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A study of the kinetic properties of the stable semiquinone of the reaction-center secondary acceptor in chromatophores of non-sulfur purple bacteria

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Flash-induced absorption changes at 450 nm were investigated in isolated chromatophores of *Rhodopseudomonas sphaeroides* and *Rhodospirillum rubrum* non-sulfur purple bacteria to follow the redox changes of the semiquinone species of the secondary quinone acceptor of the photosynthetic reaction center. Excitation of a dark-adapted chromatophore suspension by a series of successive flashes in the presence of electron donors capable of rapidly reducing the photooxidized reaction-center pigment causes the formation of a stable semiquinone species (Q_B^-) with a lifetime which is shown to be proportional to the amount of the oxidized redox mediator in the incubation medium. It is shown that the disappearance of the flash-induced absorption changes at 450 nm on lowering the ambient redox potential (E_h) to 200–300 mV is the result of increasing the lifetime of Q_B^-, as the amount of the oxidized mediator diminishes; consequently, in these circumstances, the 2–5 min dark interval between the flash cycles appears insufficient for Q_B^- recovery. After the addition of redox mediators with a low midpoint potential, acting as an oxidant for Q_B^-, the flash-induced redox changes of Q_B^- were observed at low E_h values unless E_h reached a value at which Q_B underwent reduction at equilibrium to form Q_BH_2. The data provide evidence that reaction centers with a fully oxidized secondary acceptor can donate electrons to the cyclic electron-transport chain only after two turnovers, leading to the formation of the doubly reduced ubiquinone species (Q_BH_2) of the secondary acceptor.

Introduction

Light activation of chromatophores of non-sulfur purple bacteria causes a photo-induced separation of charges in the pigment-protein complex of a photosynthetic reaction center (RC) [1–4]. A photo-excited electron is transferred from the primary donor (P-870) via the complex of porphyrin pigments to the primary quinone (Q_A), subsequently to the secondary quinone (Q_B), both being attached to the reaction-center proteins [3–5]. In contrast to the primary electron acceptor, Q_A, the secondary quinone, Q_B, is able to accept two electrons [3–8].

In dark-adapted chromatophores and RC preparations of non-sulfur bacteria, binary oscillations of the formation of a semiquinone anion, Q_B^-, have been observed after excitation by a series of light flashes [3–14]. Stable semiquinone anions, Q_B^-, are formed after odd numbered flashes and disappear after even numbered flashes. Their disappearance is attributed to the reduction of Q_B^-, producing a doubly reduced form, Q_BH_2, which is able to donate electrons to the subsequent electron carriers of the cyclic electron transport chain [9,10,13]. The binary oscillations of Q_B^- can be observed by absorbance changes at 450 nm, the

Abbreviations: TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; RC, reaction center.

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wavelength of the maximum absorption of Q_B^- in the visible portion of the spectrum [15].

The binary oscillations were observed at high redox potentials (E_h) and were found to disappear when E_h was lowered to approximately 300–200 mV [3,9,10,13]. The cause of their disappearance and the mechanism of electron removal from the RC at low E_h is as yet unclear. As an explanation of the observation, some investigators [9,13] have suggested the redox-potential-dependent coupling of the RC and the cytochrome bc_1 complex (QH_2/ferrycytochrome c_2 oxidoreductase) at low E_h values which enables electron exchange between the Q_B^- molecules and the carriers of the bc_1 complex to take place.

As has been formulated [11–14,16], for the occurrence of the binary oscillations of Q_B^- formation, the following conditions must be satisfied:
(a) An effective electron donor must exist, capable of reducing the photooxidized P-870 for the time between the flashes, In native preparations, this is cytochromes c_2 [4]. In chromatophores and RC preparations, where the cytochrome c_2 are partly washed out during the preparation procedure, this role may be performed by reduced redox mediators;
(b) There must be a sufficient dark-adaptation time interval to allow all the RCs to return to the initial state in which the pigment is reduced and the quinone are oxidized;
(c) The intensities of the flashes must be saturating and their duration must be shorter than the time of a single turnover of the RC;
(d) The time between the flashes must be sufficiently long to allow the photooxidized P-870 to undergo reduction before the next flash but must be shorter than the lifetime of a semiquinone anion Q_B^- under the study conditions;
(e) The extent of oxidation of the electron carriers in the cyclic electron transport system (especially, that of the quinone pool) must be fairly high, a necessary condition for the oxidation of the reduced Q_BH_2 formed after even flashes.

Obviously, the lowering of the E_h potential from 400–350 mV to 300–200 mV should not violate the conditions (a), (c) and (e). On the other hand, it is possible that the disappearance of the binary oscillations of Q_B^- may be the consequence of an enhancement of its lifetime at low E_h, as our previous observations suggest [11]. In this case, the dark-adaptation time between the flash cycles (typically 2–5 min), which is sufficient for the oxidation of Q_B^- to Q_B at high E_h, may appear too short for its oxidation at low E_h, i.e., the condition (b) is no longer fulfilled.

In the present work using chromatophores of non-sulfur purple bacteria *Rhodopseudomonas sphaeroides* and *Rhodospirillum rubrum*, we investigated the dependence of the Q_B^- lifetime on E_h and on the concentrations of various redox mediators with different midpoint potentials in order to elucidate conditions that control the kinetics of the dark relaxation of the stable semiquinone secondary acceptor.

Materials and Methods

Cells of *Rps. sphaeroides* (wildtype, strain R-1) and *R. rubrum* (wild-type no. 1, Moscow University) were grown at 30°C in a medium of Ormerod and Gest [17] containing malic acid, peptone and vitamins [18]. Cells were sedimented from 2–3-day-grown cultures. Chromatophores were isolated by the method of Samuilov and Kondrat'eva [19]: after ultrasonic disintegration, the unbroken debris was sedimented by centrifugation at $40\,000 \times g$ for 15 min; chromatophores were sedimented from the supernatant by centrifugation at $144\,000 \times g$ for 1 h.

