Photosynth Res (2014) 120:237–246
DOI 10.1007/s11120-013-9961-8

REGULAR PAPER

Modulation of the redox state of quinones by light in *Rhodobacter sphaeroides* under anaerobic conditions

André Verméglio · Pierre Joliot

Received: 25 September 2013 / Accepted: 16 December 2013 / Published online: 31 December 2013

Abstract Illumination of intact cells of *Rhodobacter sphaeroides* under anaerobic conditions has a dual effect on the redox state of the quinone pool. A large oxidation of the quinone pool is observed during the first seconds following the illumination. This oxidation is suppressed by the addition of an uncoupler in agreement with a light-induced reverse electron transfer at the level of the complex I, present both in the non-invaginated part of the membrane and in the chromatophores. At longer dark times, this illumination increases the reducing power of the cells leading to a significant reduction of the others reaction centers (RCs). From the observation that a significant proportion of RCs could be reduced by the preillumination without affecting the numbers of charge separation for the RCs, we conclude that there is no rapid thermodynamic equilibrium between the quinones present in the non-invaginated part of the membrane and those localized in the chromatophores. Under anaerobic conditions where the chromatophores quinone pool is fully reduced, we deduce, on the basis of flash-induced fluorescence kinetics, that the reduced RCs are exclusively reoxidized by the quinone generated at the Q_o site of the cyt bc_1 complex. The supramolecular association between a dimeric RC-LHI complex and one cyt bc_1 complex allows the confinement of a quinone between the RC-LHI directly associated to the cyt bc_1 complex.

Keywords *Rhodobacter sphaeroides* · Fluorescence · Anaerobiosis · Quinones · Supercomplexes · Complex I

This article is dedicated to the memory of Pr. Roderick Clayton (23 October 2012) who introduced André Verméglio to the wonderful world of photosynthetic bacteria.

A. Verméglio (✉)
CEA-Cadarache, DSV-SBVME, Laboratoire de Bioénergétique Cellulaire, UMR 7265, CNRS-CEA-Aix-Marseille II, 13108 Saint Paul lez Durance Cedex, France
e-mail: avermeglio@cea.fr

P. Joliot
CNRS UPR 1261, Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France

Introduction

Sixty years ago, L.N.M. Duysens described in his doctoral thesis the first measurement of a small absorbance decrease linked to the photo-oxidation of the primary electron donor of the reaction center (RC) in intact cells of the photosynthetic bacterium *Rhodospirillum rubrum* (Duysens 1952). This was also the clear demonstration of the remarkable potency of flash-induced absorption spectroscopy to study the effects of light in photosynthetic organisms. Since that time, great progress has been made in our understanding and knowledge of the photochemistry and structure of the bacterial RC. A crucial step was the first isolation of a photosynthetic reaction complex from *Rhodobacter* (*Rba.*) *sphaeroides* R26 by Reed and Clayton (1968). This was followed by an extensive characterization of the composition and properties of this RC complex by Feher (1971, 1998). Continuous progress in our knowledge of the bacterial RC was made thanks to the improvements of the time resolution of flash spectroscopy and electron paramagnetic resonance together with the developments of genetic methods (Marrs 1974; Bylina and Youvan 1987). But a major breakthrough was obtained when Michel (1982) and Deisenhofer et al. (1984, 1985) crystallized and resolved the structure of the RC of *Rhodopseudomonas* (now *Blastochloris*) *viridis* by X-ray crystallography. The structure of the RC of *Rba. sphaeroides* was obtained

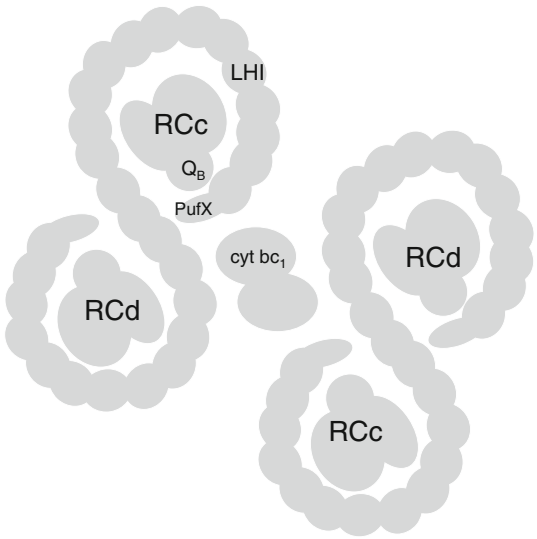
subsequently by Allen et al. (1986, 1987). Concomitantly several advancements in the description of the light-induced cyclic electron transfer between RCs, cyt bc_1 complex and soluble carriers in the membrane and the periplasm were made. Today, kinetics of each step of this cyclic electron transfer is established, and functional and structural information is available for each of the individual complexes of the photosynthetic chain (light harvesting complexes, RC, cyt bc_1 complex, etc.) (see e.g., The Purple Photosynthetic Bacteria 2009).

The light-induced cyclic transfer implies a constant number of charges in the circuit. The functioning of the RC requires a primary electron acceptor in the oxidized form while the functioning of the cyt bc_1 complex implies both the supply of quinol at site Q_o and of quinone at site Q_i. Under oxidizing conditions, the rate of the cyclic electron flow should be limited by the local concentration of quinol (QH_2) in the vicinity of the Q_o site of the cyt bc_1 complex. Under reducing conditions, the turnover of the system is limited by the transfer of the quinone formed at site Q_o of the cyt bc_1 complex to the Q_B site of the RC. Under anaerobic conditions, photosynthetic bacteria have, therefore, to maintain most of the primary electron acceptor Q_A in its oxidized state for an optimal light-induced electron transfer. This control could be achieved by different mechanisms: use of exogenous electron acceptors like TMAO, NO_3^-, etc.; the presence of a significant membrane potential in the dark which could partly prevent reduction of the quinone pool by NADH at the level of complex I (McEwan et al. 1985).

The first evidence for the reduction of NAD^+ by light in intact photosynthetic bacteria was obtained by Duysens and Sweep (1957), Olson et al. (1959), and Olson and Amesz (1960) using the blue fluorescence properties of NADH. Since that time, a reverse electron flow occurring between succinate and NAD^+ has been observed in both intact cells and isolated chromatophores from various photosynthetic bacteria species (Knaff 1978 and refs therein). This reverse electron flow is inhibited by uncouplers or inhibitors of the cyclic electron transfer or of the complex I (Knaff 1978). The definitive demonstration for a key role of complex I in the photosynthetic process is the inability of complex I-deficient mutants to grow under anaerobic photosynthetic conditions (Dupuis et al. 1997; Herter et al. 1998; Tichi et al. 2001).

