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Fast oxidation of the primary electron acceptor under anaerobic conditions requires the organization of the photosynthetic chain of *Rhodobacter sphaeroides* in supercomplexes

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Abstract

The kinetics of reoxidation of the primary acceptor Q_a has been followed by measuring the changes in the fluorescence yield induced by a series of saturating flashes in intact cells of *Rhodobacter sphaeroides* in anaerobic conditions. At 0 °C, about half of Q_a^- is reoxidized in about 200 ms while reoxidation of the remaining fraction is completed in several seconds to minutes. The fast phase is associated with the transfer of ubiquinone formed at site Q_o of the cytochrome bc_1 complex while the slowest phase is associated with the diffusion of ubiquinone present in the membrane prior to the flash excitation. The biphasic kinetics of Q_a^- oxidation is interpreted assuming that the electron chain is organized in supercomplexes that associate two RCs and one cyt bc_1 complex, which allows a fast transfer of quinone formed at the level of cyt bc_1 complex to the RCs. In agreement with this model, the fast phase of Q_a^- reoxidation is inhibited by myxothiazol, a specific inhibitor of cyt bc_1. The PufX-deleted mutant displays only the slowest phase of Q_a^- oxidation; it is interpreted by the lack of supramolecular organization of the photosynthetic chain that leads to a larger average distance between cyt bc_1 and RCs.

Keywords: Electron transfer; *Rb. sphaeroides*; Reaction center; Cytochrome bc_1 complex; Supercomplex

1. Introduction

The electron transfer chain of the photosynthetic apparatus of purple bacteria includes two major membranous complexes: the cytochrome bc_1 complex (cyt bc_1) and the reaction center (RC) that collects light energy from the light harvesting complexes (LH) localized in its vicinity. Following light absorption, a charge separation occurs at the RC level. This initiates a cyclic electron transfer where the RC and cyt bc_1 are connected via cyt c_2 in the periplasmic space and ubiquinone (UQ) molecules in the membrane. This light-induced electron transfer leads to the formation of an electrochemical proton gradient and switches on the ATP synthesis.

Thermodynamic analysis of electron transfer reactions on the donor side of the RC led us [1] to propose that the photosynthetic electron transfer chain of *Rhodobacter* (*Rb.*) *sphaeroides* is organized in a supercomplex that includes a dimer of RCs, one cyt bc_1 and one cyt c_2 molecule. This organization agrees with the overall stoichiometry between the RC and cyt bc_1 (RC/cyt bc_1~2) measured for the intracytoplasmic membrane. The thermodynamic equilibrium between the primary donor of the RC and the high-potential carriers of cyt bc_1 (cyt c_1 and Rieske protein) is mediated in less than 1 ms by the cyt c_2 trapped in the supercomplex [1]. On the contrary, the thermodynamic

Abbreviations: AFM, atomic force spectroscopy; cyt, cytochrome; LH, light-harvesting; P_{870}, primary electron donor; Q_a, Q_b, primary and secondary electron acceptors; *Rb.*, *Rhodobacter*; RC, reaction center; UQ, ubiquinone

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equilibration between different supercomplexes is a much longer process that takes more than 1 s [1]. The organization in supercomplexes also explains the occurrence of a light-induced cyclic electron transfer at temperature as low as −20 °C in the absence of antifreeze [2]. In these conditions, the aqueous phase inside the chromatophores and in the periplasmic space is fully frozen, preventing a fast long-range diffusion of cyt c_2. In the same line of evidence, a very efficient electron transfer between the RC and cyt bc_1 of *Rb. capsulatus* is mediated by cyt c_y, a cytochrome that is tightly attached to the membrane and therefore should not diffuse rapidly [3–6].

In *Rb. sphaeroides* chromatophores under oxidizing conditions, Drachev et al. [7] conclude that the reaction between UQH_2 produced upon excitation by the second flash and the cyt bc_1 complex is monomolecular. This result, which implies that quinone exchange between RC and cyt bc_1 complex occurs via a local pool of quinone, can also be interpreted in the frame of the supercomplex hypothesis.

In addition to these thermodynamic and kinetic arguments, biochemical and structural approaches have provided information on the supramolecular organization of the photosynthetic components. Mutants of *Rb. sphaeroides* deleted in LH2 form tubular rather than vesicular intracytoplasmic membranes [8–10]. Freeze-fracture electron microscopy pictures of these tubular membranes reveal a well-ordered arrangement of dimeric particles of 110 Å in diameter. Analysis of negatively stained samples discloses a dimeric association of the RCs surrounded by an open ring of the LH1 but gives no clear evidence for the presence of a cyt bc_1 complex [11]. More recently, a careful analysis of the biochemical composition of these tubular membranes has demonstrated that they do not contain cyt bc_1 complexes [12]. By a biochemical approach, Francia et al. [13] have provided further proof for the dimeric association of RCs in *Rb. sphaeroides*. After detergent solubilization of chromatophores, these authors found two membrane complexes corresponding to monomeric and dimeric RC–LH1 complexes in addition to isolated LH1 and LH2 complexes.

The dimeric association of RCs and the formation of an incomplete LH1 ring require the presence of the PufX polypeptide [13]. This has been recently clearly visualized by AFM studies [12,14]. The PufX polypeptide, encoded by the *pufX* gene localized in the *puf* (photosynthetic formation unit) operon [15,16], is a membrane protein closely associated with the RC–LH1 complex in a 1:1 ratio [14]. The presence of this polypeptide is essential for anaerobic photosynthetic growth in both *Rb. capsulatus* [15] and *Rb. sphaeroides* [16]. The PufX polypeptide is strictly required for the isolation of dimeric RC–LH1 complexes [14]. In addition, Freese et al. [17] have shown by linear dichroism measurements on oriented native membranes that the polypeptide PufX is required for the formation of supramolecular photosynthetic units in a long-range regular array. It is therefore tempting to speculate that this protein plays an essential role in the formation of the open LH1 structure observed in vivo. In the absence of PufX, the RC is surrounded by a complete ring of LH1, which could restrict the diffusion of quinone and therefore the connection between the RC and the cyt bc_1 complex. This hypothesis would readily explain the inability of a mutant deleted in *pufX* (PufX−) to grow under phototrophic anaerobic conditions. However, photosynthetic growth and the light-induced cyclic electron transfer are restored in this mutant when the UQ pool is partially oxidized by the addition of an electron acceptor as trimethylaminooxide (TMAO) or dimethylsulfoxide (DMSO) [18]. These results imply that the supramolecular organization of the photosynthetic apparatus and (or) the open structure of the LH1 in *Rb. sphaeroides* are necessary for an efficient cyclic electron transfer only under stringent anaerobic conditions, i.e., when the UQ pool is mainly reduced.

Is this supramolecular organization of the photosynthetic apparatus of *Rb. sphaeroides* an evolutionary benefit of functional interest? An obvious consequence of the sequestration of the soluble carrier cyt c_2 in the supercomplex is an increase in the rate of electron transfer between membranous complexes due to the increase in the local substrate concentration [1]. However, in the case of the PufX- mutant, whose photosynthetic electron chain is not organized in supercomplexes, the transfer of positive charges from the donor side of the RCs to the cyt bc_1 complex occurs in a similar time range as for the wild type (WT) [18,19]. This implies that the diffusion of cyt c_2 on the surface of the membrane is a fast process, which does not limit the overall rate of the cyclic electron transfer process. On the other hand, the cyclic electron transfer is likely limited by the reoxidation of the primary electron acceptor Q_a^-, owing to the large reduction of the UQ pool under anaerobic conditions.

