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Organization of electron transfer components in *Rhodobacter sphaeroides* forma sp. *denitrificans* whole cells

Monique Sabaty [a], Jocelyne Jappé [a], Jacqueline Olive [b], André Verméglio [a,*]

[a] *CEA, DPVE / SBC, CE de Cadarache, 13108 Saint-Paul-lez-Durance Cedex, France*
[b] *Institut Jacques Monod, 2, Place Jussieu, 75005 Paris, France*

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Abstract

Two pools of cytochrome c_t can be observed in whole cells of *Rhodobacter sphaeroides* forma sp. *denitrificans* upon excitation by continuous light or saturating flashes. The first pool is connected only to the photosynthetic chain. The second one is preferentially coupled to the respiratory and the denitrifying chains. This second pool is in large excess compared to the first when cells are grown under denitrifying and/or aerobic conditions. These two pools equilibrate in less than 50 ms at pH lower than 7.5, but not at higher pH or in the presence of glycerol or divalent cations. For the first pool, the rate of electron transfer between cytochrome c_1 and cytochrome c_2 is not affected by the medium viscosity. Measurements of cytochrome c_t re-reduction in the presence of subsaturating concentrations of myxothiazol show that a given cytochrome c_2 can only react with a single bc_1 complex. This is interpreted in terms of a supramolecular organization of the photosynthetic electron transfer components. Under conditions where the synthesis of the photosynthetic chain is repressed, i.e., addition of nitrate or dark semi-aerobic conditions, the LHII/LHI ratio decreases. This induces the formation of tubular membranes. Freeze-etching pictures of these tubes show a well-ordered dimeric organization of the membrane proteins.

Keywords: Electron transfer; Supercomplex; Reaction center; Cytochrome c_2; bc_1 complex; (*Rb. sphaeroides* forma sp. *denitrificans*)

1. Introduction

The non-sulfur photosynthetic bacteria are the most versatile microorganisms. They are able to perform, depending on the environmental conditions, photosynthesis or respiration with oxygen or compounds like trimethylaminooxide (TMAO), dimethylsulfoxide (DMSO) or nitrate as electron acceptors [1]. Electron and proton transfer components involved in these processes are located on the same internal membrane and in the periplasmic space. Their activities induce a proton electrochemical gradient across the cytoplasmic membrane for ATP generation [2]. Several electron transfer components are common to these different bioenergetic chains. This is in particular the case for ubiquinone molecules, bc_1 complex and cyt c_2, which are involved in photosynthesis, aerobic respiration and denitrification [1,3]. Competition for electrons via diffusible electron transfer components like ubiquinone molecules and cyt c_2 plays an important role in the regulation of these different bioenergetic processes [4–7]. The thermodynamic control exerted by the photoinduced or respiratory-induced membrane potential is also an essential part of the regulation mechanism [4,8,9]. These regulations allow the bacteria to use preferentially the available acceptor which possesses the more positive redox potential, i.e., the most energetically favorable. Light energy is therefore used preferentially. Consequently, photosynthetic activity strongly inhibits aerobic respiration, denitrification or the reduction of DMSO [10–13]. The use of the same electron transfer components in different bioenergetic processes is a simple way for the bacteria to respond rapidly to variations in the environmental factors. Nev-

Abbreviations: R., Rhodobacter; Rh., Rhodopseudomonas; E., Ectothiorhodospira; RC, reaction center; cyt, cytochrome, TMAO, trimethylaminooxide; DMSO, dimethylsulfoxide.

* Corresponding author. Fax: +33 42 254701; e-mail: VERMEGLIO@DSVCAD.CEA.FR.

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ertheless, it has been shown recently that two compartments of cyt c_2 are out of equilibrium under certain conditions in whole cells of *Rhodobacter sphaeroides* Ga [14]. The evidence comes from the observation of two clearly distinct phases in the kinetics of cyt c_t (cyt c_1 + cyt c_2) photo-oxidation at high pH or in the presence of divalent cations. Strong arguments show that one pool of cyt c_t is coupled to the respiratory chain in the part of the cytoplasmic membrane while the other is located in the highly invaginated intracytoplasmic membrane. Joliot et al. [15] have suggested that the electron transfer components of the photosynthetic chain in the intracytoplasmic membrane are organized in supercomplexes composed of two RC, one cyt c_2 and one bc_1 complex. Two main arguments are in favor of such an organization. The first comes from the observation of a high apparent equilibrium constant between the primary (P) and the secondary donors (FeS, cyt c_1 and cyt c_2) demonstrating a lack of thermodynamic equilibrium between the photosynthetic electron transfer components [15]. Second, the addition of sub-saturating concentrations of myxothiazol, an inhibitor of the bc_1 complex at the Q_Z site [16], induces the decrease of the amplitude of the slow electrogenic phase of the carotenoid band-shift without affecting its half-time [14]. This implies that diffusion of cyt c_2 is restricted to domains containing a single bc_1 complex. This conclusion differs from that of Fernàndez-Velasco and Crofts [17], who observed that, in isolated chromatophores, a small proportion of bc_1 complex can catalyze the full reduction of all the photo-oxidized cyt c_2.

Two distinct pools of cyt c_t have also been observed in *R. sphaeroides* forma sp. *denitrificans* cells [18]. The first pool is photo-oxidized by the first two flashes of a series, for cells grown both in the presence and in the absence of nitrate. The full photo-oxidation of the second pool requires a large number of actinic flashes and the efficiency of this process is strongly decreased in the presence of glycerol. The relative amount of this second pool, compared to the first one, is highly enhanced when the cells have been grown in the presence of nitrate [18].

In the present report we have investigated the organization of the electron transfer components for whole cells of *R. sphaeroides* forma sp. *denitrificans*. We show that the ratio between the two pools of cyt c_t depends highly upon the growth conditions. These two pools respond in a similar fashion to physico-chemical parameters in both *R. sphaeroides* forma sp. *denitrificans* and *R. sphaeroides* Ga cells [14]. Analysis of cyt c_t re-reduction in function of myxothiazol concentration strongly supports a supramolecular organization of the photosynthetic chain. Growth in the presence of nitrate or under dark semi-aerobic condition induces a decrease in the LHII/LHI ratio and the formation of tubular membranes. A high degree of organization of the membrane complexes is observed by freeze-etching in this part of the internal membrane.

2. Materials and methods

R. sphaeroides forma sp. *denitrificans*, a generous gift of Prof. Satoh, was grown at 30°C in Hutner medium. For phototrophic conditions, the bacteria were exposed to a white light provided by incandescent bulbs (75 μmol photons m^{-2} s^{-1}). The culture was performed either in the presence or in the absence of 20 mM nitrate. Growth was also realized under dark anaerobic conditions in the presence of 20 mM nitrate or sparged with N_2O. For aerobic cultures, 250 ml Erlenmeyer flasks filled with 100 ml of medium were incubated on a rotary shaker (200 rpm). The cells were harvested after 24 h, i.e., in the exponential phase, regardless of the growth conditions.

