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THE INTERACTION OF THE REACTION CENTER SECONDARY QUINONE WITH THE UBIQUINONE-CYTOCHROME c_2 OXIDOREDUCTASE IN *RHODOPSEUDOMONAS SPHAEROIDES* CHROMATOPHORES

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(1) Two populations of reaction centers in the chromatophore membrane can be distinguished under some conditions of initial redox poise (300 mV $< E_h <$ 400 mV): those which transfer a reducing equivalent after the first flash from the secondary quinone (Q_{II}) of the reaction center to cytochrome *b* of the ubiquinone-cytochrome c_2 oxidoreductase; and those which retain the reducing equivalent on Q_{II}^- until a second flash is given. These two populations do not exchange on a time scale of tens of seconds. (2) At redox potentials higher than 400 mV, Q_{II}^- generated after the first flash is no longer able to reduce cytochrome *b*-560 even in those reaction centers associated with an oxidoreductase. Under these conditions, doubly reduced Q_{II} generated by a second flash is required for cytochrome *b* reduction, so that the Q_{II} effectively functions as a two-electron gate into the oxidoreductase at these high potentials. (3) At redox potentials below 300 mV, although the two populations of Q_{II} are no longer distinguishable, cytochrome *b* reduction is still dependent on only part of the reaction center population. (4) Proton binding does not oscillate under any condition tested.

Introduction

A heterogeneous population of ubiquinone is known to be functional at several loci in the cyclic electron-transfer system of photosynthetic bacteria [1]. Ubiquinone is particularly appropriate for these multiple functions because its numerous possible states of reduction and protonation can provide a wide variety of functional redox couples. In *Rhodopseudomonas sphaeroides*, photosynthetic electron transfer within the reaction center occurs when reducing equivalents from the photo-oxidized bacteriochlorophyll special pair [$(BChl)_2^+$] are transferred via the intermediate bacteriopheophytin to the primary quinone (Q_I), subsequently to the secondary quinone (Q_{II}), and finally from Q_{II} out of the reaction center [1]. Absorbance changes at 450 nm which oscillate with flash number have been attributed to Q_{II}, and have been described in isolated reaction centers [2–5]. This behavior purportedly results from the formation of a semiquinone anion after the first flash, and formation after the second flash of a doubly reduced quinol species (essentially invisible at 450 nm) which rapidly gives up two reducing equivalents at once, to form quinone, returning the system to its initial state. Consequently, the formation of the ubisemiquinone anion as measured by an absorbance increase at 450 nm [6] is observed to oscillate with flash number displaying a periodicity of two.

In view of the large oscillations of Q_{II} reduction observed in isolated reaction centers, the role that Q_{II} plays in the chromatophore system, where reducing equivalents are transferred from Q_{II} to cytochrome *b*-560 of the ubiquinone-cytochrome c_2 oxidoreductase, is particularly significant. Based on observations of binary oscillations of the 450 absorbance, proton

Abbreviations: BChl, bacteriochlorophyll; Tricine, *N*-tris(hydroxymethyl)methylglycine; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.

binding [7], the carotenoid bandshift [8], and cytochrome *b* reduction [9], several investigators have concluded that Q_{II} acts as a two-electron gate between the reaction center and the oxidoreductase. Since there is an excess of reaction centers in the chromatophore (about three reaction centers for every two functional oxidoreductases [10]), it is important to ascertain if all the reaction centers are involved in any gating process, or if the distribution of the ubiquinone-cytochrome c_2 oxidoreductase among the reaction centers can alter the reactions of the flash-produced Q_{II}^- in some of the reaction centers. This possibility has been explored by examining the flash number dependence of reactions which occur in the reaction center (Q_{II}^- formation, antimycin-insensitive H^+ binding) or in the ubiquinone-cytochrome c_2 oxidoreductase (cytochrome *b* reduction, antimycin-sensitive H^+ binding) as a function of initial redox poise. The results presented in this paper suggest that two populations of reaction center are present in the chromatophore membrane: those which are bound to a ubiquinone-cytochrome c_2 oxidoreductase in a relatively long-lived association, and those which are not.

Materials and Methods

Preparation of chromatophores from photosynthetically grown *Rps. sphaeroides* Ga [11] and methods of redox potentiometry were as previously described [12]. Cytochrome *b* reduction was measured spectrophotometrically at 560–540 or 560–570 nm, in the presence of valinomycin to eliminate any contribution from the antenna bacteriochlorophyll bandshift [13,14]. Measurement of the absorbance change at 450 nm attributed to semiquinone anion formation was also carried out in the presence of valinomycin, which effectively eliminated a sizeable contribution from the carotenoid bandshift at this wavelength. Proton-binding reactions were measured as previously described [15,16]. A reduced minus oxidized extinction coefficient of 10.5 $mM^{-1} \cdot cm^{-1}$ was used for cytochrome *b* at 560–540 nm (Prince, R.C., unpublished data) in some of the experiments presented here, and 8.5 $mM^{-1} \cdot cm^{-1}$ for the ubisemiquinone anion [17]. Typically, about 5 min dark time between flash cycles were used, although dark times up to 20 min did not alter the results.

Results

The transfer of reducing equivalents from the reaction center to the ubiquinone-cytochrome c_2 oxidoreductase has been investigated by analysis of cytochrome *b* reduction, Q_{II}^- formation and the binding of protons to sites in the reaction center and the oxidoreductase.