A home-made differential dual-wavelength spectrophotometer, with a xenon flash as an activation source [20] was used to measure light-induced absorption changes. Excitation pulses were 50 μs in duration. The cuvette system used in the experiments (optical length, 1 cm) was provided with facilities for a simultaneous measurement of E_h and had a magnetic stirrer. The kinetic curves presented in Fig. 4a, b and curve 1 in Fig. 4c were obtained with a differential two-beam spectrophotometer [21] designed and constructed in our laboratory. During the dark intervals the measuring beam was switched off.

Flash-induced redox changes of Q_B^- in *R. rubrum* chromatophore preparations were recorded at 450 nm against the 480 nm reference, when measurements were made with the double-wavelength spectrophotometer, and at 450 nm, when the two-beam spectrophotometer was used. Since

in the 450–480 nm region carotenoids contribute significantly to the absorption changes observed in *Rps. sphaeroides* chromatophores [9], the reference wavelength was selected around 475 nm such that a absorption changes of the carotenoids at 450 nm were compensated by those at the reference wavelength. To obtain anaerobic conditions, a glucose-glucooxidase system was employed, which was prepared in the following way. 1 mg glucooxidase (P-L Biochemicals, 280000 U/g) and 1 μl catalase (Fluka, 260000 U/ml) were dissolved in 0.3 ml of the incubation medium (containing no mediators). In each experiment, 20 μl of this mixture and a glucose solution were added to the incubation medium, which was 3 ml in volume (final concentration of glucose of 4%). Sodium dithionite ('Sigma') was used to lower E_h, and the aeration of the suspension, to increase E_h.

Results

Fig. 1a shows the 450 nm absorption changes of an *R. rubrum* chromatophore suspension induced by four successive flashes in the presence of the redox mediator TMPD, $E_m = 260$ mV [22]. The changes reflect the redox changes of Q_B and show patterns similar to those reported in [Refs. 6–13]. The formation of a stable semiquinone Q_B^- anion, relaxing slowly in an exponential way, was observed in response to odd flashes (Fig. 1b) and was found to disappear in response to even flashes (the photooxidized P-870 under our conditions is reduced in a time shorter than the resolution time of the instrument; therefore, the observed long-lived absorption changes are associated only with the oxidationreductions of Q_B^-). With lowering of E_h, the lifetime of Q_B^- becomes longer, as is seen from Fig. 1c. The half-lifetime of Q_B^- formed after the first flash is reciprocally proportional to the amount of oxidized TMPD ($TMPD_{ox}$) (Fig. 1c), suggesting that Q_B^- is oxidized by the $TMPD_{ox}$ and that the observed prolongation of the Q_B^- lifetime (Fig. 1b) is a consequence of a smaller amount of $TMPD_{ox}$ at lower E_h.

The observed dependence of the Q_B^- lifetime on the $TMPD_{ox}$ content gives good reasons to suggest that the disappearance of the binary oscillations observed in [9,10,13] upon lowering E_h was a consequence of a longer lifetime of Q_B^-, resulting from the smaller amount of oxidized molecules of the redox mediators used in the reactions. Indeed, for *R. rubrum* chromatophores the bimolecular rate constant for Q_B^- oxidation is 3.2 ± 0.1 $mM^{-1} \cdot s^{-1}$, as follows from Fig. 1c. This means that the lifetime of Q_B^- is 12 s at $E_h = 260$ mV and 50 μM TMPD. At $E_h = 160$ mV it will reach as high as

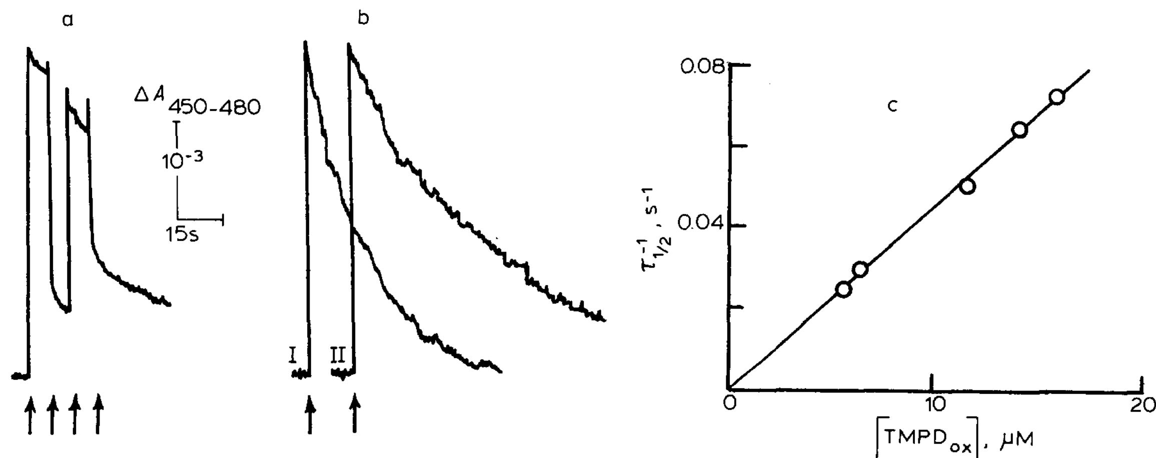


Fig. 1. Flash-induced absorption changes at 450 nm in an *R. rubrum* chromatophores suspension as a function of the concentration of oxidized TMPD. (a) Flash-induced binary oscillations of Q_B^-, the semiquinone species of the secondary acceptor, $E_h = 215$ mV; (b) the effect of E_h on the characteristic time of Q_B^- dark recovery, $E_h = 250$ mV (1) and $E_h = 220$ mV (2); (c) the reciprocal half-time of Q_B^- dark recovery as a function of the amount of oxidized TMPD. Incubation medium: 100 mM KCl/20 mM phosphate buffer (pH 6.8)/40 μM TMPD. In calculating the concentration of $TMPD_{ox}$, the midpoint potential, E_m, of TMPD was taken as 260 mV [22]. The dark interval between the flash cycles was 5 min. In all the figures throughout the article, flashes are shown by arrows.

