SECONDARY ELECTRON TRANSFER IN CHROMATOPHORES OF *RHODOPSEUDOMONAS CAPSULATA* A1a pho$^+$

Binary out-of-phase oscillations in ubisemiquinone formation and cytochrome b_{50} reduction with consecutive light flashes

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1. Introduction

It is now well established that in isolated photochemical reaction centres of *Rhodopseudomonas sphaeroides* strain R26, a gating mechanism operates in the reduction of exogenous ubiquinone (a 2 electron carrier) by the primary acceptor complex (a 1 electron carrier) [1,2].

In chromatophores the situation is less well defined. An oscillation in proton uptake with flash number (1–2 H^+/reaction centre taken up on even numbered flashes, none on odd numbered flashes) was observed [3] in *Rps. sphaeroides* carotenoidless strain R26 at pH 7.5 but not in strain Ga which contains carotenoid. In *Rhodospirillum rubrum* chromatophores, no oscillation in proton uptake with flash number was observed [4], but proton release to a carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) accessible phase oscillated with a periodicity of 2. Binary oscillations in the semiquinone signal and in the slow phase of the electrochromic shift of the carotenoid spectrum with flash number have been observed in chromatophores of *Rps. sphaeroides* w.t. and in the neurosporene containing GlC mutant but only at redox potentials of $\geqslant$300 mV and in the presence of 50 μM TMPD [5]. In addition, binary oscillations in the semiquinone signal in cells of *Rhp. rubrum* were observed in aerobic cells but not in anaerobic cells. We have suggested that cytochrome b_{50} is reduced by electrons delivered from the primary acceptor complex, either directly by Q_{II} of the acceptor complex or by way of a component of equivalent stoichiometry, but without the interaction of the large pool of ubiquinone which can act as a secondary acceptor pool during multiple flash excitation in the presence of excess donor to the reaction centre [6,7]. We report here binary oscillations in cyt b_{50} reduction with flash number under conditions in which binary but out-of-phase oscillations in semiquinone formation are also observed.

Abbreviations: DAD, 2,3,5,6,-tetramethyl-*p*-phenylenediamine; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; MOPS, morpholinopropane sulphonate; cyt b_{50}, cytochrome b_{50}; cyt c_2, cytochrome c_2; *P*-870, reaction centre bacteriochlorophyll dimer; UQ, ubiquinone 10; $UQ^{\cdot -}$, ubisemiquinone 10 anion; $E_{h(7.0)}$, ambient reduction oxidation potential at pH 7.0; ϵ^{red-ox}, extinction coefficient difference between reduced and oxidised forms

2. Methods

Rhodopseudomonas capsulata, carotenoidless mutant A1a pho$^+$ was grown anaerobically in the medium described [8] at 29°C in 1 l Roux bottles placed about 10 cm from 40 W fluorescent strip lights in a temperature-controlled cabinet with forced air circulation. Cells were subcultured 3 times before being subcultured into the 1 l bottles, and they were harvested after ~24 h growth. Chromatophores were prepared by one passage through a

French Pressure Cell at 13 000–14 000 lb/in^2. Unbroken cells and large fragments were removed by centrifugation for 20 min at 17 000 × g and for 45 min at 35 000 × g.

For kinetic measurements flash excitation was provided by a Xe flash, 90–95% saturating, of ~5 μs pulse width, and absorbance changes recorded using a rapidly responding single beam kinetic spectrophotometer [9]. Transients were averaged after being read into a PDP 11/34 computer (Digital Equipment Corp.) via a Datalab DL905 Transient Recorder (Data Labs, Mitcham, Surrey, CR4 4 HR). The spectrophotometer was equipped with a shutter so that chromatophores were only exposed to the measuring light for the few seconds during which absorbance changes were recorded. For redox titrations, a stirred anaerobic redox cuvette was used [10], but for recording spectra of the flash number dependency of excitation-induced absorbance changes a redox poised reservoir in an anaerobic flow system was used to provide dark-adapted material. The whole system was computer controlled through a DEC Laboratory Peripheral System 11 using an appropriate assembly language program.

