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\textsubscript{B}\textsuperscript{−}) 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\textsubscript{h}) to 200–300 mV is the result of increasing the lifetime of Q\textsubscript{B}\textsuperscript{−}, 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\textsubscript{B}\textsuperscript{−} recovery. After the addition of redox mediators with a low midpoint potential, acting as an oxidant for Q\textsubscript{B}\textsuperscript{−}, the flash-induced redox changes of Q\textsubscript{B}\textsuperscript{−} were observed at low E\textsubscript{h} values unless E\textsubscript{h} reached a value at which Q\textsubscript{B} underwent reduction at equilibrium to form Q\textsubscript{B}H\textsubscript{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\textsubscript{B}H\textsubscript{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\textsubscript{A}), subsequently to the secondary quinone (Q\textsubscript{B}), both being attached to the reaction-center proteins [3–5]. In contrast to the primary electron acceptor, Q\textsubscript{A}, the secondary quinone, Q\textsubscript{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\textsubscript{B}\textsuperscript{−}, have been observed after excitation by a series of light flashes [3–14]. Stable semiquinone anions, Q\textsubscript{B}\textsuperscript{−}, are formed after odd numbered flashes and disappear after even numbered flashes. Their disappearance is attributed to the reduction of Q\textsubscript{B}\textsuperscript{−}, producing a doubly reduced form, Q\textsubscript{B}H\textsubscript{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\textsubscript{B} can be observed by absorbance changes at 450 nm, the
wavelength of the maximum absorption of $Q_\text{B}^-$ 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$_2$/ferricytochrome $c_2$ oxidoreductase) at low $E_h$ values which enables electron exchange between the $Q_\text{B}^-$ 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 $Q_\text{B}^-$ 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 $Q_\text{B}^-$ 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 $Q_\text{B}^-\text{H}_2$ 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 $Q_\text{B}^-$ 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 $Q_\text{B}^-$ to $Q_\text{B}$ 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 $Q_\text{B}^-$ 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 × g for 15 min; chromatophores were sedimented from the supernatant by centrifugation at 144 000 × 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 $Q_\text{B}^-$ 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 aerobic conditions, a glucose-glucooxidase system was employed, which was prepared in the following way. 1 mg glucooxidase (P-L Biochemicals, 280 000 U/g) and 1 μl catalase (Fluka, 260 000 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 QB 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 oxidation reductions 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 $3.2 \pm 0.1$ mM$^{-1}$ 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

![Figure 1](image-url)
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 $\text{TMPD}_\text{ox}$ becomes smaller and the lifetime of $Q_B^-$ becomes larger; it is evident that the amount of $\text{TMPD}_\text{ox}$ 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 $\text{TMPD}_\text{ox}$ 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 ($\Delta E_h$) (by adding a reductant). Immediately after this, the chromatophore suspension is illuminated by another series
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 ($\Delta E_h$ 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 repetitive 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 TMPDox 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 ($E_m,\gamma = 11$ 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 $\mu$M 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).](image)
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$^{-1}$ 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. 5a).

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.

6b), 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$_A$ at equilibrium yielding Q$_A$H$_2$ 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 $E_h$ ≈ 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$^{-1}$·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:

\[
\begin{align*}
(1) \quad P^+Q_B & \xrightleftharpoons{k_1} PQ_B \\ (2) \quad P^+Q_B & \xrightleftharpoons{k_{-1}} PQ_B
\end{align*}
\]

\[
\begin{align*}
k_1 & = k_1[M_{\text{red}}] = \frac{k_1[M_o]}{1 + \exp \left( \frac{E_h - E_m(M)}{nF} \right)} \\
k_{-1} & = k_{-1}[M_{\text{ox}}] = \frac{k_{-1}[M_o]}{1 + \exp \left( \frac{E_m(M) - E_h}{nF} \right)} \\
m & = m[M_{\text{ox}}] = \frac{m'[M_o]}{1 + \exp \left( \frac{E_m(M) - E_h}{nF} \right)}
\end{align*}
\]

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 1 can be determined from the equilibrium concentrations of the different redox states of the mediator:

Here $[M_o]$ is the total concentration of the mediator in the system; $[M_{\text{ox}}]$ and $[M_{\text{red}}]$ 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 \( i \)th state \((i = 1, 2, 3, 4)\) at time \( t \) (see Scheme I). By solving the set of linear differential equations:

\[
\frac{dp_2}{dt} = k_1 p_1 &- (k_1 + k + m) p_2 \\
\frac{dp_4}{dt} = k_1 p_2 &- (m + k_-) p_4; \quad p_2(0) + p_4(0) = 1
\]

(2)

describing the transitions of the RC, as detailed in Scheme I [14,32], we find that the probability of \( \text{QB} \) being the semiquinone after the flash, \( p(\text{QB}) \), is

\[
p(\text{QB}) = p_2 + p_4 = Ae^{-\lambda t} + Be^{-\mu t}
\]

(3)

where

\[
A = \frac{k_1 + k_- + m - \lambda}{k_1} \text{(k}_1 + m - \mu \text{)} p_4(0) - k_1 p_2(0) \\
B = \frac{k_1 + k_- + m - \mu}{k_1} k_1 p_2(0) - (k_1 + m - \lambda) p_4(0)
\]