Cytochrome b reduction

Fig. 1 shows the flash-induced reduction of cytochrome *b* in chromatophores both in the presence and absence of antimycin. At 360 mV, approx. 80% of the maximal cytochrome *b* reduction occurs after the first flash and no trace of a binary periodicity is evident. At 435 mV, the amount of first-flash cytochrome *b* reduction is approx. 20% of maximal, and the second flash results in more cytochrome *b* reduction, suggesting that some binary gating process might be occurring. A redox titration of the amount of cytochrome *b* reduction following the first (●) and second (○) flashes is shown in Fig. 2A, and the pH dependence of the apparent E_m values in Fig. 2B. The decrease in the amount of second-flash cytochrome *b* reduction simply follows the oxidation-reduction curve of the bacteriochlorophyll dimer ($(BChl)_2$, $E_m = 450$ mV, pH independent, $n = 1$) of the reaction center (i.e., prior chemical oxidation of $(BChl)_2$ inactivates the reaction center). However, the attenuation of first-flash cytochrome *b* reduction follows an apparently simple Nernst curve ($E_m = 400$ mV, pH independent, $n = 1$). This midpoint corresponds to no

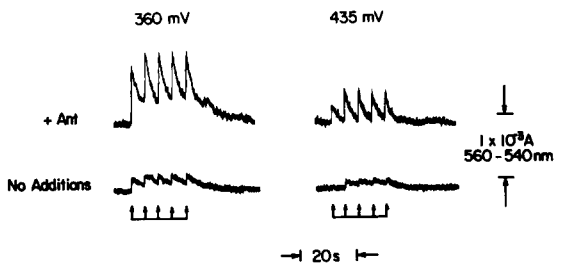

Fig. 1. Cytochrome *b* reduction at 'high' E_h. Chromatophores (160 nM reaction center) in 100 mM KCl, 20 mM Mes, 1 mM $MgCl_2$, 1 μM valinomycin, 0.5 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$, pH 6.0. Where indicated, 1 μM antimycin (Ant) was added. Absorption changes were measured at 560–540 nm, using a 50 ms time constant.

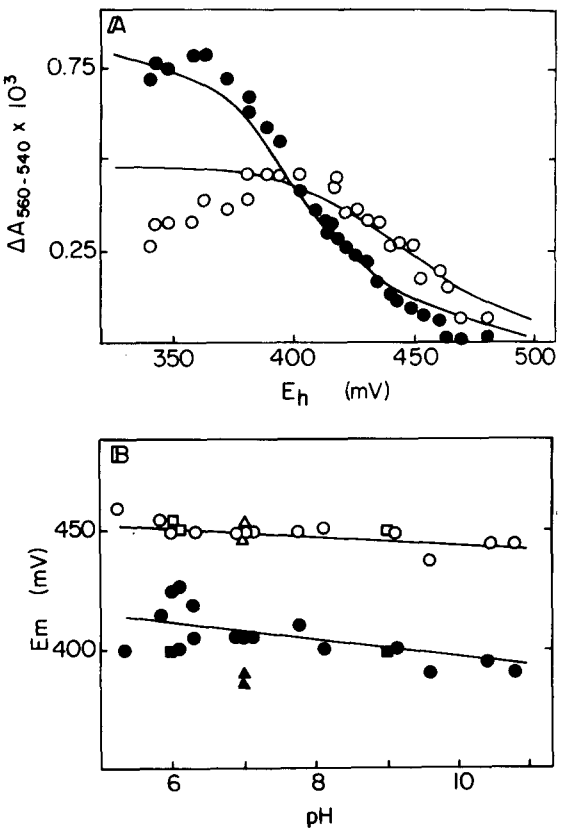

Fig. 2. A redox titration of cytochrome *b* reduction at 'high' E_h. Conditions as in Fig. 1 except 20 mM Mops (pH 6.5–7.5), Tricine (pH 7.5–9.0) or glycine (pH 9.0–11.0) were substituted for Mes (pH 5.5–6.5). (A) pH 7.0, antimycin was present at 1 μM; (●) first-flash cytochrome *b* reduction; (○) second-flash cytochrome *b* reduction. (B) The same symbols are used as in A, but also, (▲) first flash, no antimycin; (△) second flash, no antimycin; (■) first flash, no valinomycin; (□) second flash, no valinomycin.

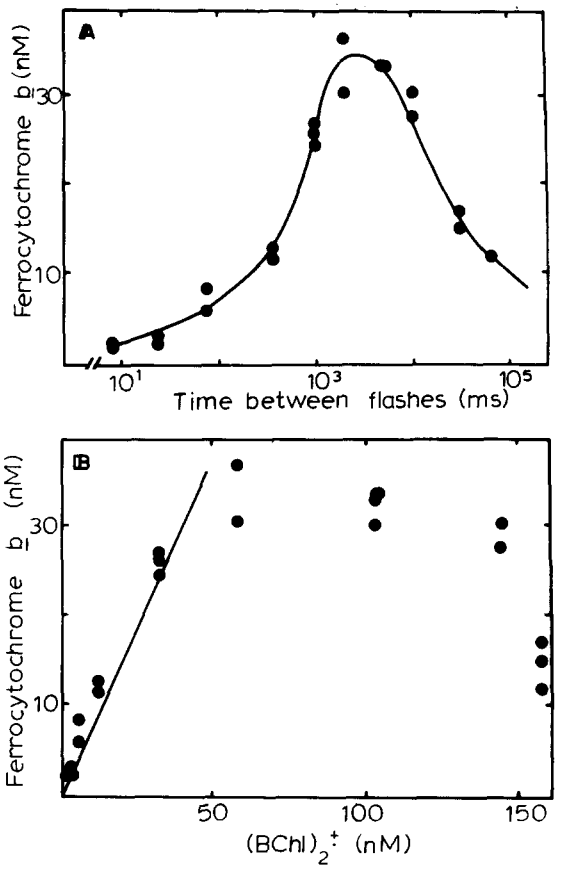

Fig. 3. Second-flash cytochrome *b* reduction at 423 mV. Chromatophores present at 208 nM reaction center, other conditions as in Fig. 2, pH = 7.0. (A) The total cytochrome *b* reduction after the first flash at 360 mV was 118 nM, and at 423 mV it was 13 nM. (B) The amount of $(BChl)_2$ oxidation on the second flash was measured from the absorption change at 605–540 nm. The solid line in B corresponds to 0.75 cytochrome b/e^-.

known component of the reaction center or the ubiquinone-cytochrome c_2 oxidoreductase.

