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Photo-induced electron transfer in intact cells of *Rubrivivax gelatinosus* mutants deleted in the RC-bound tetraheme cytochrome: Insight into evolution of photosynthetic electron transport

André Verméglio [a,b,c,*], Sakiko Nagashima [d], Jean Alric [e], Pascal Arnoux [a,b,c], Kenji V.P. Nagashima [d]

[a] CEA, DSV, IBEB, Laboratoire de Bioénergétique Cellulaire, Saint-Paul-lez-Durance, France
[b] CNRS, UMR 7265, Biologie Végétale et Microbiologie Environnementales, Saint-Paul-lez-Durance, France
[c] Aix-Marseille Université, Saint-Paul-lez-Durance, France
[d] Department of Biological Science, Tokyo Metropolitan University, Minamiohsawa 1-1, Hachioji, Tokyo 192-0397, Japan
[e] Unité Mixte de Recherche 7141 CNRS-Université Paris 6, Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France

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ABSTRACT

Deletion of two of the major electron carriers, the reaction center-bound tetrahemic cytochrome and the HiPIP, involved in the light-induced cyclic electron transfer pathway of the purple photosynthetic bacterium, *Rubrivivax gelatinosus*, significantly impairs its anaerobic photosynthetic growth. Analysis on the light-induced absorption changes of the intact cells of the mutants shows, however, a relatively efficient photo-induced cyclic electron transfer. For the single mutant lacking the reaction center-bound cytochrome, we present evidence that the electron carrier connecting the reaction center and the cytochrome bc_1 complex is the High Potential Iron–sulfur Protein. In the double mutant lacking both the reaction center-bound cytochrome and the High Potential Iron–sulfur Protein, this connection is achieved by the high potential cytochrome c_8. Under anaerobic conditions, the halftime of re-reduction of the photo-oxidized primary donor by these electron donors is 3 to 4 times faster than the back reaction between P^+ and the reduced primary quinone acceptor. This explains the photosynthetic growth of these two mutants. The results are discussed in terms of evolution of the type II RCs and their secondary electron donors.

1. Introduction

In purple photosynthetic bacteria, the proton-motive force required for ATP synthesis is generated by translocation of protons, from the cytoplasmic to the periplasmic side of the membrane, coupled to the light-induced cyclic electron transfer. Two membrane-associated components are involved in this process: a photosynthetic reaction center (RC) and a cytochrome (cyt) bc_1 complex. The primary reactions of photosynthesis are induced by absorption of a photon, followed by a transmembrane charge separation between the primary electron donor (P), a special pair of bacteriochlorophylls, and the primary and secondary quinone acceptors (Q_A and Q_B). The secondary electron donor to the photo-oxidized primary donor, P^+, can be either a RC-bound cyt subunit or a water-soluble periplasmic electron carrier. This periplasmic electron carrier ensures a connection between the cyt bc_1 complex and the RC to complete the light-induced cyclic electron transfer. Similarly, the connection between the RC-bound cyt and the cyt bc_1 complex is established by a soluble electron carrier in the periplasm.

For an optimal photochemical efficiency, the reduction of P^+ by the secondary donor has to be faster than the back reaction between P^+ and Q_A^- (halftime of a few milliseconds or tens of milliseconds depending upon the nature of Q_A). This requirement is realized for both species possessing a RC-bound cyt like *Blastochloris (B.) viridis* or *Rubrivivax (R.) gelatinosus* [1] and species lacking the cyt like *Rhodobacter (R.) sphaeroides* where P^+ is directly reduced by a soluble electron carrier [2]. In both cases, the re-reduction of the primary electron donor P^+ is completed in less than 10 μs, much faster than the back reaction between P^+ and the reduced primary electron acceptor Q_A^- or between P^+ and Q_B^- (halftime of hundreds of milliseconds). Therefore the absence of a RC-bound cyt does not impede an efficient photosynthetic electron transfer. Consequently it was proposed that the acquisition of highly reactive mobile cyt *c* in some species might have rendered the bound cyt subunit superfluous and subject to deletion [3,4]. This proposition is in agreement with the phylogenetic studies of Matsuura and coworkers [5,6], who showed that species lacking the cyt subunit are randomly distributed among purple bacteria, suggesting that the RC-bound cyt subunit would have been lost independently in various lines during evolution [5,6].

Abbreviations: *B.*, *Blastochloris*; cyt, cytochrome; HiPIP, High Potential Iron–sulfur Protein; HP, high potential; LP, low potential; RC, reaction center; *R.*, *Rhodobacter*; *R.*, *Rubrivivax*

* Corresponding author at: CEA, DSV, IBEB, Laboratoire de Bioénergétique Cellulaire, Saint-Paul-lez-Durance, France. Tel.: +33 442254630; fax: +33 4 42254701.
E-mail address: andre.vermeglio@cea.fr (A. Verméglio).

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The presence of an unusual RC in *Rhodovulum sulfidophilum*, which possesses a triheme cyt subunit instead of the common tetraheme subunit [7], could reflect a part of this evolutionary process.

Another evidence of the non-essential role of the RC-bound cyt subunit was demonstrated by the disruption of the *pufC* gene coding for the RC-bound cyt subunit of *R. gelatinosus* [9]. The corresponding mutant strain, denoted C244, is able to grow under anaerobic photosynthetic conditions although at a rate slower than the WT. The RC-bound cyt subunit is therefore dispensable for the photosynthetic growth in *R. gelatinosus*.

Electron carriers connecting the cyt bc_1 complex and the RC-bound cyt are diverse. The nature of the operative electron donor (cyt c_2, HP cyt c_8, HiPIP) depends upon the considered species and several environmental parameters. In *B. viridis*, the cyt c_2 is the operative electron donor, while it is likely auracyanin in the green filamentous bacteria, *Chloroflexus* species [10,11]. HiPIP is the only high potential putative electron donor in species such as *Rhodovibrio salinarum*, and *Ectothiorhodospira shaposhnikovii* [12]. In species like *R. gelatinosus* and *Rhodoferax fermentans*, HiPIP is the operative electron donors to the RC-bound cyt [13,14]. However the nature of the electron donor switches from HiPIP, for cells grown anaerobically in the light, to HP cyt c_8 for dark semi-aerobic conditions [15] for *R. gelatinosus*. The operative electron donor switches from HiPIP to HP cyt c_8 for *Allochromatium vinosum* cells grown under anaerobically in the light depending on the composition of the growth medium [16], or depending on the imposed redox potential for *Rhodocyclus tenuis* cells [17]. This reveals the plasticity and adaptability of the photosynthetic bioenergetic processes to environmental conditions. Less diversity is however observed for the electron donor to RCs that do not possess a bound cyt, which is usually a cyt c_2 [18]. For example, no species using HiPIP as direct electron donor to the RC has been described thus far although *in vitro* kinetic study demonstrated that *R. gelatinosus* RC complex without the cyt subunit was reduced by HiPIP [19].