Another important element for an efficient cyclic electron transfer is the supramolecular organization of the components involved. Several years ago we proposed that the key elements of the photosynthetic chain of *Rba. sphaeroides* R26 (LH, RC, cyt bc_1, and cyt c_2) are organized in supercomplexes (Joliot et al. 1989). This proposal was based on the observation that the measured apparent equilibrium constants between the electron transfer components of the photosynthetic chain (RC, cyt c_2, and cyt bc_1) were much lower than the equilibrium constants deduced from their mid-point potentials (Joliot et al. 1989). This low apparent equilibrium constant was proposed to be due to a restricted diffusion of 1 cyt c_2 between 2 RCs and 1 cyt bc_1 complex forming a supercomplex. Crofts and coworkers have proposed an alternative model to explain the low apparent equilibrium constant measured in chromatophores or the inhibitor titrations (Fernandez-Velasco and Crofts 1991; Crofts et al. 1998). In their model, the supercomplex behavior is explained by heterogeneity of chromophores and does not invoke a particular interaction between RCs and cyt bc_1 complexes. These authors, however, recognized that, in contrast to the results obtained with chromatophores (Fernandez-Velasco and Crofts 1991), inhibitor titrations experiments performed on intact cells indicate a restricted diffusion of cyt c_2 supporting the supercomplex model (Crofts et al. 1998). Although biochemical and AFM studies have demonstrated the presence of dimeric RC-LHI complexes in membranes of *Rba. sphaeroides* (Francia et al. 1999; Jungas et al. 1999; Scheuring et al. 2004; Siebert et al. 2004; Bahatyrova et al. 2004) in agreement with the supercomplex hypothesis, their association with cyt bc_1 complex has not been observed by these approaches.

The relative arrangement of membranous complexes of photosynthetic chain could be of prime importance for the confinement of the mobile quinone molecules. Such confinement increases the local concentration of QH_2 (in



Scheme 1 Putative supramolecular organization of photosynthetic unit of *Rhodobacter sphaeroides*. The RCcs correspond to RCs connected to the cyt bc_1 complex. Their reoxidation corresponds to the fast fluorescence phase. The RCds are not connected directly connect to the cyt bc_1 complex. Their reoxidation corresponds to the slow fluorescence phase

oxidizing condition) or oxidized Q (in reducing condition) in the vicinity of the Q_o or Q_B site, respectively, necessary for an optimal cyclic electron transfer as discussed above. Recently, Comayras et al. (2005) concluded from a kinetic approach that quinone molecules in the chromatophore membrane of *Rba. sphaeroides* are confined to domains containing no more than six RCs . We also provided evidence for a confinement of the quinones from fluorescence flash-induced measurements on cells of *Rba. sphaeroides* under anaerobic conditions at 0 °C (Joliot et al. 2005). The rate of reoxidation of the reduced primary electron acceptor Q_A^- presents a fast and a slow phase of equal amplitude. The rapid reoxidation phase of Q_A^- was specifically inhibited by the cyt bc_1 inhibitor myxothiazol. We interpreted this behavior in the context of the supercomplex model where the fast phase corresponds to the transfer of quinone formed at site Q_o to the Q_B pocket of the RC in direct contact with the cyt bc_1 of the supercomplex and the slow phase to the reoxidation of the other RC of the supercomplex (Scheme 1).

In the present work, we have investigated, by fluorescence measurements, the effect of continuous and flash illumination on the redox state of the primary acceptor Q_A under physiological conditions for intact cells of *Rba. sphaeroides* Ga placed under anaerobic conditions.

Materials and methods

Bacterial growth

Rhodobacter sphaeroides Ga cells were grown in the light in Hutner's medium at 30 °C under anaerobic conditions.

Fluorescence measurements

Bacteriochlorophyll fluorescence changes were measured with a JTS spectrophotometer (Biologic). To avoid long-term effect of sedimentation, the cuvette ($\Phi = 8$ mm) was placed horizontally. The yield of fluorescence was detected through a Schott filter (RG 780) and sampled using short detecting flashes (440 nm, 4 µs duration) with negligible actinic effects. The sample was subjected to continuous light (630 nm, 620 µE/m^2 s) and saturating actinic flashes (laser YAG Quantel, 532 nm, 10 ns duration). In all experiments, the fluorescence level was normalized to the F_0 level of aerobic dark-adapted cells. We define $F = (F - F_0)/F_0$ and $F_i = (F_i - F_0)/F_0$ the fluorescence level measured in the dark. The flash-induced fluorescence changes following a saturating flash were detected from 1.2 ms to several seconds after the actinic flash. The first detection time (1.2 ms) is long enough to allow the complete reduction of P_{870} and the oxidation of Q_A^- of RCs in the Q_AQ_B or $Q_AQ_B^-$ states before flash. If F_{ext} is the fluorescence level extrapolated from times longer than 1.2 ms to time 0, the fluorescence changes induced by a flash is denoted $\Delta F_{ext} = (F_{ext} - F_i)/F_0$. This change is nearly proportional to the concentration of RCs in the state $Q_AQ_BH_2$ before flash excitation.

Light-induced absorbance change measurements

Light-induced absorbance changes associated with membrane potential were measured using the same JTS spectrophotometer, as the difference between the light-induced variations of absorbance measured at 505 and 490 nm using weak monochromatic detection flashes.

Results

Modulation of the internal redox state of *Rba. sphaeroides* Ga by continuous illumination

We followed the redox state of the primary electron acceptor Q_A by monitoring the bacteriochlorophyll fluorescence yield. The redox state of Q_A can be determined either in the dark (F_i) or 1.2 ms after a saturating exciting flash (ΔF). In the experiment of Fig. 1, intact cells of *Rba. sphaeroides* Ga were placed under aerobic condition in a closed cuvette. Starting from F_0, the normalized fluorescence yield F_i gradually increases in the dark due to the development of anaerobic conditions associated to the oxygen consumption. The amplitude of this increase was found to be variable among the batches of cells which may correspond to different levels of internal reducing power. In the case of the batch used in Fig. 1, F_i reaches a maximum and stable value of 0.055 after 10 min of dark incubation (data not shown). This fluorescence increase reflects the reduction of a small fraction of Q_A during the course of the anaerobiosis process. During a continuous illumination of 2 min, the fluorescence level reaches a value of 0.32. After cessation of the light, the fluorescence drops rapidly with a fast phase completed in $\sim$1 s to a minimum level equal to the F_0 level measured in the presence of oxygen showing that all Q_A are reoxidized (Fig. 1). The cells are then submitted to a series of saturating flashes fired at various times (Fig. 1). The flash-induced fluorescence (ΔF) corresponds to the amount of RCs in the state $Q_AQ_BH_2$ prior to flash excitation. The low value of ΔF measured after 5 s of dark implies a high proportion of RCs in the states Q_AQ_B and $Q_AQ_B^-$ for which Q_A is reoxidized in less than 1.2 ms. We thus conclude that the pool of quinone is largely oxidized during the first seconds that follow the cessation of the illumination. In longer times, one observes an increase of ΔF completed in 100 s while the increase in F_i is a slower process

