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The photoinduced cyclic electron transfer in whole cells of *Rhodopseudomonas viridis*

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Light-induced electron transfer has been studied in whole cells of *Rhodopseudomonas viridis*. No photooxidation of the low-potential hemes of the RC bound cytochrome is observed under the more reducing physiological conditions used in this study, i.e. anaerobiosis. On the other hand, photooxidation of the high-potential heme c_{556} of the RC-bound cytochrome is observed 10 μs after flash excitation for cells placed both in aerobic or anaerobic conditions. The photooxidized c_{556} is re-reduced by the soluble cytochrome c_2. This electron transfer occurs with an half-time of 110 μs and 40 μs under aerobic and anaerobic conditions, respectively. The half-time of re-reduction of the soluble cytochrome c_2 is also dependent on the redox state of the cells (25 ms and 8 ms under aerobiosis and anaerobiosis, respectively). This re-reduction process is inhibited by addition of 1 mM myxothiazol or stigmatellin, showing that the bc_1 complex is involved in this reaction. Participation of the bc_1 complex in the cyclic electron transfer is also confirmed by the observation of photooxidation of a cytochrome *b* under anaerobic conditions. Varying the flash intensity or the viscosity of the medium did not affect the half-time of the oxidation of cytochrome c_2. On the other hand, the rate of re-reduction of cytochrome c_2 is strongly affected under such conditions. We conclude from this series of experiments that the soluble cytochrome c_2 forms a stable complex with the RC tetraheme cytochrome. Once oxidized, the cytochrome c_2 diffuses and is re-reduced via a cyclic electron transfer involving the bc_1 complex.

Introduction

The first step of the transformation of light into chemical energy in purple photosynthetic bacteria is a cyclic electron transfer linked to the transport of protons across the intracytoplasmic membrane. This process involved two membrane-bound multisubunit complexes, the photosynthetic reaction center (RC) and the cytochrome bc_1 complex. Two soluble components, ubiquinol in the hydrophobic domain of the membrane and cytochrome c_2 in the periplasmic space, connect these two transmembrane complexes on the acceptor and donor sides of the reaction center, respectively [1]. For one type of RC, the electron is directly transferred from the cytochrome c_2 to the photooxidized dimeric primary donor (P^+). This is, for example, the case of species like *Rhodobacter sphaeroides, Rhodobacter capsulatus, Rhodospirillum rubrum, Rhodopseudomonas palustris* or *Erythrobacter longus* [1–4]. In other species (*Rps. viridis* and *Rps. sulfoviridis, Chromatium vinosum* and *C. tepidum, Rhodocyclus gelatinosus, Rps. acidophila* or *Roseobacter denitrificans* previously named *Erythrobacter* OCH114 and *Ectothiorhodospira* sp.) a tetraheme cytochrome *c*, more or less tightly bound to the RC, contains two high-potential and two low-potential hemes [1–10]. Each of these hemes can serve as electron donor to the photooxidized primary donor, depending on the redox conditions and the temperature. It is generally admitted that cytochrome c_2 or a related cytochrome subsequently re-reduces the photooxidized high-potential hemes of this tetraheme cytochrome. This conclusion was drawn from reconstitution experiments between isolated reaction centers (or chromatophores) and purified cytochrome c_2 in the case of *Rps. viridis* [11,12] or *Rhodocyclus gelatinosus* [13] or studies of the light-induced electron transfer pathway in whole cells (*Chromatium vinosum* [14]).

The determination of the X-ray crystal structure of *Rps. viridis* RC provided much information on the organization of the different prosthetic groups [15,16]. The four hemes of the RC-bound cytochrome are arranged approximately linearly. Their thermodynamic

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; RC, reaction center.

and kinetics properties were deduced from measurements of light-induced absorbance changes and redox potentiometry. The midpoint redox potential of the four hemes c_{559}, c_{556}, c_{552} and c_{554} * have been found to be equal to +380 mV, +310 mV, +20 mV and −60 mV, respectively [17–19]. Heme c_{559} reduces the photooxidized primary donor in about 0.19 μs [20,21]. This heme is subsequently re-reduced by heme c_{556} with an half-time of 2.5 μs [20,21]. This kinetic behaviour, EPR [22] and linear dichroism [18,19,23] studies are consistent with the following order: P, c_{559}, c_{552}, c_{556} and c_{554} for the organization of the donor side of the *Rps. viridis* RC.