In the present study, we have investigated the *in vivo* kinetics of the light-induced electron transfer in mutants of *R. gelatinosus* deleted in the RC-bound cyt and in a mutant lacking both the RC-bound cyt and the HiPIP.

2. Materials and methods

2.1. Molecular biology

Deletion of the RC-bound cyt subunit of *R. gelatinosus* was obtained through a removal of the *pufC* gene without any insertions of antibiotics-resistance genes as described in Ref. [8]. The gene coding for HiPIP or HP cyt c_8 was also knocked out as described in Ref. [8].

2.2. Bacterial growth

For growth tests *R. gelatinosus* cells were aerobically grown in PYS medium [9] to the exponential growth phase for further use as precultures. The preculture was inoculated to the fresh PYS medium (1% inoculum) to start the photosynthetic growth measurement. The measurement vessel is a screw-capped glass tube (18 mm in diameter) filled with the medium and placed about 30 cm apart from a 60 W tungsten lamp. The temperature was kept at 30 °C with a water bath and a circulator. The cell growth was monitored as an optical density at 660 nm in the test tube. Five tubes derived from the same preculture were prepared for each strain. The average of three out of the five measurements omitting the minimum and the maximum was plotted versus the time.

For light-induced absorbance changes measurements, *R. gelatinosus* wild type and the mutants were grown photoheterotrophically (for 24 h for the WT and 48 h for the two mutants) or semi-aerobically in the dark (24 h) in Hutner medium. Some experiments were performed with a spontaneous mutant with a depressed production of the LH2 complex, denoted IL144RL2 [20].

2.3. Preparation of the periplasmic fraction

Cells were harvested by centrifugation at 4000 *g* for 10 min, resuspended in 50 mM Tris–HCl (pH 8) in the presence of 0.45 M sucrose, 1.3 mM EDTA, and 1 mg/ml lysozyme. After 2 h of incubation at 30 °C, the suspension was spun down for 20 min at 4000 *g*. The supernatant and the pellet constitute the periplasmic and the membrane fractions, respectively.

2.4. Redox titrations and determination of the relative concentration of HP cyt c

Redox titrations of the periplasmic fractions were performed in 20 mM Tris–HCl (pH 7) with a cocktail of mediators as described in Ref. [15]. The redox potential (E_h) was varied by the addition of small aliquots of potassium fericyanide (10 mM), sodium ascorbate (10 mM) or dithionite (10 mM). The concentration of soluble cyt *c* was determined by measuring the optical density at 550 nm for the periplasmic fraction as a function of E_h, with a U-2000 spectrophotometer (Hitachi). The experimental data were fitted with ($n=1$) Nerst curves. The concentration of HP cyt *c* corresponds to the Nerst component with an E_m of +295 mV using an extinction coefficient of 20 $mM^{-1}cm^{-1}$ [15] and the concentration of the RC was determined by measurements of light-induced absorption changes at 430 nm on the membrane fraction using extinction coefficients of 30 $mM^{-1}cm^{-1}$ for (P^+–P).

2.5. EPR spectroscopy on whole cells

EPR spectra were taken at 10 K with an X-band Varian E-109 spectrometer fitted with an Oxford instruments ESR 900 helium-flow cryostat. Instrument settings: microwave frequency 9.2 GHz, microwave power 1 mW, modulation amplitude 1 mT. EPR spectra were integrated and compared to a standard HiPIP solution of known concentration. Continuous illumination of cells with infrared light was performed at room temperature before freezing in liquid nitrogen.

2.6. Light-induced optical absorption spectroscopy on whole cells

Optical absorbance changes were measured with a laboratory-built spectrophotometer as described previously [21]. The cells were re-suspended in fresh medium and either oxygenated by flowing air or anaerobically adapted. Actinic excitation was provided by a single saturating xenon flash (3 μs half-time duration) filtered though a Kodak Wratten filter 89B which passes near infra red light and blocks wavelengths below 680 nm.

2.7. Structural models of the RC, HiPIP and HP cyt c_8 of R. gelatinosus

Primary sequences of the different proteins from *R. gelatinosus* were blasted against the Protein Data Bank. We then used the best hits to construct a model using Swiss-Model and Swiss-PdbViewer. The best hits were the structure of the cyt c_{551} from *Pseudomonas aeruginosa* (PDB ID: 351C; e-value = 3.10^{-18}) [22], of the HiPIP from *Rhodoferax fermentans* (PDB ID: 1HLQ; e-value = 8.10^{-12}) [23] and of the RC from *Thermochromatium tepidum* (PDB ID: 1EYS; e-values = 3.10^{-106}, 5.10^{-125}, 5.10^{-55} for the L, M an H subunits respectively) [24]. The 3D structure images were generated using the program Pymol.

3. Results

3.1. Photosynthetic growth of mutants deleted in pufC, HiPIP or (and) HP cyt c_8

Both the single mutant deleted for the gene encoding the RC-bound cyt (Δ*pufC* mutant) and the double mutant deleted for genes encoding the RC-bound cyt and the HiPIP (Δ*pufC-HiPIP* mutant) are

able to grow under anaerobic photosynthetic condition (Fig. 1). Nevertheless, the growth rate is strongly affected by these deletions: the doubling times estimated from the exponential growth phases are 4.0 h for the WT, 13.3 h for Δ*pufC* and 47.8 h for Δ*pufC-HiPIP* mutants. The triple mutant deleted for the gene encoding the HP cyt c_8 in addition to the two deletions (Δ*pufC-HiPIP-HP cyt* c_8 mutant) is still capable of photosynthetic growth but its doubling time is further increased (82.5 h). When grown under dark aerobic conditions, the deletion of the RC-bound cyt, both the RC-bound cyt and HiPIP, or all the three proteins including HP cyt c_8 does not affect the doubling time which is 2 h as for the WT (data not shown). To better understand the photo-induced electron transfer underlying the photosynthetic growth of these various mutants, we have investigated the light-induced absorbance changes in intact cells as described in the following paragraphs.