The amount of cytochrome *b* reduction following the second flash at $E_h > 400$ mV is strongly dependent on the interval between the first and second flashes (see Fig. 3A). The submaximal extent of cytochrome *b* reduction at intervals of less than 10^3 ms after the first flash is due simply to the predominantly oxidized state of $(BChl)_2$ at the time of the second flash, preventing any useful photochemistry. This reasoning is quantitatively demonstrated in Fig. 3B, where the amount of second-flash cytochrome *b* reduction is plotted, instead of against time, as a function of the amount of photo-oxidizable $(BChl)_2$ at various times after the first flash. The amount of second-flash cytochrome *b* reduction increases linearly with the number of available reducing equivalents until approx. 50 nM $(BChl)_2^+$. However, the amount of cytochrome *b* reduced on the second flash decreases as the time between flashes is increased further, reaching the level after the first flash (12.8 nM) after approx. 1 min. This regeneration of the initial state has a $t_{1/2}$ of 12 s and is not directly dependent on the re-reduction of $(BChl)_2^+$.

ΔA_{450}

Flash-induced absorption changes at 450 nm at three different redox potentials are shown in Fig. 4. Because a sizeable absorption increase due to $(BChl)_2^+$ is also present at 450 nm (approx. 90% as large as the absorbance decrease at 605 nm), Fig. 4 also shows the flash-activated absorption change at 605 nm, which is due to $(BChl)_2^+$ alone. While both the amount of $(BChl)_2^+$ formed after each flash and its rate of re-reduction vary with redox potential, the contribution from $(BChl)_2^+$ at 450 nm will duplicate the 605 nm change, and any deviation from this must be due to a contribution from another component. Thus, it is clear that the ΔA_{450} changes at 360 mV, shown in Fig. 4, contain a contribution from a component which has a binary dependence on the number of the flash, which is not evident at 260 mV, and at 445 mV it appears that the ΔA_{450} reaches a higher level after the first flash than after subsequent activations, suggesting that any oscillatory activity is being rapidly damped.

The identity of the component responsible for the oscillations at 450 nm is confirmed by the spectra in Figs. 5 and 6. Fig. 5A shows the spectrum (•) of the second minus third flash ΔA observed at $E_h = 355$ mV, and Fig. 5B shows the second minus first flash ΔA when $E_h = 440$ mV. Between 420 and 490 nm, the shape of these spectra is very similar to the difference spectrum of oxidized ubiquinone minus ubisemiquinone-10 anion measured in methanol [6].

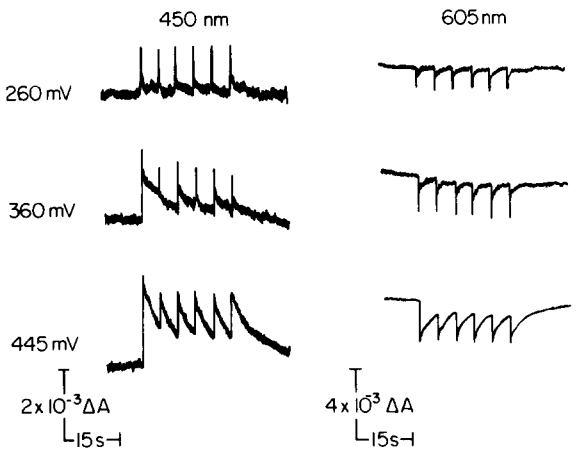

Fig. 4. Absorbance change at 450 nm. Chromatophores present at 210 nM reaction center; other conditions as in Fig. 1, except 5 μM diaminodurene also present. Single-beam absorbance changes are measured using a 50 ms time constant.

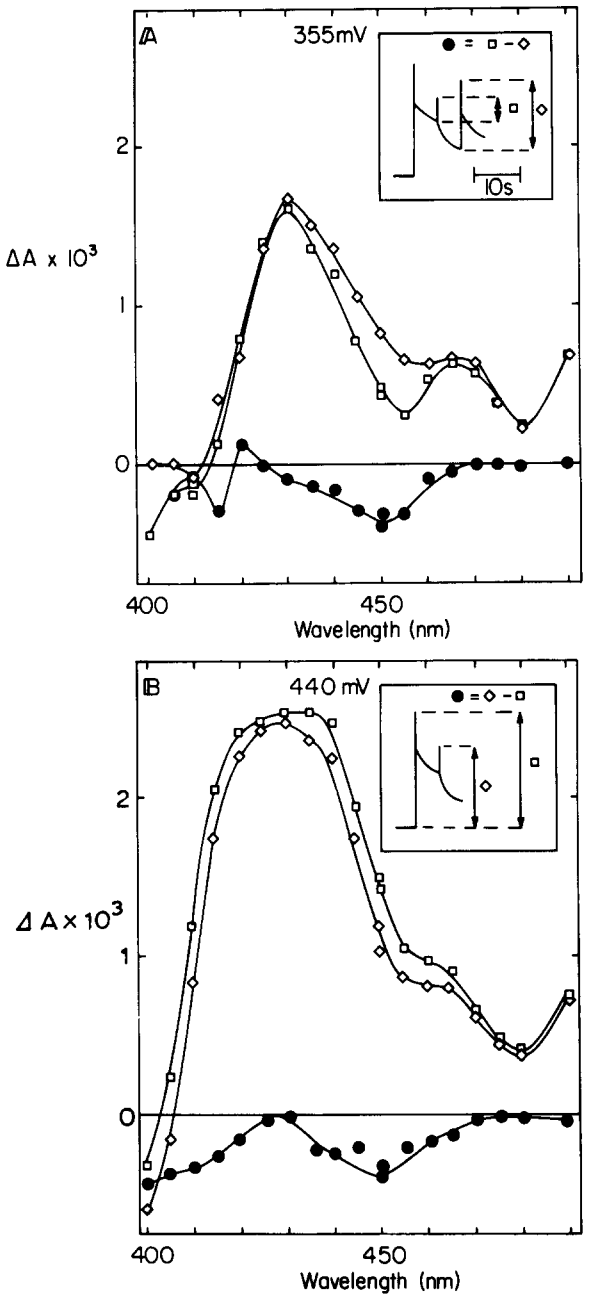

Fig. 5. Spectra of absorption changes measured near 450 nm. Conditions as in Fig. 4. (A) $E_h = 355$ mV. (B) $E_h = 440$ mV.