On the other hand, only little information on the photoinduced cyclic electron transfer in *Rps. viridis* membranes is available. Early studies by Olson and Nadler [24] on whole cells showed that the soluble cytochrome c_2 can be photooxidized. Later, Shill and Wood [11] and more recently Knaff et al. [12] reported efficient electron transfer occurring between soluble cytochrome c_2 and the high-potential heme c_{556} of the RC in reconstituted systems. The bc_1 cytochrome complex was thought for a long time to be absent in *Rps. viridis* photosynthetic membrane [25]. In fact the bc_1 complex is present but in a low amount. From protoheme analysis, Wynn et al. [26] deduced that there is no more than 1 bc_1 complex per 5 reaction centers in the *Rps. viridis* photosynthetic membrane. This bc_1 complex has been nevertheless isolated and characterized [26,27]. The complete amino-acid sequence of the different subunits forming the complex has been determined recently [28]. A high level of homology between *Rps. viridis* and *Rhodobacter capsulatus* bc_1 complexes was found [28]. From the above data and from the observation that cytochrome c_2 is not synthesized when *Rps. viridis* cells are grown under aerobic conditions [29], a photoinduced cyclic electron transfer involving reaction center, cytochrome c_2 and bc_1 complex similar to that well-characterized in species like *Rhodobacter sphaeroides* and *capsulatus* [1], is generally assumed to occur in photosynthetic membranes of *Rps. viridis*. This has, however, not been proven experimentally by kinetics studies. The study of electron flow in *Rps. viridis* has to be done with whole cells, since the lamellar structure of its membrane [30] does not retain the soluble cytochrome c_2. We have therefore undertaken a series of measurements of light-induced absorbance changes in whole cells. Another aim of this work was to find out a possible role for the two hemes c_{552} and c_{554} which, due to their low redox midpoint potentials, cannot be involved in a classical photoinduced cyclic electron transfer.

* The subscript refers to the wavelength of maximum absorption of different heme α-band at room temperature following the nomenclature of Dracheva et al. [21].

Materials and Methods

Rps. viridis wild-type strains were grown in the light at 30°C in Hutner medium under anaerobic conditions. The cells, harvested after 24 h, were suspended in fresh growth medium at pH 7.0.

Light-induced absorption changes were measured with an apparatus similar to the one described in Ref. 31. To ensure aerobic conditions, air was gently bubbled into the suspension. Anaerobiosis was obtained by addition of glucose (20 mM) and glucose oxidase (3 mg/ml) and bubbling with nitrogen. Excitation light was provided either by a xenon lamp (flash duration 3 μs) filtered through a Kodak Wratten filter 89B or by an Alexandrite laser (Schwartz Electro-Optics, model 1-2-3, 250 ns, 200 mJ, 750 nm). No difference was observed in the absorption change kinetics between the two types of excitation. The light intensity of the xenon flash could be decreased with suitable grey filters while the laser flash was attenuated with crossed polarizers.

We have taken an $\Delta\epsilon_{red-ox} = 19.6\ mM^{-1}\,cm^{-1}$ at 550.2 nm and $\Delta\epsilon_{red-ox} = 25\ mM^{-1}\,cm^{-1}$ at 556 nm for the soluble cytochrome c_2 [12] and heme c_{556} [21], respectively, to estimate their relative concentration.

CCCP, Antimycin A and myxothiazol were purchased from Sigma and dissolved in ethanol. Stigmatellin, dissolved in methanol, was purchased from Fluka.