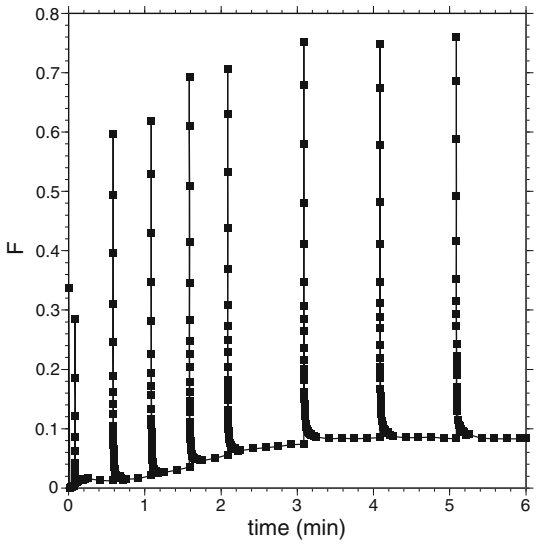


Fig. 1 Effect of a series of actinic flashes on a suspension of *Rba. sphaeroides* Ga cells placed under anaerobic conditions. The cells were illuminated by 2 min of continuous illumination followed by a series of actinic flashes. The fluorescence *F* is plotted as a function of the time following this illumination

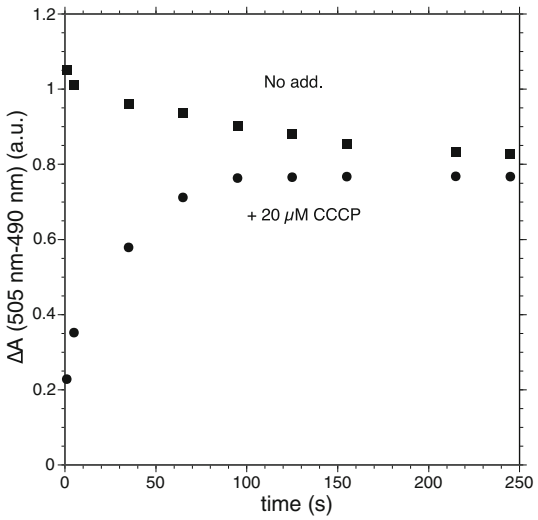


Fig. 2 Amplitude of the carotenoid band-shift ($\Delta A_{505\ nm\ -\ 490\ nm}$) of bacteria under anaerobic conditions induced by a single flash fired at various times (the first point measured at 1 s) after a continuous illumination of 2 min in the absence (*open squares*) or the presence of 20 μM CCCP (*open circles*)

completed in ~250 s. In addition, we measured the extent of charges separation by monitoring the carotenoid band-shift under the same conditions (Fig. 2, closed squares). The extent of charge separations decreases while F_i increases showing that the measurement of fluorescence yield gives a fair estimation of the number of closed RCs.

In the experiments shown in Fig. 3, the bacteria have been illuminated for 2 min (curve 1, same experiment as in

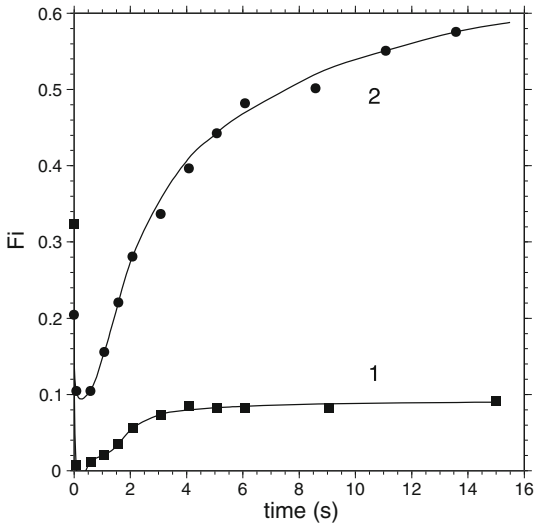


Fig. 3 Variations of the F_i level on bacteria in anaerobic conditions. *Curves 1 and 2* the fluorescence *F* is plotted as a function of the time following 2-min or 1-h continuous illumination, respectively

Fig. 1) or 1 h by continuous red light (curve 2). The fluorescence level reached after 1-h illumination (0.2) is lower than that measured after 2-min illumination (0.32) showing that the fraction of active RCs including an oxidized Q_A increases with the time of illumination. After cessation of the illumination, the level of F_i is plotted as a function of time. One second after cessation of the light, the F_i level is higher after 1-h illumination (0.105) than after 2-min illumination (0.008) suggesting that the reductive power generated in the light increases with the time illumination. For longer dark times, the F_i level is measured 10 ms before each flash of a series. The F_i level increases as a function of the time and reaches a steady-state level of 0.095 after 2-min illumination or 0.58 after 1-h illumination. This shows that the reductive power is an increasing function of the time of illumination.

Illuminating the cells under anaerobiosis has, therefore, a dual effect on the redox state of Q_A. Shortly after the cessation of illumination, all Q_A and part of the quinone pool are oxidized. For longer times, illumination increases the reducing power of the cells that is maximal after 1-h illumination. This reducing power is likely generated by the light-induced oxidation of succinate via complex II and complex I.