3.2. Time-resolved spectral analysis of flash-induced optical absorbance changes on intact cells of ΔpufC mutant

Light-induced absorbance changes in whole cells of the Δ*pufC* mutant, grown photosynthetically, were measured at different times (20 μs, 10 ms, 30 ms) after flash excitation under both aerobic (Fig. 2A) and anaerobic conditions (Fig. 3A). The absorbance changes are dominated by those related to the carotenoid bandshift. The carotenoid bandshift is an electrochromic change that responds to the membrane potential in a linear way [25]. Under anaerobic conditions, the fast phase reflects photochemical charge separation in the RC and the slow (milliseconds) rise phase reflects the electrogenic (Q-cycle) activity in the cyt bc_1 complex (Fig. 3). In addition to these field-indicating changes in the 450–550 nm region, a positive band between 420 and 440 nm and a trough at 605 nm at 20 μs after the flash are present, indicative of the formation of a photo-oxidized P (P^+). This is completely different from what is observed for the WT where no P^+ could be observed 20 μs after the actinic flash, due to its rapid re-reduction by one LP heme of the RC-bound cyt under anaerobic condition. This is clearly shown in Fig. 1 of the supplementary material where large changes of the photo-oxidized LP heme could be observed at 20 μs after the actinic flash in the Soret and the α-band regions in addition to the carotenoid bandshift. When the Δ*pufC* mutant is placed under aerobic conditions, the half time of re-reduction of P^+ is 6 ms (Fig. 2B) much faster than the back reaction between P^+ and Q_B^- ($k = 0.25–0.30\ s^{-1}$ between pH 3.5 and 8) [26,27]. The disappearance of the P^+ signal is therefore too fast to be linked to this back reaction. This implies that P^+ has been reduced by a secondary electron donor. This secondary electron donor is not a cyt since no negative signal could be observed in the Soret region around 420 nm (Fig. 2A). Under anaerobic conditions, the halftime of re-reduction of P^+, measured at 605 nm, is about 3 ms (Fig. 3C). Under these anaerobic conditions the quinone pool is fully reduced, and the partner of P^+ for the back reaction is Q_A^- (half time 35 ms, [26]). The rate of P^+ reduction is therefore only 6 to 10 times faster than this back reaction between P^+ and Q_A^-. One consequently expects a competition between the re-reduction of P^+ by a secondary electron donor and its re-reduction by back reaction with Q_A. The small transient decrease of the carotenoid bandshift, measured as the difference absorption change at 535-minus-518 nm, in the first several milliseconds following the initial rise phase linked to charge separation is indicative of such back reaction between P^+ and Q_A^- (Fig. 3B). Despite this back reaction occurring for a part of the RCs (about 20%), the observation of a slow rise in the carotenoid bandshift under anaerobic condition implies an effective turnover of the cyt bc_1 complex. Note that due to the fast decay observed under aerobic condition (Fig. 2), the slow rising phase could not be observed in these conditions. Under anaerobic condition, the half time of the slow phase of this carotenoid bandshift (15 ms, Fig. 3B) is very similar to what has been observed for intact WT cells of *R. gelatinosus* [13]. The relative slow rise time of this phase are interpreted as due to the relatively low amount of cyt bc_1 complex compared to the RC. The negative changes observed in the Soret region (Fig. 3) are attributed to both the carotenoid bandshift and cyt b_H oxidation as discussed below. Altogether, this series of experiments implies that a photo-induced cyclic electron transfer, with kinetic parameters not too different from those of the WT strain, occurs in the Δ*pufC* mutant of *R. gelatinosus*. This accounts for the photosynthetic anaerobic growth of this mutant.

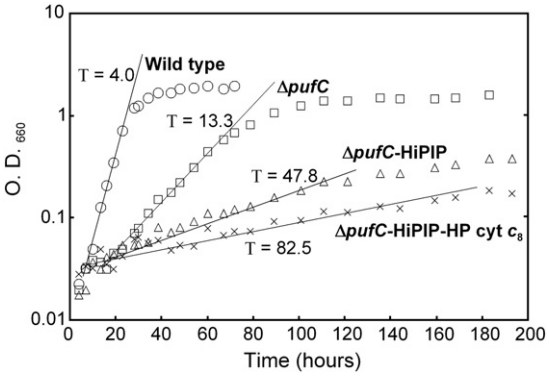

Fig. 1. Growth curves of wild type (WT, ❍) and mutants lacking RC-bound cytochrome subunit (Δ*pufC*, ❒) or both RC-bound cytochrome subunit and HiPIP (Δ*pufC*-HiPIP, △), and the triple mutant (Δ*pufC*-HiPIP-HP cyt c_8, X) of *R. gelatinosus* grown under photosynthetic conditions. Doubling times (T) were calculated by exponential regressions to the early-to-mid exponential growth phases, 0.1 < O.D. < 0.8 for the WT and Δ*pufC*, 0.05 < O.D. < 0.3 for Δ*pufC*-HiPIP mutant and 0.05 < O.D. < 0.2 for Δ*pufC*-HiPIP-HP cyt c_8 mutant.