Results and Discussion

Light-induced difference spectra following laser excitation in whole cells of Rps. viridis

Fig. 1A shows the light-induced difference spectrum observed in the visible region for different detection times (10 μs, 7 ms, 20 ms and 100 ms) after flash excitation for an anaerobic suspension of *Rps. viridis* whole cells. The light-induced difference spectrum, measured 10 μs after the laser excitation, is characteristic of oxidation of heme c_{556} of the RC-bound cytochrome [20,21]. No absorbance changes linked to the photooxidation of the low potential hemes of the RC-bound cytochrome can be observed at this detecting time. This finding is consistent with the observation that the low potential hemes are not reduced when cells are placed under anaerobiosis (data not shown). The difference spectrum measured 7 ms after the actinic flash peaks at 551 nm. These absorption changes cannot be linked to the photooxidation of the low potential hemes of the RC-bound cytochrome as discussed above. They correspond to the oxidation of cytochrome c_2 in agreement with their wavelength spectrum position. At longer times of detection, the

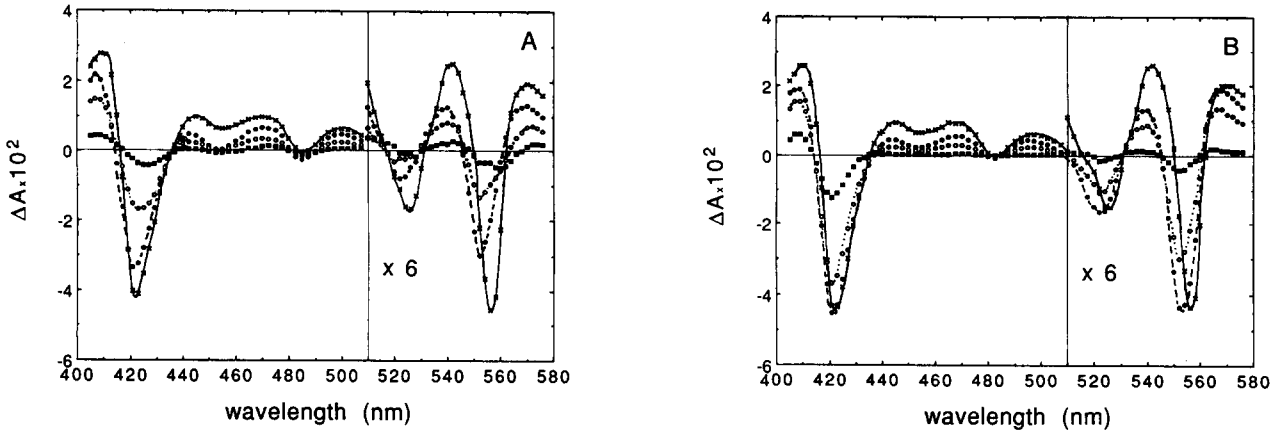

Fig. 1. Difference absorption spectra recorded at different times after an actinic flash for *Rps. viridis* whole cells ($OD_{960\ nm}$ 0.7). (A) anaerobic conditions; (B) aerobic conditions. ×, 10 μs; •, 7 ms; ○, 20 ms; ■, 100 ms. Note that from 510 to 580 nm the absorbance changes have been enhanced by a factor as indicated by ×6.

absorption changes present, in addition to some oxidized cytochrome *c*, an α-band centered at 560 nm and a Soret band peaking at 428 nm. These wavelengths are characteristic of the high-potential cytochrome *b* of the bc_1 complex [27].

The internal redox potential of the cells could be varied to some extent by making the suspension aerobic by gentle air-bubbling. This did not affect significantly the shape of the light-induced absorption changes (Fig. 1B). The main differences reside in the disappearance of the changes linked to cytochrome *b* oxidation in the long time range of detection and a lower rate of cytochrome c_2 re-reduction. From the relative amplitudes of the changes linked to heme c_{556} and cytochrome c_2 photooxidations measured at 10 μs and 1 ms, respectively, we have calculated that one molecule of cytochrome c_2 is photooxidized per RC both in anaerobic and aerobic conditions.

It has been reported that *Rps. viridis* membranes do not present a carotenoid band shift [24]. The light-induced difference spectra depicted in Fig. 1A and B in the 440–520 nm region are, however, not characteristic of cytochrome oxidation but, although of small amplitude, more typical of a carotenoid bandshift. Several features of these absorption changes (effect of uncouplers, kinetics) are in agreement with this attribution. They will be presented in a subsequent paper.