In this paper, we investigate the consequence of the supramolecular organization of the photosynthetic chain of *Rb. sphaeroides* on the kinetics of Q_a^- oxidation after flash excitation under anaerobic conditions. We conclude that most of the UQ molecules formed at the Q_o site of cyt bc_1 are trapped in the supercomplex in the case of the WT but not in the case of the PufX− strain.

2. Materials and methods

The experiments have been performed with *Rb. sphaeroides* Ga (WT) and the PufX− strains. The WT cells are grown in Hutner medium at 30 °C under light anaerobic conditions. Ga cells were grown for 24 h ("young" cells) or for 72 h ("old" cells). The PufX− mutant was grown for 24 h under dark semiaerobiosis.

Spectrophotometric and fluorescence measurements have been performed in the apparatus developed by D. Béal and P. Joliot, adapted to measurements of light-induced spectral changes in biological material at temperatures between 30 and −50 °C as described in Ref. [2]. The biological material

is placed in measure and reference cuvettes 0.2-mm thick (1.35-cm^2 surface). The absorption level and fluorescence yield are sampled using 2-μs monochromatic flashes given at various intervals after excitation.

The amplitude of the carotenoid shift, proportional to the transmembrane potential, was measured by the difference $\Delta I/I$ (500 nm–490 nm). The relative amount of active RCs has been determined by measuring the membrane potential change 100 μs after a saturating flash excitation. Actinic illumination was provided by a Xenon flash filtered through far-red filter Wratten 89B. The measuring photodiodes are protected from the actinic illumination by a blue Schott filter BG39.

The fluorescence yield is sampled by weak 2-μs flashes at 425 nm. Actinic illumination was provided by a Xenon flash filtered through a blue filter Schott BG39. The measuring photodiodes are protected from the actinic illumination by a far-red filter Wratten 89B.

For both absorption and fluorescence measurements, the cells were suspended in growth medium and dark-adapted at 0 °C for more than 20 min in aerobic condition. Such a procedure induces full oxidation of the primary acceptor Q_a and oxidation of most of the secondary acceptor Q_b. The cells are then placed in the cuvettes and maintained in the dark at 20 °C for ~5 min. Anaerobic conditions, reached in less than 5 min, induced the reduction of most of the UQ pool but not that of the primary acceptor Q_a. Following this incubation period, the temperature of the cuvettes is set at 0 °C, in order to decrease the rate of the cyclic electron transfer, allowing a better time resolution of the light-induced events.

3. Results and discussion

3.1. Flash-induced fluorescence yield of cells placed under anaerobic conditions

The flash-induced redox changes of the primary acceptor Q_a of intact cells of *Rb. sphaeroides* have been determined by measuring the fluorescence yield. A flash excitation induces a fluorescence yield increase, associated with both the oxidation of the primary donor P_{870} and the reduction of the primary acceptor Q_a. In anaerobic conditions and at 0 °C, the reduction of P_{870} measured at 603 nm is completed in less than 3 ms (not shown). Thus, following flash excitation, the fluorescence yield sampled beyond 4 ms monitors exclusively the concentration of Q_a^-.

In a first set of experiments, we have determined the redox state of both the primary and secondary quinonic electron acceptors in dark-adapted cells placed under anaerobic conditions. No change in the fluorescence yield is observed when cells are subjected to transition from aerobic to anaerobic conditions, showing that all RCs include an oxidized primary acceptor Q_a. The redox state of the secondary acceptor Q_b has been determined by comparing the fluorescence yield sampled at 4 ms after one, two or three flashes given 5 ms apart. A time of 4 ms is long enough to achieve the reoxidation of Q_a^- for RCs in the $Q_a^-Q_b$ or $Q_a^-Q_b^-$ states, but too short for those in $Q_a^-Q_bH_2$ state. The fluorescence yields measured after the first and second flashes are 0.87 and 0.92 of the maximum fluorescence yield, respectively. Taking into account a miss coefficient of 0.07, we estimate that, in dark-adapted cells in anaerobic conditions, about 93% of the RCs are in the $Q_aQ_bH_2$ state while the remaining fraction is in the Q_aQ_b state.

Fig. 1 shows the kinetics of the fluorescence yield measured on dark-adapted young cells of *Rb. sphaeroides* Ga strain submitted to a series of four saturating flashes given 20 s apart. The fluorescence decay is multiphasic, irrespective of the flash number. A first phase is completed in less than 200 ms with a half time (20 to 30 ms) independent of its amplitude. The second phase is completed in more than 20 s. The amplitude of both phases oscillates with a periodicity of two with a maximum after each odd flash. The amplitude of the fast phase induced by the first flash is close to the half of the initial fluorescence increase. The same experiment per-

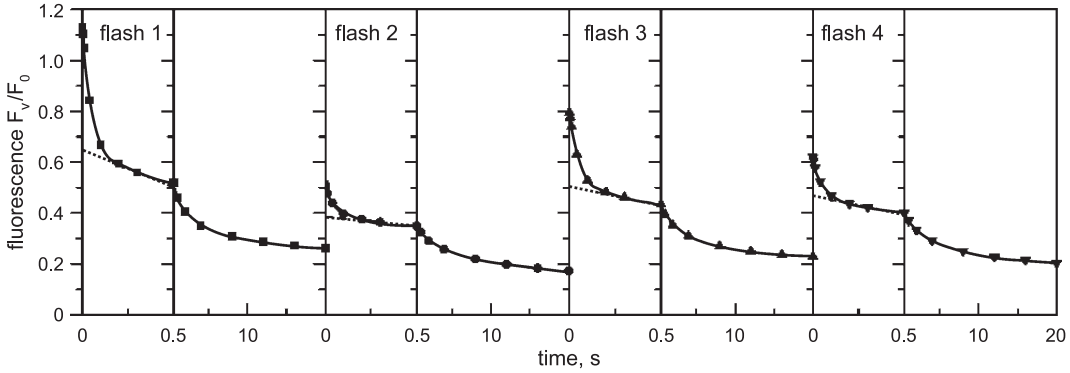


Fig. 1. Fluorescence yield changes induced by a series of four saturating flashes given 20 s apart to dark-adapted Ga cells. First illumination of dark-adapted cells (see Sections 3.1). After each actinic flash, fluorescence yield at time zero is computed by extrapolation of the decay kinetics.

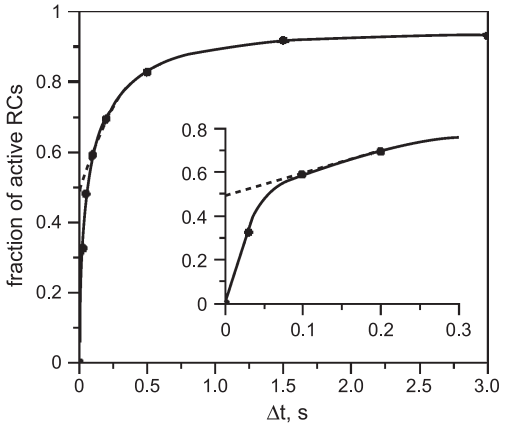


Fig. 2. Concentration of active RCs measured during a series of saturating flashes as a function of the time interval Δt between flashes. The cells are submitted to a series of 20 flashes separated by 1-min dark. The relative concentration of active RCs is determined by measuring the absorption change $\Delta I/I$ (500–490 nm) sampled 100 μs after each actinic flash. The concentration of active RCs is normalized to the maximum concentration of active centers present after 1-min dark. The concentration of active RCs reaches a steady-state value after the fifth flash whatever the time interval Δt between flashes. This steady-state value is plotted as a function of Δt. A full reoxidation of Q_a^- is reached for Δt~10 s.

formed at room temperature (data not shown) displays a similar behavior, but with faster kinetics (about fourfold).