Unless stated otherwise, for the measurement of light-induced absorption changes the bacteria were exposed to 200 μM of benzoquinone, centrifuged at low speed (5 min, $6000 \times g$) and then resuspended in 20 mM Tris buffer adjusted to different pH values with HCl. This benzoquinone treatment inhibits the respiration [15] and the denitrification activities (unpublished results) by slowing down the input of electrons without affecting the terminal oxidases. This treament overcomes the reduction of the primary acceptor observed under anaerobic conditions for untreated cells. An optimal redox state for the quinone pool and the cyt c_t is obtained by addition of KCN [15]. The half-times of the slow phase of the carotenoid band-shift, measured for benzoquinone treated cells in the presence of 1 mM KCN and for untreated cells placed under anaerobiosis, are equal to 1.5 ms in both cases. This demonstrates that the rate of cyclic electron transfer is not affected by the benzoquinone treatment and is optimal in these conditions. Anaerobiosis was obtained by addition of 20 mM glucose plus 3 mg/ml of glucose oxidase and bubbling argon in the cell suspension.

The light-induced absorbance changes were performed with an apparatus similar to the one described in Ref. [19]. Excitation was provided by xenon flashes (3 μs half-time) or a 24 V quartz lamp filtered through two Kodak Wratten 89B filters and suitable neutral filters to decrease the flash or the continuous illumination energy when required.

The photo-oxidation of cyt c_2 and c_1, denoted cyt c_t, was measured as the difference $\Delta A_{551nm} - \Delta A_{542.2nm}$ for cells treated with saturating concentration of myxothiazol. For the experiment of Figs. 8 and 9, the photo-oxidation of cyt c_t was measured as the difference $\Delta A_{550.5nm} - (\Delta A_{545nm} + \Delta A_{556nm})/2$. This difference eliminates the contribution of cyt b_H photo-

oxidation. The photo-oxidation of the primary donor P was measured at 542.2 nm. Extinction coefficients for cyt c_t and P were taken to be equal to 19.5 mM^{-1} cm^{-1} and 10.3 mM^{-1} cm^{-1} respectively.

Low-temperature absorption spectra were recorded with a modified Cary 14 spectrophotometer equipped with a Helium gas cryostat.

For electron microscopy, the cells were centrifuged for 5 min at 4000 × *g*. They were then rinsed with 0.1 M cacodylate buffer (pH 7.2), $CaCl_2$ 10 mM. The bacteria were fixed with glutaraldehyde 2% for 2 h. Post-fixation and staining were performed with $KMnO_4$ 2% and $K_3(Fe(CN)_6)$ 0.8% overnight at 4°C. The bacteria were then included in agarose 2%. Small cubes of agarose (approx. 1 mm^3) were progressively dehydrated by successive baths in ethanolic solutions (from 10 to 100%). The dehydrated cubes were then impregnated in Spurr resin. Polymerization is realized at 70°C for 7 h. Ultra-thin sections (80 nm) were post-stained by uranyl acetate and lead citrate and examined in a Jeol electron miscroscope (model 12000-EX-2).

Freeze-fracture was realized as follows. Bacteria were frozen in Freon 22 on gold holders and stored in liquid nitrogen. Freeze fracturing and platinum carbon shadowing were performed at −150°C. A quartz crystal was used in order to obtain platinum and carbon layers of 2 and 20 nm, respectively. Replicas were examined in a Philips CM12 electron microscope.

3. Results

3.1. Kinetics of cyt c_t and P photo-oxidation in R. sphaeroides forma sp. denitrificans: effect of pH and glycerol

Photo-oxidation of cyt c_t and P, induced by continuous illumination, has been measured at different pH for benzoquinone treated cells of *R. sphaeroides* forma sp. *denitrificans* grown under phototrophic conditions in the presence or in the absence of nitrate. Fig. 1 shows these kinetics in the case of cells grown in the presence of nitrate at pH 7 and pH 8.6. As already observed for *R. sphaeroides* Ga cells [14], a clear biphasic photo-oxidation of cyt c_t is observed when the pH is higher than 7.5. The dependence of the half-time of the slow phase of cyt c_t photo-oxidation in function of the pH suspension is plotted in Fig. 2. The very steep change in less than one pH unit for the kinetics of cyt c_t photo-oxidation implies that several protonable groups of about the same p*K* around 7.8, are involved in that transition. At pH 7, biphasicity can be induced by the addition of 30% glycerol (Fig. 1) or divalent cations like Mg^{2+} (see Figs. 3 and 7). To demonstrate that high pH or addition of Mg^{2+} does not mistreat the cells, we have performed the following experi-

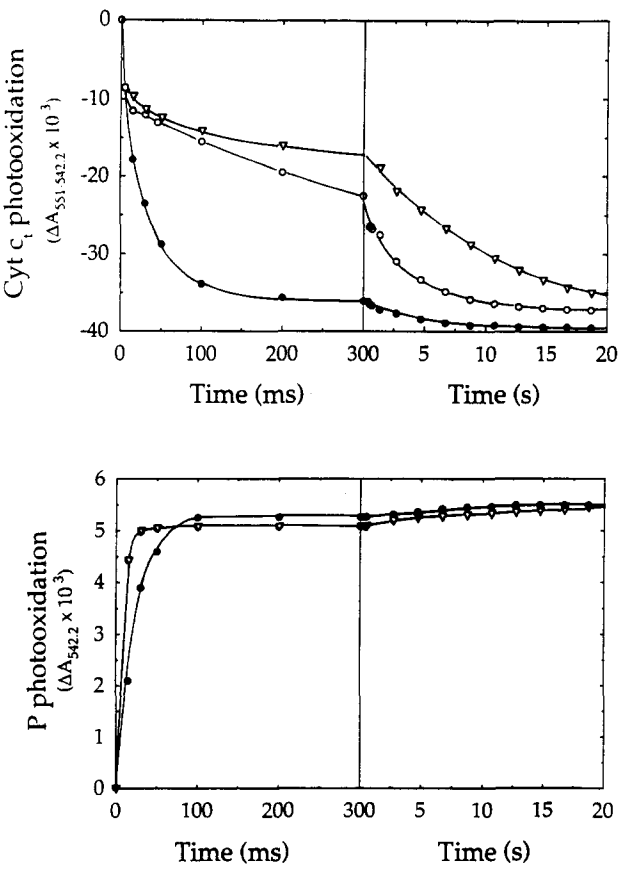

Fig. 1. Kinetics of the cyt c_t and the primary donor P oxidation induced by continuous illumination of benzoquinone-treated *R. sphaeroides* forma sp. *denitrificans* cells. The cells were grown phototrophically in the presence of nitrate. 20 μM myxothiazol, 1 mM KCN, 20 mM Tris-HCl buffer pH 7 (filled circle), pH 7 in the presence of 30% glycerol (inverted open triangle) and pH 8.6 (open circle).