In the following paragraphs, we will successively discussed participation of cytochrome *b*, kinetics of oxidation and reduction of cytochrome c_2 under aerobic and anaerobic conditions and absence of photooxidation of low-potential-hemes RC-bound cytochrome.

Participation of a bc_1 complex in the cyclic electron transfer of Rps. viridis whole cells

The possible participation of a cytochrome *b* in the cyclic electron transfer of *Rps. viridis* cells is suggested by the light-induced difference spectrum observed in the hundreds of millisecond time range under anaerobic conditions (Fig. 1A). This difference spectrum corresponds to the oxidation of both a cytochrome *b* and a cytochrome *c* which can be either the soluble cytochrome c_2 or the membrane-bound cytochrome c_1. Several observations and experiments support the hypothesis that this cytochrome *b* is part of the bc_1 complex. First of all, addition of increasing concentration of a specific inhibitor like myxothiazol [32] (Fig. 2) or stigmatellin [33] (not shown) progressively slows down the cytochrome c_2 re-reduction. A complete inhibition of cytochrome c_2 reduction in the tens of millisecond time scale is observed for concentration greater than 1 μM of myxothiazol (Fig. 2). The amplitude of flash-induced oxidized cytochrome c_2 is only slightly increased (5%) in presence of stigmatellin in

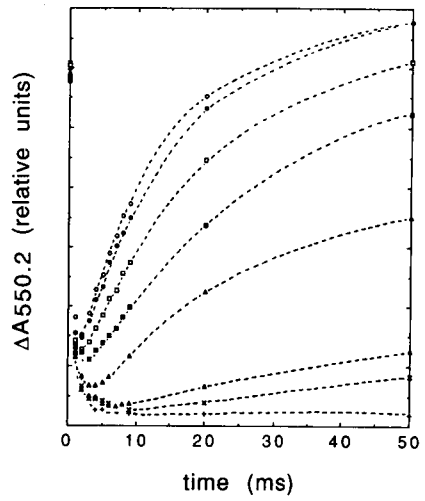

Fig. 2. Flash-induced absorption changes linked to cytochrome c_2 re-reduction measured at 550.2 nm in *Rps. viridis* whole cells under anaerobic conditions as a function of myxothiazol concentration. ○, No addition; •, 0.1 μM; □, 0.2 μM; ■, 0.3 μM;, △, 0.4 μM; ▲, 0.5 μM; ×, 0.7 μM; +, 1 μM.

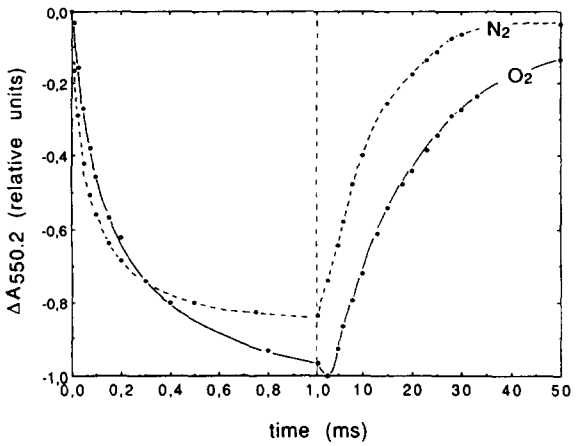

Fig. 3. Kinetics of oxidation and re-reduction of the cytochrome c_2 measured at 550.2 nm under aerobic (○, O_2) or anaerobic (●, N_2) conditions. Note the change of time scale.