Following a pre-illumination under continuous light or by a flash series given to dark-adapted cells in anaerobic conditions, two unexpected results arose. First, a fraction of Q_a^- (15 to 20%) stays reduced irrespective of the duration of subsequent dark-adaptation. Second, illumination by a series of saturating flashes leads to period-2 oscillations with an inverted phase (shown in Fig. 8) when compared to the oscillations measured with dark-adapted material (Fig. 1). The behavior observed on a dark-adapted sample can be obtained again only by aeration of the sample.

3.2. Turnover of the cyclic electron transfer chain

In Fig. 2, the fraction of centers with an oxidized Q_a has been determined by measuring the flash-induced membrane potential. The cells have been submitted to a series of 20 flashes separated by 1-min dark period. Beyond the fifth flash, the concentration of active RCs at the time the flash is fired reaches a steady-state level whatever is the time interval Δt between flashes. This steady-state level is plotted as a function of Δt. As shown in Fig. 2, about half of Q_a^- is reoxidized in less than 200 ms ($t_{1/2}$~20–30 ms). The other half is reoxidized according to multiphasic kinetics completed in ~10 s. This agrees with fluorescence measurements performed in the same experimental conditions (not shown). We therefore conclude that, in the conditions we used, the fluorescence yield is close to be proportional to the concentration of Q_a^-.

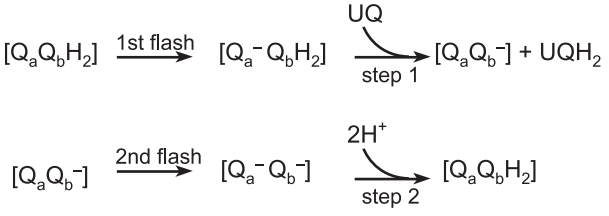
3.3. Kinetics of flash-induced fluorescence yield of young and old cells under anaerobic conditions

In Fig. 3, the kinetics of the fluorescence decay after a single saturating flash has been compared for dark-adapted young (curves 1) and old (curves 2) cells. One observes (Fig. 3A) that both the half time and the amplitude of the fast phase are similar whatever the growth conditions. For young cells, the initial rate of the fast phase is ~20 times larger than that of the slow phase. The initial rate of the slow phase is about 1.6 times faster for old than for young cells. Moreover, the fraction of Q_a^- that remains reduced 60 s after the flash is ~4 times larger for the young than for the old culture (Fig. 3B). These results suggest that even in anaerobic conditions the membrane includes a small amount of oxidized UQ, the concentration of which is larger in old than in young cells. It implies that the redox potential within the cell is higher for old than for young cells, a difference that may reflect the exhaustion of all the organic reserves in old cells.

To interpret the data obtained with dark-adapted samples, let us assume that the rate of Q_a^- reoxidation is a diffusion-limited process that depends on the concentration of (oxidized) UQ within the membrane prior to flash excitation. If the concentration of UQ is larger than that of the RCs, all the Q_a^- formed by a flash will be eventually reoxidized. Since most of the RCs are initially in the $Q_aQ_bH_2$ state (as discussed in Section 3.1), a series of saturating flashes induces the following reactions (Scheme 1):

Step 1 is a diffusion-limited process while step 2 involves fast electron transfer within the RCs. This model predicts period-2 oscillations of the fluorescence yield when detected at 4 ms, i.e., after completion of step 2, provided the time between flashes is longer than the time required for the completion of step 1.

It is worth noting that similar oscillations are predicted if the quinone formed at the level of site Q_o of the cyt bc_1 complex is taken into account. When the RCs and cyt bc_1 complexes are randomly distributed in the membrane, the quinone UQf formed at the level of the cyt bc_1 complex and the quinone UQd already present in the membrane are both randomly distributed and behave in a similar way. In this model, oscillations with no damping will occur if the sum of UQd+UQf is larger than the concentration of the RCs and if the time interval between flashes is longer than that required for completion of step 1. For shorter time intervals, a fraction



Scheme 1.

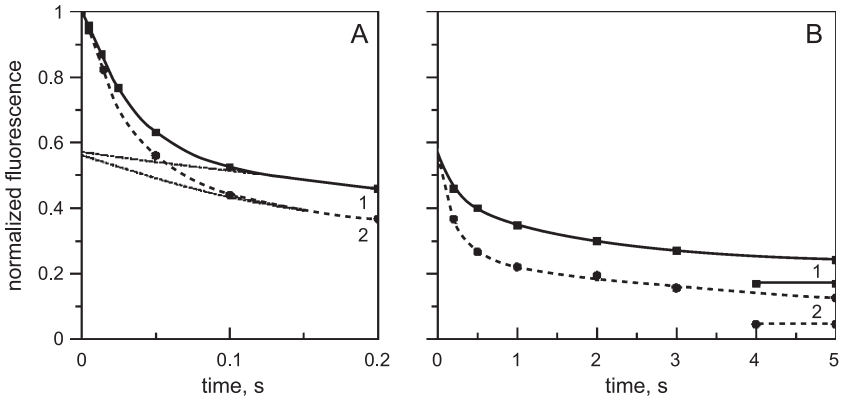


Fig. 3. Kinetics of the fluorescence decay induced by a single saturating flash given to dark-adapted young or old Ga cells (see Section 2). Curve 1: Young cells. Curve 2: Old cells. (A) Dashed lines, extrapolation to time zero of the slow phase. (B) Slow phase of the decay kinetics. Horizontal lines 1 and 2, fluorescence yield reached 60 s after the flash for young and old cells, respectively.

of the RCs stays in the inactive Q_a^- state that introduces a rapid damping of the oscillation. The amplitude of the oscillations only depends on the ratio between the RCs present in a $Q_aQ_bH_2$ or $Q_aQ_b^-$ state before the flash series, and is independent of the stoichiometry between RCs and cyt bc_1 complexes. According to this model, the biphasic fluorescence decay implies that the ratio of the concentration of UQ in a "fast" and a "slow" compartment is equal to the ratio of the initial rates of the fast and slow phases, i.e., a factor ~20 in the case of young cells. (Fig. 3A, curve 1).

3.4. Flash-induced fluorescence yield in the function of the flash intensity

According to Scheme 1, the relative amplitude of the fast and slow phases must be independent of the flash energy, as a weak or strong flash is expected to sample equally the fast and slow compartments.

Fig. 4A shows the fluorescence decay measured with dark-adapted cells submitted to a single flash of different energies. As shown in Fig. 4B, in which the maximum fluorescence yield has been normalized to 1, the relative amplitude of the fast phase is a decreasing function of the flash energy, which is not consistent with Scheme 1. We are thus led to propose that the localization of UQf formed at site Q_o of the cyt bc_1 complex differs from that of UQd present in the membrane prior to the flash excitation. According to a Q-cycle process [20,21], electron transfer reactions within the cyt bc_1 complex are triggered by the transfer of a positive charge from the donor side of RC to cyt c_1 (Scheme 2).

