In vivo Participation of a High Potential Iron−Sulfur Protein as Electron Donor to the Photochemical Reaction Center of *Rubrivivax gelatinosus*

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ABSTRACT: We have found that the only high redox potential electron transfer component in the soluble fraction of *Rubrivivax gelatinosus* TG-9 is a high-potential iron−sulfur protein (HiPIP). We demonstrated the participation of this HiPIP in the photoinduced electron transfer both *in vivo* and *in vitro*. First, the addition of HiPIP to purified membranes enhanced the rate of re-reduction of the photooxidized reaction center. Second, the photooxidation of HiPIP was observed in intact cells of *Ru. gelatinosus* TG-9 under anaerobic conditions by EPR and absorption spectroscopies. Analysis of flash-induced absorption changes showed that the equilibration of positive equivalents between the reaction center and HiPIP occurs in less than 1 ms after flash excitation. The complete re-reduction of the photooxidized reaction center is achieved in tens of milliseconds. The turnover of a cyt bc_1 is also involved in this reaction, as shown by a slow electrogenic phase of the membrane potential linked to this process.

In purple bacteria, the photosynthetic electron transfer chain operates according to a cyclic electron flow linked to proton translocation across the intracytoplasmic membrane. Two transmembrane complexes, the reaction center (RC)[1] and the cytochrome (cyt) bc_1 complex, are connected via quinone molecules in the lipid phase and soluble electron carriers in the periplasm to perform this cyclic electron transfer. For some species, the electron is transferred directly from a soluble cyt c_2 to the photooxidized primary electron donor (P^+). This is the case of *Rhodobacter (Rb.) sphaeroides, Rb. capsulatus, Rhodospirillum rubrum, Rhodopseudomonas (Rp.) palustris*. However, for most species [*Rp. viridis, Rp. acidophila, Chromatium vinosum, Rubrivivax (Ru.) gelatinosus, Rhodocyclus* (*Rc.*) *tenuis*], a tetrahemic cyt *c*, more or less tightly bound to the RC, is the direct reductant of P^+.

Compared to the large body of information available on *Rb. sphaeroides* and *Rb. capsulatus*, the study of the photoinduced electron transfer of species containing a RC-bound tetrahemic cyt *c* has received little attention. Several reasons can be invoked. First, production of site-directed mutants is widely utilized for the elucidation of electron transfer mechanisms in *Rb. sphaeroides* and *Rb. capsulatus* (Marrs, 1974; Sistrom et al., 1984), but the only species containing RC-bound tetrahemic cyt for which an efficient transformation protocol has been developed is *Rp. viridis* (Lang & Oesterhelt, 1989a,b; Laussermaier & Oesterhelt, 1992). However, this species grows extremely slowly under semiaerobiosis in the dark (Lang & Oesterhelt, 1989a), an essential condition to obtain cells of photosynthetically incompetent mutants. Secondly, most of these RC-bound cyt species contain very low amounts of bc_1 complex compared to the RC, and cyclic electron transfer is therefore difficult to study. Finally, studies on the photoinduced electron transfer pathway have to be performed with intact cells because, in most cases, soluble periplasmic electron carriers are lost during purification of the intracytoplasmic membrane. This is due to the lamellar structure of the intracytoplasmic membrane (e.g., *Rp. viridis*) or the small amount of invaginations. This latter situation is encountered, for example, in *Ru. gelatinosus* [formerly *Rhodocyclus gelatinosus* and *Rhodopseudomonas gelatinosa* (Trüper & Imhoff, 1992)].