comparison to the extent measured with myxothiazol (not shown). Myxothiazol blocks the electron transfer between the Q_z site and the FeS center while stigmatellin inhibits the FeS to cyt c_1 transfer. This difference in the mode of action of myxothiazol and stigmatellin is explained by the observation that stigmatellin [33] but not myxothiazol [32] raises the E_m value of the Rieske protein. Consequently, the small difference observed between the samples inhibited by myxothiazol or stigmatellin implies that only few positive equivalents are stored on the FeS center in the presence of myxothiazol. This is in agreement with the low level of bc_1 complex present in *Rps. viridis* membranes [26]. When cells are placed under aerobiosis, the light-induced difference spectra show no change linked to the oxidation of cytochrome *b* (Fig. 1B). This suggests that the oxidation state of this cytochrome *b* is modulated by the respiratory activity in agreement with its identification with the high-potential cytochrome *b* of the bc_1 complex. We expect a photoinduced reduction of cytochrome *b* under oxidizing conditions specially after addition of antimycin A. This is, however, not observed (data not shown). Possible explanations are that oxidized cytochrome c_2 obscures the reduction of the cytochrome *b* or that antimycin A is ineffective in whole cells due to a permeability barrier.

Kinetics of oxidation and reduction of cytochrome c_2

We have measured the kinetics of electron transfer between heme c_{556} and cytochrome c_2 and the re-reduction of this last component by detecting laser-induced absorption changes at 550.2 nm, a wavelength where the contribution of heme c_{556} is very small (see Fig. 1). Fig. 3 compares such an absorption change for cells placed under anaerobiosis or aerobiosis. Under anaerobiosis, the cytochrome c_2 oxidation kinetics after laser excitation are multiphasic. The half-time is about 40 μs but complete oxidation requires 1 ms. The half-time of cytochrome c_2 re-reduction takes about 8 ms under such redox conditions. Similar kinetics for the oxidation and reduction of cytochrome c_2 are observed after addition of $2 \cdot 10^{-4}$ M KCN, which inhibits 70% of the total respiratory activity by blocking the oxidase (data not shown). Under aerobic conditions (Fig. 3) the kinetics of cytochrome c_2 oxidation are slower ($t_{1/2} = 110$ μs) than under anaerobic conditions. The re-reduction of cytochrome c_2 is also 2–3-times slower under aerobiosis compared to anaerobiosis.

In order to check if these electron transfer reactions are limited by diffusion we have modulated the relative concentration of photooxidized c_{556} and reduced cytochrome c_2 by decreasing the laser light intensity. Fig. 4A shows that hitting only 28% of RC does not affect the rate of cytochrome c_2 oxidation. On the other hand, the half-time of cytochrome c_2 re-reduction is decreased by a factor 2. Similarly, no effect on the kinetics of cytochrome c_2 oxidation is observed under

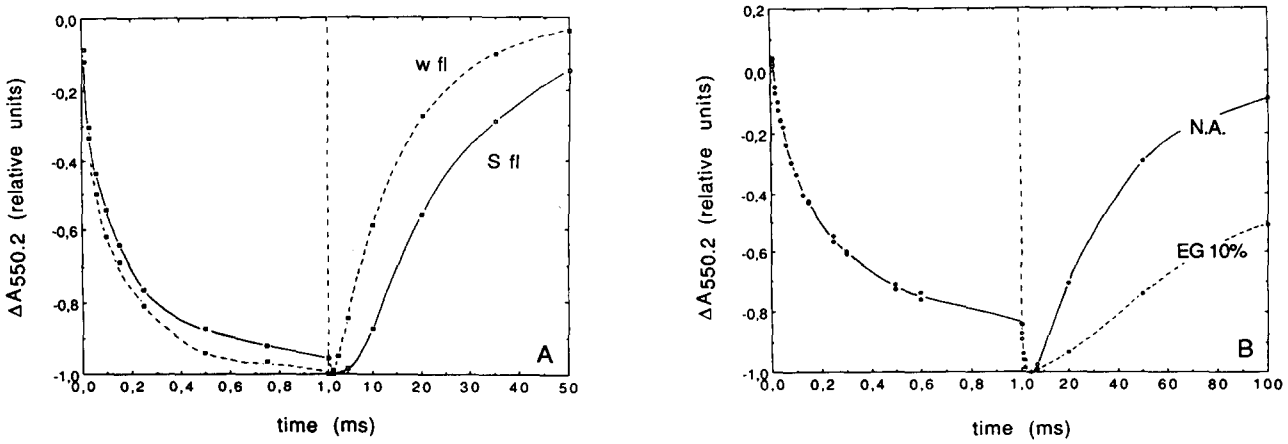

Fig. 4. Same as for Fig. 3 under aerobic conditions. (A) strong flash (S fl, □); weak flash (w fl, ■). The two curves have been normalized at 3 ms. (B) No addition (NA, ○); addition of 10% (v/v) ethyleneglycol (E.G.10%, ●).