350 s if the only dark relaxation process of Q_B^- is its oxidation by the mediator, which can easily be shown (for details see the Discussion). In the latter case, most of the Q_B^- population undergoes no oxidation during the 3–5 min dark period between the flash cycles. The presence of the semiquinones in a portion of the RCs before the flash cycle is applied will cause a decrease of the 450 nm absorbance in response to flash activation, since the absorption changes produced by Q_B molecules initially in a fully oxidized state will be compensated by the absorption changes of opposite sign arising from the redox conversion of Q_B molecules that were not oxidized during the dark interval and are present in the semiquinone form before the flash cycle.

To verify our hypothesis about the cause of the previously observed [9,10,13] disappearance of the binary oscillations of the 450 nm absorption changes at low E_h, oxidation-reduction titrations of the flash-induced absorption changes in *Rps. sphaeroides* chromatophores were performed under conditions similar to those reported in Ref. 9 at two concentrations of TMPD (Fig. 2). As seen from Fig. 2a, there is a decrease in the flash induced 450 nm absorbance on lowering E_h, the extent of decrease being dependent on the TMPD concentration: the higher the concentration the lower E_h at which the 450 nm absorption changes disappear (Fig. 2b). A plausible explanation of this is that at low E_h the amount of $TMPD_{ox}$ becomes smaller and the lifetime of Q_B^- becomes larger; it is evident that the amount of $TMPD_{ox}$ is higher when the total content of TMPD in the incubation medium is higher.

The experimental points corresponding to the 450 nm absorbance decrease following the first flash at low E_h fit closely the Nernst curve ($n = 1$) (Fig. 2b). Similar data obtained in Ref. 13 were interpreted as suggesting that at low E_h there occurs a reduction of some redox group which makes a fast electron exchange between Q_B^- and the bc_1 complex possible.

We calculated theoretically how the 450 nm absorbance must change following the first flash under conditions where Q_B^- has a longer lifetime as a result of lowering E_h and diminishing the $TMPD_{ox}$ content. Corresponding theoretical reductive titration curves were plotted (Fig. 3; a method for calculating the titration curves is detailed in the Appendix). The following experimental situation was modelled (Fig. 3, inset). Dark-adapted chromatophores (fully oxidized Q_B) are illuminated by six successive flashes spaced at $\theta = 7.5$ s. In all RCs, the Q_B^- is observed to oscillate with a periodicity of two, which is seen as binary oscillations of the 450 nm absorbance. Following the flash cycle, there is a dark period lasting 20 $\theta = 150$ s and the ambient redox potential is lowered somewhat (ΔE_h) (by adding a reductant). Immediately after this, the chromatophore suspension is illuminated by another series

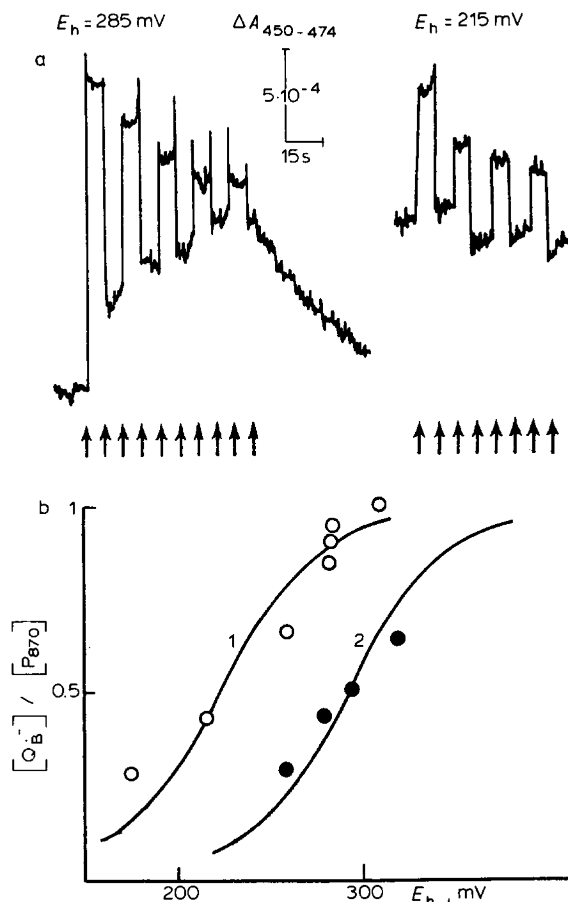


Fig. 2. Flash-induced absorption changes at 450 nm of an *Rps. sphaeroides* chromatophore suspension at different E_h levels (a), and E_h-dependence of the magnitude of the 450 nm absorption change induced by the first flash (b). TMPD concentration: (a) 66, (b), 66 (1) and 17 (2). RC concentration, 0.3 μM. Incubation medium was 50 mM phosphate buffer. Dark interval between the flash cycles was 3 min.

of six flashes. The procedure is repeated in this way. In plotting the titration curve for a sample illuminated by several flash cycles (with a 150 s dark period between the cycles) at each given E_h, magnitudes of the 450 nm absorption changes induced by the first flashes of every last flash cycle were used after which E_h was lowered to the next specified value.

It has appeared from the model titration that the behavior pattern of the titration curves strongly depends on the amount of E_h change (ΔE_h value), on the concentration of the redox mediators (compare curves 2 and 3 of Fig. 3), on the rate constant of Q_B^- oxidation by the mediator, on the number of repeative absorbance measurements at each E_h (compare curve 1 with curves 2 and 3 of Fig. 3). A comparison of curves 1 and 4 of Fig. 3 shows that the model titration curve I plotted as described in the Appendix does not virtually differ from theoretical Nernst curve (4), a fact in good agreement with the observed similarity between the experimental curves of Fig. 2, or the curves presented in Ref. 13, and theoretical $n = 1$ Nernst curves.