Chromatophores were routinely suspended in 50 mM MOPS, 100 mM KCl (pH 7.0), with valinomycin added to 2 μM to prevent build up of a membrane potential.

An ϵ^{red-ox} of 19.5 mM^{-1} cm^{-1} at 605 nm, calculated from [11] was used to estimate the extent of *P*-870 photo-oxidation. This value was determined in [11] for *Rps. sphaeroides* Ga. No value is available for *P*-870 in chromatophores in *Rps. capsulata* A1a pho$^+$; however our results suggest 18.5 ± 3.6 mM^{-1} cm^{-1}.

A minimum of 2 min dark time was allowed between measurements. Increasing the dark time did not significantly affect the oscillatory patterns observed except at the lower end of the redox potential range under study (≤300 mV).

Cytochrome b_{50} kinetics were recorded at 560–570 nm, a wavelength pair which almost entirely eliminates ΔA due to *P*-870 and cyt c_2 in *Rps. capsulata* A1a pho$^+$ (J.R.B., unpublished observations). Since most measurements of oscillations were made at 370–400 mV, where mediators such as TMPD and DAD had little effect on the *P*-870 rereduction kinetics in the presence of 0.5–1.0 mM potassium ferrocyanide (result not shown), potassium ferrocyanide was the only exogenous electron donor present except where indicated. This also avoided complications arising from rapid ΔA due to mediator oxidation and reduction encountered [5]. Under these conditions *P*-870 rereduction was extremely slow (85% complete after 10 s at $E_{h(7.0)}$ 450 mV rising to ~100% complete after 5 s at $E_{h(7.0)} \leqslant 370$ mV. In order that significant reaction centre rereduction and cyt b_{50} reoxidation could occur between flashes, the time between each flash in a train was 5–10 s. Although some *P*-870 rereduction by the return of the electron from the acceptor complex may have occurred over this time scale, it was not sufficient to obscure oscillations.

3. Results and discussions

3.1. *Binary oscillations in cytochrome* b_{50} *kinetics*

The traces of fig.1 show the cyt b_{50} reduction kinetics in a train of 4 flashes at $E_{h(7.0)}$ 370 mV and $E_{h(7.0)}$ 300 mV. Both the extent and initial rate of cyt b_{50} reduction showed a binary oscillation with very little cyt b_{50} being reduced on flash 1 and 3 at $E_{h(7.0)}$ 370 mV, and considerably more going reduced on flashes 2 and 4. The oscillation had almost disappeared at 300 mV, due to an increase in the extent of cyt b_{50} reduction on flash 1 and a decrease in that on flash 2. Detailed spectra of the flash-induced change on each flash in a train of 4 flashes from the dark-adapted state were recorded in the spectral region 530–580 nm where the α band of cyt b_{50} is located. After correction for the reaction centre change, they showed that the component(s) going reduced on flashes 2, 3 and 4 had identical spectral characteristics.

In the absence of antimycin A the extent of cyt b_{50} reduction on flashes 2, 3 and 4 was considerably reduced, presumably due to a rapid antimycin-sensitive reoxidation process, but fig.3A,B shows that oscillations could still be observed.

3.2. *Binary oscillations in the redox state of UQ*

At least 4 redox components can show flash number-dependent $\Delta A_{420-480}$ where ubisemiquinone redox changes are recorded. These are *P*-870, UQ, cyt b_{50} (γ band) and cyt c_2 (γ band). At 446 nm, close to the peak of the $UQ^{\cdot -}$–UQ difference

spectrum in methanol [12], ΔA due only to *P*-870 and UQ should be observed. Since there was no flash number-dependent variation in the extent of *P*-870 photo-oxidation at <390 mV (fig.1) oscillations in the ΔA_{446} should reflect the redox state of UQ. The results shown in fig.1 suggest that $UQ^{\cdot -}$ was formed on odd numbered flashes and disappeared on even numbered flashes, i.e., out of phase with cyt b_{50} reduction. This oscillation showed a similar redox potential dependency as the cyt b_{50} oscillation, i.e.,