The absorption parameters used to obtain the spectra in Fig. 5 were selected first to eliminate the

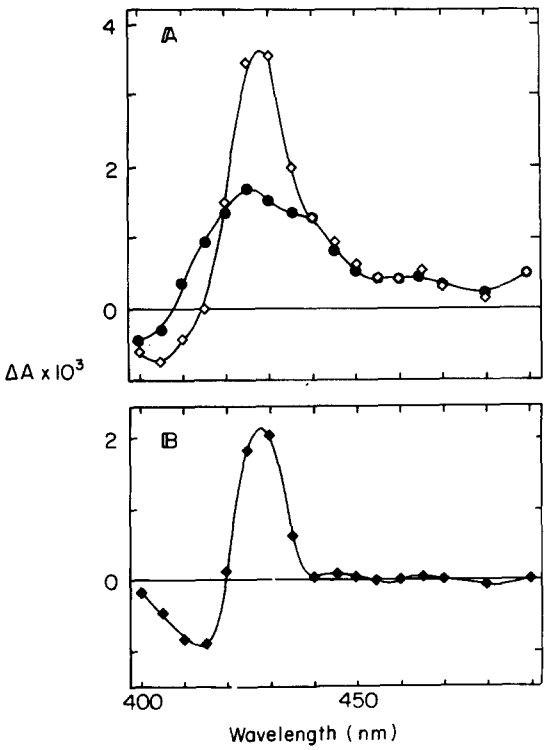

Fig. 6. Antimycin difference spectrum. Conditions as in Fig. 1, except chromatophores present at 210 nM reaction center, $E_h = 440$ mV. (A) (•) Second-flash absorbance changes in the absence of antimycin; (◇) second-flash absorbance changes in the presence of 1 μM antimycin. (B) Difference spectrum of the absorbance changes measured in A.

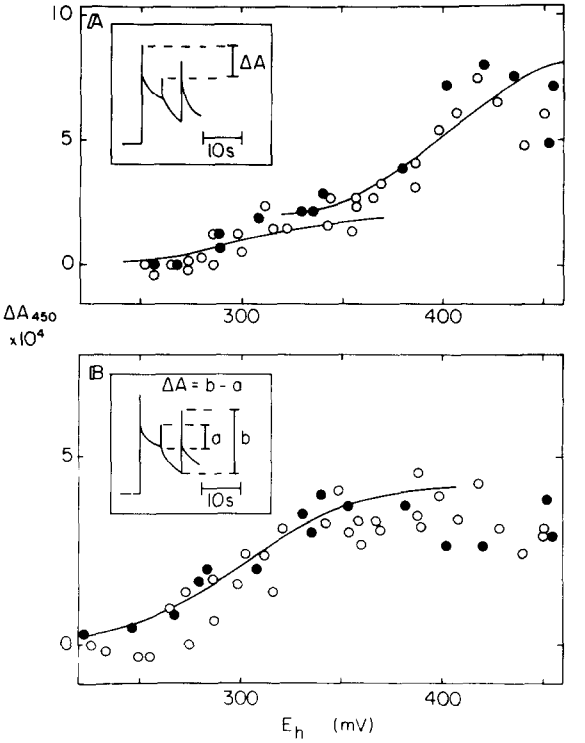

Fig. 7. Redox titration of the absorbance change at 450 nm. Conditions as in Fig. 1, except chromatophores present at 190 nM reaction center, and 10 μM diaminodurene and 5 μM *N*-methylphenazonium methosulfate were present. (○) pH 6.4, (•) pH 7.8. At high E_h (above 380 mV) the points are corrected to account for the amount of $(BChl)_2$ which is chemically oxidized.

contribution of $(BChl)_2^+$ to the differences, and second, to minimize any contributions from cytochrome oxidation and reduction reactions. Little or no cytochrome c_2 oxidation could be measured at 355 mV or higher, and the lack of antimycin kept the contribution from cytochrome *b* reduction minimal. The difference in the second-flash absorption change at 440 mV before and after addition of antimycin (Fig. 6A) should only change the contribution in the spectrum from cytochrome *b* (note that antimycin has no effect on the ΔA_{450} changes, see Fig. 8). This difference spectrum (Fig. 6B) of cytochrome *b* demonstrates that the oscillatory changes at 450 nm cannot be a result of a contribution from cytochrome *b* at this wavelength.

Fig. 7 shows redox titrations of 450 nm absorption differences as measured in Fig. 5. The first minus second flash difference (Fig. 7A) appears with an apparent $E_m = 300$ mV, and exhibits a second increase with apparent $E_m = 400$ mV (note the similarity to the E_m of the decrease in first-flash cytochrome *b* reduction in Fig. 2). The third minus second flash absorption changes (Fig. 7B) appear with an apparent $E_m = 300$ mV, but no further change occurs at 400 mV, consistent with observations that the large changes in ΔA_{450} at above 400 mV are most evident only after the first two flashes.