In anaerobic conditions, most of the cyt bc_1 complexes include a reduced cyt b_h and an oxidized cyt b_l. The formation of cyt c_1^+ triggers the formation of UQ at site Q_o (reaction (1)). In reaction (2), UQ is reduced at site Q_i, which leads to the oxidation of both *b* cytochromes. We assume that all of the UQ formed by reaction (1) is transferred to site Q_i. Indeed, if a fraction of UQ formed by reaction (1) were transferred to site Q_b, a corresponding fraction of the cyt bc_1 complex would be in a state including both b hemes reduced and thus unable to undergo further fast turnover reactions in the presence of a reduced quinone pool. When a second positive charge is transferred to cyt c_1, a second concerted process leads to the formation of a second UQ at site Q_o (reaction (3)). This reaction leads to the reduction of cyt b_h^+ while cyt b_l stays oxidized. In this condition, UQ formed at site Q_o (UQf) cannot be reduced at site Q_i and is thus available for the oxidation of $Q_a^{-\cdot}$. The fast reoxidation of Q_a^- implies that UQf is produced at a high concentration in the vicinity of the RCs and, consequently, that cyt bc_1 complexes are localized at short distance of the RCs. This is the case if the electron transfer chain is organized in supercomplexes that associate two RCs, one cyt bc_1 and one cyt c_2 (see Section 1). In the supercomplex model, a saturating flash given to dark-adapted cells induces the formation of two Q_a^- on the acceptor side of the RC's dimer and two positive charges on the donor side. These two positive charges induce a double turnover of the cyt bc_1 complex (reactions (1)–(3)) that leads to the formation of a single UQf that induces the fast oxidation of only half of Q_a^-, as actually observed (Figs. 1, 3, 4)). On the other hand, under sub-saturating flash excitation, a fraction of the supercomplexes undergoes a single charge separation and a single turnover of the cyt bc_1 complex (reactions (1) and (2)) that do not lead to the formation of UQf. Thus, the amplitude of the fast phase will decrease more rapidly than the number of RCs excited by the flash as shown in Fig. 4B. For the fraction of supercomplexes that undergoes a double charge separation, the kinetics of the fast phase should be independent of its amplitude. This is shown in Fig. 4B, in which the fast phase, close to an exponential function, displays a half time (~30 ms) independent of the flash energy. The kinetics of formation of UQf at site Q_o of the cyt bc_1 complex has been estimated from the analysis of the kinetics of reduction of cyt c_1+cyt c_2. At 0 °C, this process is completed in ~100 ms, a time close to the duration of the fast reoxidation phase of Q_a^-. It suggests that this phase is controlled by the rate of both quinone formation and

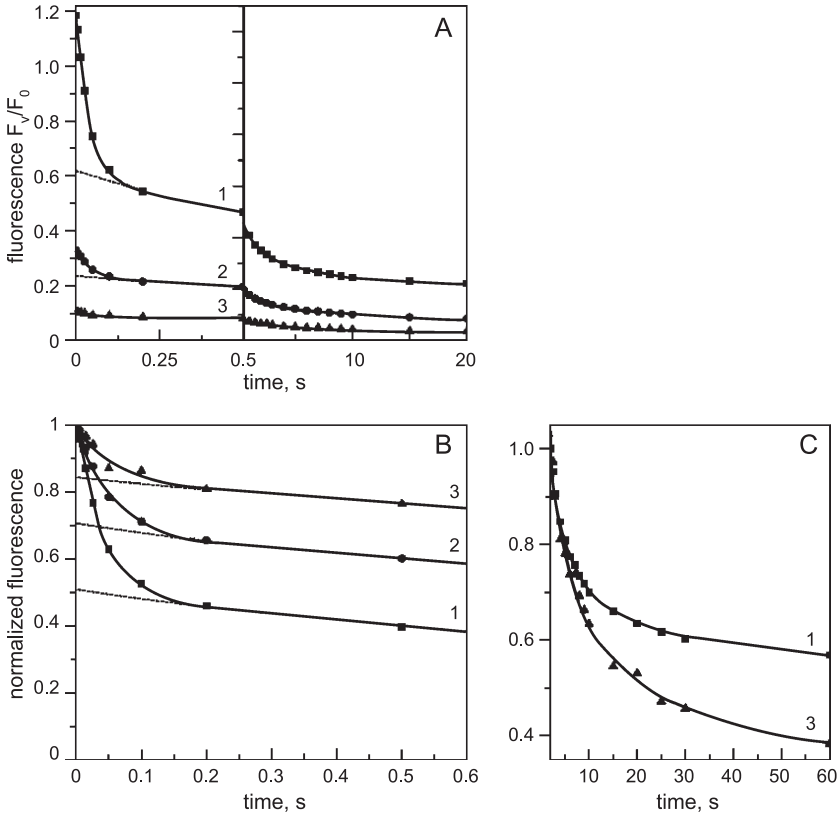
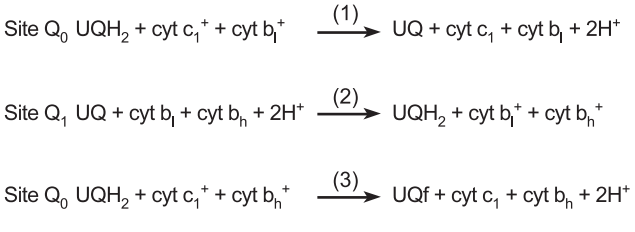


Fig. 4. Kinetics of fluorescence decay induced by a single flash of different energies given to dark-adapted Ga cells. (A) Curve 1: saturating flash. Curve 2: subsaturating flash hitting ~29% of the RCs. Curve 3: subsaturating flash hitting ~9.5% of the RCs. (B) The maximum fluorescence yield for subsaturating flashes has been normalized to the fluorescence yield measured after a saturating flash. (C) Slow phase of the fluorescence decay after normalization at t=2 s. Curve 1, saturating flash. Curve 3, subsaturating flash hitting ~9.5% of the RCs.

quinone diffusion from the site Q_o of the cyt bc_1 complex to the site Q_b of the RC. Thus, the half time for the transfer of UQf to the Q_b site is likely shorter than the half time of the fast phase.

We propose that the slow phase is associated with the presence of quinone UQd, randomly distributed in the membrane prior to flash excitation and thus present at low concentration in the vicinity of the RCs. The concentration of UQd is proportional to the extent of the slow oxidation phase of Q_a^-, i.e., 0.32 and 0.51 of the concentration of the RCs in the case of young and old cells, respectively (see Fig. 3B). In Fig. 4C, the kinetics of the slow phase measured after a saturating flash or a subsaturating flash have been compared after normalization at time 2 s. As expected for a bimolecular reaction involving Q_a^- and UQd, the fluorescence decay is faster after the weak than after the saturating flash with equal initial slopes. In addition, in the case of the weak flash, the fluorescence decay is expected to be close to an exponential function because of the excess of UQd compared to Q_a^-. In Fig. 5, the kinetics of the slow phase has been analyzed over a dark period of 4 min for dark-adapted Ga cells submitted to a flash excitation hitting ~0.06 of the RCs. Despite the large excess of UQd with respect of Q_a^-, the fluorescence decay is highly multiphasic, which suggests that UQd is not homogeneously distributed in the membrane. Moreover,



Scheme 2.