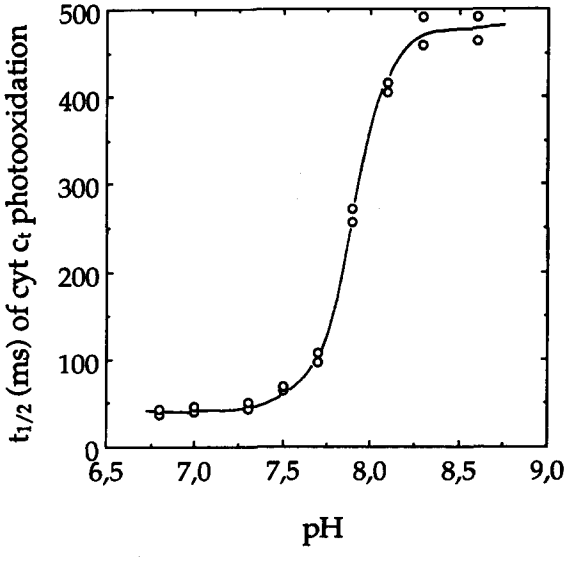

Fig. 2. Half-time (in ms) of the slow phase of cyt c_t photo-oxidation as a function of the pH of the suspension. The cells were grown phototrophically in the presence of nitrate. 20 μM myxothiazol, 1 mM KCN, 20 mM Tris-HCl buffer at different pH.

ments. First, we have resuspended the cells subjected to high pH or addition of Mg^{2+} in a pH 7 buffer. The normal kinetics of cyt c_t photo-oxidation observed at this pH are restored (data not shown). Moreover, the addition of 50 mM KCl reverses the effect of high pH on the cyt c_t photo-oxidation (data not shown and Ref. [14]). Secondly, the biphasic behavior of the cyt c_t photo-oxidation can be observed for cells suspended at pH 7, even in the absence of Mg^{2+}, when a high intensity excitation light is used. This is shown in Fig. 3 where we have plotted the function [P] = f[cyt c_t] for different values of the excitation light intensity. For the highest intensity used (400 photons/RC/s), a clear biphasic pattern is observed in the presence and in the absence of Mg^{2+}. At lower light intensity, this biphasic pattern is much less pronounced when Mg^{2+} is omitted. The high dependence of the function [P] = f[cyt c_t] upon the light intensity at pH 7 in the absence of Mg^{2+} implies that the rate of photo-oxidation of the secondary electron donors is limited by a dark diffusional process in agreement with the behavior observed in the presence of glycerol [18] (Fig. 1). On the other hand, the independence of the function [P] = f[cyt c_t] towards light intensity in the presence of Mg^{2+} (Fig. 3) or at high pH (data not shown) shows that the rate of photo-oxidation of the secondary electron donors is limited by the rate of the photochemical process in these conditions.

From the values of the absorption coefficients of cyt c_t and P, we have calculated that the ratio cyt c_t/P is equal to 3.6 for the cells used in Fig. 1. 32% of the

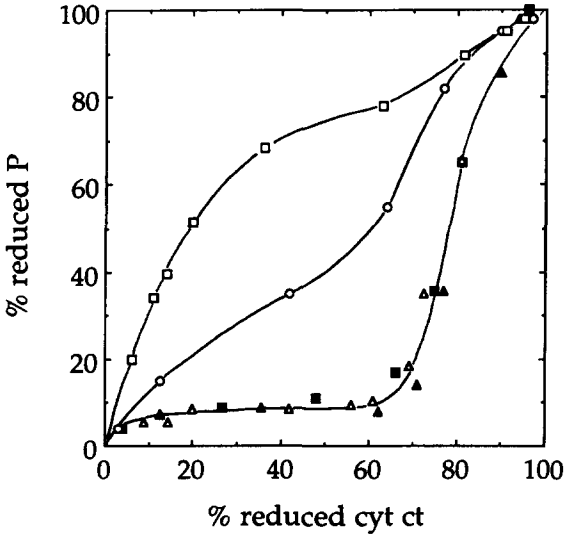

Fig. 3. Reduced P as a function of reduced cyt c_t in experiments similar to those reported in Fig. 1 for different intensities of actinic light. *R. sphaeroides* forma sp. *denitrificans* cells were suspended either in 20 mM Tris-HCl buffer (pH 7), 20 μM myxothiazol, 1 mM KCN, in the absence (open symbols) or in the presence of 20 mM $MgCl_2$ (filled symbols); 400 (open triangle, filled triangle), 200 (open circle) and 80 photons (open square, filled square) per RC per s.

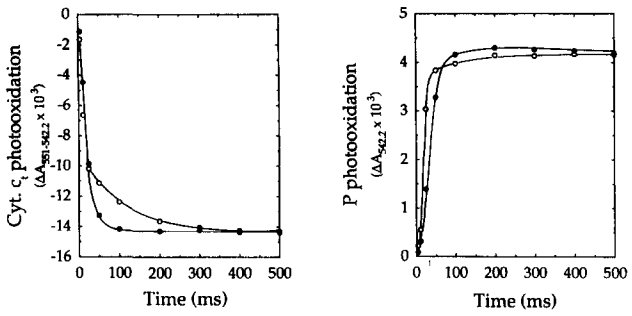

Fig. 4. Same as Fig. 1 but for cells grown phototrophically in the absence of nitrate.

total cyt c_t is photo-oxidized during the fast phase, while more than 85% of the total P is involved in that process (Fig. 1). These percentages varied from 30 to 35% for the cyt c_t and from 80 to 90% for P depending on the batch of cells. On the other hand, the stoichiometric ratio between cyt c_t and P, involved in the fast phase, was rather constant and equal to 1.05 ± 0.05. This ratio lies between 10 and 30 for the slow phase depending on the batch of cells. As already observed for whole cells of *R. sphaeroides* Ga [14], the light-induced difference spectra for the fast and slow phases of cyt c_t photo-oxidation peak at slightly different wavelengths, 551 and 550 nm respectively (data not shown).

When cells are grown phototrophically in the absence of nitrate (Fig. 4), more than 70% of the cyt c_t is photo-oxidized during the fast phase. The ratio between the total cyt c_t and P is equal to 1.7 for cells used in the experiment of Fig. 4. For the fast phase of cyt c_t photo-oxidation, this ratio is equal to 0.95 ± 0.05.