The data presented in Figs. 1–3 well support the idea that the longer lifetime of Q_B^- in the presence of a smaller amount of $TMPD_{ox}$ is responsible for the disappearance of the binary oscillations of the 450 nm absorbance. With this consideration in mind, it was believed that a low-potential mediator (i.e., that with a low midpoint potential E_h), when added to the incubation medium, will be oxidized at all redox potentials higher than its E_m and will therefore be able to oxidize Q_B^-. Different low-potential mediators were investigated; phenazine methosulfate, phenazine ethosulfate, Neutral red, indigo tetrasulfonate. The most effective oxidant for Q_B^- appeared to be Methylene blue ($E_{m,7} = 11$ mV [22]). In both *Rps. sphaeroides* and *R. rubrum* chromatophores, its addition sharply reduced the lifetime of Q_B^- (Fig. 4b, c) and in its presence the binary oscillations of Q_B^- formation are observed at relatively low E_h values (Fig. 5a).

These data demonstrate that conditions under which Q_B^- can be rapidly oxidized in the dark can be achieved by adding a low-potential mediator to

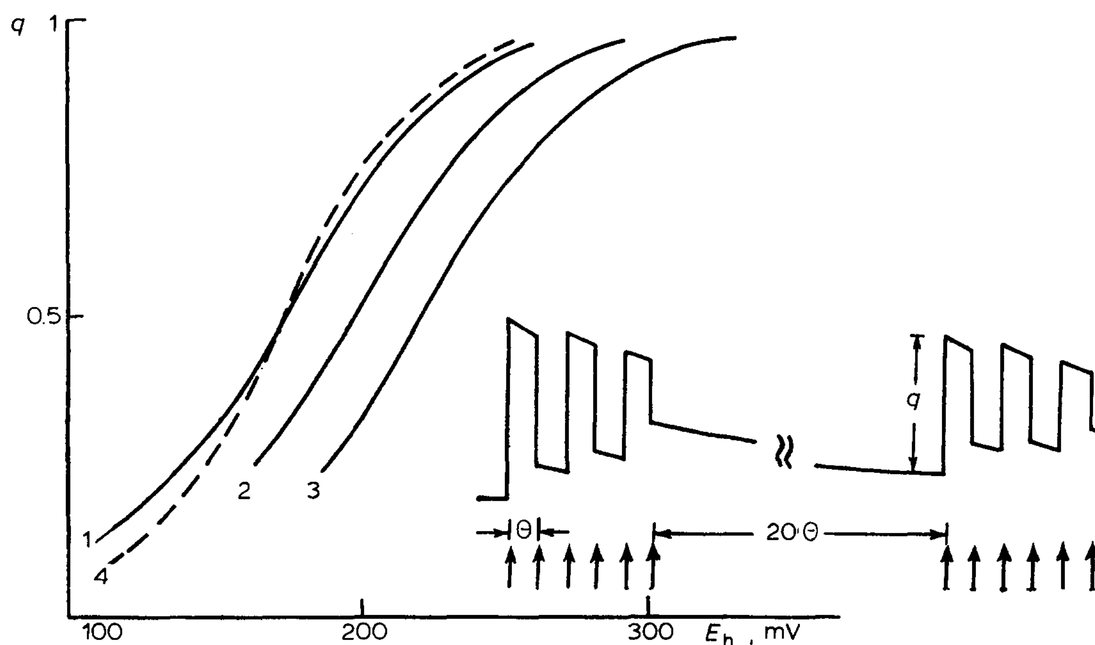


Fig. 3. Theoretical reductive titration curves, normalized to unity, of the Q_B^- changes following the first flash. The derivation of curves 1–3 as described in the text. TMPD concentration: 50, 66 and 17 μM for curves 1, 2 and 3, respectively. Number of measurements at a given E_h: one for curve 1; two for curves 2 and 3; 4, $n = 1$ titration curve for a one-electron carrier with $E_m = 174$ mV. Inset: sequence of events used in the calculation of the amount of flash-induced change of Q_B^- formation (see text).

the incubation medium and that the best mediator for this purpose is Methylene blue. In order to be able to estimate the dark-adaptation time for each case (at a given Methylene blue concentration), we determined the rate constant for Q_B^- oxidation by Methylene blue in both types of chromatophore preparation used in the study. This was done in the following way. Sodium ascorbate was added in the dark to a chromatophore suspension containing TMPD as an electron donor (the incubation conditions were such that binary oscillations of Q_B^- formation could be observed upon flash activation). The final concentration of the added ascorbate was 0.5 mM. The addition of ascorbate caused lowering of E_h and reduction in TMPD. The suspension was then illuminated by a flash. This caused the formation of Q_B^- molecules with a lifetime greater than 5 min (Fig. 4a) (a more accurate determination of their lifetime was impossible in view of the instrument null stability limitations).

Such a large lifetime of Q_B^- is indicative of an almost full reduction of the TMPD. Under such conditions, the oxidation of Q_B^- by this mediator may be disregarded. After the second (Fig. 4a) and subsequent flashes, normal binary oscillations of Q_B^- formation were observed, suggesting that in the presence of a relatively large amount of sodium ascorbate the two-electron gating process is operating normally. After the addition of Methylene blue (Fig. 4b), the dark recovery of Q_B^- was seen to proceed at a much faster rate, the rate increasing with increasing the content of Methylene blue (Fig. 4b, c). The system containing chromatophores, ascorbate and Methylene blue has a redox potential of about 130–150 mV. At this potential the TMPD present is fully reduced and the Methylene blue is fully oxidized. Fig. 4c shows the dependence of the rate of Q_B^- oxidation on the concentration of Methylene blue in *R. rubrum* and *Rps. sphaeroides* chromatophores. The data from Fig. 4, were used to estimate the rate constant for Q_B^- oxidation and yielded about 43 $mM^{-1} \cdot s^{-1}$ for *R. rubrum* chromatophores and about 5.5 mM^{-1} s^{-1} for *Rps. sphaeroides* chromatophores.