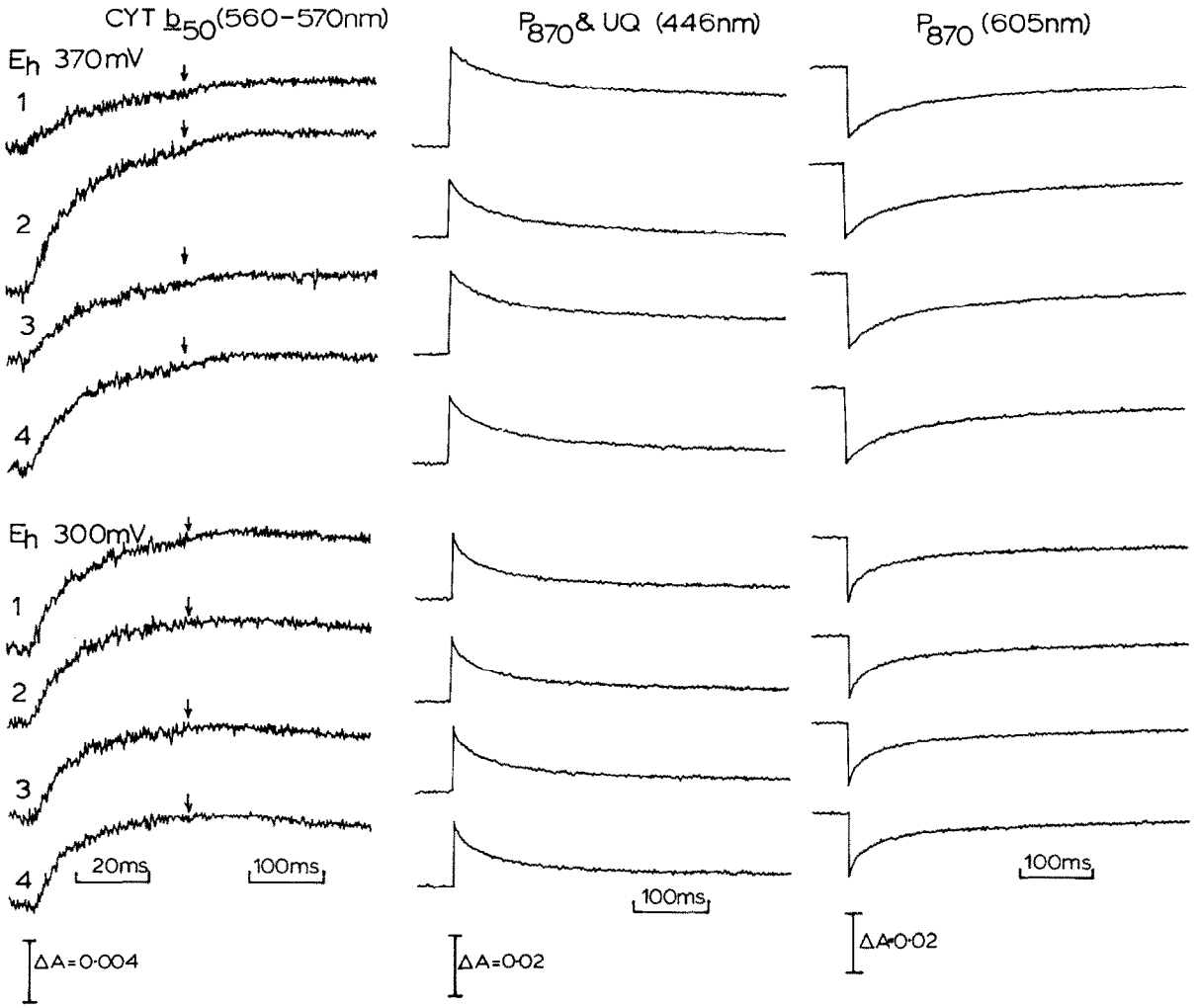

Fig.1. Flash number dependency of $\Delta A_{560-570}$, ΔA_{446} and ΔA_{605}. For the cyt b_{50} measurements, chromatophores were suspended to ~1.4 μM reaction centres in 50 mM MOPS, 100 mM KCl (pH 7.0) containing a total of 6 mM potassium ferrocyanide, 6 mM potassium ferricyanide, 10 μM TMPD, 2 μM valinomycin and 2 μM antimycin A, in an anaerobic redox cuvette. Signals were not averaged. The instrument response time was 200 μs. The time between flashes in a group was 5 s. The dark time between groups was 2 min. The vertical arrows indicate where the transient recorder time base was changed. For the 446 nm and *P*-870 measurements, chromatophores were suspended to ~1.4 μM reaction centres in 50 mM MOPS, 100 mM KCl (pH 7.0) containing a total of 2 mM potassium ferrocyanide and ferricyanide, 2 μM valinomycin and 2 μM antimycin A in an anaerobic redox cuvette. Signals were not averaged. The instrument response time was 500 μs. The time between flashes in a group was 10 s. The dark time between groups was 2 min.

binary oscillations were marked at 370 mV but they gradually damped as the potential was lowered to 300 mV. Antimycin A had no effect on the UQ oscillation. The difference between the rapid kinetics of the ΔA_{446} on flash 1 and flash 2 should reflect the rate of conversion of $UQ^{\cdot -}$ to ubiquinol (fig.2). This process had a $t_{1/2}$ 30–50 μs, considerably faster than the rates observed for $Q_I^{\cdot -}$ to $Q_{II}^{\cdot -}$ transfer in *Rps. sphaeroides* R26 reaction centres [13,14].