The LHI complex of *Ru. gelatinosus* has been purified and characterized in terms of pigment composition and spectroscopic properties (Jirsakova & Reiss-Husson, 1993). The photochemical reaction center and the soluble electron transfer components of the periplasm have been characterized. Although EPR signals arising from a Rieske protein have been observed on isolated membranes (Nitschke and Rutherford, personal communication), detailed information on the cyt bc_1 complex is not yet available. The *Ru. gelatinosus* RC possesses a polypeptide composition very similar to those reported in other photosynthetic bacteria (Clayton & Clayton, 1978; Fukushima et al., 1988; Agalidis & Reiss-Husson, 1992). However, contrary to species like *Chromatium vinosum* or *Rp. viridis*, the tetrahemic cyt subunit is less tightly bound to the RC and lost during purification (Clayton & Clayton, 1978; Fukushima et al., 1988; Matsuura et al., 1988; Agalidis & Reiss-Husson, 1992). As observed for many different species, this tetrahemic cyt possesses two hemes with rather high midpoint potentials (E_m) and two hemes with low E_m (Fukushima et al., 1988; Nitschke et al., 1992). The high-potential (HP) hemes have E_m values of 300 and 320 mV, respectively, and their α-band

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[1] Abbreviations: cyt, cytochrome; RC, reaction center; HiPIP, high-potential iron−sulfur protein; HP, high potential; LP, low potential; bc_1, quinol−cytochrome *c* oxidoreductase; E_m, midpoint potential; TNBT, tri-(*N*-butyl)tin; *Ru.*, *Rubrivivax*; *Rb.*, *Rhodobacter*; *Rp.*, *Rhodopseudomonas*; *Rc.*, *Rhodocyclus*.

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is centered at 555 nm. The E_m values of the low-potential (LP) hemes are 70 and 130 mV, and their α-band absorbs at 551 nm. Depending upon redox conditions, HP and/or LP hemes can be photooxidized (Dutton, 1971). The alignment of the amino acid sequences of the tetrahemic cyt subunit from *Ru. gelatinosus* and *Rp. viridis* proposed by Nagashima et al. (1994) shows strong conservation of numerous stretches throughout the entire sequence. It is therefore very likely that the overall arrangement of the tetrahemic cyt observed in the RC of *Rp. viridis* is conserved for *Ru. gelatinosus* (Nitschke & Rutherford, 1994).

Many electron transport proteins, cyt c_{551}, cyt c', cyt c_2, and HiPIP, can be found in the periplasmic space of *Ru. gelatinosus*. Some of the characteristics of these electron transfer components isolated from *Ru. gelatinosus* or other species have been studied. The HiPIP of *Ru. gelatinosus* has been characterized in terms of E_m (+330 mV) (De Klerk & Kamen, 1966; Mizrahi et al., 1980) and amino acid composition (Tedro et al., 1976). The three-dimensional structure of HiPIP has been determined for *Chromatium vinosum* (Carter et al., 1974), *Rc. tenuis* (Rayment et al., 1992), *Ectothiorhodospira halophila* (Breiter et al., 1991), and *Ectothiorhodospira vacuolata* (Benning et al., 1994). Biochemical and structural characteristics have been obtained for cyt c' isolated from several species [see Moore and Pettigrew (1990) for a review]. The E_m of the cyt c' isolated from *Ru. gelatinosus* TG-9 has been determined to be equal to 55 mV (B. Schoepp, unpublished results). Cyt c_{551} isolated from *Ru. gelatinosus* differs from the cyt c_2 type cyt in its amino acid sequence (Ambler et al., 1979a,b). Senn and Wüthrich (1983) have studied this cyt by NMR techniques, but its three-dimensional structure has not been determined. Its E_m is rather low, around 30 mV (B. Schoepp, unpublished results and T. E. Meyer and M. A. Cusanovitch, personal communication).

HiPIP has been found in all strains of *Ru. gelatinosus* studied so far. On the other hand, the presence of high potential cyt *c* depends on the considered strain. According to Meyer (1970), *Ru. gelatinosus* ATH 2.2.1 does not contain any soluble high redox potential cytochrome *c*. Matsuura et al. (1988) have purified a cytochrome c_2 from the supernatant of disrupted cells of strain *Ru. gelatinosus* IL144. Although these components are putative electron donors for the RC-bound tetrahemic cyt, electron transfer between a cytochrome c_2 and the HP heme c_{555} of the tetrahemic cyt has been reported only in reconstitution experiments with isolated pigment−protein complexes of *Ru. gelatinosus* (Matsuura et al., 1988). On the other hand, Kennel et al. (1972) have shown that HiPIP could be an *in vitro* electron donor to the RC of *C. vinosum*.