Note that for the *R. rubrum* chromatophores the rate constant was determined from the slope of the curve (Fig. 4c) for a fairly high concentration of the dye because at a low concentration there is a deviation from the linearity, perhaps, due to the nonspecific binding of the dye to the chromatophores, which makes its interaction with the Q_B^- molecules more difficult.

In *Rps. sphaeroides* chromatophores in the presence of methylene blue or any other low-potential redox mediator, the flash-induced binary oscillations of the 450 nm absorbance were observed at as low E_h as 100 mV (Fig. 5a). At lower E_h, the amplitude of the absorbance change after the first flash becomes smaller and no binary periodicity is observed: in response to the second flash the 450 nm absorbance increases or decreases a little (Fig.

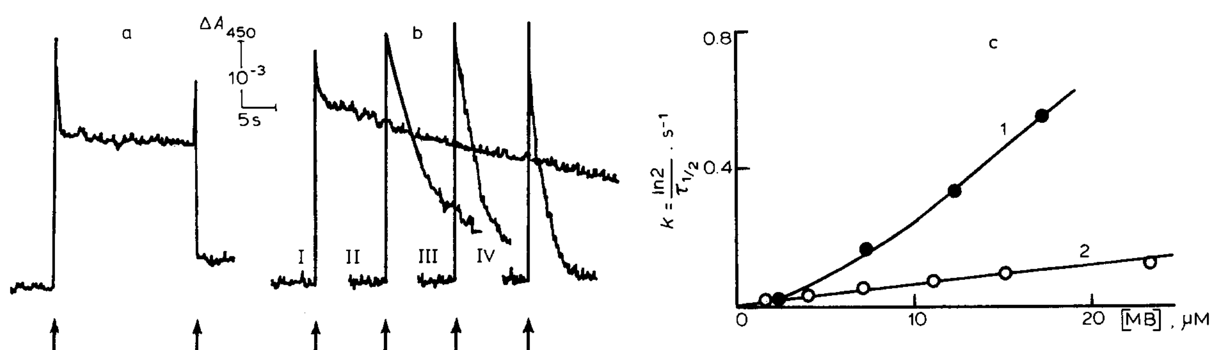


Fig. 4. Flash-induced 450 nm absorption changes of an *R. rubrum* chromatophore suspension in the absence (a) and presence (b) of Methylene blue (μM): 2.5 (I), 7.5 (II), 12.5 (III), 17.5 (IV). Incubation medium: 25 mM phosphate buffer (pH 7.0)/50 μM TMPD/0.5 mM sodium ascorbate. (c) Rate constant of Q_B^- dark recovery as a function of the concentration of Methylene blue in *R. rubrum* (1) and *Rps. sphaeroides* (2) chromatophores. The incubation medium of *Rps. sphaeroides* chromatophores was 50 mM phosphate buffer (pH 7.0)/40 μM TMPD/0.5 mM sodium ascorbate. The incubation medium of *R. rubrum* chromatophores was as in (a) and (b). The dark interval between the flash cycles was 10 min.

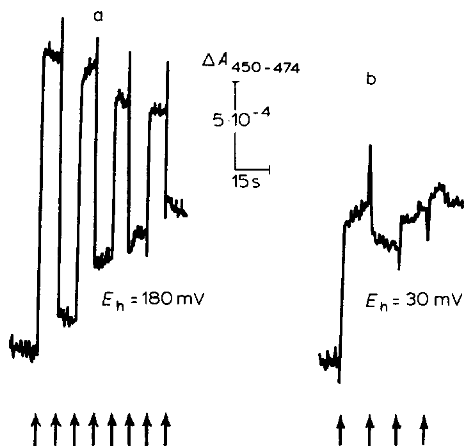


Fig. 5. Flash-induced 450 nm absorption changes of a *Rps. sphaeroides* chromatophore suspension in the presence of low-potential mediators at different E_h values. (a) Incubation medium: 50 mM phosphate buffer (pH 7.0)/66 μM TMPD/3 μM Methylene blue. (b) Incubation medium: 50 mM phosphate buffer (pH 7.0)/40 μM TMPD/10 μM Methylene blue/8 μM phenazine methasulfate/40 μM indigo tetrasulfonate. The dark interval between the flash cycles was 5 min.

5b), depending on the value of E_h.

In *R. rubrum* chromatophores, the 450 nm absorbance in the presence of low-potential mediators changes in a similar way on lowering E_h, as demonstrated in our previous investigations [23,24].

In all likelihood, the flash-induced 450 nm absorption changes observed at low E_h are a reflection of the reduction of Q_B at equilibrium yielding Q_BH_2 and resulting in flash-induced reduction of Q_A molecules. Like Q_B^-, the semiquinone species of Q_A has the absorption maximum at 450 nm within the visible spectrum, but its apparent extinction coefficient is smaller [24,25]. A detailed discussion of the E_h-dependent behavior of the 450 nm absorption changes after the first flash in the presence of a low-potential mediator and also its relation with the pattern of the redox titration curve of Q_B has been made in Ref. 23.

Discussion

The data show that the observed disappearance of the flash-induced binary oscillations of Q_B^- on lowering the E_h to 300–200 mV in the absence of low-potential mediators [3,9,10,13] is unlikely to be due to the redox-dependent binding of the bc_1 complex to a reaction center, as suggested in Refs. 9, 13. The cause is the increase of the lifetime of the Q_B^- molecules on diminishing the amount of the oxidized mediator which oxidizes Q_B^-. As seen from Figs. 2a and 5a, the binary oscillations of the 450 nm absorbance gradually decay with the number of the flashes and the half of the secondary acceptors appear as the semiquinone species (the characteristics of the decay have been described in Refs. 11, 12, 14). The result of the increased lifetime of Q_B^- at low E_h and with a small amount of the oxidized mediator is that some portion of the Q_B^- population cannot be oxidized during the dark period between the flash cycles, the reflection of which is the drop of the amplitude of the 450 nm absorption oscillations (Fig. 2b). Ultimately, when the Q_B^- lifetime is very long, no 450 nm absorption changes are observed after flash activation because about the half of the Q_B^- population already exists as the semiquinone species before the flash cycle.