These experiments demonstrate the presence of two pools of cyt c_t out of equilibrium at high pH or at low pH in the presence of Mg^{2+} or glycerol, especially for cells grown under denitrifying conditions. These two pools of cyt c_t can also be evidenced following a series of actinic flashes. Matsuura et al. [18] have already reported that, for cells grown under photodenitrifying conditions, an increase of the medium viscosity does not affect the amount of cyt c_t photo-oxidized on the first two flashes of a series but strongly reduces this amount on the following flashes. A similar effect is observed when the pH of the suspension is higher than 7.5. This is shown in Fig. 5 where the photo-oxidation of P and cyt c_t are compared at pH 7 and 8.6 for *R. sphaeroides* forma sp. *denitrificans* cells grown phototrophically in the presence of nitrate. At pH 7, the full oxidation of cyt c_t necessitates more than 10 flashes. After each flash, the photo-oxidized P is rapidly re-reduced by the cyt c_t (Fig. 5). At pH 8.6, a significant amount of cyt c_t is only photo-oxidized on the first two flashes. On the following flashes, a low efficiency for the cyt c_t photo-oxidation is observed al-

though a large fraction of P is in the oxidized state (Fig. 5). This implies that reduced cyt c_t, although in excess, can interact, at high pH (Fig. 5) or in the presence of glycerol [18], with only a small part of the photo-oxidized RC. These results show that the equilibration between the two pools of cyt c_t is possible at low pH in a time range of 50 ms, the dark time between two excitation flashes. This equilibration is prevented at high pH or in the presence of glycerol in the second time range. When cells are grown in the absence of nitrate (Fig. 6), most of the cyt c_t and the P are photo-oxidized on the first two flashes even at high pH. This is in agreement with the results of Matsuura et al. [18] and with the observation that the percentage of the fast phase of cyt c_t photo-oxidation is particularly high in these growth conditions (Fig. 4).

3.2. Relation between the respiratory and denitrifying chains and the two pools of cytochrome c_t

The large amount of the slowly photo-oxidized cyt c_t in conditions where the denitrification pathway is strongly induced (Fig. 1) [18] suggests that this pool of cyt c_t could be related to this bioenergetic process. To verify this hypothesis, we have measured the ratio between the cyt c_t involved in the slow and fast photo-oxidation phases (R = cyt c_t slow/cyt c_t fast) for different growth conditions. The lowest values are measured when the denitrifying chain is present in very low concentration, for example when cells are grown in the light in the absence of nitrate ($R = 0.1$–0.3). On the other hand, when the denitrifying chain is strongly expressed by the addition of nitrate, this ratio lies between 1.85 and 3. The highest value ($R = 2.6$–3) is obtained under dark semi-aerobic conditions in the presence of nitrate, i.e., when the photosynthetic chain is repressed while the respiratory and denitrifying chains are synthesized in large quantities [20,21].

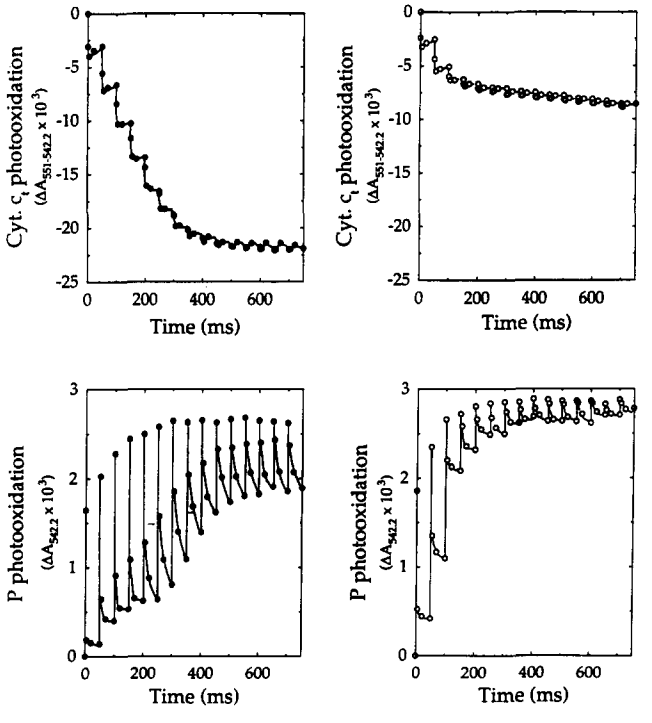

Fig. 5. Flash-induced photo-oxidation of cyt c_t and P for *R. sphaeroides* forma sp. *denitrificans* cells grown phototrophically in the presence of nitrate. The saturating excitation flashes were fired every 50 ms. 20 μM myxothiazol, 1 mM KCN, 20 mM Tris-HCl buffer pH 7 (filled circle) or pH 8.6 (open circle).

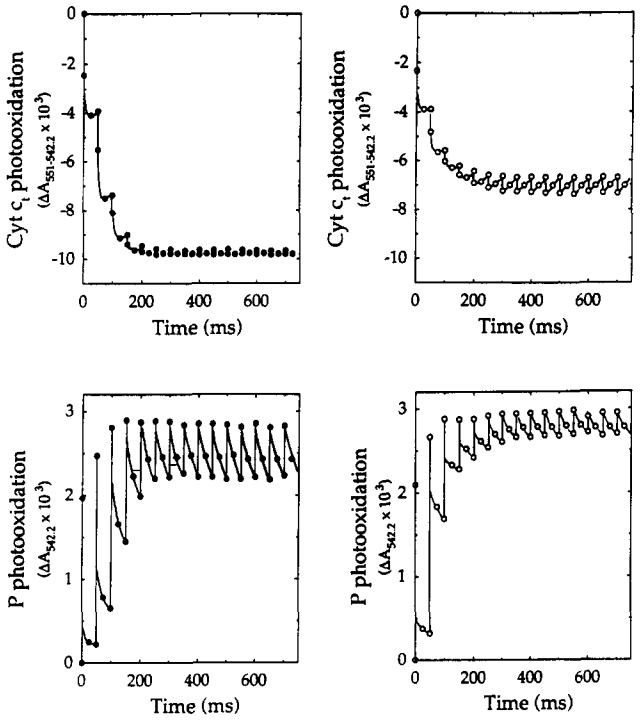

Fig. 6. Same as Fig. 3 but for cells grown phototrophically in the absence of nitrate.