(4)

and \( \lambda \) and \( \mu \) are roots of the characteristic equation

\[
v^2 + pv + q = 0
\]

(5)

in which

\[
-p = k_1 + k_- + k + 2m; \quad q = (k_1 + m)(k + m) + mk_1
\]

(6)

Typically, in realistic system

\[
k_1 > k_- \gg k, m
\]

(7)

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

\[
\lambda = -p = k_1 + k_- \\
\mu = \frac{-q}{p} = m + \frac{k_- t}{k_1 + k_-}
\]

(8)

(9)

Hence, the kinetics of \( \text{QB}^- \) 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 \( \text{QB}^- \) 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 \( \text{QB}^- \) can proceed in two ways: either by direct oxidation by the mediator (with the rate constant \( m = m'(\text{Mox}) \)) or by the backward transfer of an electron to P-870 (with the effective rate constant of \( k[k_1/(k_1 + k_-)] = kP^+ \), where \( P^+ \) in a given case is the relative amount of the reaction-center pigment in the oxidized state in the dark at equilibrium:

\[
p^+ = \frac{1}{1 + \exp \left( \frac{E_m(P) - E_b}{RT} \right)}
\]

(10)

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

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 \( \mu \)M TMPD and at \( E_b = 250 \text{ mV} \) (see Fig. 1b), the characteristic time of \( \text{QB}^- \) dark recovery (the lifetime) is 20 s. At this \( E_b \), the relative amount of \( \text{P}^-870 \) oxidized in the dark is \( 3.4 \cdot 10^{-4} \) (the value of \( E_a(P) \) used in the estimation was +450 mV [33]). Using \( k = 0.15 \text{ s}^{-1} \) [11], it is easy to evaluate \( kP^+ \) in Eqn. 9: \( kP^+ \approx 5 \cdot 10^{-5} \text{ s}^{-1} \). If the dark recovery of \( \text{QB}^- \) were by way of backward electron transfer to P-870, then at \( E_b = 250 \text{ mV} \) 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, \( \text{QB}^- \) recovery is through its oxidation by the mediator. Hence, at fairly low \( E_b \) values whenever \( \text{QB}^- \to \text{P}^-870 \) electron-transfer reaction can be neglected, the \( \text{QB}^- \) dark recovery can be described by an exponential curve with the power exponent being proportional to the concentration of the oxidized mediator:

\[
p(\text{QB}^-) = Be^{-\mu t} = e^{-mt} = \exp \left[ \frac{m'(\text{Mox})t}{1 + \exp \left( \frac{E_m(M) - E_b}{RT} \right)} \right]
\]

(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 TMPDox 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 \( \mu \)M at \( E_h = 285 \) mV, it is easy to find that the corresponding bimolecular rate constant is equal to 0.61 mM\(^{-1}\) 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 nonsulfur purple bacteria were used:

\[
p(t) = p(n \theta + \tau) = p_0(n) = \frac{\alpha e^{-mt}}{\alpha_1 + \beta_1} \left[ 1 - \left( \frac{\alpha_1}{\alpha_1 + \beta_1} \right)^n \right] \]

\[
\alpha_1 = \alpha e^{-m\theta}; \quad \beta_1 = 1 - (1 - \beta) e^{-m\theta}; \quad p_0(0) = 0 \quad (A-1)
\]
where \( p(t) = p(n\theta + \tau) = p_\ast(n) \) is the probability for the secondary quinone being in the semiquinone form at time \( t \) (\( t \) the time elapsed after the first flash; \( \theta \) is the time between two successive flashes; \( \tau \) is the time after the last flash; \( n \) is the flash number). \( \alpha \) and \( \beta \) are probabilities of electron transfer from P-870 to QB when QB is fully oxidized and when QB is in the semiquinone form, respectively. \( \alpha_1 \) and \( \beta_1 \) are the corresponding probabilities \( \alpha \) and \( \beta \) for a situation when some QB molecules have undergone oxidation to QB for the time, \( \tau \), 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 QB 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]:

\[
p_\ast(n) = \frac{ae^{-mr}[1 - (1 - (\alpha_1 + \beta_1))^n]}{\alpha_1 + \beta_1} + [1 - (\alpha_1 + \beta_1))^n]p_0(0) \quad (A-2)
\]

The calculation was made in the following way. Assuming that at \( E_h = 360 \text{ mV} \) the QB population is entirely oxidized, the concentration of QB immediately after the \( n \)th flash (\( p_0(n) \)) was found from Eqn. A-1. Then, from the relation \( p_{1\theta} = p_0(n)e^{-m\theta} \), the diminution of the QB concentration during the intercycle dark period (\( I\theta \)) was determined. At the end of the dark period, the redox potential was changed stepwise by a value of \( AE_h \). Values of \( m \), \( \alpha_1 \), and \( \beta_1 \), 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 QB after the cycle of \( n \) flashes, assuming that \( p_0(0) = p_{1\theta} \). In this manner the procedure was continued.

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

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