It is clear from the data presented in Fig. 1 and Fig. 4 that in the presence of an effective electron donor (that rapidly reduces the photooxidized reaction-center pigment and thus prevents the backward return of an electron from Q_B^- to P-870), the lifetime of Q_B^- depends only on the amount of oxidized mediator. When the latter is small, the Q_B^- lifetime can be very long, amounting to as much as several minutes. There is therefore little probability of the electron exchange between the Q_B^- and the bc_1 complex, as has been postulated in Refs. 9, 13. Also, there seems little probability that Q_B^- can be oxidized by the ubiquinone pool [26], or that there is an interaction between the Q_B^- molecules belonging to different RCs [3].

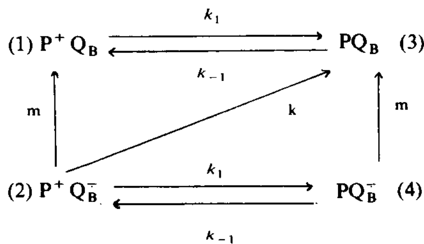
According to our data, over the entire region of E_h, at which the secondary acceptor is oxidized in the dark (to $E_h \approx 100$ mV), flash activation of dark-adapted chromatophores produces one Q_B^- molecule per RC (the value of the millimolar extinction coefficient of Q_B^- used in the calculation was taken as 6.5 $mM^{-1} \cdot cm^{-1}$ [27]). The population of generated Q_B^- almost entirely oxidizes in the dark in the presence of an oxidized mediator. We interpret this as an indication of the kinetic nature of the stabilization of the semiquinone, because Q_B^- molecules are little exposed to the exogenous electron acceptors, rather than its thermodynamic nature (see Refs. 3, 28 for discussion).

The present data indicate that the two-electron

gating process mediates electron transfer between RC and bc_1 complexes not only at high E_h, as postulated by several investigators [3,8–10,13], but also at low E_h values, down to the potential at which Q_B is reduced to form Q_BH_2. The cause of the disappearance of the binary oscillations of Q_B^- under certain conditions is the disconcordance of the redox states of individual Q_B molecules in different RCs. The electron transport via the secondary reaction-center quinone in chromatophores of purple bacteria can be presented schematically as shown in Fig. 6 [29]. Following the absorption of a light quantum, the photo-excited electron is transferred onto a Q_B molecule. This produces a stable semiquinone species which is incapable of exchanging electrons with the carriers of the cyclic electron transport chain. Electrons are delivered to the cyclic electron transport chain from the RC only after the absorption of a second light quantum by the RC leading to the formation of an ubiquinone molecule, which can freely move out of the RC.

The high kinetic stability of Q_B^- permits the RC to operate normally and to accumulate reducing equivalents in elementary steps even at low light intensities. This may have physiological significance, since the non-sulfur purple bacteria exist under low illumination conditions as a rule [18,30].

Let us analyze quantitatively the Q_B^- dark recovery process in a system containing chromatophores and a redox mediator. In the simplest case, whenever cytochrome c_2, a physiological electron donor for P-870, is absent (a typical situation for *R. rubrum* chromatophores prepared by the method used by us [31]), this process involves the following transitions, assuming that the electron localized on Q_B^- can either return to P-870 or move onto the oxidized mediator:



Scheme I

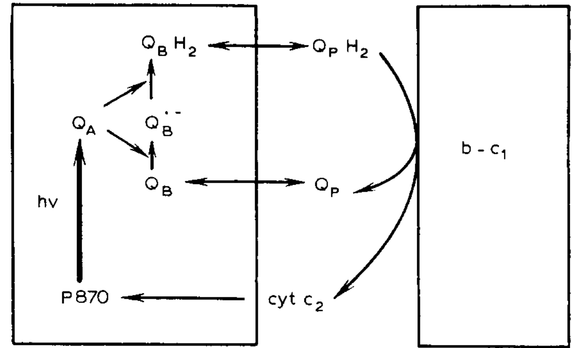


Fig. 6. The photosynthetic electron transport routes in non-sulfur purple bacteria.

where P, P^+ represent the primary reaction center electron donor (P-870) in the reduced and oxidized state, respectively; k is the rate constant for the backward electron transfer from Q_B^- to P^+; m is the rate constant of Q_B^- oxidation by the mediator; k_1 and k_{-1} are the rate constants of P^+ reduction by the mediator in the forward and backward reactions, respectively.

We also assume that the concentration of the mediator, both in the oxidized and reduced state, is greater than the amount of RCs in the system (this condition is as a rule satisfied in the experiment). Then, values of the rate constants appearing in Scheme I can be determined from the equilibrium concentrations of the different redox states of the mediator:

$$k_1 = k_1'[M_{red}] = \frac{k_1'[M_o]}{1+\exp\frac{\{E_h - E_m(M)\}nF}{RT}} \quad (1a)$$

$$k_{-1} = k_{-1}'[M_{ox}] = \frac{k_{-1}'[M_o]}{1+\exp\frac{\{E_m(M) - E_h\}nF}{RT}} \quad (1b)$$

$$m = m'[M_{ox}] = \frac{m'[M_o]}{1+\exp\frac{\{E_m(M) - E_h\}nF}{RT}} \quad (1c)$$

Here $[M_o]$ is the total concentration of the mediator in the system; $[M_{ox}]$ and $[M_{red}]$ are the concentrations of the mediator in the oxidized and reduced state, respectively; $E_m(M)$ is the midpoint redox potential of the mediator; n is the number of reducing equivalents; F is the Faraday constant; R is the universal gas constant; T is the

absolute temperature; bimolecular rate constants are denoted with a prime.