Other evidence for the involvement of the slow photo-oxidized cyt c_t in the respiratory and denitrifying chains is described in Fig. 7. In curve a, we have measured the kinetics of cyt c_t photo-oxidation for an anaerobic suspension of cells, grown in the light in the presence of nitrate, at pH 7 in the presence of $MgCl_2$ and myxothiazol but without benzoquinone. The clear biphasic photo-oxidation of cyt c_t (Fig. 7) shows that the two pools of cyt c_t can also be observed for cells which have not been treated with benzoquinone. When the suspension is made aerobic, only the fast oxidation phase of cyt c_t is observed (Fig. 7, curve c). Under anaerobic conditions but in the presence of N_2O (Fig. 7, curve b), the slow phase is present but its amplitude is decreased by about 44%. This relatively low amount of cyt c_t oxidized by the addition of N_2O (Fig. 7, curve b) is due to the low efficiency of the N_2O reductase. The experiments described in Fig. 7 demonstrate that

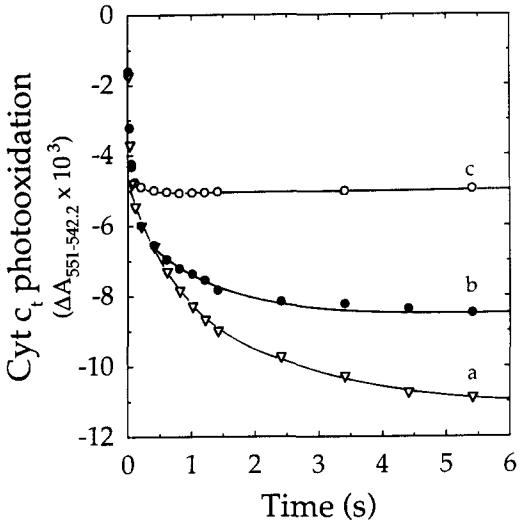

Fig. 7. Kinetics of the cyt c_t oxidation induced by continuous illumination of *R. sphaeroides* forma sp. *denitrificans* cells grown phototrophically in the presence of nitrate. The cells have not been treated with benzoquinone. 20 mM Tris-HCl buffer (pH 7), 20 μM myxothiazol, 20 mM $MgCl_2$. Curve a: anaerobic conditions; curve b: in the presence of N_2O; curve c: aerobic conditions.

only the cyt c_t involved in the slow phase is readily oxidized by the respiratory activity and to a much lesser extent by the N_2O reductase activity. This is in agreement with the results of Itoh et al. [3] who have demonstrated that the bc_1 complex and the cyt c_2 are involved in the N_2O reduction. The above results are also in line with those obtained with *R. sphaeroides* Ga cells in which the cyt c_t involved in the slow photo-oxidation phase is preferentially coupled to the respiratory chain [14].

3.3. Reduction kinetics of cyt c_t in function of myxothiazol concentration

Several arguments have been given in favor of the organization of the electron transfer components of the photosynthetic chain in a supercomplex [14]. Each supercomplex contains two RC, one cyt c_2 and one bc_1 complex. This supercomplex model explains in particular the low value of the apparent equilibrium constant observed between P and cyt c_t during continuous illumination [15]. The observation that the half-time of the slow phase of the carotenoid band-shift is not affected by the addition of sub-saturating concentrations of myxothiazol, while its amplitude is decreased, is also a strong argument for the supercomplex model [14]. A similar approach has been applied to *R. sphaeroides* forma sp. *denitrificans* cells grown phototrophically in the presence of nitrate. We have measured the rate of cyt c_t re-reduction after flash excitation for different sub-saturating concentrations of myxothiazol (Fig. 8). Even at a high concentration of myxothiazol (5 μM) a significant part (15%) of the cyt c_t is re-reduced with a half-time of about 10 ms. The observation of a fast re-reduction of cyt c_t, insensitive to myxothiazol, suggests that the involved reducing pathway bypasses the bc_1 complex. A similar situation has been observed for cells of *R. sphaeroides* Ga [14] and *Rhodospirillum rubrum* [22]. To take into account this phenomenon, we have subtracted the kinetics measured at 5 μM of

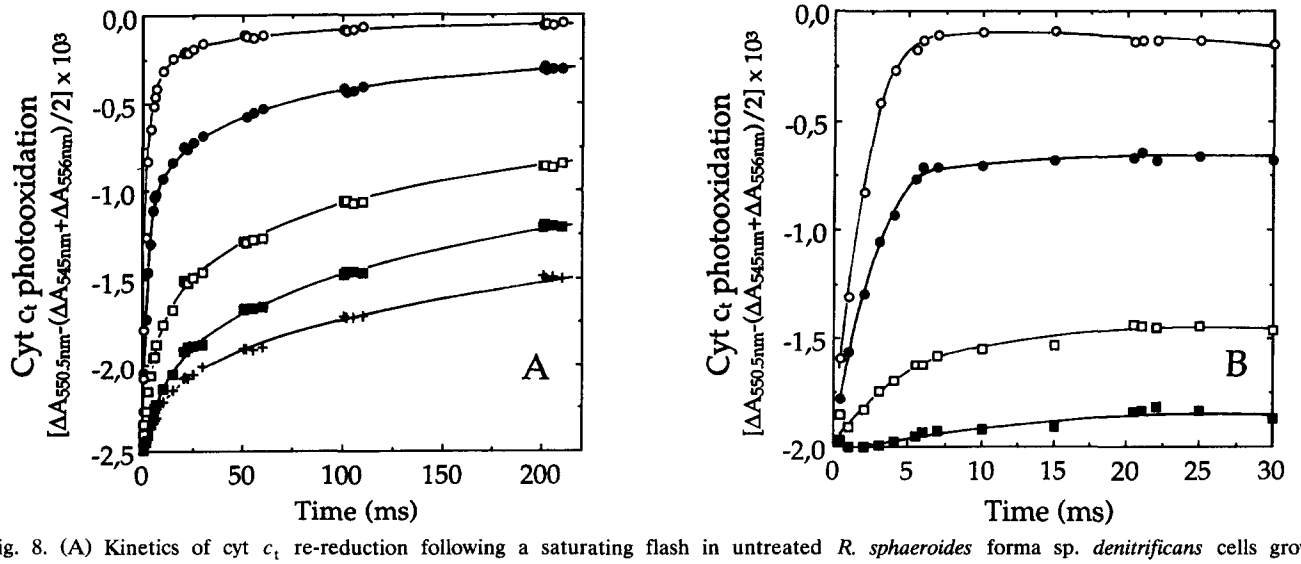

Fig. 8. (A) Kinetics of cyt c_t re-reduction following a saturating flash in untreated *R. sphaeroides* forma sp. *denitrificans* cells grown phototrophically in the presence of nitrate. 20 mM Tris-HCl buffer (pH 7), 1 mM KCN, 20 mM $MgCl_2$: no addition (open circle), 0.2 μM myxothiazol (filled circle), 0.64 μM myxothiazol (open square), 1 μM myxothiazol (filled square), and 5 μM myxothiazol (+). (B) Same as A after subtraction of the 5 μM myxothiazol kinetics from the kinetics obtained for sub-saturating concentrations of myxothiazol (see text for further explanation).