Comparison of cytochrome b reduction, H^+ binding, and Q_{II}^- reduction

Fig. 8 shows the flash number dependence of cytochrome *b* reduction, H^+ binding, and Q_{II}^- (as ΔA_{450}) changes at 260, 380 and 450 mV, and clearly demonstrates (a) the out-of-phase relationship between cyto-

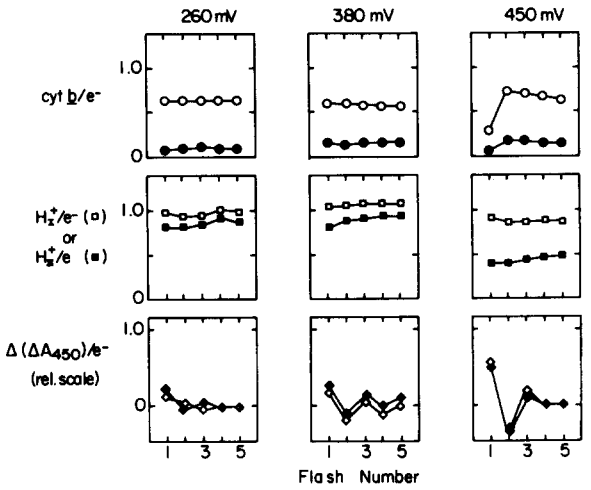

Fig. 8. Flash number dependence of cytochrome *b* (cyt b) reduction, proton binding, and ΔA_{450}. Chromatophores (140 nM reaction center) in 100 mM KCl, 1 mM $MgCl_2$, 2 μM valinomycin, 0.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$, 5 μM diaminodurene, pH 6.0. 50 μM chlorophenol red was present for the H^+-binding experiments. Open symbols indicate the presence of 2 μM antimycin. 5 s dark time between flashes were used. The corrections used to obtain $\Delta(\Delta A_{450})/e^-$ take into account both the contributions of $(BChl)_2^+$ at 450 nm, and the number of reducing equivalents produced on each flash: $\Delta(\Delta A_{450}) = \Delta A_{450(n)}/\Delta A_{605(n)} - \Delta A_{450(n-1)}/\Delta A_{605(n-1)}$ where n = flash number and $\Delta A_{450(0)}/\Delta A_{605(0)} = 0$.

chrome *b* reduction and ΔA_{450} formation at 450 mV, (b) the binary oscillations in ΔA_{450} at 380 mV, independent of cytochrome *b* reduction, (c) the total absence of flash number dependence of both H_I^+ and H_{II}^+ binding, and (d) the absence of any binary dependence at 260 mV.

Using the $\epsilon_{red-ox} = 8.5$ determined by Wraight et al. [17] and the procedures which eliminate $(BChl)_2^+$ contributions from the ΔA_{450} (as for Figs. 5 and 7), we have quantified in Table I the amount of Q^- responsible for the 450 nm changes. While at $E_h \approx$ 350 mV, 0.15 Q^-/e^- disappear after the second flash, at $E_h = 440$ mV, the second flash elicits a much greater disappearance of Q_{II}^- (0.48–0.60 Q_{II}^-/e^-). Unfortunately, the amount of Q^- formed on the first flash cannot be directly measured because the $(BChl)_2^+$ contributes to the total change at 450 nm. Nevertheless, the corrections used to obtain the data in Fig. 8 (bottom row) provide a means for estimating

TABLE I

E_h (mV)	Flash number	$\Delta Q_{II}^-/e^-$	Source (Fig.)
350	2	−0.15	7A
350	3	+0.25	7B
355	3	+0.20	7A
440	2	−0.60	7A
440	2	−0.48	5B

the amount of first-flash stable Q_{II}^- formation (relative to the second-flash disappearance) as 0.2–0.3 Q_{II}^-/e^- at about 350 mV and 0.8–1.0 Q_{II}^-/e^- at 450 mV.

Ubiquinone-cytochrome c_2 oxidoreductase mobility

Pertinent to the problem of a heterogeneous population of oscillating Q_{II}^- is the mobility of the ubiquinone-cytochrome c_2 oxidoreductase among the reaction center population. In Fig. 9, the number of

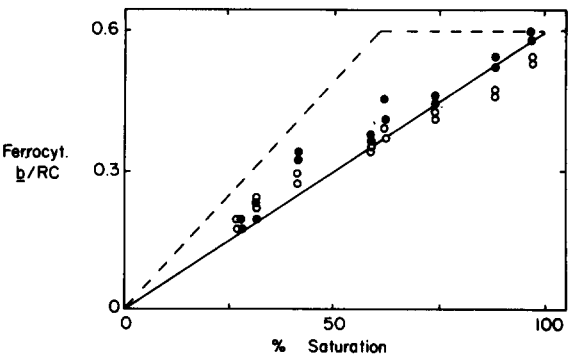

Fig. 9. Cytochrome *b* reduction as a function of flash saturation. Chromatophores (160 nM reaction center) in 100 mM KCl, 20 mM Mes, 1 mM $MgCl_2$, 3 μM valinomycin, 1 μM antimycin, 5 μM diaminodurene, 5 μM *N*-methylphenazonium methosulfate, pH 7.0. Absorbance changes at 560–570 nm were measured. Flash saturation was varied using neutral density filters, and determined at $E_h = 450$ mV by the amount of $(BChl)_2$ photo-oxidation after the first flash, compared to the amount of $(BChl)_2$ photo-oxidation after eight flashes. The solid line illustrates the case where cytochrome *b* is tightly bound to the reaction center; the dashed line illustrates the case where the cytochrome *b* is freely mobile among the reaction centers. (•) $E_h = 360$ mV, (○) $E_h = 230$ mV. Ferrocyt, ferrocytochrome; RC, reaction center.

reducing equivalents available from the reaction center was varied by using flashes of varying intensity [20]. Since in this experiment the ratio of cytochrome *b* to reaction center was 0.6, if the oxidoreductase were freely mobile among the entire reaction center population, a 60% saturating flash would provide enough reducing equivalents to reduce all of the cytochrome *b* (dashed line). Alternatively, a population of tightly bound reaction center-oxidoreductase complexes would not undergo full cytochrome *b* reduction unless a flash was 100% saturating (solid line). The data clearly correspond to the latter possibility, both when Q_{II}^{-} oscillations occur (360 mV, solid circles) and when they do not (230 mV, open circles).

Discussion

Considerations on the observation of oscillatory behavior

The oscillatory appearance and disappearance of semiquinone after single-turnover activation of photosynthetic systems is unique in its simplicity when compared to the complex kinetic descriptions necessary to describe oscillations in other chemical and biochemical systems [18,19]. The quinone can simply be visualized as a binary switch or gate, which receives a single reducing equivalent on each turnover but releases a two-electron equivalent only after even-numbered turnovers. The experimental observation of this gating in the $(BChl)_2$-Q_{II} system can be rationalized by a few simple considerations:

(I) Donor-acceptor correspondence. (a) Electron availability – the reaction center $(BChl)_2$ must be reduced before a flash can elicit electron transfer through Q_I to Q_{II}. (b) Homogeneity of the quinone species – all of the reaction center Q_{II} must be in the same redox state prior to any turnover.