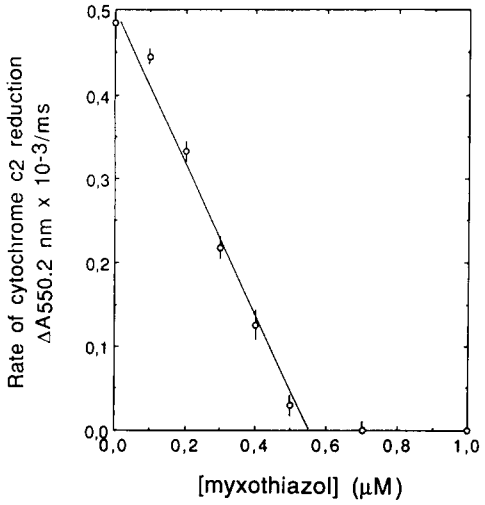

Fig. 5. Initial rates of re-reduction of cytochrome c_2 in function of the concentration of added myxothiazol. These rates have been computed from the data of Fig. 2.

anaerobiosis when the laser intensity is decreased, in particular their multiphasic character is preserved (not shown). These experiments suggest that the electron transfer between cytochrome c_2 and heme c_{556} is not limited by diffusion, i.e., a stable complex is present between reduced cytochrome c_2 and the RC tetrahemic cytochrome c. On the other hand, the kinetics of cytochrome c_2 re-reduction depends strongly upon the relative concentrations of the reactants in agreement with a diffusion process. The diffusional behaviour of this reaction is also proved by the relationship between the initial rate of cytochrome c_2 re-reduction and the concentration of added myxothiazol at subsaturating concentrations (Fig. 2). The initial rate of cytochrome c_2 re-reduction, calculated from the data of Fig. 2, is depicted in Fig. 5. The linear dependence of this rate for subsaturating concentration of myxothiazol is in agreement with a second-order process for the re-reduction of cytochrome c_2. A similar behaviour has been observed for chromatophores of *Rhodobacter sphaeroides* Ga, implying the free diffusion of oxidized cytochrome c_2 in the chromatophore [34]. The faster re-reduction of oxidized cytochrome c_2, observed under anaerobiosis or in presence of KCN (Fig. 3), compared to aerobiosis is also in agreement with a second-order process for this reaction, since the concentration of reduced quinone molecules is expected to increase under the former conditions. The kinetics of re-reduction of oxidized cytochrome c_2 are, however, nearly exponential both under aerobic and anaerobic conditions. This pseudo-first-order behaviour is in agreement with the larger concentration in the cells of the cytochrome c_2 compared to its reductant, the cytochrome c_1. Many turnovers of the bc_1 complex and diffusion of cytochrome c_2 are therefore expected for the complete re-reduction of cytochrome c_2.

To further check these two hypotheses: (1) stable association between reduced cytochrome c_2 and RC and (2) diffusion of the oxidized cytochrome c_2, we have performed experiments in presence of ethyleneglycol to increase the internal viscosity of the cells [35,36]. These experiments, however, cannot be done under anaerobic conditions because the addition of even only 10% of ethyleneglycol induces the partial reduction of the low-potential heme of the RC-bound cytochrome (data not shown). Under aerobic conditions addition of 10% ethyleneglycol has no effect on the rate of oxidation of cytochrome c_2 while its re-reduction is slowed down by a factor 3 (Fig. 4B), in agreement with the two proposed hypotheses. Similarly, addition of 20% glycerol has no effect on the rate of oxidation of cytochrome c_2 but slowed down its reduction by a factor 4 (data not shown).