In this work we studied, by light-induced absorbance changes on whole cells and isolated membranes of the *Ru. gelatinosus* strain TG-9 wild type and the carotenoidless mutant strain TG-9 Cad, the nature and the kinetic characteristics of the electron donor to the RC-bound tetrahemic cyt. Part of these results have been presented elsewhere (Schoepp et al., 1994).

MATERIALS AND METHODS

In order to study electron transfer in whole cells of *Ru. gelatinosus* TG-9 free of electrochromic components in absorbance change measurements, we have produced a blue-green carotenoidless mutant (TG-9 Cad) from the wild-type TG-9. The mutation was obtained after treatment of an exponential photoheterotrophic culture of the wild type with 1-methyl-3-nitro-1-nitrosoguanidine (570 μM). The carotenoidless mutant *Ru. gelatinosus* TG-9 Cad presents very small absorbance bands between 450 and 550 nm correlated with a strong decrease of the 800−850 nm Q_y peaks of the LHII antennae (data not shown).

Cells of *Ru. gelatinosus* TG-9 wild-type or carotenoidless mutant Cad were grown 24 h, in Hutner medium at 30 °C, in anaerobic conditions under continuous illumination (75 μmol of photons m^{-2} s^{-1}).

Purification of the Soluble Carriers and Preparation of Membranes. Harvested cells, resuspended in 50 mM Tris-HCl (pH 8) and 5 mM EDTA, were disrupted by French Press at 50 MPa. The remaining intact cells were eliminated by centrifugation at 10000*g* for 10 min as the sedimented fraction. Membrane fragments were collected by ultracentrifugation at 255000*g* for 90 min. The electron carriers were purified from the supernatant after slight modification of the method of Tedro et al. (1976). The supernatant fraction was adjusted to pH 6 and centrifuged again at 255000*g* for 90 min. The pellet was discarded and the supernatant loaded on a cellulose CM-23 column, equilibrated with 20 mM Tris-HCl (pH 6) at 4 °C. The column was washed with two column volumes of the same buffer. The different soluble carriers were recovered by applying a stepwise salt gradient. HiPIP, c_{551}, and c' were eluted from the column at 25, 50, and 75 mM NaCl, respectively. Each of these three fractions was concentrated by ultrafiltration with PM 5000 membranes and dialyzed against 20 mM Tris-HCl (pH 6). The final purification step for each carrier involved a Sephadex G-100 gel filtration chromatography, using 20 mM Tris-HCl (pH 6) buffer at room temperature. The final purity was determined by gel electrophoresis and by absorption spectroscopy using the following index: $A_{280}/A_{388red} = 2.15$ for HiPIP (Tedro et al., 1976) and $A_{280}/A_{424red} = 0.38$ for cyt c' (Meyer, 1970).

Absorption and EPR Spectroscopies. The photoinduced absorbance changes were recorded with a home-built Joliot-type flash kinetics spectrophotometer (Joliot et al., 1980). Whole cells were suspended in fresh growth medium. Isolated membranes were resuspended in 50 mM Tris-HCl (pH 8), and the redox potential was imposed by addition of DAD 20 μM/sodium ascorbate 20 μM (E_h = 260 mV). In most experiments, the excitation light was provided by an Alexandrite laser (780 nm, 100 ns flash duration, 50 mJ, SEO, Laser 1-2-3, Schwartz Electro Optics) since we have observed that excitation by a xenon flash (flash duration 2−3 μs) induced 30% double hits (data not shown).

EPR spectra were obtained with an X-band Varian E-109 spectrometer. An Oxford Instruments ESR 900 Helium-flow cryostat was used to adjust the EPR sample temperature. Quantification of spin concentrations was performed by double integration and comparison with a sample of purified *C. vinosum* HiPIP of known concentration. Continuous illumination of whole cells was performed directly in the EPR tube at room temperature with a 120 W incandescent lamp filtered through Kodak filter 89B. The tube was immediately frozen in liquid nitrogen and then transferred into the cryostat.