Let $p_i = p_i(t)$ be the probability of the reaction-center complex being in the ith state (i = 1, 2, 3, 4) at time t (see Scheme I). By solving the set of linear differential equations:

$$\frac{dp_2}{dt} = k_{-1}p_4 - (k_1 + k + m)p_2$$

$$\frac{dp_4}{dt} = k_1 p_2 - (m + k_{-1})p_4; \quad p_2(0) + p_4(0) = 1 \tag{2}$$

describing the transitions of the RC, as detailed in Scheme I [14,32], we find that the probability of Q_B being the semiquinone after the flash, $p(Q_B^-)$, is

$$p(Q_B^-) = p_2 + p_4 = Ae^{-\lambda t} + Be^{-\mu t} \tag{3}$$

where

$$A = \frac{k_1 + k_{-1} + m - \lambda}{k_1} \frac{(k_{-1} + m - \mu)p_4(0) - k_1 p_2(0)}{\lambda - \mu}$$

$$B = \frac{k_1 + k_{-1} + m - \mu}{k_1} \frac{k_1 p_2(0) - (k_{-1} + m - \lambda)p_4(0)}{\lambda - \mu} \tag{4}$$

and λ and μ are roots of the characteristic equation

$$\nu^2 + p\nu + q = 0 \tag{5}$$

in which

$$-p = k_1 + k_{-1} + k + 2m; \quad q = (k_{-1} + m)(k + m) + mk_1 \tag{6}$$

Typically, in realistic system

$$k_1 > k_{-1} \gg k, m \tag{7}$$

It can be shown that then $\lambda \gg \mu$. Disregarding the negligibly small constants, k and m, we have

$$\lambda \approx -p \approx k_1 + k_{-1} \tag{8}$$

$$\mu \approx \frac{-q}{p} \approx m + \frac{k k_{-1}}{k_1 + k_{-1}} \tag{9}$$

Hence, the kinetics of Q_B^- dark recovery following the flash are characterized by two times: one $(1/\lambda)$ reflects the fast establishment of equilibrium between states (2) and (4) in Scheme I; the other $(1/\mu)$ is the time of Q_B^- dark oxidation. Using Eqn. 7, it is easy to show that in Eqn. 3 $B \gg A$, with the A/B ratio tending to zero when $k \to 0$.

Eqn. 9 shows that the dark recovery of Q_B^- can proceed in two ways: either by direct oxidation by the mediator (with the rate constant $m = m'[M_{ox}]$) or by the backward transfer of an electron to P-870 (with the effective rate constant of $k[k_{-1}/(k_1 + k_{-1})] = kP^+$, where P^+ in a given case is the relative amount of the reaction-center pigment in the oxidized state in the dark at equilibrium:

$$P^+ = \frac{1}{1 + \exp\frac{\{E_m(P) - E_h\}F}{RT}}$$

E_m(P) is the midpoint potential of P-870. It is important to note that the value of $(kP^+)^{-1}$ characterizes the maximum possible lifetime of Q_B^- at a given E_h.

In estimating the contribution of each of these two processes for a system containing *R. rubrum* chromatophores and the mediator TMPD, in the presence of 40 μM TMPD and at $E_h = 250$ mV (see Fig. 1b), the characteristic time of Q_B^- dark recovery (the lifetime) is 20 s. At this E_h, the relative amount of P-870 oxidized in the dark is $3.4 \cdot 10^{-4}$ (the value of E_m(P) used in the estimation was +450 mV [33]). Using $k = 0.15$ s^{-1} [11], it is easy to evaluate kP^+ in Eqn. 9: $kP^+ \cong 5 \cdot 10^{-5}$ s^{-1}. If the dark recovery of Q_B^- were by way of backward electron transfer to P-870, then at E_h = 250 mV the process would proceed with a characteristic time of about $2 \cdot 10^4$ s. In reality, the process goes much faster, as can be seen from Fig. 1b. The implication is that, under the conditions considered, Q_B^- recovery is through its oxidation by the mediator. Hence, at fairly low E_h values whenever te $Q_B^- \to$ P-870 electron-transfer reaction can be neglected, the Q_B^- dark recovery can be described by an exponential curve with the power exponent being proportional to the concentration of the oxidized mediator:

$$p(Q_B^-) \approx Be^{-\mu t} = e^{-mt} = \exp\frac{m'(M_o)t}{1 + \exp\frac{\{E_m(M) - E_h\}nF}{RT}} \tag{10}$$

This conclusion agrees well with the experimen-

tally observed linear dependencies of the Q_B^- lifetime on the concentration of oxidized TMPD (Fig. 1c) and on the concentration of oxidized Methylene blue (Fig. 4c).

Eqns. 1–10 were derived for the simplest system consisting of RCs and a redox mediator. It is clear, however, that they hold for a system in which cytochromes c_2 are present on the donor side of the RCs (the case of *Rps. sphaeroides* chromatophores) provided that the redox equilibrium between the cytochrome c_2, P-870 and the redox mediator is set up much more quickly than the oxidation of Q_B^- by a mediator occurs or than the time of electron return from Q_B^- to P^+-870. This condition is fulfilled in a *Rps. sphaeroides* chromatophore suspension containing TMPD as a redox mediator (Fig. 2): Q_B^- are oxidized on a seconds time-scale (Fig. 2a); the time of electron return to P^+-870 is about 5 s, according to our estimates [34]; this is much longer than the time of an electron exchange between cytochromes c_2 and P-870 [35], and also much longer than the time of interaction between the TMPD and the donor reaction-center moiety in *Rps. sphaeroides*, which is tens or hundreds of milliseconds under the study conditions [36].