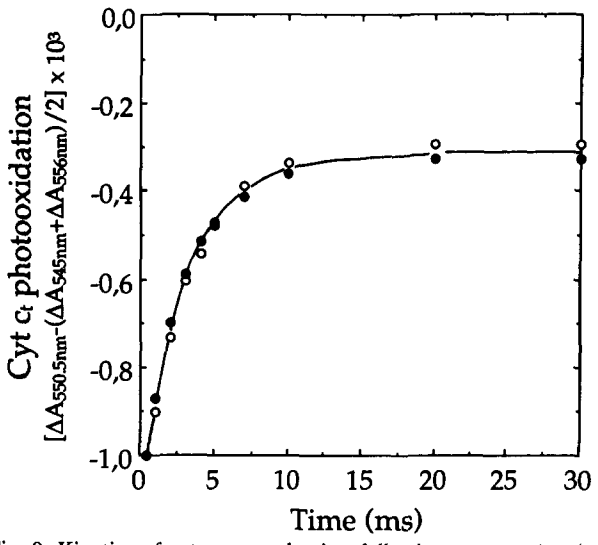

Fig. 9. Kinetics of cyt c_t re-reduction following a saturating (open circle) or a sub-saturating (filled circle) flash for untreated *R. sphaeroides* forma sp. *denitrificans* cells grown phototrophically in the presence of nitrate. The two curves have been normalized at 500 μs. The light intensity of sub-saturating flash was decreased to photo-oxidize only 25% of the RCs. 20 mM Tris-HCl buffer (pH 7), 1 mM KCN, 0.2 μM myxothiazol.

myxothiazol from those observed at sub-concentrations (0.2, 0.6 and 1 μM). The results are presented in Fig. 8B. At all concentrations of myxothiazol the kinetics of cyt c_t re-reduction are clearly biphasic (Fig. 8B). They are composed of a fast phase the half-time of which is almost unaffected by the addition of myxothiazol and of a slow decaying phase the amplitude of which increases with increasing concentration of the inhibitor. If the cyt c_2 could diffuse among different bc_1 complexes, we would expect that all the photo-oxidized cyt c_2 should be able to be re-reduced by the uninhibited bc_1 complexes. Therefore, the addition of sub-saturating concentrations of myxothiazol should only slow down the rate of cyt c_t re-reduction but not change the total amount of cyt c_t re-reduced. This is clearly not the case in Fig. 8B. On the other hand, if the cyt c_2 can react with only a given bc_1 complex, the cyt c_2 connected with an inhibited bc_1 complex will remain oxidized, while those connected with an uninhibited complex will be rapidly re-reduced. This is the situation observed in Fig. 8B. Another way to check that the cyt c_2 can only react with a given bc_1 complex is to decrease the intensity of the excitation flash. In the experiment of Fig. 9, we have compared the effect of reducing the intensity of the excitation flash on the rate of reduction of cyt c_t for cells partially (30%) inhibited by 0.2 μM myxothiazol. Decreasing the flash intensity in order to photo-oxidize only 25% of the total cyt c_t photo-oxidized by a saturating flash does not affect the ratio between the fast and the slow phases of cyt c_t re-reduction (Fig. 9). In a diffusional model for the cyt c_2, one expects a larger inhibition after a saturating flash than after a sub-saturating flash because the number of photo-oxidized cyt c_2 is smaller than the number of uninhibited bc_1 complex in this last condition. On the other hand, the behavior observed in Fig. 9 is expected if the cyt c_2 can react with only one bc_1 complex. The main effect of decreasing the flash intensity is to accelerate the fast phase of cyt c_t reduction (Fig. 9). This acceleration is in agreement with the lower amount of bc_1 complex compared to RC. Indeed for such stoichiometry, we need more turn-overs of the bc_1 complex under saturating flash excitation than under sub-saturating excitation. The result of the experiment of Fig. 9, performed at pH 7 in the absence of Mg^{2+}, shows, moreover, that RC, cyt c_2 and bc_1 complex also associate into a supercomplex in these conditions.

3.4. Kinetics of cyt c_t photo-oxidation after a single saturating flash in the presence of glycerol

In the supercomplex model, we expect a small effect of increasing the medium viscosity on the electron transfer rates between cyt c_2 and P or between cyt c_1 and cyt c_2 because the cyt c_2 is confined between the RCs and the bc_1 complex. We indeed observe that the 100 μs phase of cyt c_t oxidation, which corresponds to the electron transfer reaction between cyt c_1 and cyt c_2^+ and between cyt c_2 and P^+, is not affected by the addition of 30% glycerol (Fig. 10). The only observable effect of the addition of glycerol on the cyt c_t oxidation after a single flash is a decrease of the total amplitude

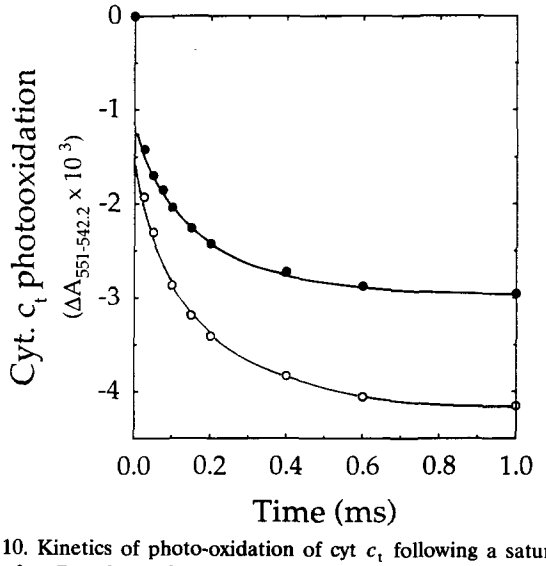

Fig. 10. Kinetics of photo-oxidation of cyt c_t following a saturating flash for *R. sphaeroides* forma sp. *denitrificans* cells grown phototrophically in the presence of nitrate. 20 μM myxothiazol, 1 mM KCN, 20 mM Tris-HCl buffer (pH 7): control experiment (open circle); after addition of 30% glycerol (filled circle).

of the amount of cyt c_t photo-oxidized. This corresponds with a partial dissociation of the cyt c_2 from the RC induced by the addition of glycerol.

3.5. Ultrastructure of R. sphaeroides forma sp. denitrificans cells grown in the presence and in the absence of nitrate

By varying the growth conditions it has been shown that the photosynthetic chains of *R. sphaeroides* cells are essentially located in the intracytoplasmic membrane and that the number of these invaginations is decreased when the synthesis of the photosynthetic chain is repressed [23,24]. For *R. sphaeroides* forma sp. *denitrificans* cells, Satoh and coworkers have reported that the addition of nitrate in the culture medium induces a decrease in the number of intracytoplasmic membrane [25] and a decrease in the LHII/LHI ratio [26]. We have confirmed (Fig. 11) that the number of invaginations is decreased by a factor of 2–3 when cells are grown under photodenitrifying conditions. In addition to this decrease, long tubular structures are induced (Fig. 11). The diameter of these tubes is about 100 nm and they can be as long as 2 μm. They may impair cell division (see Fig. 11). These tubes are also observed when the cells are grown under dark anaerobic denitrifying conditions with either nitrate or N_2O as terminal acceptor or under dark semi-aerobic conditions in the absence of nitrate (data not shown). They are therefore not related to the induction of the denitrifying pathway but more likely to the decrease in the LHII/LHI ratio observed in these conditions [26] (Fig. 12). Indeed, similar tubes have been observed for different mutants of *R. sphaeroides* lacking the LHII complexes [27–29].