Effect of CCCP on the internal redox state of *Rba. sphaeroides* Ga

The dual effect on the quinones redox state of a continuous illumination could possibly be due to the reverse electron transfer occurring at the level of the complex I induced by the light-induced membrane potential (Dupuis et al. 1997;

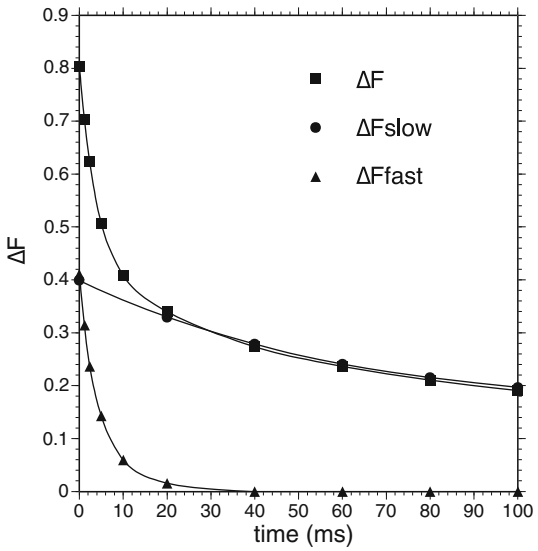


Fig. 4 Fluorescence decay following a flash for bacteria placed under anaerobic conditions. The bacteria were placed under anaerobic conditions and excited by two saturating flashes spaced by 30 s after 5-min dark adaptation. *Closed squares* ΔF following the first 100 ms after the flash. *Closed circles* deconvolution of the slow phase of the fluorescence extrapolated to time 0. *Closed triangles* fast phase of the fluorescence obtained by subtracting the slow phase from the total fluorescence change. The kinetics shown is the average of the kinetics measured after the two successive flashes. This averaging cancels the effect of the two small-period oscillations observed during a series of flashes

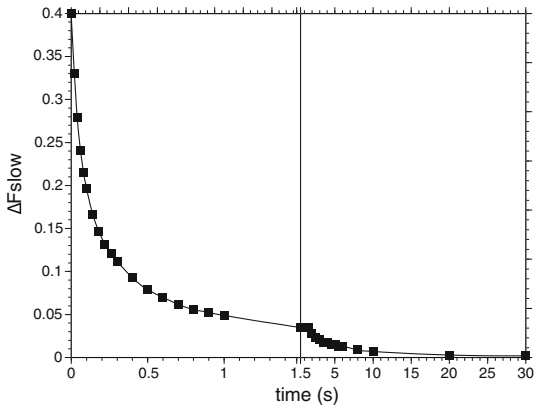


Fig. 5 ΔF_{slow} for the experiment shown in Fig. 4 but for longer times

Herter et al. 1998; Tichi et al. 2001). This reverse process induces the formation of NADH by oxidizing the quinone pool in the presence of a membrane potential. In order to confirm the implication of the electrochemical gradient in the control of the redox state of the ubiquinone we analyzed the effect of the addition of CCCP, an uncoupler known to induce protons leak through the membrane. In the presence of 20 μM CCCP, the membrane potential measured during 2-min illumination is ∼15 % of that measured in the absence of inhibitor and, after switching off the light, collapse to zero in less than 1 s (data not shown). In the presence of CCCP, only 22 % of the total numbers of RCs are open after 1 s of dark following 2-min illumination, as shown by the measurement of the number of charge separations (Fig. 2, close circles). This is at variance to the observation in the absence of CCCP where all RCs are oxidized (Fig. 2, closed squares). During the following dark period, the number of open RCs increases reaching a steady state in ∼2 min, slightly lower than that measured in the control.

Analysis of the kinetics of the decay of the flash-induced fluorescence

In the experiments described in Figs. 4 and 5, the bacteria were placed under anaerobic conditions and excited by two saturating flashes spaced by 30 s after 5-min dark adaptation. The kinetics shown in Figs. 4 and 5 are the average of the kinetics measured after these two flashes. This averaging cancels the effect of the two small-period oscillations observed during a series of flashes. Extrapolation to time zero of the decay kinetics is proportional to the fraction of RCs in the $Q_AQ_BH_2$ state prior to the flash (see Materials and methods). The fluorescence decay displays multiphasic kinetics with two phases of equal amplitude, a fast phase completed in ∼20 ms (Fig. 4) and a slow phase completed in ∼30 s highly multiphasic and close to a second-order process (Fig. 5). We have previously interpreted the biphasism and the equality between the amplitude of the fast and slow phases in a model in which a dimer of RCs and 1 cyt bc_1 complex are organized in supercomplexes. This point will be discussed in more details in the Discussion section.

Figure 6 displays the fluorescence decay kinetics measured after flashes given at different F_i levels. Curve 1 corresponds to the experiment reported in Figs. 4 and 5. Curves 2, 3, and 4 correspond to the kinetics measured 15 min after a 2-min illumination, and 2 min 30 s or 15 min after 1-h illumination, respectively. One striking point in this experiment is that the amplitude and kinetics of ΔF are nearly identical although a large difference in the F_i level is observed, i.e., 0.017, 0.092, and 0.367, respectively. This implies that the RCs involved in the flash-induced fluorescence increase do not experience the same redox potential than those linked to the F_i increase. This suggests that a rapid thermodynamic equilibrium does not occur between quinones in the intracytoplasmic membrane. Such equilibrium slowly occurs between 2 min 30 s and 15 min of dark after 1-h illumination as shown by the 30 % decrease in the ΔF (Fig. 6, curve 4). We propose that this lack of thermodynamic equilibrium is due to the different membrane localizations of the RCs, with a large number of RCs in the chromatophores and a minor fraction in the non-

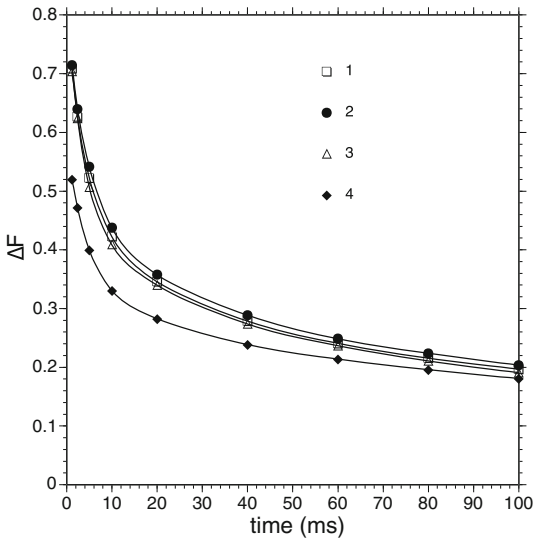


Fig. 6 Comparison of the flash-induced fluorescence decay kinetics for dark-adapted or preilluminated samples. *Curve 1* 5-min dark adaptation in anaerobiosis; *curve 2* after 15 min of dark following a 2-min preillumination; *curve 3* after 2 min 30 s of dark following 1-h preillumination; *curve 4* after 15 min of dark following 1-h preillumination