3.3. Nature of the electron carrier to P^+ for ΔpufC cells grown under photosynthetic conditions

For Δ*pufC* cells grown under photosynthetic conditions, the electron carrier connecting RC and cyt bc_1 complex presents no obvious spectral characteristic. The spectral changes featured in Figs. 2A and 3A appear to reflect essentially the carotenoid change and the oxidation of P. In line with the very low amount of HP cyt c_8 synthesized under these growth conditions as determined by measurement of the periplasmic content (not shown, see M and M section), we estimate that less than 10% of P^+ re-reduction involves a cytochrome as electron donor. In agreement with this view, an analysis of the light-induced changes of a Δ*pufC* mutant containing a low amount of antennae (IL144RL2) shows that the absorbance changes detected in the 30–100 ms range in the Soret region under anaerobic condition are partially due to the carotenoid bandshift (data not shown) and probably to cyt b_H oxidation during the light-induced cyclic electron transfer. To explain the absence of absorbance changes linked to the electron donation to the RC, it is natural to surmise an involvement of the HiPIP in the photo-induced electron transfer. Indeed, redox changes of HiPIP are difficult to measure from optical absorption changes because of its weak, broadband, reduced-minus-oxidized spectrum. Moreover the role of HiPIP as an electron carrier connecting the RC and the cyt bc_1 complex has already been established in the case of the WT strain [13,28]. Its putative photo-oxidation in intact cells of *R. gelatinosus* Δ*pufC* mutant was therefore investigated by EPR spectroscopy. Fig. 4 shows EPR spectra of either dark-adapted or illuminated whole cells of the Δ*pufC* mutant grown under the anaerobic photosynthetic conditions in the field region 300 to 340 mT. The spectrum taken in the light exhibits features absent from the spectrum of the dark-adapted cells. The two signals at $g = 2.11$ and 2.04 are indicative of the photo-oxidation of HiPIP [13]. The two additional peaks around a *g*-value of 2 are attributed to the photo-oxidation of the primary donor (P^+) of the RC.

To analyze further the involvement of HiPIP as direct electron donor to P^+ in the mutant lacking the RC-bound cyt, a double mutant deleted in both *pufC* and *HiPIP* gene, denoted Δ*pufC-HiPIP*, was constructed. As

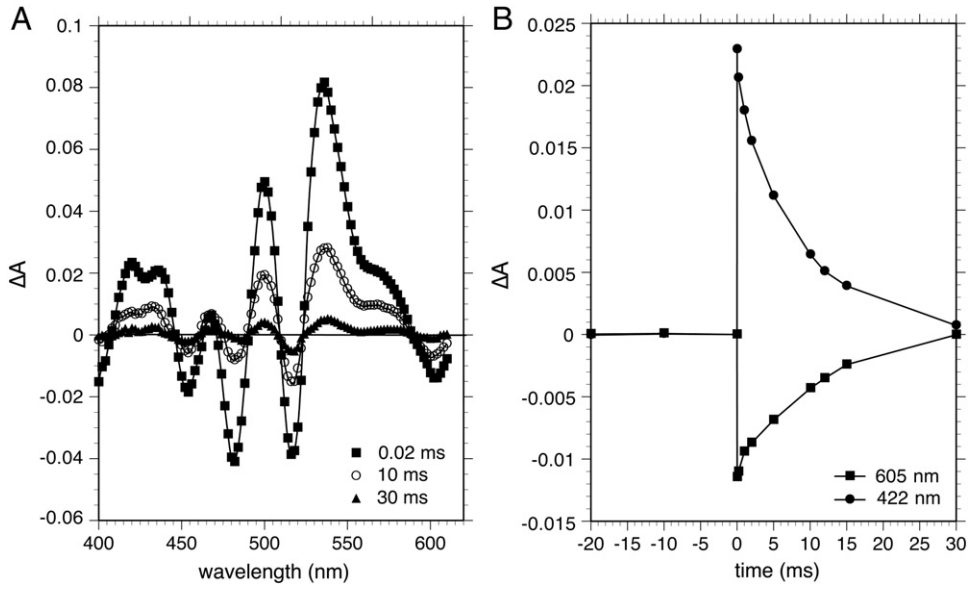

Fig. 2. Light-induced absorbance changes induced by a single turnover flash for intact cells of the Δ*pufC* mutant grown under anaerobic condition in the light. The cells were placed under semi-aerobic conditions by a gentle air flow. For panel B, the flash was fired at t = 0. Panel A: Absorbance changes detected at 0.02 ms (■), 10 ms (○), 30 ms (◆) after the actinic flash. Panel B: Kinetics measured at 605 nm (■) and 422 nm (●).

shown in Fig. 1, this double mutant grows under anaerobic photosynthetic conditions although at reduced rate compared to the single mutant Δ*pufC* (a factor of 3). This significant retard in the growth rate is a strong argument in favor for HiPIP being the direct electron donor to P^+ in the single mutant Δ*pufC*. Analysis of the light-induced absorbance changes for intact cells of this double mutant, grown photosynthetically and placed under anaerobiosis, shows a large negative signal around 425 nm 10 ms after the actinic flash (Fig. 5A). We attribute a part of this signal to the oxidation of a cyt that re-reduces P^+. This re-reduction occurs with a halftime of 3 ms (Fig. 5C). The slow phase of the carotenoid bandshift occurs with a half time of 12 ms demonstrating the occurrence of an efficient light-induced cyclic electron transfer (Fig. 5B). As observed for the Δ*pufC* mutant, a transient dip, linked to the back reaction between P^+ and Q_A^-, is observed in the carotenoid bandshift kinetics (Fig. 5B). In the absence of HiPIP, this electron donor is replaced by a cyt. This situation is reminiscent to the one observed in a mutant deleted in HiPIP where the HP cyt c_8 acts as the direct electron donor to the RC-bound cyt [28]. We therefore surmise that this cyt is the HP cyt c_8. Several arguments support this hypothesis. First, we compared the relative amount of the HP cyt c_8 present in the single mutant Δ*pufC* and in the double mutant Δ*pufC-HiPIP* when both are grown under anaerobic conditions in the light as described in the M and M section. The synthesis of the HP cyt c_8 is enhanced by a factor of 3 in the double mutant Δ*pufC-HiPIP* compared to the single mutant Δ*pufC* (data not shown). Moreover we have measured light-induced absorbance changes for the cells of the Δ*pufC* mutant grown under dark aerobic condition in order to check whether HP cyt c_8 could donate electrons directly to P^+. Indeed, it was shown even in WT strain that under such growth condition, the HP cyt c_8 is the major electron donor to the RC-bound cyt due to enhancement of its synthesis [15].