3.3. *Oscillatory* $\Delta A_{420-480}$

Figure 3A–D show spectra of the flash-induced ΔA at various times after the flash for flashes 1 and 2 from the dark-adapted state in the absence and presence of antimycin A. The band peaking at 430 nm had the kinetics of cyt b_{50} (traces not shown). In resolving the spectra it was assumed that the ΔA recorded 1 ms after the flash on flash 2 in the absence of antimycin A would be largely due to *P*-870, since the spectra were recorded at $E_{h(7.0)}$ 370–390 mV where the extent of rapid cyt c_2 oxidation in this strain is small [10]. From these data a set of normalising factors was calculated to correct other spectra for the *P*-870 change using 540 nm as a reference wavelength at which only *P*-870 changes would be observed. Figure 3EF, show the spectra of the changes recorded on flash 1 and 2 in the presence of antimycin A, at 1 ms and 50 ms, respectively, after the flash, cor-

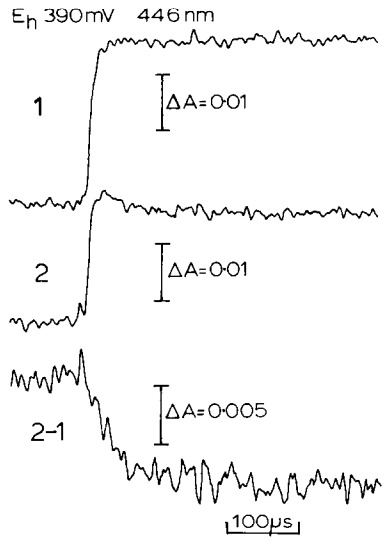

Fig.2. Rapid kinetics of the change recorded at 446 nm on the first 2 excitations from the dark-adapted state, and the difference kinetics of the 2 absorption changes. The traces were obtained using the same set up as in fig.3C,D. Traces (32) are averaged. The instrument response time was 1 μs.