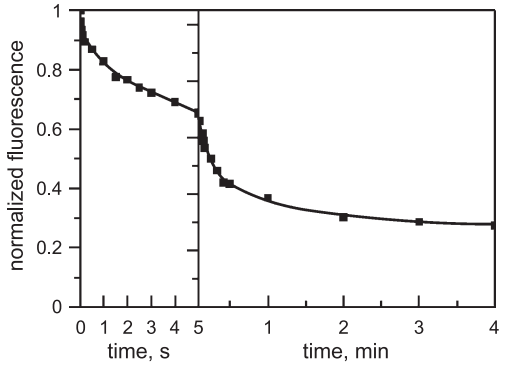


Fig. 5. Kinetics of the fluorescence decay induced by a subsaturating flash hitting ~6% of the RCs, given to dark-adapted young Ga cells.

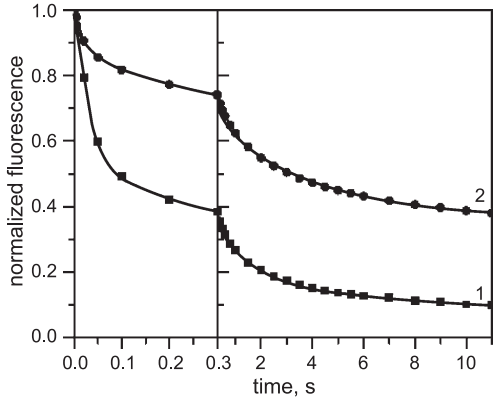


Fig. 6. Effect of myxothiazol on the kinetics of fluorescence decay. Curve 1, control. Curve 2, 200 μM myxothiazol.

~23% of the RCs have not been reoxidized after 4-min dark, which implies that these RCs are localized in membrane regions devoid of UQd. A similar structural heterogeneity has been previously reported for the distribution of the plastoquinone (PQ) in the thylakoid membrane of chloroplasts [22–24]. On the basis of a kinetics analysis of oxygen and fluorescence emission, it has been proposed that membrane proteins act as barriers to the diffusion of PQ. The surface fraction occupied by these proteins is above the two-dimensional percolation threshold (~50%), creating a network of small isolated domains of various sizes including an average of three to four RCs. There is a broad distribution in the stoichiometry between PQ and PSII centers among these domains. We thus propose that the bacterial membranes present similar limitation to quinone diffusion than the thylakoid membrane of chloroplasts. A study of the photoreduction of the UQ pool in chromatophores of *Rb. sphaeroides* Ga has led Comayras and Lavergne to the same conclusion (personal communication).

Assuming similar sizes of the domains in chloroplasts and in bacteria and according to a Poisson distribution of UQd among domains, one expects large variations of UQd/RCs ratio. In addition, a significant fraction of domains will not include any quinone.

3.5. Effect of myxothiazol on the fluorescence decay kinetics

Fig. 6 shows the fluorescence decay following a saturating flash given to dark-adapted cells in the absence (curve 1) or the presence of myxothiazol (curve 2). Myxothiazol induces a large inhibition of the fast phase that is associated with an increase of the fraction of Q_a^- not reoxidized at the end of the slow phase. This experiment demonstrates that UQf is formed at a short distance of RCs while UQd is randomly distributed in the membrane. Excitation by four saturating flashes given 3 min apart induces the reduction of ~55% of Q_a^- that stay reduced for period longer than 10 min (data not shown). It implies that no enzymatic reaction is able to form UQd and that the cyt *bc*1 turnover is fully blocked in this range of time. In the presence of myxothiazol and upon repetitive flash illumination (time interval from flashes 500 ms to several minutes), a fast ($t_{1/2}$~20 ms) reoxidation phase of Q_a^- of small amplitude (~8% of the variable fluorescence) is observed. In the same experimental conditions, the flash-induced membrane potential changes display a decaying phase with similar half time (data not shown). On the contrary, in the absence of myxothiazol, a large increasing phase of the membrane potential is observed in the 100-ms time range that is associated with cyt bc_1 turnover. It suggests that the decaying phase observed in the presence of myxothiazol is associated with a back reaction involving P_{870}^+ and Q_a^-. The half time of this back reaction ($t_{1/2}$~20 ms) is similar to that we previously measured in whole cells (23 ms, [2]) but about three times shorter than that measured in isolated RCs [25]. We propose that repetitive flash excitation induces simultaneously the reduction of Q_a^- and a partial oxidation of the secondary donors (cyt c_1, cyt c_2 and Rieske protein), owing to the inhibition of cyt bc_1 complex. For the small fraction of RCs associated with fully oxidized secondary donors, the reduction of P_{870}^+ can occur only via a back reaction involving Q_a^-.

The fast phase of small amplitude observed in curve 2 can be associated with the presence of a small fraction of non-inhibited cyt bc_1 complexes and/or with a small fraction of the RCs that are not connected to cyt bc_1 complexes and thus in which Q_a^- is rapidly reoxidized by a back reaction.

3.6. Comparison of the kinetics decay measured with Ga and PufX− strains

In Fig. 7, the comparison of the kinetics of fluorescence decay for dark-adapted samples of Ga and PufX− strains after a single saturating flash highlights the role of the supramolecular organization of the photosynthetic unit in the process of Q_a^- reoxidation. The initial rate of the fluorescence decay is ~10 times slower for the PufX− strain than for the Ga strain, showing that the turnover of the RCs is slowed down in the PufX− strain, in agreement with previous works [18]. In the absence of supercomplexes,

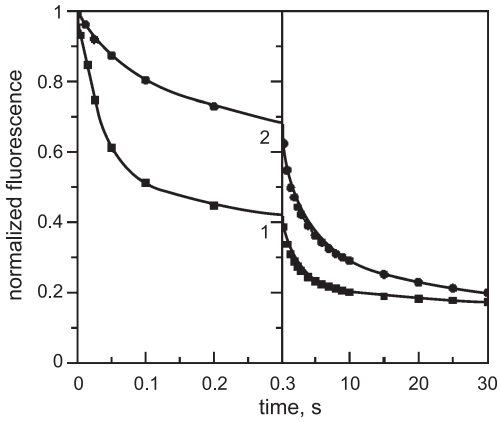


Fig. 7. Kinetics of the fluorescence decay induced by a single saturating flash given to dark-adapted young Ga (curve 1) or PufX− (curve 2) cells.

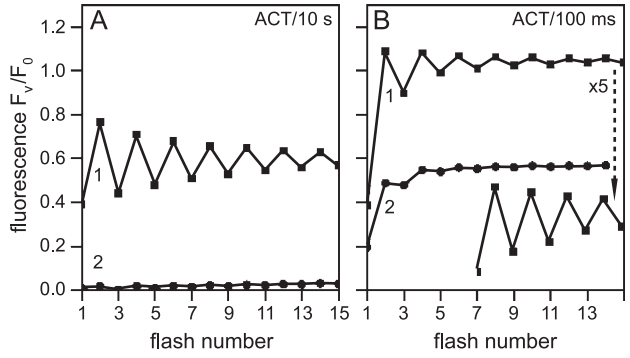


Fig. 8. Fluorescence yield oscillations measured on preilluminated Ga cells submitted to a series of saturating flashes. The cells are illuminated as follows: 1-s saturating continuous light, 30-s dark, 15 saturating flashes, 30-s dark. (A) Time interval between flashes Δt=10 s. (B) Δt=100 ms. Curves 1: Fluorescence yield measured 4 ms after each flash. Curves 2: Fluorescence yield measured 100 μs before the subsequent flash.