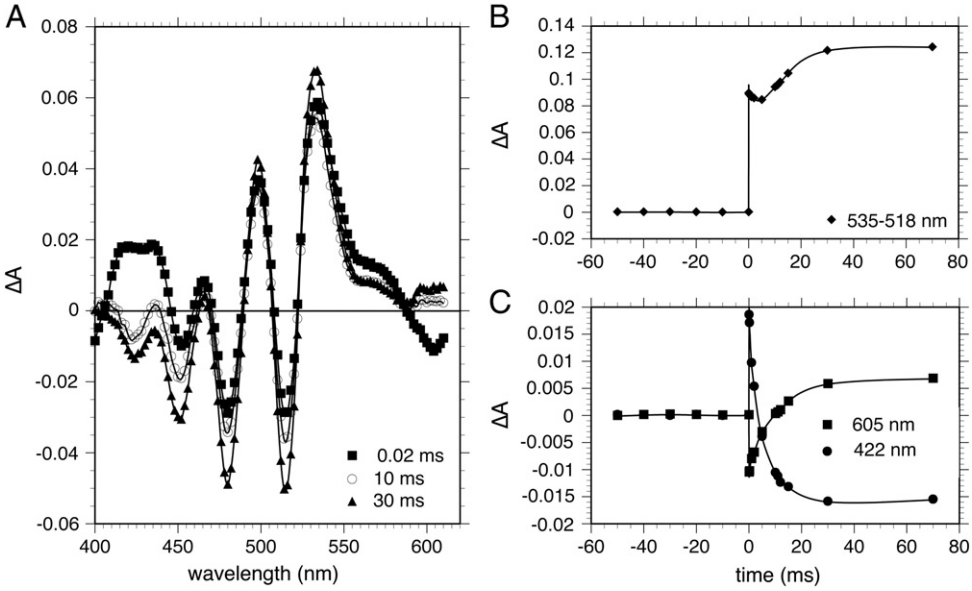

Fig. 3. Light-induced absorbance changes induced by a single turnover flash for intact cells of the Δ*pufC* mutant grown under anaerobic condition in the light. The cells were placed under anaerobiosis by N_2 flushing. For panels B and C, the flash was fired at t = 0. Panel A: Absorbance changes detected at 0.02 ms (■), 10 ms (○), 30 ms (◆). Panel B: Kinetics of the carotenoid bandshift measured at 535–518 nm (◆). Panel C: Kinetics measured at 605 nm (■) and 422 nm (●).

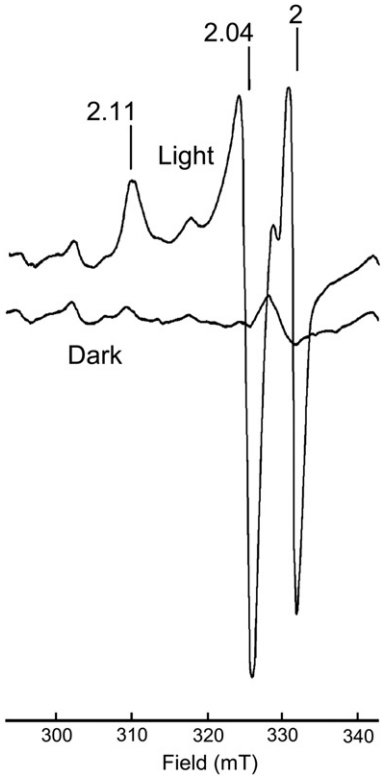

Fig. 4. X-band EPR spectra for a suspension of intact cells of the Δ*pufC* mutant placed under anaerobic condition. The cells were either subjected to 5 min of continuous illumination (light) or dark-adapted for 5 min (dark) at room temperature prior cooling. Spectra were recorded at 10 K. The light-induced EPR signal is characteristic of oxidized HiPIP ($g = 2.11$ and 2.04) and oxidized primary electron donor of the RC ($g = 2.00$).

Here we confirm that the synthesis of the HP cyt c_8 is also enhanced for the Δ*pufC* mutant grown under dark aerobic conditions compared to cells grown under anaerobic condition in the light (data not shown). Fig. 6A shows the light-induced absorbance changes for Δ*pufC* cells grown under such conditions and placed under anaerobiosis. Detection of the light-induced changes at various times after the flash clearly shows a trough in the Soret region, indicative of a photo-oxidation of a cyt. Kinetics of absorbance at 425 nm shows that the cyt photo-oxidation occurs with a halftime of 5 ms concomitant with the P^+ reduction measured at 605 nm (Fig. 6B). The kinetics of both the P^+ re-reduction and the cyt oxidation are similar to those observed for the double mutant Δ*pufC-HiPIP*. Note however that due to the growth condition of semi-aerobically in the dark, the chemical nature of the carotenoids has been modified. As a consequence, the absorbance changes linked to the carotenoid bandshift are of much less amplitude than those observed for cells grown under anaerobic condition in the light (Fig. 3A). Another argument in favor of the HP cyt c_8 acting as electron donor to P^+ is provided by the observation that deletion of the HP cyt c_8 gene in addition to the deletions of PufC and HiPIP genes induces a decrease by a factor 2 in its growth rate compared to the double mutant (Fig. 1). We conclude from these experiments that the HP cyt c_8 could be a direct electron donor to P^+ as efficient as the HiPIP.

4. Discussion

4.1. Light-induced electron transfer in ΔpufC mutant

Although both Δ*pufC* and Δ*pufC-HiPIP* mutants grow at lower rate than the WT (4 and 12 times respectively) under photosynthetic conditions, this photosynthetic growth is a clear evidence for the occurrence of a light-induced cyclic electron transfer in these two strains. Several characteristics of this light-induced cyclic electron transfer are revealed by measurements of the light-induced absorbance changes in the visible range, from 400 to 610 nm, on intact cells. When cells are grown anaerobically in the light or aerobically in the dark, the re-reduction of the P^+ in the Δ*pufC* mutant is fast ($t_{1/2} = 3$ to 6 ms) compared to its rate of back reaction with the reduced acceptor side. This implies that the P^+ is directly reduced by a secondary electron donor without an intermediation by the RC-bound cyt. The complete cyclic electron transfer occurs with a half time around 15 ms under anaerobic conditions as shown by measurements of the slow electrogenic phase of carotenoid bandshift. Under these anaerobic conditions, where the quinone pool is reduced prior to the flash excitation, the re-reduction of P^+ by the secondary donor competes with the back reaction between P^+ and Q_A^-. Nevertheless, more than 80% of the RCs are rereduced by the secondary donor.