(II) Semiquinone-quinol lifetimes. (a) Q_{II}^{-} must be long lived relative to the time between flashes. (b) Any $Q_{II}H_2$ (or Q_{II}^{2-}) produced must be reoxidized in the time between flashes, replenishing Q_{II}.

Any circumstances where donor-acceptor correspondence does not occur will result in mixing of different quinone species (Q_{II}, Q_{II}^{-}, $Q_{II} \cdot H$, and $Q_{II}H_2$) in the Q_{II} population, leading to 'damping' of the oscillations. Although not explicitly stated, the requirement for homogeneity of the quinone species (Ib) further demands that only one Q_{II} is present in each reaction center, and that a nearly saturating flash is necessary. No oscillations will be observed when the semiquinone-quinol lifetimes are not as described above. For instance, if Q_{II}^{-} were rapidly oxidized, no stable Q^{-} formation would be observed. With these considerations in mind, we can analyze the conditions outlined in Results and evaluate possible reasons for the patterns of oscillatory behavior observed.

Reaction center-oxidoreductase interactions

Van den Berg et al. [10] have determined that the ubiquinone-cytochrome c_2 oxidoreductase is present in the chromatophore membrane at 0.7 ± 0.1 per reaction center protein [10]. Others have demonstrated that components of the oxidoreductase (cytochrome *b*-560, Q_z, Rieske iron-sulfur protein, cytochromes c_1 and c_2) are present in amounts ranging from 0.6 to 0.8 per reaction center, suggesting that the oxidoreductase contains one each of these components [14,20–25]. At redox potentials between 300 and 400 mV, the amount of cytochrome *b* reduction (with antimycin present) after every flash, and the amount of oscillating Q_{II}^{-}, correspond to the total amount of oxidoreductase and the amount of excess reaction center, respectively. Since Fig. 9 implies that the oxidoreductase is not freely mobile among the reaction center population, we should therefore consider that under these conditions, the secondary quinones which are oscillating are in those reaction centers which are not associated with an oxidoreductase (a lone reaction center).

The role of Q_{II} (either Q_{II}^{-} or $Q_{II} \cdot H$) in the photoreduction of cytochrome *b*-560 when cytochrome c_2 is initially oxidized seems well established. Since no other pathway for cytochrome *b* reduction is available under these conditions (such as oxidant-induced reduction, see Ref. 10), in the presence of antimycin the Q_{II} semiquinone generated in most of the reaction centers is almost entirely oxidized by cytochrome *b*-560 with $t_{1/2} \approx 15$ ms [14]. Oxidation of only some of the Q_{II} semiquinone violates the oscillatory requirement for a long-lived semiquinone (IIa), and for homogeneity of the quinone species (Ib), but only when the Q_{II} population as a whole is considered. By considering two independent populations of reaction centers, this problem is no longer relevant. In Fig. 10A, the dashed line illustrates that

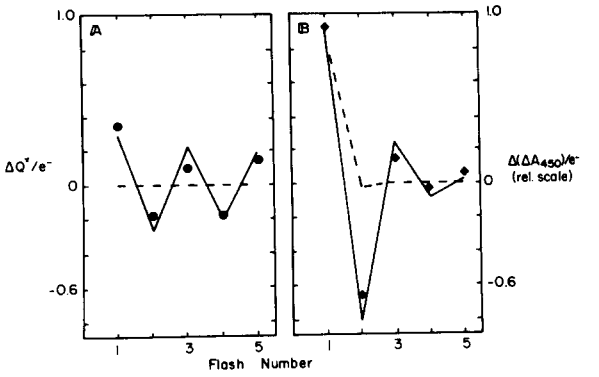

Fig. 10. Predicted changes in Q_{II}^{-}/e^{-} compared with actual absorption changes measured at 450 nm. Experimental conditions as in Fig. 8. Flash saturation of approx. 95% was measured, and this value was used in all calculations. Experimental points in both A and B are plotted on the same relative scale. It is assumed that any Q_{II}^{-} which reduces cytochrome *b* (+ antimycin) is not measured, and that any Q_{II}^{-} which is doubly reduced is available as fully oxidized Q_{II} on the next turnover. The predicted changes in Q_{II}^{-}/e^{-} were calculated using the formula:

$$x_n = R(1 - x_{n-1}) + (x_{n-1} - Rx_{n-1}),$$

where x is the amount of Q^{-} generated by a flash, n is the flash number ($x_0 = 0$) and R is the damping factor. (A) E_h = 350 mV, the solid line indicates the predicted change in Q_{II}^{-} for each reducing equivalent produced by the reaction center if the ratio of reaction center oxidoreductase pairs to lone reaction centers was 0.7, a long-lived Q_{II}^{-} (longer than 50 ms) was produced only in those reaction centers, and reaction center-oxidoreductase pairs did not mix between flashes 1 and 5. The dashed line would result if the ubiquinone-cytochrome c_2 oxidoreductase were free to redistribute among the total reaction center population in the time between flashes. (B) E_h = 440 mV. It is assumed that the component with E_m = 400 mV is 83% oxidized, so a stable Q_{II}^{-} will be produced in 83% of the reaction center-oxidoreductase pairs, as well as in all of the lone reaction centers. Because of the slow reduction of $(BChl)_2^{+}$ only about 65% of the reducing equivalents available on the first flash are available on the second and subsequent flashes. The dashed line shows the result if the re-reduction (after each flash) of $(BChl)_2^{+}$ is equally probable for $(BChl)_2^{+}$ which was photo-oxidized and $(BChl)_2^{+}$ which was initially chemically oxidized. The solid line shows the result if the re-reduced $(BChl)_2^{+}$ is only that which was photo-oxidized.

no change in Q^{-}/e^{-} would occur after each flash if the oxidoreductase were freely mobile in the time between flashes (since cytochrome *b*, can be more than 60% reoxidized in the time between flashes, ferricytochrome *b* could oxidize the remaining Q_{II}^{-} in the lone reaction center population with $t_{1/2} \approx 15$ ms). A much closer fit to the data is provided by the solid line which assumes that 30% of the reaction center population is unable to interact with an oxidoreductase, and oxidation of doubly reduced Q_{II} is not coupled to cytochrome *b* reduction. The oxidant of the latter Q_{II} could be a member of the ubiquinone 'pool' (approx. 19 Q/reaction center, E_{m7} = 90 mV, n = 2) which is known to become photoreduced under these conditions, possibly by exchange with, or two-electron oxidation of, fully reduced Q_{II} [24].