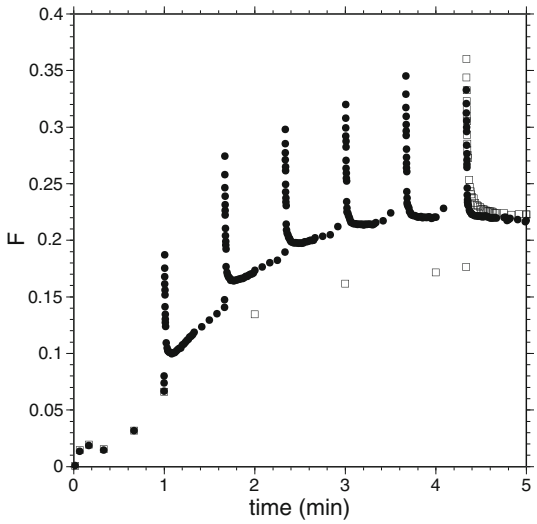


Fig. 7 Variation of F for bacteria placed under anaerobic conditions and excited by a series of flashes or by a single flash fired after 4 min 20 s after the end of the preillumination. To obtain a stationary state, the sample was subjected to light–dark cycles (2-min light to 9-min dark). Note that the first detection times (from 1.2 ms to 300 ms) of fluorescence level following an exciting flash are omitted for sake of clarity. The maximum flash-induced fluorescence varies from 0.867 to 0.929

invaginated part of the membrane. The maximal value of F obtained after a full reduction of all Q_A following a continuous illumination of a sample in the presence of 10 μM PMS is equal to 2.03 (data not shown). We assume that, after few mins of dark following 1-h illumination, the fluorescence increases ($F_i = 0.34$) corresponding to the reduction of most of the Q_A localized in the non-invaginated part of the membrane while most of Q_A localized in the chromatophore are still oxidized. On this basis, the RCs present in the non-invaginated part of the membrane correspond to ~17 % of the total number of RCs. This value well matches with the estimate of the fraction of RCs localized in non-invaginated part of the membrane (Verméglio et al. 1993).

The RCs present in the non-invaginated part of the membrane in the $Q_AQ_B^-$ state are progressively reduced by the quinone pool during the first minutes of dark that follows a continuous illumination (Fig. 7, curve 1). This reduction occurs according to the equations $Q_AQ_B^- \Leftrightarrow Q_A^-Q_B$ and $Q_A^-Q_B + QH_2 \Leftrightarrow Q_A^-Q_BH_2 + Q$ (Kleinfeld et al. 1984). The RCs which were initially in the Q_AQ_B state are converted slowly to the $Q_AQ_BH_2$ state by the reduced quinone pool during the dark period. These RCs are responsible for the irreversible (in the 30 s time scale) fluorescence increase induced by a single flash fired 4 min 20 after the preillumination (Fig. 7, curve 1). We propose that these RCs remain in their reduced form after flash excitation because the oxidized quinones formed by the cyt bc_1 complex are rapidly reduced by complexes I present in a large concentration in this part of the membrane and could not, therefore, interact with the reduced RCs. If the sample is subjected to a series of flashes (Fig. 7, curve 2) the RCs in the $Q_AQ_BH_2$ state are progressively photoreduced in the $Q_A^-Q_BH_2$ state and as expected, the same F_i level is obtained after a single flash or a series of flashes.

Analysis of the kinetics of the decay of flash-induced fluorescence as a function of the dark time following a continuous illumination

In Figs. 8 and 9 the bacteria were submitted to several cycles, 2-min light to 2-min dark. A stationary state for F_i and ΔF values is obtained after more than four cycles. The kinetics of the fluorescence decay has been studied after a saturating flash fired at various times following the cessation of the continuous illumination. The fluorescence decay always displays a multiphasic kinetics with a fast phase completed in ~20 ms and a slow phase. The amplitudes of the fast (ΔF_{fast}) and of the slow phases (ΔF_{slow}) are plotted as a function of the dark time after the cessation of illumination (Fig. 10). In less than 5 s of dark, ΔF_{fast} reaches an amplitude close to its maximum value. ΔF_{slow} increases from nearly zero to its maximal value with a $t_{½}$ ~10 s.

The kinetics of the fast fluorescence phase ($t_{½} = 3.5$ ms, Fig. 4 ΔF_{fast}) is close to an exponential function and do not depend on the time between the preillumination and the flash excitation (Fig. 8). The close similarity between this fast

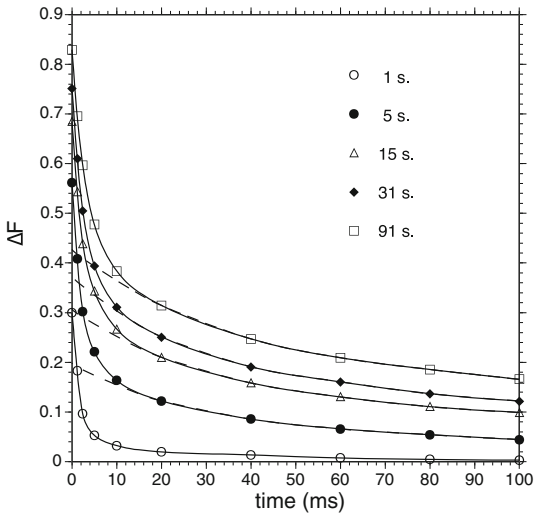


Fig. 8 Kinetics of fluorescence changes induced by a single flash fired at various times (from 1 s to 91 s) after a 2-min continuous illumination for an anaerobic suspension of cells. The *dashed lines* represent the extrapolation of the contribution of the slow phase in the short time scale

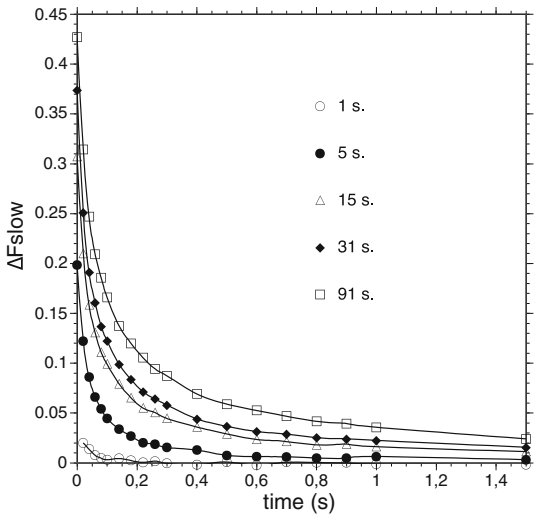


Fig. 9 Kinetics of the fluorescence slow phases (ΔF_{slow}) measured in the same experiment as Fig. 8