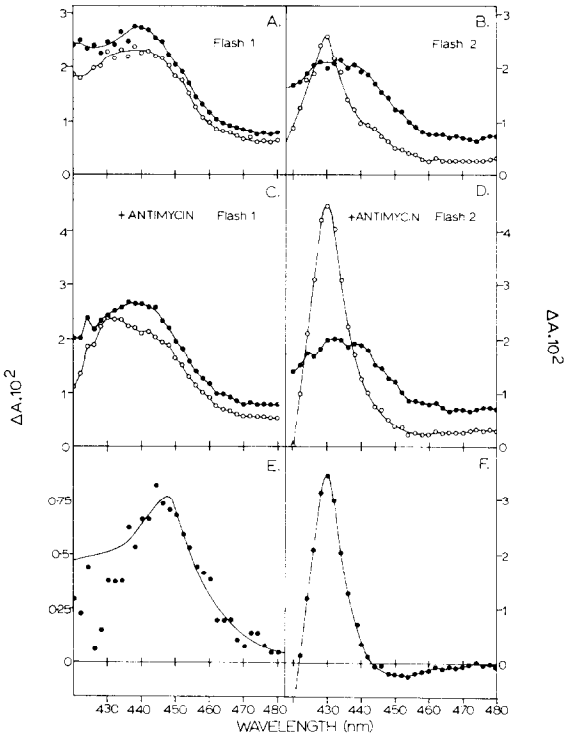

Fig.3. (A–D) Flash number dependency of the spectra of the excitation-induced $\Delta A_{420-480}$ from the dark-adapted state. (A,B) Chromatophores were suspended ~1.4 μM reaction centres in 50 mM MOPS, 100 mM KCl (pH 7.0) containing a total of 1 mM potassium ferricyanide, 1 mM ferrocyanide and 2 μM valinomycin in a 25 ml total vol. in an anerobic flow system. The $E_{h(7.0)}$ was 390 mV. The time between flashes in a group was 10 s. The spectra show changes recorded 1 ms (•) and 50 ms (○) after both flash 1(A) and flash 2(B). Signals were not averaged. The instrument response time was 200 μs. (C,D) As for (A,B) except that antimycin A was present at 2 μM. (E) The points (•) show the excitation-induced ΔA 1 ms after flash 1 in a train in the presence of antimycin A (as in fig.3C) corrected for the reaction centre changes as described in the text, using the change recorded at 540 nm as a measure of the reaction centre change alone. Superimposed is the difference spectrum for $UQ^{\cdot -}$–UQ in methanol (from [12]). (F) Spectrum of the flash-induced ΔA 50 ms after flash 2 in a train in the presence of antimycin A (as in fig.3D) corrected for the reaction centre change as above and in the text.

rected for *P*-870 changes. Figure 3F represents the difference spectrum of cyt b_{50}. Superimposed on fig.3E is the difference spectrum for $UQ^{\cdot -}$–UQ in methanol [12]. The fit is good at >440 nm, but there appears to be some additional spectral complexity at 420–440 nm.

3.4. *Oscillations in the rate of* P-870 *rereduction*

The rate of *P*-870 rereduction also showed binary oscillations, being more rapid after even numbered flashes than after odd numbered flashes. This oscillation was most clearly evident in the absence of antimycin A, and presumably again reflects the operation of a 2 electron gate, and its effect on the availability of electrons to the chain reducing *P*-870.

3.5. *Correlation of the oscillations in* ΔA_{446} *and* $\Delta A_{560-570}$

It seems likely that the oscillations in ΔA reflect a 2 electron gate acting between the primary acceptor complex and cyt b_{50}. This may be described by the following scheme, which ignores protonation events:

$$Q_I Q_{II} b_{50}^{ox} \cdot A^{ox} \xrightarrow{h\nu_1} Q_I^{\cdot -} Q_{II} b_{50}^{ox} \cdot A^{ox} \xrightarrow{\text{dark}}$$

$$Q_I Q_{II}^{\cdot -} b_{50}^{ox} \cdot A^{ox}$$

$$Q_I Q_{II}^{\cdot -} b_{50}^{ox} \cdot A^{ox} \xrightarrow{h\nu_2} Q_I^{\cdot -} Q_{II}^{\cdot -} b_{50}^{ox} \cdot A^{ox} \xrightarrow{\text{dark}}$$

$$Q_I Q_{II}^{2-} b_{50}^{ox} \cdot A^{ox} \rightarrow Q_I Q_{II} b_{50}^{red} A^{red}$$

where A is an unidentified acceptor.