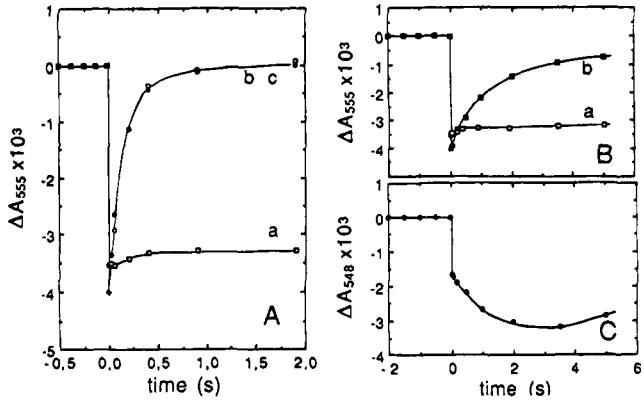

FIGURE 1: Kinetics of flash-induced absorbance changes recorded in cyt α-band on purified membrane fragments of *Ru. gelatinosus* TG-9 Cad suspended in 50 mM Tris-HCl (pH 8) and 5 mM EDTA. The redox potential was poised at 260 mV by addition of 20 μM DAD and 20 μM sodium ascorbate. The membrane fragments concentration was 390 nM RC equivalent. (Part A) (a) Membrane fragments without addition, (b) supplemented with the total soluble fraction, and (c) supplemented with 1 μM HiPIP. (Part B) (a) Membrane fragments without addition and (b) supplemented with 100 μM horse heart mitochondrial cyt *c*. (Part C) Oxidation of soluble cyt *c* measured at 548 nm under conditions of curve b of part B.

RESULTS

Identification of the Electron Donor to the HP Hemes of Tetrahemic Cyt. Flash excitation of purified membranes of *Ru. gelatinosus* TG-9 Cad, poised to a redox potential around 260 mV, induces the photooxidation of one of the HP hemes (Figure 1A, curve a). In the absence of additional electron donor, the photooxidized HP heme, monitored at 555 nm, is not re-reduced in the time scale of observation, i.e., 5 s (Figure 1A,B, curves a). Upon addition of the total soluble fraction, a fast re-reduction of the photooxidized HP heme is observed (Figure 1A, curve b). This indicates that the physiological electron donor to the HP heme is present in this fraction. To determine the nature of this electron donor, we have analyzed the content in electron transfer components of this soluble fraction (see Materials and Methods). We have found no evidence for the presence of cyt c_2 in the soluble fraction of *Ru. gelatinosus* TG-9 cells in agreement with the observation of Meyer (1970) with strain ATH 2.2.1. Cyt c' and HiPIP can be found in large quantities in this fraction. We have estimated the relative amount of these compounds by combining EPR and absorption spectroscopies on whole cells and the periplasmic fraction. Although some variations may be observed depending on the batch of cells, there is typically about 1.3 molecule of HiPIP and 0.3 molecule of cyt c' per RC. A very small amount of low E_m cyt *c* (c_{551}) could also be detected in the periplasmic fraction (about 0.05 cyt c_{551} per RC). Each of these electron transfer components has been purified to homogeneity (see Materials and Methods) and added separately to purified membranes. The addition of micromolar concentrations of cyt c_{551} or cyt c' does not affect the re-reduction rate of the HP heme, in agreement with the fact that these components are in the oxidized form under the redox conditions imposed in the experiment (data not shown). On the other hand, the re-reduction rate of the HP heme is strongly increased upon addition of 1 μM HiPIP, demonstrating that this component is a good electron donor to the HP heme (Figure 1A, curve c). These two observations, absence of cyt c_2 in the periplasmic fraction of *Ru. gelatinosus* TG-9 Cad and re-reduction of the HP heme only by HiPIP, lead us to conclude that this electron transfer component is the physiological electron donor to the RC in this species. Morever, although horse heart mitochondrial cyt *c* can transfer electrons to the photooxidized HP heme, as shown by their concomitant oxidation and reduction observed at 548 and 555 nm, respectively (Figure 1, parts B and C), this cyt is a very poor donor. First, addition of cyt *c* only slightly increases the rate of re-reduction of the photooxidized HP heme. Secondly, the acceleration does not saturate even at very high concentrations of cyt *c* (100 μM) (Figure 1B, curve b). This observation is in contrast with the results of Matsuura et al. (1988), who proposed that cyt *c* is a good electron donor to the HP heme of *Ru. gelatinosus* strain IL144. The different behavior observed in their study and in the present work is perhaps related to the distinct nature of the two strains used. The phylogenetic affiliations of the different species of the β branch are still in progress (Trüper & Imhoff, 1992; Nagashima et al., 1993; Hiraishi, 1994). The two strains *Ru. gelatinosus* IL144 and *Ru. gelatinosus* TG-9 could be distinct in this respect.