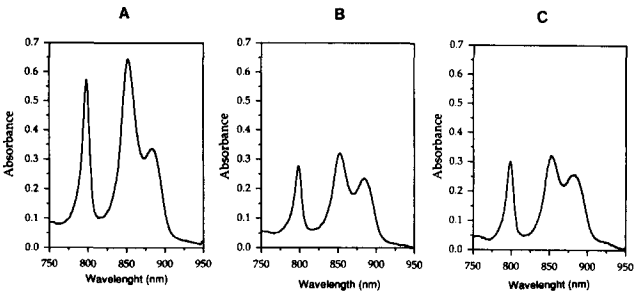

Fig. 12. Absorption spectra at 105 K of cell free extracts of bacteria grown under light anaerobic conditions in the absence (A) or in the presence (B) of 20 mM nitrate or under dark aerobic conditions (C) (0.1 mg/ml of protein).

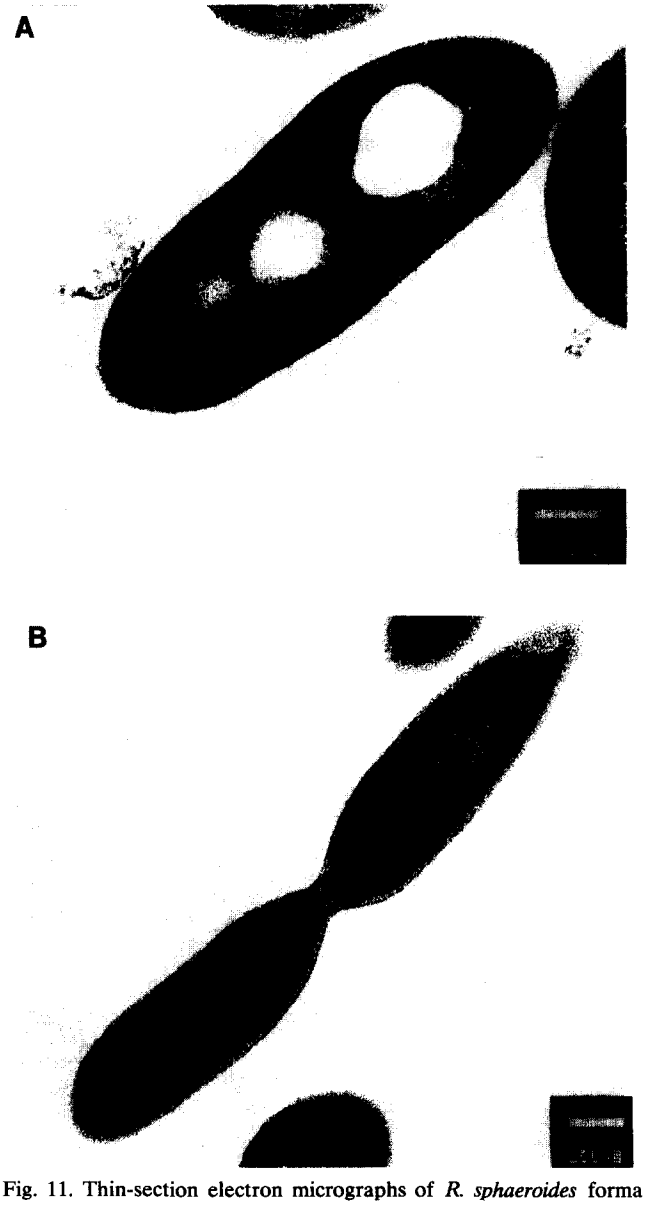

Fig. 11. Thin-section electron micrographs of *R. sphaeroides* forma sp. *denitrificans* cells. The top and bottom panels show cells grown phototrophically in the absence and in the presence of nitrate, respectively. Note that the tubules are running between two parts of a dividing cell.

Freeze-etching pictures of these tubes (Fig. 13) present a regular arrangement of membrane complexes. Particles with a dimension of about 100 × 100 Å, are arrayed 2 by 2. Similar structures have been observed in the cells of *R. sphaeroides* M_{21}, a mutant lacking the LHII complex, where tubular membranes are also present (R.A. Niederman, personal communication). The size of each particle (100 Å) is similar to the RC-to-RC distance measured in the photosynthetic membranes isolated from *Rhodopseudomonas viridis*, *Ectothiorhodospira halochloris* (130 Å) or *Rhodopseudomonas marina* (100 Å) [30–32]. In membranes of *Rh. viridis*, *E. halochloris* and *Rh. marina*, a hexagonal lattice is observed in agreement with the *p6* symmetry of the LH ring. We did not observe such a hexagonal lattice in