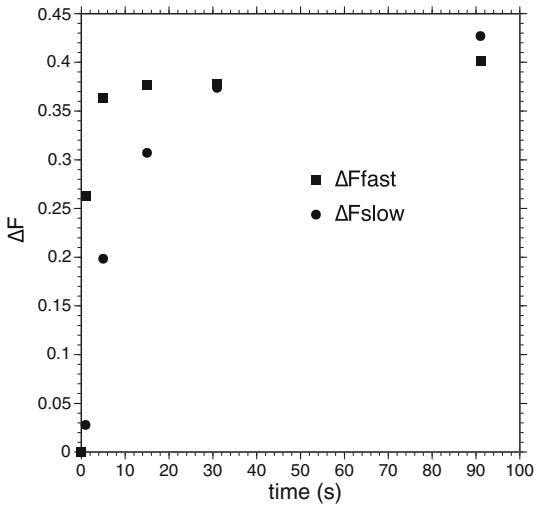


Fig. 10 Amplitudes of the fast phase (ΔF_{fast}, *closed circles*) and the slow phase (ΔF_{slow}, *closed triangles*). Data from the experiments presented in Fig. 8

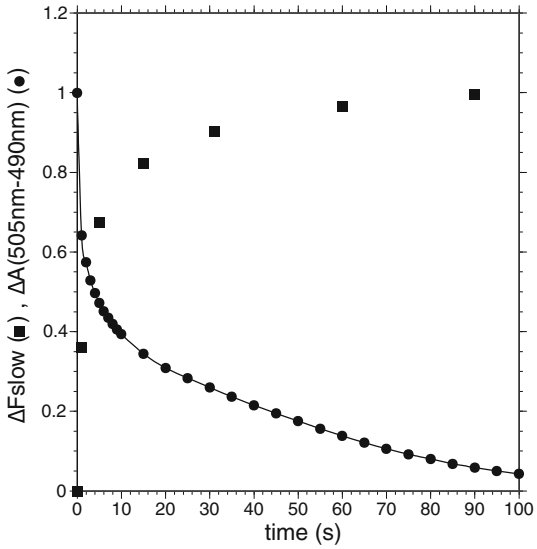


Fig. 11 Comparison between the amplitude of the decrease of the carotenoid bandshift ($\Delta A_{505\ nm\ -\ 490\ nm}$, *closed circles*) after a 2-min continuous illumination and the amplitude of the slow fluorescence phase (ΔF_{slow}, *closed squares*) as a function of the dark time after light cessation. In both cases the first measurement is done 1 s after the end of the illumination

kinetics of Q_A^- oxidation and the kinetics of the flash-induced increasing phase of the membrane potential linked to the cyt bc_1 complex turnover (data not shown) suggests that Q_A^- is oxidized by the quinone formed at the cyt bc_1 level. This implies that about half of the RCs is localized at close proximity of a cyt bc_1 complex.

The other fraction of RCs is slowly reduced by the quinone present in the membrane (Fig. 9). The lack of slow phase after 1-s dark reflects the presence of an excess of free oxidized quinones and implies that these RCs are in the Q_AQ_B state. The increase of the amplitude of the slow fluorescence phase observed in the min time range indicates a progressive increase of the fraction of the RCs in the $Q_AQ_BH_2$ state at the expense of the centers in the Q_AQ_B state due to the reduction of the quinone pool. This decrease in quinone concentration is also associated with an increase of the half time of the slow phase (Fig. 10).

In Fig. 11, we compare the increase of the amplitude of the slow phase as a function of the dark time following a 2-min continuous illumination to the decrease of

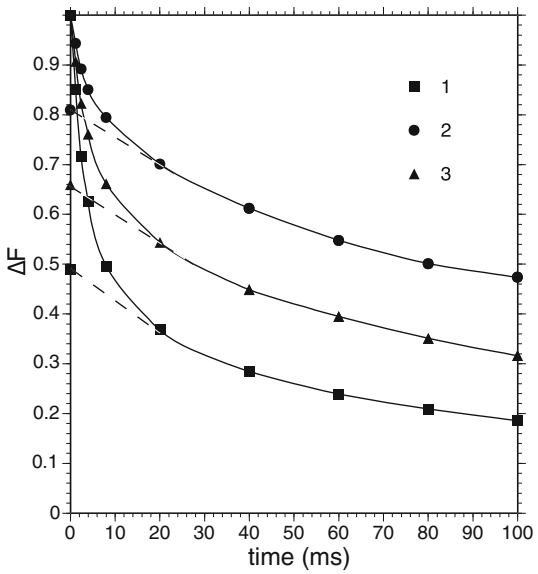


Fig. 12 Kinetics of fluorescence changes induced by a single flash of various intensities. The bacteria were first illuminated by 2-min continuous light, dark-adapted for more than 10 min and illuminated by single flashes of three different intensities. *Curve 1* corresponds to kinetics observed after excitation by a saturating flash, *curve 2* and *curve 3* after a flash that hits 6 and 30 % of the RCs, respectively. These three kinetics are normalized to emphasize the relative amplitude of the fast and slow phases

membrane potential measured in the same condition. We thus propose that, at the end of the illumination, the light-induced membrane potential induces the oxidation of a fraction of the quinone pool via complex I in less than 1 s. In a longer time scale, the increase in the amplitude of the slow phase that reflects the decrease in the quinone concentration correlates with the decay of the membrane potential in agreement with the hypothesis that the latter induces the oxidation of quinones.

Relative amplitude of the fast phase and slow phases as a function of the flash energy

In Fig. 12, the bacteria were first illuminated by 2-min continuous light and dark-adapted for more than 10 min in order to get a steady-state concentration of oxidized Q_A. The bacteria are then illuminated by single flashes of various intensities. Curve 1 is the kinetics measured after a saturating flash that displays a fast and a slow phase of equal amplitude as already shown. If the exciting flash hits only 6 % of the RCs, the relative amplitude of the fast phase is largely reduced (19 % of the total) (Fig. 12 curve 2). For an exciting flash that hits 30 % of the RCs, the relative amplitude of the fast phase is 34 % (Fig. 12 curve 3). This experiment will be interpreted in the Discussion section.

Discussion

Organization of the photosynthetic chains

The supramolecular organization of the photosynthetic chain in *Rba. sphaeroides* is still a matter of debate. We previously proposed that the photosynthetic electron chains localized in the chromatophores are organized in supercomplexes. This model based on kinetics and functional studies has been challenged by Crofts et al. (1983, 1998) and Fernandez-Velasco and Crofts (1991) who proposed that cyt c_2 and quinols visit up to 5 cyt bc_1 dimers. Moreover, analysis of the membrane organization of photosynthetic complexes of *Rba. sphaeroides* by AFM (Bahatyrova et al. 2004) showed dimeric RC-LH1 complexes closely associated to LH2 complexes, but no cyt bc_1 complexes could be detected.