Two possible means for functionally dividing the reaction center population are: (a) all reaction centers are equivalent but the oxidoreductase remains bound to some of them for long periods of time (probably minutes at least), (b) some of the reaction centers are never able to bind an oxidoreductase, and the lifetime of the reaction center-oxidoreductase pairs may be either long or very brief. We cannot yet distinguish between these possibilities.

The disappearance of Q_{II} oscillations

Previous investigators [8,9] have noted the disappearance of Q_{II}^{-} oscillations in chromatophores at $E_h < 300$ mV, and this apparent E_m is also notable for the p*K* shifts and changes in the rate of cytochrome *b* reduction and H^{+} binding which occur thereabouts [14–16,26]. The change in the rate of $(BChl)_2^{+}$ re-reduction as a function of the redox state of cytochrome c_2 (about 100 μs when ferrocytochrome c_2 is present, and hundreds of milliseconds when ferricytochrome c_2 is present) provides a possible source of these phenomena:

(a) Cytochrome c_2 oxidized – stabilization of Q_{II}^{-} (i.e., raised E_m) by electrostatic interaction with $(BChl)_2^{+}$ may slow the oxidation of Q_{II}^{-} by cytochrome *b* [14,16] in the reaction center-oxidoreductase pairs, and prevent oxidation or protonation of Q_{II}^{-} in lone reaction centers.

(b) Cytochrome c_2 reduced – rapid re-reduction of $(BChl)_2^{+}$ removes the electrostatic stabilization of Q_{II}^{-}; reduction of cytochrome *b* is faster, and in the lone reaction centers, H_I^{+} can be transferred directly to Q_{II}^{-}, yielding $Q_{II} \cdot H$. Since the extinction coefficient of $Q \cdot H$ at 450 nm is only about 15% that of Q^{-} [6], the observed oscillations would be greatly diminished.

The above scheme requires that cytochrome c_2 is mobile or at least randomly distributed with respect to the reaction center population. Some evidence would suggest that ferrocytochrome c_2 is in fact immobile, however the mobility of ferricytochrome c_2 was not measured [20]. The distribution of cytochrome c_2 has never been assessed.

Secondary quinone gating of cytochrome b reduction

The demonstration that the decrease in first-flash cytochrome *b* reduction and increase in Q_{II}^- formation both occur with an apparent $E_m = 400$ mV suggests that some redox component with this E_m is responsible for the change. Since both above and below 400 mV we can account for nearly all of the reducing equivalents produced by the reaction center as being in Q_{II} and (in the presence of antimycin) cytochrome *b*, it seems most likely that the oxidized 400 mV component is not an acceptor of a reducing equivalent on the first or subsequent flashes, but that oxidation of this component raises the E_m of Q_{II}/Q_{II}^- such that reduction of cytochrome *b* is no longer possible except by doubly reduced Q_{II}. The disappearance of Q_{II}^- after the second flash implies that the 1 s back reaction from Q_{II}^- to $(BChl)_2^+$ [1] is not the major source of $(BChl)_2^+$ re-reduction and is consistent with the E_m of the Q_{II}/Q_{II}^- couple being higher under these conditions. The reaction center Fe^{2+}, known to interact with both Q_I and Q_{II}, is a possibility for a couple (Fe^{2+}/Fe^{3+}) with an $E_m = 400$ mV, the oxidation state of which could have a pronounced effect on the midpoints, of, and electron transfer between, Q_I and Q_{II} [1,30]. However, no change in the light-induced *g* 1.82 signal was observed in isolated reaction center over this E_h range, suggesting that the reaction center Q_I-Fe interaction is undisturbed above 400 mV (Tiede, D.M., unpublished observation).

Unlike the situation between 300 and 400 mV, at $E_h > 400$ mV the large ΔA_{450} indicates that some quinone in the reaction center-oxidoreductase pairs is stably reduced to the semiquinone anion after the first flash (presumably Q_{II}^- although possibly Q_I^-, see Ref. 30). Reduction of cytochrome *b* by $Q_{II}H_2$ after the second flash would require that another electron acceptor was available for the second reducing equivalent from $Q_{II}H_2$ so that oxidized Q_{II} could be replenished [9]. The damping of Q_{II} oscillations under these conditions is so severe that only the first and the second flashes exhibit pronounced oscillatory behavior. Two possible damping mechanisms are examined in Fig. 10B. With some of the reaction center $(BChl)_2$ chemically oxidized prior to flash excitation, a situation where Q_{II}^- is generated in only some of the reaction centers occurs (see Ia). With random re-reduction (by ferricyanide) of both the chemically and photo-oxidized $(BChl)_2$ taking place between flashes, the quantity of new Q_{II}^- formed at the second flash would be almost as great as the amount of first-flash generated Q_{II}^- which becomes doubly reduced at the second flash. This situation results in almost no net change of Q_{II}^- after the second flash and is illustrated by the dashed line in Fig. 10B. The solid line in Fig. 10B provides a better fit to the data and comes from the rather surprising assumption that only the photo-oxidized $(BChl)_2^+$ is re-reduced between flashes. Damping then stems from the only partial re-reduction of $(BChl)_2^+$ between flashes. More complete re-reduction requires time intervals between flashes comparable to the decay $t_{1/2}$ of Q_{II}^- (12 s, from Fig. 3) and results in even greater damping (data not shown). Selective reduction of photo-oxidized $(BChl)_2$ over the chemically oxidized species would further imply that the E_m value of the $(BChl)_2/(BChl)_2^+$ couple is actually higher than the 450 mV value obtained in equilibrium titrations, a possibility which will be explored in future experiments.