The kinetics of electron transfer in a stable complex between cytochrome c_2 and the RC are expected to be exponential. This is approximately the case for the cytochrome c_2 oxidation under aerobic conditions (Fig. 3). However, under anaerobic conditions, the electron transfer between cytochrome c_2 and heme c_{556} is clearly multiphasic and this multiphasicity is still observed under low-intensity excitation as already stated (Fig. 3). By measuring the kinetics of oxidation of cytochrome c_2 in the presence of both KCN (1 mM) and myxothiazol or stigmatellin (2 μM), we checked that this departure from exponentiality was not due to truncation of the signal induced by the fast reduction of cytochrome c_2 (not shown). The multiphasicity of cytochrome c_2 oxidation is not related to changes in relative concentration of cytochrome c_2 and heme c_{556}, since anaerobiosis does not induce change in the redox state of any of the cytochromes c of *Rps. viridis* cells (not shown). It is also not due to the influence of the membrane potential on the rate constant, since addition of 10 μM CCCP in presence of benzoquinone did not affect the kinetics (not shown). Addition of CCCP in presence of KCN ($2 \cdot 10^{-4}$ M) induced the reduction of the low-potential hemes of the RC-bound cytochrome, therefore, this experiment has to be done in the presence of benzoquinone (50 μM) which overcomes this reduction. We have no straightforward explanation for the multiphasic character observed for the oxidation of cytochrome c_2. Such behaviour has been observed for several other reactions in the photosynthetic RC [37–39] and has been interpreted as due to different conformational states. Multiple first-order rates have been also observed in flash-induced electron transfer between cytochrome c and Zn-substituted cytochrome-c peroxidase [40]. If one follows this type of interpretation the difference observed between anaerobiosis and aerobiosis would suggest that the electron transfer between cytochrome c_2 and heme c_{556} can occur from a great number of different relative positions of the

two hemes in the case of anaerobiosis than under aerobiosis. Although this ad hoc hypothesis has to be postulated to interpret the multiphasic behaviour of the cytochrome c_2 oxidation, the lack of any effect of flash intensity or the viscosity on the kinetics of cytochrome c_2 oxidation is a strong evidence for a reduced cytochrome c_2 forming a complex with the RC. The existence of a stable complex between cytochrome c_2 and the cytochrome subunit of *Rps. viridis* RC and the high transfer rate between these electron carriers ($t_{1/2} = 40$ μs–110 μs) contrast with the results obtained by Knaff et al. [12] who have studied the electron-transfer kinetics between isolated RC and cytochrome c_2. In their in vitro study Knaff et al. found that the kinetics of electron transfer between cytochrome c_2 and heme c_{556} has an hyperbolic dependence on the concentration of cytochrome c_2. This suggests the formation of a complex between cytochrome c_2 and RC. The best fit was obtained with a rate constant of electron transfer equal to 270 s^{-1} and a dissociation constant equal to 30 μM [12]. The results we have obtained in vivo and those obtained in vitro may be reconciled if one considers that the concentration of cytochrome c_2 in the *Rps. viridis* cells is, like in the case of *Rhodobacter sphaeroides* chromatophores, in the millimolar range [41], i.e., at least one order of magnitude higher than in the in vitro experiment. On the other hand, the rate of electron transfer we have measured between the soluble cytochrome c_2 and the tetraheme RC cytochrome compares well with that measured by Van Grondelle et al. [14] in whole cells of *Chromatium vinosum* for the electron donation between the soluble cytochrome c_{551} and the heme c_{555} of the bound cytochrome ($t_{1/2} = 300$ μs).

We conclude from this series of experiments that reduced cytochrome c_2 forms a stable complex with the tetraheme cytochrome *c* of the photosynthetic RC. After oxidation, the cytochrome c_2 leaves the RC and is re-reduced by a bc_1 complex in a second-order process. We have not been able to determine the kinetics of rebinding of reduced cytochrome c_2 to the reaction center because of the low ratio (1.3) between the total photooxidable cytochrome c_2 and the RC in whole cells. This ratio has been determined by analysing the absorption changes following a series of five actinic flashes spaced by 100 ms in presence of 2 μM myxothiazol (data not shown).

These results, therefore, demonstrate the occurrence of a cyclic electron transfer in whole cells of *Rps. viridis* similar to the one described in other photosynthetic bacteria [1].

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