In the present study, we confirm that, at room temperature, the primary acceptors of these RCs are reoxidized according to a fast and a slow phase of equal amplitude as already observed at a lower temperature (Joliot at al. 2005). Under anaerobic condition, the photo-reduced RC could only be reoxidized by the quinone formed at the level of the cyt bc_1 complex. Several arguments are supportive of a close proximity between the RCs associated with the fast oxidation and cyt bc_1 complexes. First, the kinetics of the fast phase is very similar to those of the slow phase of the membrane potential linked to the cyt bc_1 complex turnover. Second, the fast reoxidation kinetics is independent of the redox state of quinone pool as shown in Fig. 8. Taking into account that both RC-LH1 and cyt bc_1 complexes are organized in dimers, that the fast oxidation phase is linked to half of the RCs, and that there are about two RCs per cyt bc_1 complex in the chromatophores (Crofts et al. 1983), one deduces that all the cyt bc_1 complexes present in the chromatophores are localized at proximity of a dimeric RC-LH1 complexes in agreement with the supercomplex model (Scheme 1). In this model, the fast fluorescence phase corresponds, therefore, to the reoxidation of the Q_A^- of the RC-LH1 complex, directly connected to the cyt bc_1 complex (named RCc). The other RC of the RC-LH1 dimer, which is open on the membrane and named RCd, can only be reoxidized by the oxidized quinones formed by the cyt bc_1 complex connected to the RCcs in the $Q_AQ_B^-$ state prior to the flash excitation for which the Q_A does not necessitate a reoxidation. Their reoxidation corresponds to the slow fluorescence phase.

In a model where quinones could diffuse, an alternate possibility to explain the fast and slow fluorescence phases is the presence of RC-LH1 complexes both near and distant from cyt bc_1 complexes. Since both RC-LH1 and cyt bc_1 complexes are organized in dimers this proposal implies a

ratio of 1 cyt bc_1 complex per RC at odds with the classical view ruling out this possibility.

Another aspect of the supercomplex model is the confinement a single cyt c_2 trapped between 2 RC-LH1 and 1 cyt bc_1 complex (Joliot et al. 1989). This confinement has important consequences on the kinetics of Q_A^- oxidation in function of the flash intensity. If one assumes that under anaerobic conditions the cyt b_{HP} is reduced for all the cyt bc_1 complexes, the oxidation of Q_A^- required the formation of an oxidized quinone by the cyt bc_1, i.e., a double turnover of this complex. In the supercomplex model, only half of the RCs will be oxidized by the Q formed at the cyt bc_1 level. For a flash which hits 30 % of the RCs, the probability to excite both RCs of a dimer is equal to 9 %. Therefore, a double turnover of the cyt bc_1, *i.e.,* a fast oxidation of Q_A^- will occur for these 9 % of supercomplexes. The relative amplitude of the fast phase will lead to a ratio of 0.09/0.3 = 0.30. Applying the same reasoning for a flash hitting 6 % of RCs, the amplitude of the fast phase will be 6 % of the total changes. In the diffusional model, the probability for transferring two or more positive charges between an oxidized cyt c_2 and a given cyt bc_1 complex could be computed according to the Poisson distribution. For a flash that hits 6 % or 30 % of the RCs, about 1 % or 12 % of the cyt bc_1 would have trapped two or more positive charges. The relative amplitudes of the fast phase measured on Fig. 12 are equal to 0.34 and 0.19 for flashes exciting 30 % and 6 % of RCs, respectively. These values are much higher than those deduced for a diffusional model; therefore, ruling out this hypothesis. The experimental value for a flash hitting 30 % of RCs (0.34) is slightly higher than the value (0.30) computed for the supercomplex model. For a flash hitting 6 % of the RCs, the experimental value (0.19) is significantly higher than the computed one (0.06). This indicates the presence of a oxidized cyt b_{HP} for a small part of the cyt bc_1 complexes (about 10 %) which, therefore, does not require a double turnover for quinone production. In any case, a much better agreement is obtained between the experimental and computed value for the supercomplex model than for the diffusional model.

Modulation of redox state of quinones by light under anaerobiosis

The fluorescence measurements described in the present paper clearly show that illuminating *Rba. sphaeroides* cells under anaerobiosis has a dual effect on the redox state of Q_A. Shortly after the cessation of illumination, all Q_A and part of the quinone pool are oxidized. Then the reducing power of the cells slowly increases and reaches a maximal level after few tens of seconds or few minutes depending of the batch of cells and the length of illumination, being maximal after 1 h. This dual effect on the quinones redox state by a continuous illumination is linked to the reverse electron transfer induced by the light-induced membrane potential occurring at the level of the complex I (Dupuis et al. 1997; Herter et al. 1998; Tichi et al. 2001). Such reverse process induces the reduction of NAD^+ by oxidizing of the quinone pool. This process could induce a large and progressive reduction of NAD^+ during illumination at the expense of reduced carbon sources like succinate via complex II (Keister and Yike 1967). Our proposal is in agreement with the effect of uncouplers like CCCP. First, the lifetime of the oxidation phase correlates with the lifetime of the light-induced membrane potential. Moreover, addition of CCCP completely modified the redox state of the RCs during and after a 2-min illumination. 78 % of the RCs are closed after 1 s of dark following a 2-min illumination in the presence of CCCP while they are all oxidized in a control experiment. The observation that the quinones of the chromatophores compartment are rapidly oxidized implies that some complexes I are localized in this membrane compartment. This is in agreement with previous data which have shown that reduction of NAD^+ occurs in isolated chromatophores when subjected to continuous illumination (Knaff 1978; Nore 1989).

References

Allen JP, Feher G, Yeates TO, Rees DC, Deisenhofer J, Michel H, Huber R (1986) Structural homology of reaction centers of *R. sphaeroides* and *R. viridis* as determined by X-ray diffraction. Proc Natl Acad Sci USA 83:8589–8593

Allen JP, Feher G, Yeates TO, Komiya H, Rees DC (1987) Structure of the reaction center from *Rhodobacter sphaeroides* R-26 II. The protein subunits. Proc Natl Acad Sci USA 84:6162–6166

Bahatyrova S, Frese RN, Siebert CA, Olsen JD, van der Wer KO, van Grondelle R et al (2004) The native architecture of a photosynthetic membrane. Nature 430:1058–1062

Bylina EJ, Youvan DC (1987) Genetic-engineering of herbicide resistance-saturation mutagenesis of Isoleucine-229 of the reaction center L-subunit. Z Naturforsch J Biosci 42:769–774

Comayras R, Jungas C, Lavergne J (2005) Functional consequences of the organization of the photosynthetic apparatus in *Rhodobacter sphaeroides*—I Quinone domains and excitation transfer in chromatophores and reaction center center antenna complexes. J Biol Chem 280:11203–11213