Proton binding

The binding of one H_I^+ to each reaction center after each flash, at the same time as a significant portion of the Q_{II} population is oscillating between the fully oxidized and the semiquinone anion form, casts serious doubt on the earlier supposition that H_I^+ is bound directly to Q_{II} [15]. Wraight [5], in similar findings with isolated reaction centers, concluded that the proton was not bound directly to Q_{II}, but to a protein side group which had undergone a downward pK_a shift in response to the proximity of Q_{II}^-. Use of this mechanism in the chromatophore would insure that the reductant of cytochrome *b* is always Q_{II}^- [14,28].

Antimycin-sensitive proton binding (H_{II}^+) also exhibits no binary dependence on flash number, although at $E_h > 400$ mV, the amount of H_{II}^+ binding decreases (0.8 to 0.4 H^+/e^-, see Fig. 8). This is con-

sistent with the findings of Petty et al. (Fig. 1, Ref. 26) and is suggestive that the stoichiometry of H^+_{II} binding is decreased (approx. 50%) by the changes in the coupling between Q_{II} and cytochrome *b*-560 at redox potentials above 400 mV.

Previous investigators have reported binary out-of-phase oscillations in proton binding in *Rps. sphaeroides* R-26 [7]. High concentrations (100 μM) of diaminodurene were used in the earlier study, however to avoid potential redox dye interactions in the present study, the concentration of diaminodurene never exceeded 10 μM, and generally was not added at all. This precaution insured that proton-binding results were not altered by the interaction of redox dyes with the reaction center.

Functional E_m values and redox mediator interactions

As pointed out in previous papers [14,29], the equilibrium midpoint values of the $Q_{II}/Q_{II} \cdot H$, $Q_{II} \cdot H/Q_{II}H_2$ and Q_{II}/Q^-_{II} couples [31] have only limited value in explaining all of the reactions of cytochrome *b*-560, a notable exception being the apparent equilibrium between cytochrome *b*-560 and the $Q_{II}/Q_{II} \cdot H$ couple which occurs in the absence of antimycin [14]. One model proposes that the reducing equivalents which are unaccounted for in the absence of antimycin (i.e., not measurable as Q^-_{II} or ferrocytochrome *b*-560, see Fig. 8) have been transiently transferred from Q^-_{II} to cytochrome *b*-560, along with H^+_I; subsequent equilibration of both proton and electron between ferrocytochrome *b*-560 and Q_{II} yields equilibrium concentrations of ferrocytochrome *b* and the essentially invisible $Q_{II} \cdot H$ [28]. However, the p*K* and E_m shifts of cytochrome *b*-560 as a result of an apparent electrostatic interaction with the oxidized reaction center bacteriochlorophyll dimer [16,26] suggest that the functional E_m of this component may be quite different from that measured in an equilibrium titration, and could be constantly varying during photosynthetic electron transfer. As mentioned previously, similar shifts could occur in the E_m of the Q_{II} semiquinones, and the heterogeneity of the reaction center population may be a further source of diversity in the functional E_m values.

On the time scale of seconds, as used in this and other studies [7–9], significant equilibrium could occur with components of the reaction center and oxidoreductase and the redox mediators between flashes. To some extent, this problem can be minimized by utilizing relatively hydrophilic mediators (such as ferri/ferrocyanide) or low concentrations of mediators, yet there is a danger that membrane components will be out of redox equilibrium with the redox electrodes. Higher concentrations of redox mediators allow much faster and more complete redox equilibrium but not without danger of excessive mediator interaction with components of the electron-transfer cycle.

At redox potentials where ferrocytochrome c_2 can no longer function as a rapid donor to $(BChl)^+_2$ ($E_h >$ 300 mV), the issue of redox mediator interaction becomes critical. Without mediator re-reduction of $(BChl)^+_2$, no further electron transfer to Q_{II} would be possible. Two major alterations in the extent of Q_{II} oscillatory behavior occur at similar redox potentials as changes in the rate of $(BChl)^+_2$ re-reduction. The re-reduction $t_{1/2}$ changes from the microseconds to the milliseconds time scale with an apparent E_m of 300 mV; and the rate changes from the milliseconds to seconds time scale with an apparent E_m of approx. 400 mV (see Fig. 3). The former is the E_m of cytochrome c_2, while the latter is notably similar to the E_m of the ferricyanide/ferrocyanide couple (420 mV, see Ref. 32). Different changes in the rate of $(BChl)^+_2$ re-reduction could be responsible for some of the variability in the E_m dependence of Q_{II} oscillations reported thus far [7–9].

The absence of Q^-_{II} oscillations at redox potentials where the system is optimally coupled (Q_z reduced, cytochrome *b*-560 oxidized) has been apparent in all studies carried out in chromatophores [8,9], and is particularly notable in light of the rather remote conditions ('high' E_h) necessary before oscillations can be observed. Other investigators have proposed that the gating by Q_{II} may be operational under a wider range of conditions because a stable out-of-equilibrium Q^-_{II} can exist in half of the reaction centers when cytochrome c_2 is initially reduced. In such a situation no oscillatory behavior of Q_{II} would be observed because of the initially mixed population of Q_{II} and Q^-_{II} (see consideration Ib). These proposals have led to the conclusion that the oxidoreductase and reaction center are associated in a 1 : 2 complex [27,33]. The results of this paper do not necessarily exclude

this possibility, particularly when ferrocytochrome c_2 is initially present. We have found that although the gating by Q_{II} is not experimentally measurable under optimally coupled conditions, the oscillations of Q_{II}^{-} have proven instructive in the study of the interaction between the reaction center and the ubiquinone-cytochrome c_2 oxidoreductase.

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