UQf is not generated in the immediate vicinity of the RCs, which explains the lack of a fast reoxidation phase. The amounts of Q_a^- reoxidized after 30-s dark are about equal in the PufX− strain and the Ga strain as the total amounts of quinone available (UQf+UQd) are equal. The kinetics of the fluorescence decay in the PufX− strain is highly multiphasic and similar to that observed in the Ga strain in the presence of myxothiazol. Nevertheless, owing to the absence of UQf, the amplitude of the fluorescence decay is lower in the Ga strain in the presence of inhibitors. These results suggest that the rate constant for the oxidation of Q_a^- by UQd (second-order reaction) is similar for the PufX− and Ga strains.

3.7. Illumination by a flash series of preilluminated cells: period-2 oscillations

In Fig. 8, young Ga cells were submitted to cycles of illumination and darkness as follows: a 2-s illumination under strong light that induces the reduction of a large fraction of Q_a is followed by a 30-s dark period. During this dark period, the reoxidation of Q_a^- by quinone formed by cyt bc_1 turnover leads to the formation of an excess of $Q_aQ_b^-$ state. Then, the cells are submitted to a series of 15 flashes given 100 ms or 10 s apart. The fluorescence yield is sampled 4 ms after each flash (curves 1) or 100 μs before the subsequent flash (curves 2). Curves 1 display period-2 oscillations with an inverted phase when compared to the oscillations measured with dark-adapted material (Fig. 1). As shown in Fig. 8A, curve 2, Q_a^- is fully reoxidized in ~10 s. This implies that the slowest phases of Q_a^- oxidation (10 s to min) seen in dark-adapted cells (Figs. 3 and 5) are not present in preilluminated cells. We have no interpretation to explain this behavior which is also observed in the case of preilluminated PufX− strain.

Fig. 9 shows a similar experiment performed with the PufX− strain with time intervals between flashes of 4 s or 400 ms. One observes period-2 oscillations with the same phase as that observed in Fig. 8 but with a much more pronounced damping despite of the longer time intervals between flashes. Moreover, the fluorescence level increases as a function of the flash number, indicative of a progressive reduction of Q_a.

3.8. Modeling

Scheme 3 shows the sequence of reactions induced by saturating flashes exciting supercomplexes in initial state $[Q_aQ_b^-/Q_aQ_b^-b_h]$.

This model has been applied to simulate the behavior of cells subjected to a flash series following a preillumination under saturating light (Fig. 10). We have computed the oscillations of Q_a^-, assuming that at the time the flash series is fired, ~0.65 of the supercomplexes are in the $[Q_aQ_b^-/Q_aQ_b^-\ b_h]$ state and ~0.35 in the $[Q_a/Q_a\ b_h]$ state. In Fig. 10A, Δt is longer than the time necessary for the completion of step c (Scheme 3A) involving the diffusion of UQd. Oscillations of a same amplitude would be obtained if the complexes were not organized in supercomplexes but randomly distributed in the membrane and if ~0.65 RCs were in the $Q_aQ_b^-$ state and ~0.35 RCs were in the Q_a state. In Fig. 10B, the time interval Δt between flashes is supposed to be longer than steps a, b, a′, b′ (Scheme 3B) and shorter than step c (Scheme 3A). A reasonable fit is

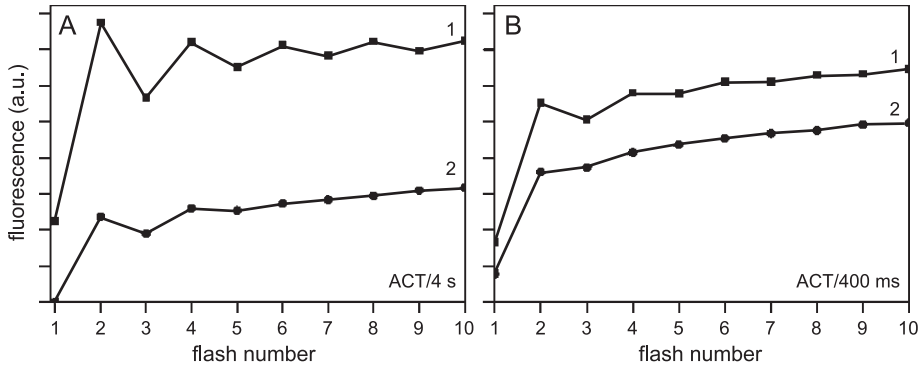
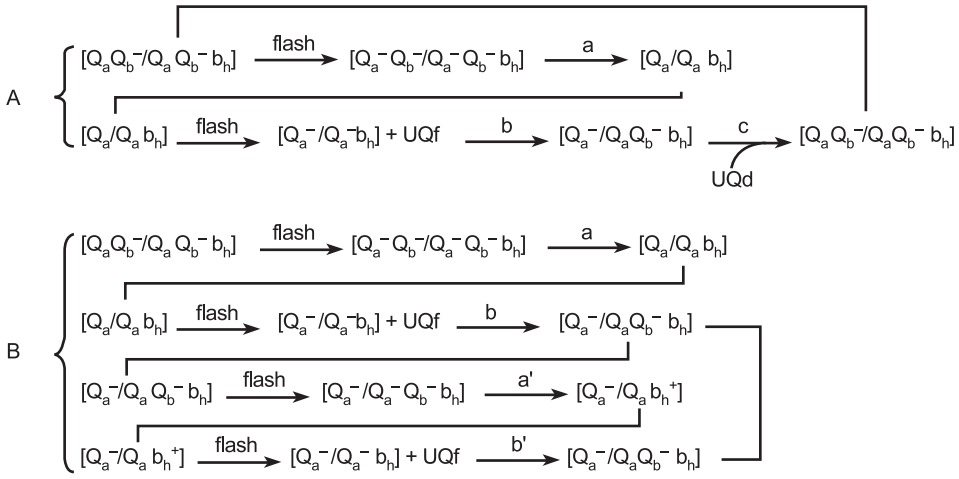


Fig. 9. Fluorescence yield oscillations measured on preilluminated PufX− cells submitted to a series of saturating flashes. The cells are illuminated as follows: 1-s saturating continuous light, 40-s dark, 10 saturating flashes, 90-s dark. (A) Time interval between flashes Δt=4 s. (B) Δt=400 ms.



Scheme 3. Redox states of the primary (Q_a) or secondary (Q_b) quinone acceptors of each of the two RCs that form the supercomplex and redox state of cyt b_h along a series of saturating flashes given Δt apart. RCs in the $Q_aQ_bH_2$ state are quoted Q_a. Steps a and a′ are completed in less than 4 ms. Steps b and b′ are completed in less than 200 ms. A: Δt is longer than step c (Δt~10 s). B: Δt is shorter than step c (Δt~100 ms). Step a′ : The quinone formed at site Q_o of the cyt bc_1 complex is trapped at site Q_i (see Scheme 2, reaction (2)).

obtained between the computed sequence (Fig. 10A and B) and the data shown in Fig. 8A and B (Δt=100 ms and 10 s, respectively). For both the experimental and the computed curves, the initial amplitude of period-2 oscillations is about two times larger for long Δt than for short Δt. It is predicted by the oscillating pattern computed from Scheme 3B which implies the organization of the photosynthetic electron transfer chain in supercomplexes.

