becomes apparent in the steady state, and not during a single turnover of the system. It is also possible that, although the experiments of Baccarini-Melandri and Melandri [18] were performed in an anaerobic, succinate poised system, the redox potential of the suspensions of UQ depleted chromatophores was still rather high, since loss of the ubiquinone pool considerably increased the time required for consumption of oxygen in the medium. At high redox potentials, the rate of Cyt c_2 reduction is slow even in native chromatophores.

Role of ubiquinone

It is apparent from our experiments that extraction of 90 – 95% of the ubiquinone from lyophilized chromatophores has a less dramatic effect than would be expected if the two reactions in which it is suggested that the quinone participates were dependent on the normal concentration of bulk quinone. The reduction of ferricytochrome b_{50} was not greatly affected by extraction, and it therefore seems likely that its reaction with the reduced primary acceptor is mediated through ubiquinone bound at a special site, possibly one of the reaction center UQs [35]. On the other hand, extraction drastically affected the size of the acceptor pool available to the photochemistry over a period of tens of ms, suggesting that over this time scale no alternative acceptor to Cyt b_{50} was available. We may take these results as showing that in normal chromatophores the large pool of acceptor available to the photochemistry is ubiquinone, as we have previously suggested [14], and that Cytochrome b_{50} is reduced rapidly and directly without the intervention of this pool.

Extraction of ubiquinone also effectively blocked the re-oxidation of Cytochrome b_{50}. This observation, together with the PMS effects noted above, suggests that the bulk quinone in some way indirectly effects the kinetics of oxidation of Cyt b_{50} by ZH·.

If component Z is ubiquinone, then it seems likely that it occupies a special environment separate from the bulk quinone, perhaps bound to a hydrophobic protein as is the case for the primary acceptor ubiquinone [30, 31] and the quinone pair which interacts with succinate dehydrogenase in the mitochondrial membrane [32]. This also seems probable from the apparent stoichiometry of Z (1 – 4 per Cyt c_2 oxidized per flash) as deduced from the second order kinetic effects observed by us [2] and by Dutton *et al.* [15].

The effects of extraction on the reduction of cytochrome c_2 were far from clear, largely because of the drastic effects of lyophilization on the cytochrome c_2 kinetics. In the presence of ubiquinone the oxidation of Cyt b_{50} and reduction of Cyt c_2 appeared to be kinetically matched, but either extraction of ubiquinone, or inclusion of PMS in the reaction mixture upset this apparent coupling between the two reactions. Reduced PMS appeared to be an effective donor both to Cyt c_2 and directly to the oxidized reaction center; neither reaction was inhibited by antimycin. Because of the inhibitory effect of lyophilization we can draw no firm conclusions as to the role of ubiquinone in the reduction of Cyt c_2 from these experiments.

Attempts to reduce the effects of lyophilization on cyclic electron transport, and to extract more tightly bound UQ molecules using more potent extraction methods are in progress.

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