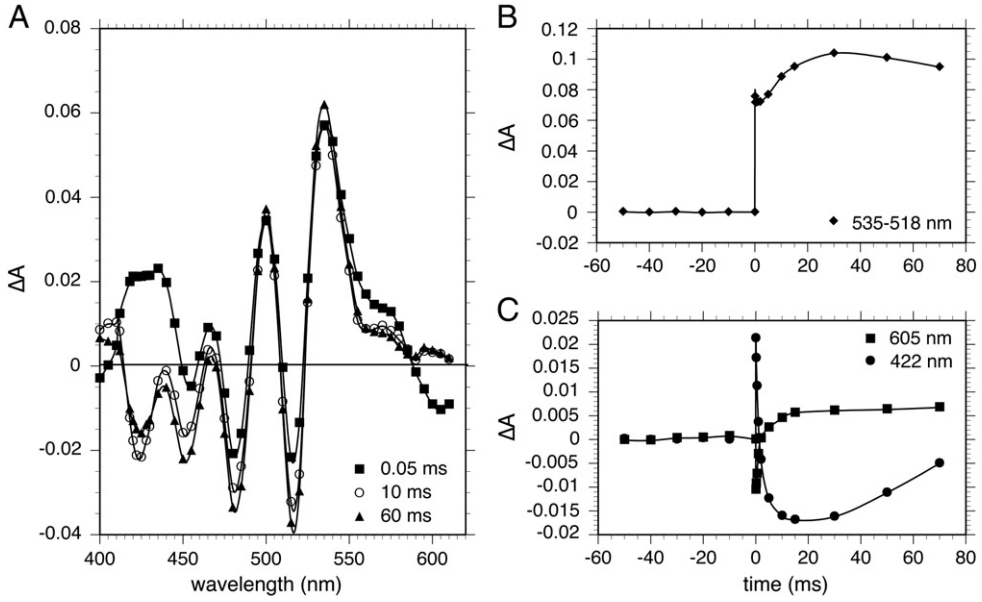

Fig. 5. Light-induced absorbance changes induced by a single turnover flash for intact cells of the double mutant Δ*pufC-HiPIP* grown under anaerobic condition in the light. The cells were placed under anaerobic conditions by N_2 flushing. For panels B and C, the flash was fired at t = 0.Panel A: Absorbance changes detected at 0.05 ms (■), 10 ms (○), 60 ms (♦). Panel B: Kinetics of the carotenoid bandshift measured at 535–518 nm (♦). Panel C: Kinetics measured at 605 nm (■) and 422 nm (●).

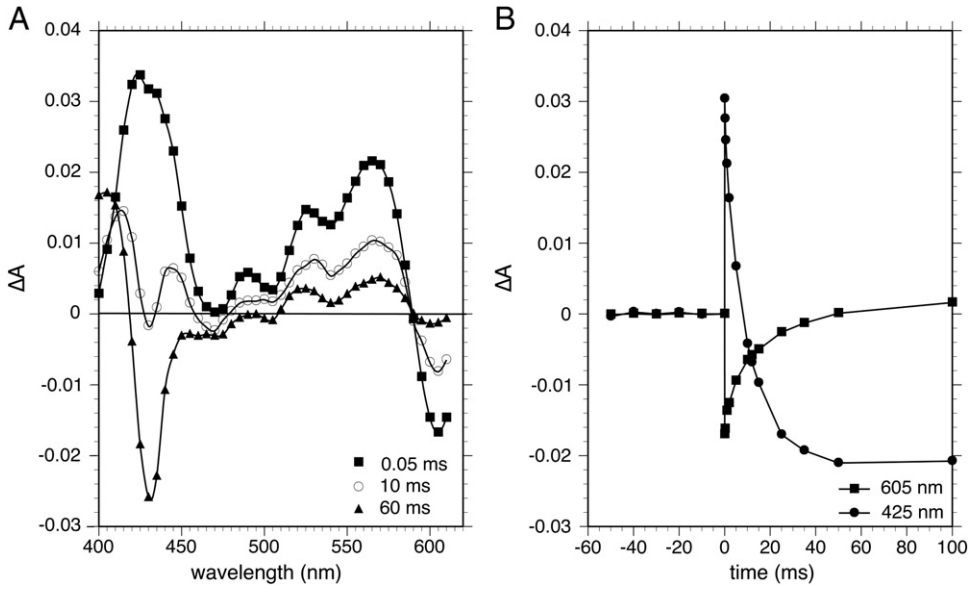

Fig. 6. Light-induced absorbance changes induced by a single turnover flash for intact cells of the Δ*pufC* mutant grown under semi-aerobic conditions. Cells were placed under anaerobiosis by N_2 flushing. For panel B, the flash was fired at t = 0. Panel A: Absorbance changes detected at 0.05 ms (■), 10 ms (○), 60 ms (♦). Panel B: Kinetics measured at 605 nm (■) and 425 nm (●).

For Δ*pufC* cells grown under the photosynthetic anaerobic conditions, we did not detect an optical signature for this secondary electron donor. We propose that this secondary electron donor is HiPIP, as in the WT, for the following reasons. First, EPR signals characteristic of HiPIP oxidation are observed after rapid freezing of intact cells illuminated at room temperature. Another argument comes from reconstitution experiments showing that HiPIP could act as electron donor to the *R. gelatinosus* RC devoid of tetraheme [19]. Moreover, the deletion of the gene encoding the HiPIP in addition to the one encoding the RC-bound cyt induces a further slowing down (by a factor of 3) of the growth rate under the photosynthetic conditions. For this double mutant Δ*pufC-HiPIP*, the mobile carrier that reacts with the RC appears to be the HP cyt c_8.