3.8.1. Damping of the oscillations

In the tail of the sequence shown in Fig. 8B, curve 1 (see insert), the damping of the oscillation is similar to that observed when isolated RCs in oxidizing conditions are submitted to a flash series. This damping results from "misses" associated with a small fraction of inactive RCs including Q_a^-, owing to the low equilibrium constant between the $Q_aQ_b^-$ and $Q_a^-Q_b$ states [26,27]. We thus assume that the damping observed in the tail of the sequence is associated with intrinsic properties of the RCs. The lack of additional damping in Fig. 8B implies that for most of the RCs, step b′ (Scheme 3B) is completed in a time interval equal to or shorter than 100 ms. This provides a further argument for the organization of the photosynthetic electron chain in supercomplexes (see Scheme 1). In contrast, in the case of the PufX− strain in which cyt bc_1 complexes and RCs are randomly distributed (Fig. 9), oscillations are rapidly damped. As discussed in Section 3.3, the damping of the oscillations increases when Δt decreases toward values shorter than those required for completion of step 1, Scheme 1 (compare Fig. 9A and B).

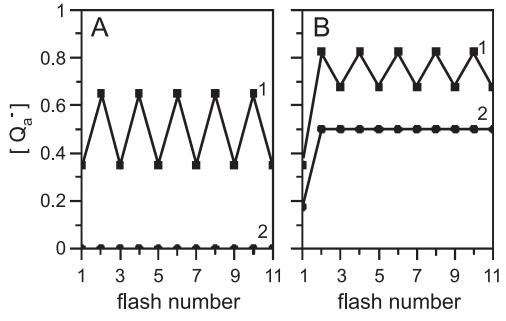


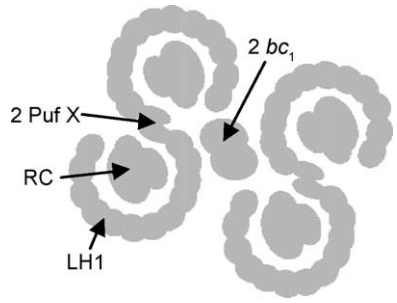
Fig. 10. Fluorescence yield oscillations induced by a series of saturating flashes given Δt apart, computed from Scheme 3. Initial states of the supercomplexes: $[Q_aQ_b^-/Q_aQ_b^-\ b_h]$ state 0.65, $[Q_a/Q_a\ b_h]$ state 0.35. (A) Δt>step c. (B) Step c>Δt>steps a, b, a′, b′ (Scheme 3). Curves 1: Fluorescence yield after completion of steps a, b and d. Curves 2: fluorescence yield Δt after the flash.

3.9. Structural models of the electron transfer chain

In the case of the PufX− mutant, the lack of the PufX polypeptide leads to a complete ring of antenna around the RC, which prevents the dimerization of RC–LH1 complexes and their supramolecular organization with the cyt bc_1 complex. According to recent structural studies [12,14], the two PufX polypeptides interconnect the two open antenna rings that surround the two RCs. Therefore, in the PufX− mutant, UQf is not formed in the vicinity of the RCs, which explains the absence of a fast phase in Q_a^- reoxidation (Fig. 7). On the other hand, the fact that similar rates of Q_a^- oxidation are observed in the PufX− strain and for the slow phase in the Ga strain suggests that the closed antenna ring around the RC does not prevent the diffusion of soluble quinone through the ring. This agrees with the fact that an efficient cyclic flow is operating in the PufX− strain when a fraction of the UQ pool is oxidized [18,28].

It thus appears that the PufX polypeptide plays an essential role in the formation of the supercomplex rather than in the opening of the antenna ring.

It is worth pointing out that the diffusion of UQ in the case of PufX− strain or UQd in the case of the Ga strain spreads over a time range of several seconds to minutes.



Scheme 4. Putative representation of the supramolecular organization of the photosynthetic chain of *Rb. sphaeroides*. The pufX polypeptide has been located according to the proposal of Scheuring et al. [14].

This suggests that a slow exchange of UQ occurs between neighboring domains.

The presence of dimers of RC–LH1 has been evidenced by biochemical and structural methods [11–14] but arguments that favor the association of such a dimer with the cyt bc_1 complex come from functional measurements only [1]. We have previously proposed a first possible structural model in which one dimer of RCs is associated with a monomer of cyt bc_1 [1,11]. This model was supported by the fact that tubular membranes of *Rb. sphaeroides*, believed to contain cyt bc_1 complexes, could accommodate only monomeric cyt bc_1 complexes in interaction with dimeric RC–LH1–PufX complexes [11]. This type of model has been challenged by Crofts and coworkers [29,30]. Since it is now demonstrated clearly that the tubular membranes do not contain cyt bc_1 complexes [12], the constraint for a monomeric cyt bc_1 complex is now released. This gets rid of a difficulty that arose in our previous model from the tilted position of the transmembrane helix of the Rieske protein in the crystallographic structure [31]. This helix would have to be stabilized by a direct interaction with the RCs. Such an interaction is unlikely due to the narrow opening in the antenna ring that offers little room for a direct contact between the cyt bc_1 complex and the RCs. We thus favor now a model of supercomplex involving the dimeric form of the cyt bc_1 complex. To satisfy the stoichiometry of two RCs per cyt bc_1, two dimers of RCs must be associated with one dimer of cyt bc_1 (Scheme 4). Such a supercomplex would behave functionally in a similar way as that involving a monomeric cyt bc_1. A similar type of supercomplex including two dimers of cytochrome-*c* oxidase and a dimer of cyt bc_1 has been structurally characterized in the respiratory chain [32]. Our previous analysis of the cyt c_2 diffusion in chromatophores [33] is consistent with a model in which two dimers of RCs are associated via a dimer of cyt bc_1. In the 1-ms time range, the diffusion of cyt c_2 is restricted to domains including a single cyt bc_1, while over a longer time range (~20 ms), the diffusion of cyt c_2 occurs in domains including two cyt bc_1.

An interesting property of the structural model shown in Scheme 4 is that one cyt bc_1 is associated with one RC of the dimer, which implies that only one of the two RCs of the supercomplex is involved in the fast oxidation of Q_a^-. The other RC is slowly reoxidized via a diffusion-limited process involving UQd. These RCs would contribute efficiently to the cyclic electron flow when a fraction of the UQ pool is oxidized.

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References

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

[2] P. Joliot, A. Verméglio, A. Joliot, Photo-induced cyclic electron transfer operates in frozen cells of *Rhodobacter sphaeroides*, Biochim. Biophys. Acta 1318 (1997) 374–384.

[3] F.E. Jenney, F. Daldal, A novel membrane-associated c-type cytochrome, cyt c_y, can mediate the photosynthetic growth of Rhodobacter capsulatus and *Rhodobacter sphaeroides*, EMBO J. 12 (1993) 1283–1292.

[4] F.E. Jenney, R.C. Prince, F. Daldal, Roles of the soluble cytochrome c_2 and membrane-associated cytochrome c_y of Rhodobacter capsulatus in photosynthetic electron transfer, Biochemistry 33 (1994) 2496–2502.