Crofts AR, Meinhardt SW, Jones KR, Snozzi M (1983) The role of the quinone pool in the cyclic electron-transfer chain of *Rhodopseudomonas sphaeroides*: a modified Q-cycle mechanism. Biochim Biophys Acta 723:202–218

Crofts AR, Guergova-Kuras M, Hong SJ (1998) Chromatophore heterogeneity explains phenomena seen in *Rhodobacter sphaeroides* previously attributed to supercomplexes. Photosynth Res 55:357–362

Deisenhofer J, Epp O, Miki K, Huber R, Michel H (1984) X-ray structure analysis of a membrane protein complex: electron density map at 3 Å resolution and a model of the chromophores

of the photosynthetic reaction center from *Rhodopseudomonas-viridis*. J Mol Biol 180:385–398

Deisenhofer J, Epp O, Miki K, Huber R, Michel H (1985) Structure of the protein subunits in the photosynthetic reaction centre of *Rhodopseudomonas viridis* at 3 Å resolution. Nature 318:618–624

Dupuis A, Peinnequin A, Darouzet E, Lunardi J (1997) Genetic disruption of the respiratory NADH-ubiquinone reductase of *Rhodobacter capsulatus* leads to an unexpected photosynthesis-negative phenotype. FEMS Microbiol Lett 148:107–114

Duysens LNM (1952) Transfer of Excitation Energy in Photosynthesis. PhD Thesis, Leiden University, The Netherlands

Duysens LNM, Sweep G (1957) Fluorescence spectrophotometry of pyridine nucleotide in photostynthesizing cells. Biochim Biophys Acta 25:13–16

Feher G (1971) Some chemical and physical properties of a bacterial reaction center particle and its primary photochemical reactants. Photochem Photobiol 14:373–388

Feher G (1998) Three decades of research in bacterial photosynthesis and the road leading to it: a personal account. Photosynth Res 55:1–40

Fernandez-Velasco J, Crofts AR (1991) Complexes or super complexes: inhibitor titrations show that electron transfer in chromatophores from *Rhodobacter sphaeroides* involves a dimeric UQH2:cytochrome *c*2 oxidoreductase, and is delocalized. Biochem Soc Trans 19:588–593

Francia F, Wang JM, Venturoli G, Melandri BA, Rigaud JL, Barz WP, Oesterhelt D (1999) The reaction center–LH1 antenna complex of *Rhodobacter sphaeroides* contains one PufX molecule which is involved in dimerization of this complex. Biochemistry 38:6834–6845

Herter SM, Kortlüke CM, Drews G (1998) Complex I of *Rhodobacter capsulatus* and its role in reverted electron transport. Arch Microbiol 169:98–105

Hunter CN, Daldal F, Thurnauer MC, Beatty JT (eds) (2009) The Purple Photosynthetic Bacteria. In: Advances in Photosynthesis and Respiration vol 28. Springer, Dordrecht

Joliot P, Verméglio A, Joliot A (1989) Evidence for supercomplexes between reaction centers, cytochrome c_2 and cytochrome bc_1 complex in *Rhodobacter sphaeroides* whole cells. Biochim Biophys Acta 975:336–345

Joliot P, Joliot A, Verméglio A (2005) Fast oxidation of the primary electron acceptor under anaerobic conditions requires the organization of the photosynthetic chain of *Rhodobacter sphaeroides* in supercomplexes. Biochim Biophys Acta 1706:204–214

Jungas C, Ranck JL, Rigaud JL, Joliot P, Verméglio A (1999) Supramolecular organization of the photosynthetic apparatus of *Rhodobacter sphaeroides*. EMBO J 18:534–542

Keister DL, Yike NJ (1967) Energy-linked reaction in photosynthetic bacteria. I. Succinate-linked ATP driven NAD+ reduction by *Rhodospirullum rubrum* chromatophores. Arch Biochem Biophys 121:415–422

Kleinfeld D, Abresch EC, Okamura MY, Feher G (1984) Damping of oscillations in the semiquinone absorption in reaction centers after successive flashes: determination of the equilibrium between $Q_A^- Q_B$ and $Q_A Q_B^-$. Biochim Biophys Acta 765:406–409

Knaff D (1978) Reducing potentials and the pathway of NAD+ reduction. In: Clayton RK, Sistrom WR (eds) The Photosynthetic Bacteria. Plenum Press, New York, pp 629–638

Marrs B (1974) Genetic recombination in *Rhodopseudomonas capsulata*. Proc Natl Acad Sci USA 71:971–973

McEwan AG, Cotton NPJ, Ferguson SJ, Jackson JB (1985) The role of auxiliary oxidants in the maintenance of a balanced redox poise for photosynthesis in bacteria. Biochim Biophys Acta 810:140–147

Michel H (1982) Three-dimensional crystals of a membrane protein complex: the photosynthetic reaction center from *Rhodopseudomonas viridis*. J Mol Biol 158:567–572

Nore BF (1989) ΔpH Driven Energy-linked NAD+ reduction in *Rhodospirillum rubrum* chromatophores. Arch Biochem Biophys 274:285–289

Olson JM, Amesz J (1960) Action spectrum for fluorescence excitation in pyridine nucleotide in photosynthetic bacteria and algae. Biochim Biophys Acta 36:14–24

Olson JM, Duysens LNM, Kronenberg OH (1959) Spectrofluorometry of pyridine nucleotide reactions in *Chromatium vinosum*. Biochim Biophys Acta 36:125–131

Reed DW, Clayton RK (1968) Isolation of a reaction center fraction from *Rhodopseudomonas sphaeroides*. Biochem Biophys Res Commun 30:471–475

Scheuring S, Francia F, Busselez J, Melandri BA, Rigaud JL, Levy D (2004) Structural role of PufX in the dimerization of the photosynthetic core complex of *Rhodobacter sphaeroides*. J Biol Chem 279:3620–3626

Siebert CA, Qian P, Fotiadis D, Engel A, Hunter CN, Bullough PA (2004) Molecular architecture of photosynthetic membranes in *Rhodobacter sphaeroides*: the role of PufX. EMBO J 23:690–700

Tichi MA, Meijer WG, Tabita R (2001) Complex I and its involvement in redox homeostasis and carbon nitrogen metabolism in *Rhodobacter capsulatus*. J Bacteriol 183:7285–7294

Verméglio A, Joliot P, Joliot A (1993) The rate of cytochrome c_2 photooxidation reflects the subcellular distribution of reaction centers in *Rhodobacter sphaeroides* Ga cells. Biochim Biophys Acta 1183:352–360