The good correlation between the P^+ reduction and the cyt oxidation in the first milliseconds is in agreement with the proposal that this cyt *c* is the immediate electron donor to P^+. However, the light-induced absorbance changes observed in the Soret region for the Δ*pufC* mutant grown under the dark semi-aerobic condition (Fig. 6) or for the double mutant Δ*pufC-HiPIP* (Fig. 5) cannot be attributed solely to the oxidation of the HP cyt c_8. If the changes observed at 422 or 425 nm were linked solely to the oxido-reduction changes of the HP cyt c_8 we would expect a disappearance of the signal in the tens of milliseconds scale concomitant to the slow phase of the carotenoid bandshift, linked to the re-reduction of the HP cyt c_8. We therefore surmise that part of the absorbance changes detected in the Soret region in the tens of millisecond scale are linked to the oxidation of the cyt b_H. Indeed, under anaerobic conditions all quinones are reduced and hemes b_L and b_H of the cyt bc_1 are oxidized (b_L^+) and reduced (b_H^-), respectively. Under flash excitation the HP cyt c_8 is oxidized by P^+. A quinol molecule is then oxidized at the Q_o site via a bifurcated reaction in which one electron reduces the oxidized HP cyt c_8 and the other the oxidized cyt b_L, leading to the formation of state $b_L^- b_H^-$. The only available oxidized quinone under these anaerobic conditions is the one released from the Q_o site of the cyt bc_1. This oxidized quinone is reduced at the site Q_i to form the state $b_L^+ b_H^+$. The dark-adapted state $b_L^+ b_H^-$ is then regenerated in the dark by the slow reduction of the oxidized cyt b_H.

From this series of experiments, we deduce that both HiPIP and HP cyt c_8 are efficient electron donors to the RC devoid of the RC-bound cyt. The rate of reaction between the P^+ and the HiPIP (or the HP cyt c_8) is fast enough to partially compete with the back reaction from Q_A^- to P^+ under anaerobic conditions and allow a light-induced cyclic electron transfer. This relatively good efficiency for the electron transfer between HiPIP (or HP cyt c_8) and the RC may be related to the nature of the docking interfaces of the partners. Tentatively, we modeled three-dimensional models of the *R. gelatinosus* RC (devoid of its tetraheme), of the soluble HiPIP and of HP cyt c_8, based on the known three-dimensional structures of homologues with high sequence identities (from 45 to 70% identity). As shown in Fig. 7, when compared to the known structure of the complex between the *R. sphaeroides* RC and its soluble cyt c_2 partner [29] it is apparent that the interacting areas are more hydrophobic in the case of *R. gelatinosus* RC that is devoid of the bound cyt subunit (Fig. 7). As concerns the RC, this is, so to say, an artifact consequence of the mutation, since the deletion of the tetraheme unit has exposed a hydrophobic region which is normally buried within the complex. Coincidentally, the docking surfaces of the HiPIP and cyt *c* happen to be relatively hydrophobic (as apparent when comparing to cyt c_2, see top panel), which may facilitate their interaction with the mutated RC.

4.2. Some considerations with respect to molecular evolution

As far as we know, purple sulfur bacteria, which are rather strict anaerobes, all contain a RC-bound cyt. In contrast, purple non-sulfur bacteria, which are able to develop under both dark aerobic and light anaerobic conditions, are more diverse. Species like *B. viridis, R. gelatinosus*, and *Rhodocyclus tenuis* for example, possess a RC-bound cyt while species like *R. sphaeroides*, *R. capsulatus* and *Rhodopseudomonas palustris*, are devoid of such a subunit. As already stated, the physiological electron donor connecting the RC-bound cyt and the cyt bc_1 complex is diverse, being HiPIP, cyt c_2 or HP cyt c_8. In contrast, only cyt c_2 (and cyt c_y in the case of *R. capsulatus* [30]) have been shown to connect the RC and the bc_1 complex in species where the RC is lacking the bound cyt subunit. No species has been shown thus far to use HiPIP or HP cyt c_8 for mediating electrons between the cyt bc_1 complex and the RC. This is surprising since HiPIP and HP cyt c_8 are widespread in photosynthetic bacteria and able to interact with the cyt bc_1 complex. Here, by directed mutagenesis, we try to gain some insights on the interaction between HiPIP (or HP cyt c_8) and the RC, following the demonstration that deletion of the *pufC* gene of *R. gelatinosus* does not inhibit photosynthetic growth due to

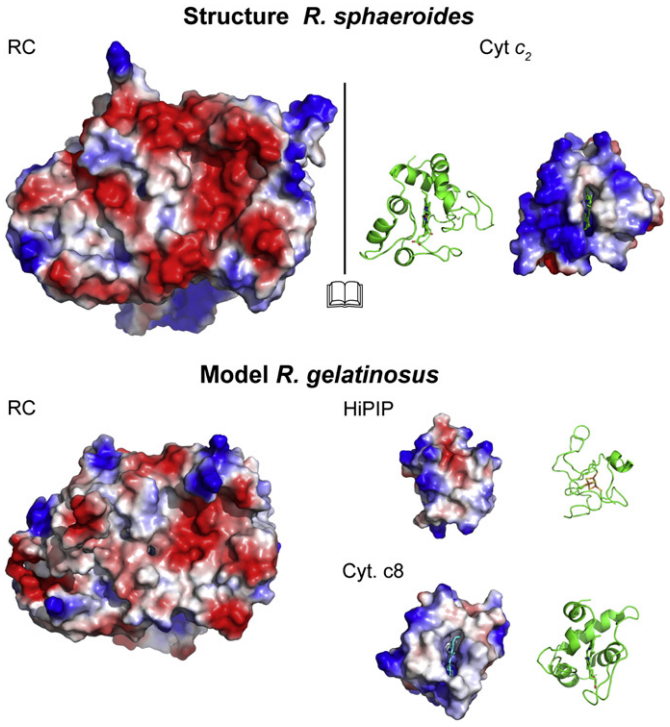

Fig. 7. Three-dimensional structures of *R. sphaeroides* RC/Cyt c_2 (top) [31] and models of the RC (without the tetraheme subunit), cyt c_8 and HiPIP from *R. gelatinosus* (bottom). In both cases, the interacting area is facing us. The surface representation is colored according to the charges and ranges from positive (blue) to negative (red) patches. Note that the interacting surfaces are highly charged and complementary in the case of the RC/cyt c_2 complex of *R. sphaeroides* whereas these putative interacting areas are more hydrophobic for the RC/HiPIP and the RC/cyt c_8 complexes of *R. gelatinosus*.

an operative light-induced cyclic electron transfer involving the direct interaction between HiPIP (or HP cyt c_8) and the RC.