This makes it possible to estimate, using Eqn. 10, the bimolecular rate constant of Q_B^- oxidation by the $TMPD_{ox}$ in *Rps. sphaeroides* chromatophores. Based on the data of Fig. 2a, an estimation of the pseudomonomolecular rate constant of Q_B^- oxidation by the mediator yields 0.029 s^{-1}. The total concentration of the mediator is known to be 66 μM at $E_h = 285$ mV, it is easy to find that the corresponding bimolecular rate constant is equal to 0.61 $mM^{-1} \cdot s^{-1}$. Even taking into account that the magnitude of this rate constant varies somewhat from preparation to preparation, it is a matter of fact that in *Rps. sphaeroides* chromatophores the rate constants of Q_B^- oxidation by Methylene blue and by TMPD are much smaller than in *R. rubrum* chromatophores, in spite of the similarity of the thermodynamic characteristics of the secondary quinone acceptors in both types of chromatophore [16,23,24]. This indicates that in *Rps. sphaeroides* chromatophores the sites of specific binding of Q_B are probably less exposed to the mediator attack than in *R. rubrum*.

In a number of known investigations concerned with flash-induced oxidation-reductions of the bc_1 complex [13,37,38], the number of electrons leaving the RC on each flash either was not monitored [38] or could not be monitored with confidence [13,37] because of the lack of the flash-induced absorption changes at 450 nm (for reasons discussed above). This makes the interpretation of the data more difficult. In the presence of a low-potential mediator, added to the incubation medium Q_B^- undergoes oxidation during the dark adaptation time, as demonstrated here. This permits one to monitor the number of electrons delivered by the RCs on each flash to the cyclic electron transport chain.

The binary oscillations of Q_B^- generation were observed not only in chromatophores but also in aerated whole cells of *R. rubrum* [9] and *Rps.sphaeroides* [25] containing no mediators. The oscillations were observed to disappear upon diminishing the content of available oxygen. Within the context of the proposed model (Fig. 6), the only way of Q_B^- relaxation in the absence of the mediator is the return of the electron to P^+-870. The effective rate constant of this process is $k[P^+]'$ where $[P^+]'$ is the relative amount of P-870 oxidized under the steady-state condition. In an aerated suspension of whole cells, some amount of P-870 can be steadily maintained in the oxidized state, because cytochrome c_2, the electron donor for P-870, is also the electron donor for the terminal oxidase [25]. With $[P^+]'$ equal to 0.1, the lifetime of Q_B^- in *Rps. sphaeroides* chromatophores will be about 50 s ($k^{-1} \approx 5$ s, as stated above) i.e., it is relatively small. The diminution of oxygen content will obviously cause the lifetime of Q_B^- to increase and the absorption changes at 450 nm to disappear.

Appendix

To obtain theoretical reductive titration curves of flash-induced absorption changes at 450 nm, relationships derived by us previously [12,14] for the binary oscillations of Q_B^- generation in non-sulfur purple bacteria were used:

$$p(t) = p(n\theta + \tau) = p_\tau(n) = \frac{\alpha e^{-m\tau}}{\alpha_1 + \beta_1}\left[1 - \{1 - (\alpha_1 + \beta_1)\}^n\right]$$

$$\alpha_1 = \alpha e^{-m\theta}; \quad \beta_1 = 1 - (1 - \beta)e^{-m\theta}; \quad p_0(0) = 0 \qquad \text{(A-1)}$$

where $p(t) = p(n\theta + \tau) = p_\tau(n)$ is the probability for the secondary quinone being in the semiquinone form at time t (t, the time elapsed after the first flash; θ is the time between two successive flashes; τ is the time after the last flash; n is the flash number). α and β are probabilities of electron transfer from P-870 to Q_B when Q_B is fully oxidized and when Q_B is in the semiquinone form, respectively. α_1 and β_1 are the corresponding probabilities α and β for a situation when some Q_B^- molecules have undergone oxidation to Q_B for the time, τ, after the current flash, with the pseudomonomolecular rate constant, m (see Eqn. 1c).

In Eqn. A-1 it is assumed that at time $t = 0$ the Q_B^- population is fully oxidized. Our interest, however, is in a more general case whenever the secondary acceptors in some RCs are in the semiquinone form at the time immediately before the next flash cycle, In this case [14]:

$$p_t(n) = \frac{\alpha e^{-m\tau}\left[1 - \{1 - (\alpha_1 + \beta_1)\}^n\right]}{\alpha_1 + \beta_1} + \{1 - (\alpha_1 + \beta_1)\}^n P_0(0) \quad \text{(A-2)}$$

The calculation was made in the following way. Assuming that at $E_h = 360$ mV the Q_B population is entirely oxidized, the concentration of Q_B^- immediately after the nth flash ($p_0(n)$) was found from Eqn. A-1. Then, from the relation $p_{l\theta} = p_0(n)e^{-m \cdot l\theta}$, the diminution of the Q_B^- concentration during the intercycle dark period ($l\theta$) was determined. At the end of the dark period, the redox potential was changed stepwise by a value of ΔE_h. Values of m, α_1, and β_1, were then calculated from Eqn. 1c for this new E_h and the results were used in Eqn. A-2 to determine the relative concentration of Q_B^- after the cycle of n flashes, assuming that $p_0(0) = p_{l\theta}$. In this manner the procedure was continued.

We have found in our earlier works [12,14] that $\alpha \approx 0.99$ and the sum $(\alpha + \beta)$, used in Eqns. A-1 and A2 is 1.92 for *R. rubrum* chromatophores [12,14] and 1.88 for *Rps. sphaeroides* chromatophores [29]. For simplicity, we assume that $\alpha = 1$ and $(\alpha + \beta) = 1.90$. Other values used were $\theta = 7.5$ s, $l = 20$, $n = 6$, $E_m(M) = 260$ mV, $\Delta E_h = 20$ mV, $m = 0.3$ $mM^{-1} \cdot s^{-1}$. Values of other parameters are given in the captions to the figures.

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