[5] H. Myllykallio, F. Drepper, P. Mathis, F. Daldal, Membrane-anchored cytochrome c_y mediated microsecond time electron transfer from the cytochrome bc_1 complex to the reaction center in Rhodobacter capsulatus, Biochemistry 37 (1998) 5501–5510.

[6] A. Verméglio, A. Joliot, P. Joliot, Supramolecular organization of the photosynthetic chain in mutants of Rhodobacter capsulatus deleted in cytochrome c_2, Biochim. Biophys. Acta 1318 (1998) 374–384.

[7] L. Drachev, M. Mamedov, A. Mulkidjanian, Y. Semenov, V. Shinkarev, M. Verkovsky, Transfer of ubiquinol from the reaction center to the bc_1 complex in *Rhodobacter sphaeroides* chromatophores under oxidizing conditions, FEBS Lett. 245 (1989) 43–46.

[8] C.N. Hunter, J.D. Pennoyer, J.N. Sturgis, D. Farrelly, R.A. Niederman, Oligomerization states and associations of light-harvesting pigment protein complexes of *Rhodobacter sphaeroides* as analyzed by lithium dodecyl-sulfate polyacrylamide-gel electrophoresis, Biochemistry 27 (1988) 3459–3467.

[9] J.R. Goleski, S. Ventura, J. Oelze, The architecture of unusual membrane tubes in the B800–850 light-harvesting bacteriochlorophyll-deficient mutant 19 of *Rhodobacter sphaeroides*, FEBS Lett. 77 (1991) 335–340.

[10] M. Sabaty, J. Jappé, J. Olive, A. Verméglio, Organization of the electron-transfer components in *Rhodobacter sphaeroides* forma sp. denitrificans whole cells, Biochim. Biophys. Acta 1187 (1994) 313–323.

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

[12] C.A. Siebert, P. Qian, D. Fotiadis, A. Engel, C.N. Hunter, P.A. Bullough, Molecular architecture of photosynthetic membranes in *Rhodobacter sphaeroides*: the role of PufX, EMBO J. 23 (2004) 690–700.
[13] F. Francia, J.M. Wang, G. Venturoli, B.A. Melandri, J.L. Rigaud, W.P. Barz, D. Oesterhelt, The reaction center–LH1 antenna complex of *Rhodobacter sphaeroides* contains one PufX molecule which is involved in dimerization of this complex, Biochemistry 38 (1999) 6834–6845.
[14] S. Scheuring, F. Francia, J. Busselez, B.A. Melandri, J.L. Rigaud, D. Levy, Structural role of PufX in the dimerization of the photosynthetic core complex of *Rhodobacter sphaeroides*, J. Biol. Chem. 279 (2004) 3620–3626.
[15] T.G. Liburn, C.A. Haith, R.C. Prince, J.T. Beatty, Pleitropic effects of pufX gene deletion on the structure and function of the photosynthetic apparatus of Rhodobacter capsulatus, Biochim. Biophys. Acta 1100 (1992) 160–170.
[16] J.W. Farchaus, H. Gruenberg, D. Oesterhelt, Complementation of a reaction center-deficient *Rhodobacter sphaeroides* pufLMX deletion strain in trans with pufBALM does not restore the photosynthetic positive phenotype, J. Bacteriol. 172 (1990) 977–985.
[17] R.N. Freese, J.D. Olsen, R. Branvall, W.H.J. Westerhuis, C.N. Hunter, R. van Grondelle, The long-range supraorganization of the bacterial photosynthetic unit: a key role for PufX protein, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 5197–5202.
[18] W.P. Barz, F. Francia, G. Venturoli, B.A. Melandri, A. Verméglio, D. Oesterhelt, Role of PufX protein in photosynthetic growth of *Rhodobacter sphaeroides*: PufX is required for efficient light-driven electron transfer and photophosphorylation under anaerobic conditions, Biochemistry 34 (1995) 15235–15247.
[19] W.P. Barz, A. Verméglio, F. Francia, G. Venturoli, B.A. Melandri, D. Oesterhelt, Role of the PufX protein in photosynthetic growth of *Rhodobacter sphaeroides*: PufX is required for efficient ubiquinone/ubiquinol exchange between the reaction center Q_B site and the cytochrome bc_1 complex, Biochemistry 34 (1995) 15248–15258.
[20] P. Mitchell, The protonmotive Q cycle: a general formulation, FEBS Lett. 59 (1975) 137–199.
[21] A.R. Crofts, S.W. Meinahrdt, K.R. Jones, M. Snozzi, The role of the quinone pool in the cyclic electron-transfer chain of Rhodopseudomonas sphaeroides. a modified Q-cycle mechanism, Biochim. Biophys. Acta 723 (1983) 202–218.
[22] P. Joliot, J. Lavergne, D. Béal, Plastoquinone compartmentation in chloroplasts: evidence for domains with different rates of photoreduction, Biochim. Biophys. Acta 1101 (1992) 1–12.
[23] J. Lavergne, J.P. Bouchaud, P. Joliot, Plastoquinone compartmentation in chloroplasts: 2. Theoretical aspects, Biochim. Biophys. Acta 1101 (1992) 13–22.
[24] H. Kirchhoff, S. Horstmann, E. Weis, Control of the photosynthetic electron transport by PQ diffusion microdomains in thylakoids of higher plants, Biochim. Biophys. Acta 1459 (2000) 148–168.
[25] R.K. Clayton, H.F. Yau, Photochemical electron transport in photosynthetic reaction centers from *Rhodopseudomonas spheroides*: kinetics of the oxidation and reduction of P-870 as affected by external factors, Biophys. J. 12 (1972) 867–881.
[26] C.A. Wraight, Electron acceptors of photosynthetic bacterial reaction centers: direct observation of oscillatory behaviour suggesting two closely equivalent ubiquinones, Biochim. Biophys. Acta 459 (1977) 525–531.
[27] A. Verméglio, Secondary electron transfer in reaction centers of Rhodopseudomonas sphaeroides: out of phase periodicity of 2 for formation of semiubiquinone and fully reduced ubiquinone, Biochim. Biophys. Acta 459 (1977) 516–524.
[28] A. Verméglio, P. Joliot, Supramolecular organization of the photosynthetic chain in anoxygenic bacteria, Biochim. Biophys. Acta 1555 (2002) 60–64.
[29] A.R. Crofts, M. Guergova-Kuras, S.S. Hong, Chromatophores heterogeneity explains phenomena seen in *Rhodobacter sphaeroides* previously attributed to supercomplexes, Photosynth. Res. 55 (1998) 357–362.
[30] A.R. Crofts, Photosynthesis in *Rhodobacter sphaeroides*, Trends Microbiol. 8 (2000) 105–106.
[31] D. Xia, C.A. Yu, H. Kim, J.Z. Xia, A.M. Kachurin, L. Zhang, L. Yu, J. Deisenhofer, Crystal structure of the cytochrome bc_1 complex from bovine heart mitochondria, Science 277 (1997) 60–66.
[32] H. Schägger, Respiratory chain supercomplexes of mitochondria and bacteria, Biochim. Biophys. Acta 1555 (2002) 154–159.
[33] P. Joliot, A. Verméglio, A. Joliot, Supramolecular organization of the photosynthetic chain in chromatophores and cells of *Rhodobacter sphaeroides*, Photosynth. Res. 48 (1996) 291–299.