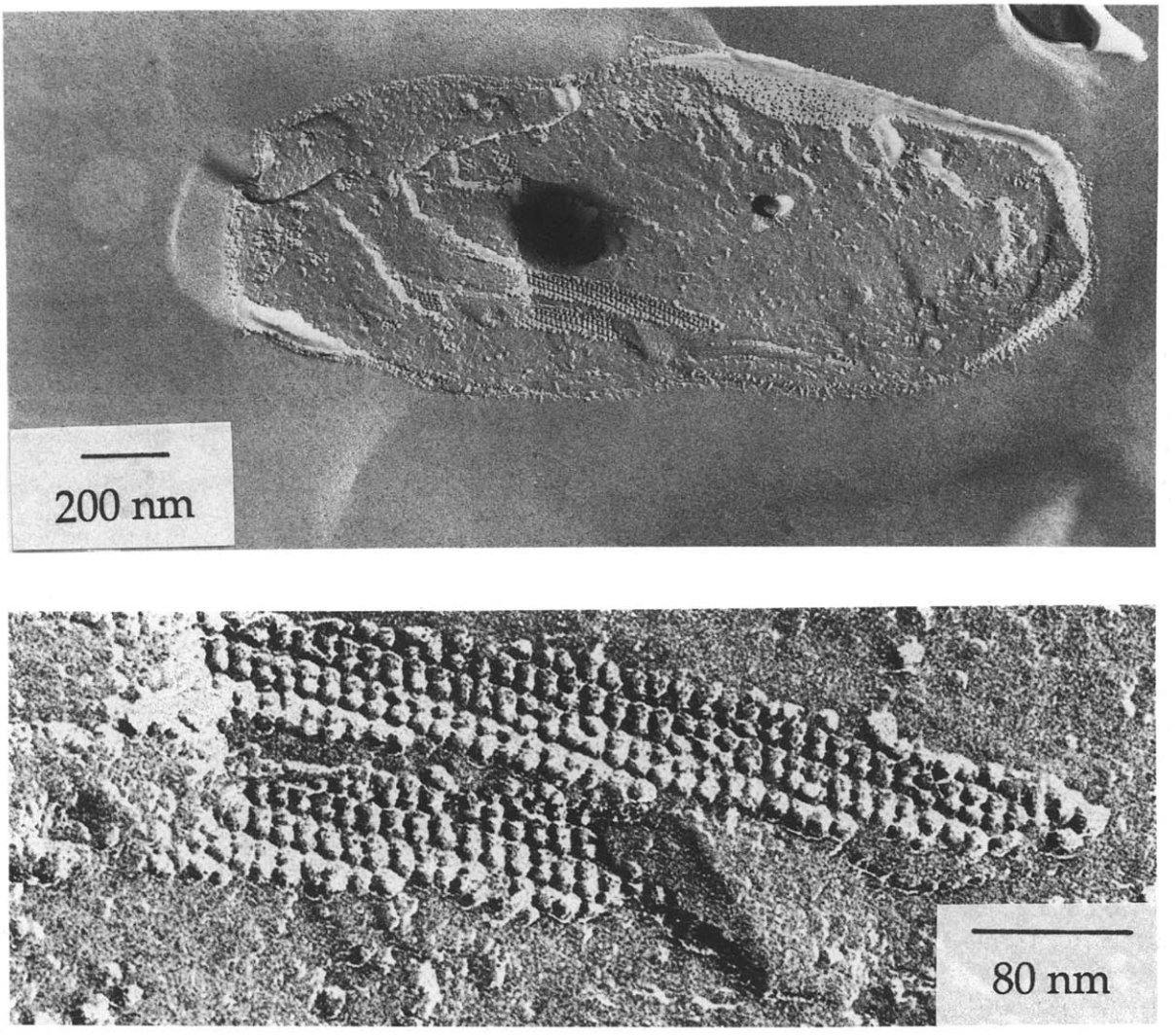

Fig. 13. Freeze-etching picture of the tubes present in cells of *R. sphaeroides* forma sp. *denitrificans* cells grown phototrophically in the presence of nitrate. Note the dimeric pattern in the regular array of membrane particles.

the case of *R. sphaeroides* forma sp. *denitrificans* cells but a clear dimeric association is apparent (Fig. 13).

4. Discussion

In this article we have precised the spectral characteristics, the functional and kinetic behavior of the two pools of cyt c_t first evidenced by Matsuura et al. [18] in *R. sphaeroides* forma sp. *denitrificans* cells. These two pools can be distinguished by their kinetic behavior under continuous illumination. One pool is rapidly photo-oxidized irrespective of the conditions (pH, medium viscosity). The second pool is slowly photo-oxidized, in particular at pH higher than 7.5, at any pH in the presence of Mg^{2+} or in the presence of glycerol (Fig. 1). These two pools of cyt c_t are also clearly evidenced following a series of actinic flashes (Fig. 5) [18]. Their relative amount depends highly on the growth conditions. As already observed in the case of *R. sphaeroides* Ga whole cells [14], these two pools differ by their localization and function. The first pool is connected to the photosynthetic electron transfer components. Its light-induced difference spectrum, centered at 551 nm, suggests that it is composed of cyt c_2 and cyt c_1 in about equal amounts. The second pool is coupled to the respiratory and the denitrifying chains as shown by the effect of these activities on its redox state (Fig. 7). It is mainly composed of cyt c_2. Electron exchange between the two pools of cyt c_2 occurs at low pH in tens of milliseconds. Because of this fast exchange, continuous illumination can oxidize the cyt c_2 connected to the respiratory and the denitrifying chains and therefore induce their inhibition [3]. At high pH, in the presence of divalent cations or when the medium viscosity is increased, redox equilibration between the two pools is prevented. In these conditions the free cyt c_2 can only be oxidized by a small number of RCs

present in the cytoplasmic part of the membrane. This induces a slow phase of cyt c_t photo-oxidation and a low efficiency under flash excitation.

The simplest model to explain these behaviors is the supposition that the cyt c_t involved in the first pool is part of a supramolecular organization composed of two RC, one cyt c_2 and one bc_1 complex. In this supercomplex, one cyt c_2 is tightly connected to two RCs and one bc_1 complex. Only a limited rotation is allowed for cyt c_2 to react with either the RCs or the bc_1 complex. Only when both cyt c_2 and cyt c_1 are photo-oxidized, the oxidized cyt c_2 can leave the supercomplex and be replaced by free reduced cyt c_2. This supercomplex model is in agreement with the observation that cyt c_t photo-oxidation occurring on the two first flashes does not depend on the physico-chemical parameters of the medium (pH, presence of divalent cations, viscosity). The observation (Fig. 10) that the rate of electron transfer between cyt c_1 and cyt c_2 is not affected by the medium viscosity is also consistent with this model. Clear-cut demonstrations of the supramolecular organization of the photosynthetic electron components are described in Figs. 8 and 9. In these experiments we show that most of the cyt c_2 can only react with a single bc_1 complex. At high pH or in the presence of Mg^{2+}, the photo-oxidized cyt c_2 is trapped in the supercomplex and therefore cannot exchange with free cyt c_2. In the presence of glycerol, diffusion of free cyt c_2 is slowed down and its exchange with the supercomplex is prevented. In *R. sphaeroides* Ga cells, it has already been observed that high pH (apparition of negative charges) or addition of Mg^{2+} (screening of negative charges) induced a stabilization of the supercomplex between RCs, cyt c_2 and bc_1 complex [14]. This paradox is apparent since only divalent cations (Mg^{2+} or Ca^{2+}) and not monovalent cations (like K^+) induce the apparition of a biphasic kinetics for the cyt c_t photo-oxidation. This suggests that these cations act as chelators between negatively charged amino acids located on the different interacting proteins.

Several growth conditions, i.e., addition of nitrate in the light [26] or dark semi-aerobiosis, induce a decrease of the LHII/LHI ratio (Fig. 12). This decrease is correlated with the formation of long tubes for the cytoplasmic membrane (Fig. 11). Similar tubes have been observed for mutants devoid of LHII complexes [27–29]. We therefore propose that the appearance of these tubes is due to a lack of LHII complexes in this region of the membrane. To our knowledge, this is the first report on the formation of tubes in the internal membrane of a Rhodobacter species induced by changes in the growth conditions. A well-ordered organization of the membrane components is observed on freeze-etching pictures for these tubes. A clear dimeric association is apparent for these particles (Fig. 13). The size of each particle corresponds to the size of the photosynthetic unit detected in species like *Rh. viridis*, *E. halochloris* and *Rh. marina* [30–32]. It is too premature to ascribe this dimeric association to the supercomplex formed by RCs and bc_1 complexes evidenced by functional approaches [14,15, this work]. Characterization of these particles in terms of the composition of proteins and electron transfer components is in progress. This will confirm or invalidate such a hypothesis.

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