A phylogenetic analysis suggests that species devoid of the cyt subunit of the RC derive from ancestors which possessed it [6]. Therefore, the Δ*pufC* mutant may be considered to be mimicking the first step of this evolutionary event. One can imagine that, following the loss of the cyt subunit, a co-evolution of both the electron donor and the RC devoid of the cyt subunit (denoted hereafter ΔcRC) would have occurred under the photosynthetic selection pressure. This hypothesis is consistent with the relatively efficient (but nevertheless suboptimal) photosynthetic growth observed for the mutant. In the present work, we show that both HiPIP and HP cyt c_8 are relatively good electron donor to the ΔcRC of *R. gelatinosus*. The question we try to address is why a wild-type phenotype of a photosynthetic electron transfer chain involving a ΔcRC and HiPIP (or HP cyt c_8) has not yet been evidenced.

The simplest reason could be that such species might exist but just have not yet been isolated. Another reason could come from the respective structural constrains for the molecular recognition between the components involved in the cyclic photo-induced electron transfer. When comparing the structures of their present forms, it is clear that the interacting area of the *R. sphaeroides* RC with the cyt c_2, with a large amount of positively charged amino acids, is less hydrophobic than its *R. gelatinosus* counterpart (Fig. 7). In the case of *R. sphaeroides*, the distributions of amino acids with opposite charges in the interacting areas of RC and cyt c_2 (Fig. 7) are of primary importance for the electrostatic capture of the cyt by the RC to form an encounter complex (see ref [31] for a review). In contrast, the interacting area of the HIPIP and the HP cyt c_8 of *R. gelatinosus* are more hydrophobic than the cyt c_2 interacting area, which is negatively charged (Fig. 7). Amino acid residues responsible for the binding between the tetraheme subunit of *R. gelatinosus* and HiPIP (or HP cyt c_8) have been determined by site-directed mutagenesis [32–34]. The binding site of both HiPIP and HP cyt c_8 (or cyt c_2) on the cyt subunit was clearly identified as the region surrounding heme 1, the most distal heme from the primary donor P. The characteristic ionic strength dependencies of the electron transfer between HiPIP (or HP cyt c_8) and the cyt subunit with various mutations have indicated that the docking of HiPIP to the cyt subunit involves hydrophobic interactions while these interactions are electrostatic for the docking of HP cyt c_8 [33–35]. The importance of the electrostatic interactions between cyts *c* and the *R. gelatinosus* cyt subunit has been demonstrated by the replacement of the 67th valine of this subunit by a glutamate [35]. Addition of a negative charge in the cyt subunit facilitates its electrostatic interaction with cyts but, at the same time, weakens the hydrophobic interaction with HiPIP. Here we have to remember that the high redox potential of HiPIPs is mainly due to hydrophobic amino acids in the vicinity of the iron–sulfur cluster buried into the protein environment [36,37]. The hydrophobic nature of the interacting surface on HiPIPs is therefore intricately linked to their redox potential and therefore might not be able to evolve toward a charged interface. This could be the main explanation why HiPIP has not been able to evolve to a more efficient electron donor to the ΔcRC.

However, this hypothesis does not apply to the case of the HP cyt c_8. One possibility is that this cyt has a common origin with a cyt c_2, the usual electron donor to P^+ among non-sulfur purple bacteria belonging to α-subgroup. The parallel evolution of these ancestral RC and cyt should have led to important changes in the amino acids composition to attain the present arrangement of RCs and cyts c_2. However, the low homology between the amino acid sequences of cyt c_2 and HP cyt c_8 does not really support this scenario. Indeed, a much higher homology is observed between cyt c_2 and mitochondrial cyt *c* than between cyt c_2 and HP cyt c_8. We therefore prefer the proposal of Meyer et al. [38]. Based on a detailed analysis of the amino acid sequences, the structures and the functions of cyts *c*, these authors propose that, in the course of evolution, purple non-sulfur bacteria possessing the RC-bound cyt subunit first acquired an aerobic pathway (alternatively aerobic bacteria may have acquired a photosynthetic pathway), which led to the emergence of cyt c_2 as the electron transfer shuttle between the bc_1 complex and cytochrome *c* oxidase. Then, the cyt c_2 of the aerobic pathway may have replaced HiPIP (and prevailed over the cyt c_8) as the soluble carrier linking the cyt subunit and the cyt bc_1 complex. Finally, the RC and cyt c_2, after deletion of *pufC,* may have co-evolved to optimize the electrostatic capture of the cyt by the RC within an encounter complex (while meeting a similar constraint for the c_2/bc_1 interaction).

To summarize, we have shown that the deletion of the RC-bound subunit does not drastically impair the photo-induced cyclic electron transfer. After such a deletion, the electron carrier connecting the RC and the cyt bc_1 complex can either be the HiPIP or the HP cyt c_8. During evolution, the loss of the RC-bound cyt has apparently not allowed the emergence of species where either HIPIP or HP cyt c_8 are the direct electron donor to the RC. In the case of HiPIP, this may be due to the hydrophobic nature of its interacting surface, which is related to its high midpoint potential. In the case of the HP cyt c_8, we propose, following the scenario of Meyer et al. [38], that the evolution of a cyt c_2, acquired before the loss of the RC-bound cyt, has been more efficient than the evolution of the HP cyt c_8 to improve its interaction with the RC. The anaerobic purple bacteria, which contain a RC-bound tetraheme, may correspond to the ancestral form of photosynthesis. It is possible that the loss of the tetraheme subunit occurred gradually: *Rhodovulum sulfidophilum*, which possesses a triheme RC-bound cyt could reflect a first event in this process. Species like *B. viridis* may also exemplify an intermediary state. This species has acquired cyt c_2 and lost HiPIP but still retains the RC-bound cyt subunit. Another "intermediary" situation is that of *Rhodopseudomonas palustris,* which has acquired the cyt c_2 and lost the cyt subunit, but still possesses two iso-HiPIPs.

What advantage there could be for an organism that has lost the tetraheme subunit over those that have retained such subunit? This is still an open question which should be addressed in the future. Since the tretraheme subunit has been lost only in photosynthetic bacteria which develop both photosynthetic and respiratory electron chains, one can however surmise that this lost could favor, via the cyt c_2, direct interactions between these two bioenergetic chains. This could allow the bacteria to rapidly switch between their two main bioenergetic processes, photosynthesis and respiration, for a fast adaptation to changes in their environment.

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