In chromatophores of *Rps. capsulata* A1a pho$^+$, binary oscillations occurred for ≥4 flashes, suggesting that $Q_{II}H_2$ (the presumed fully protonated ubiquinol) is returned to its initial state after the second flash, i.e., is oxidised to Q_{II}, giving up 2 electrons and 2 H^+ in the process. The reduction of cyt b_{50} is a 1 hydrogen transfer event below its p*K*, and the fate of $Q_{II}^{\cdot -}$ (or $Q_{II}H^{\cdot}$) generated by the proposed reduction of cyt b_{50} by $Q_{II}H_2$ is uncertain. We might consider the possibility that the extra electron reduces another molecule of cyt b_{50}, or alternatively that some other component, possibly J [15,16] or UQ acts as an acceptor in a parallel reaction. In the latter cases we could not predict whether cyt b_{50} reacts with the quinol/semiquinone couple or the quinone/semiquinone couple. Using an extinction coefficient for cyt b_{50} at 560–570 nm of 13.2 mM^{-1} cm^{-1} (determined experimentally for isolated beef heart mitochondrial cyt *b* [17] and calculated from the data in [18,19]) we determined that ≤0.6 molecules cyt b_{50}/*P*-870 oxidised were reduced in the presence of antimycin A on flash 2 at $E_{h(7.0)}$ 370 mV. The possibility that more cyt b_{50} was reduced on flash 2 but was rapidly reoxidised in an antimycin A-insensitive fashion cannot, of course, be excluded.

The pattern of cyt b_{50} reduction kinetics indicates some damping in the primary acceptor complex. This is also evident in the oscillations at 446 nm, but analysis of damping at this wavelength is complicated by the occurrence of overlapping reaction centre changes. Using $\epsilon = 8.5$ mM^{-1} cm^{-1} for $UQ^{\cdot -}$–UQ obtained with reaction centres of *Rps. sphaeroides* R26 [20] we calculated that ~1 $UQ^{\cdot -}$ was formed/reaction centre oxidised on flash 1. However, this is likely to be an overestimate, since some cyt b_{50} reduction occurred on flash 1 indicating that some centres had Q_{II} reduced before flash 1, so it may be that the above ϵ is too low.

3.6. *The diminution in oscillations with falling redox potential*

A number of factors may have contributed to the apparent disappearance of oscillations as the redox potential was lowered:

(i) A change in the mechanism of electron flow from the primary acceptor complex to cyt b_{50} such that charge accumulation no longer occurs, e.g., direct reduction of cyt b_{50} by $Q_I^{\cdot -}$ (or $Q_IH^{\cdot}$) or by $Q_{II}^{\cdot -}$ (or $Q_{II}H^{\cdot}$).

This may result from the presence before the flash of some reduced component outside the reaction centre complex, e.g., cyt c_2 or J. We intend to test whether cyt c_2 is involved using a mutant lacking cyt c_2 isolated by B. Marrs. The apparent non-chromophoric proton binding agent in isolated reaction centres of *Rps. sphaeroides* R26 appears to have a redox potential-dependent pK [13] and this might affect the gate mechanism in chromatophores.

(ii) It now appears that with cyt c_2 reduced, ~30% of the reaction centres in chromatophores may be rereduced with $t_{1/2}$ <5 μs following laser flash excitation (R. E. Overfield and C. A. Wraight, personal communication; J.R.B. unpublished

observations) and since the rate of electron transfer between Q_I and Q_{II} in chromatophores appeared to have $t_{1/2}$ 30–50 μs, it is possible that the Xe flash caused some double hits as cyt c_2 became reduced before the flash.

(iii) As the redox potential is lowered the measuring beam would become more actinic because the presence of reduced cyt c_2 would allow a more rapid turnover of the photochemistry. However, in these experiments, the brief exposure to the measuring beam (10–30 pEinstein s^{-1} cm^{-2}) before flash excitation would make this effect insignificant.

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