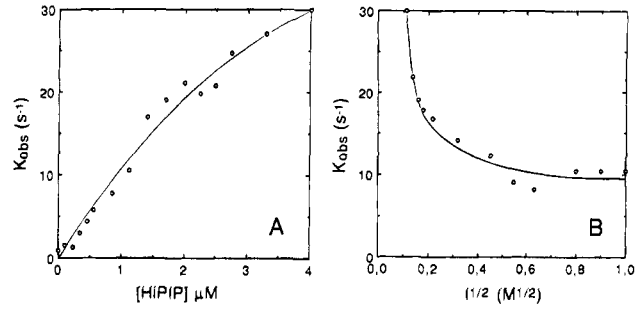

FIGURE 2: Characterization of the electron transfer reaction between HiPIP and the RC HP heme following flash excitation. Kinetics are measured on membrane fragments under conditions similar to those of Figure 1. (Part A) Relationship between HiPIP concentration and the rate of re-reduction of the HP heme. (Part B) Relationship between the ionic strength and the rate of re-reduction of the HP heme. HiPIP concentration was equal to 4 μM.

The electron donation from HiPIP to the HP heme has been studied as a function of the relative concentration of these two components and of the ionic strength of the medium by addition of various concentrations of NaCl. Figure 2A shows the dependence between the HiPIP concentration and the rate of the HP heme re-reduction in membrane fragments. This relationship can be mathematically simulated with

$$k_{obs} = k_{et}[\text{HiPIP}]/(K_d + [\text{HiPIP}])$$

where k_{et} corresponds to the electron transfer constant and K_d to the dissociation constant (Strickland et al., 1975). A good fit is obtained for k_{et} and K_d equal to 74.4 s^{-1} and 5.7 μM, respectively. This relationship between the HiPIP concentration and the k_{obs} suggests that a complex is formed between HiPIP and RC. The effect of the ionic strength on k_{obs} (Figure 2B) suggests that electrostatic interactions may be involved in the complex formation.

Light-Induced EPR and Absorbance Changes in Intact Cells. To confirm that HiPIP is the physiological electron donor to HP heme, we have measured light-induced EPR and absorption changes on intact cells of *Ru. gelatinosus* TG-9 Cad. Figure 3 (curve a) shows the EPR spectrum

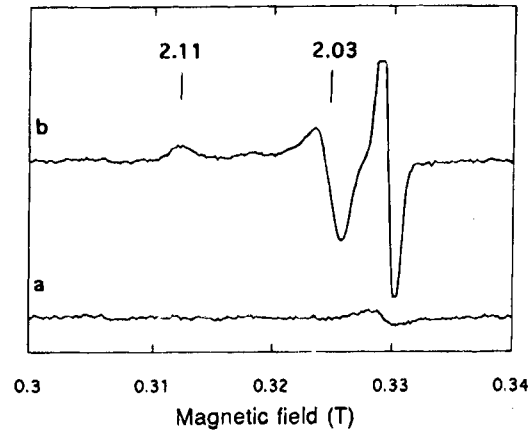

FIGURE 3: EPR spectra for a suspension of intact cells of *Ru. gelatinosus* placed under anaerobic conditions. Spectrum a was recorded after 2 min of dark adaptation and rapid cooling. For spectrum b, the suspension was subjected to 2 min of continuous illumination at room temperature before rapid cooling. Experimental conditions: modulation frequency, 100 kHz; modulation amplitude, 1 mT; microwave power, 1 mW. The temperature of the sample was 10 K.

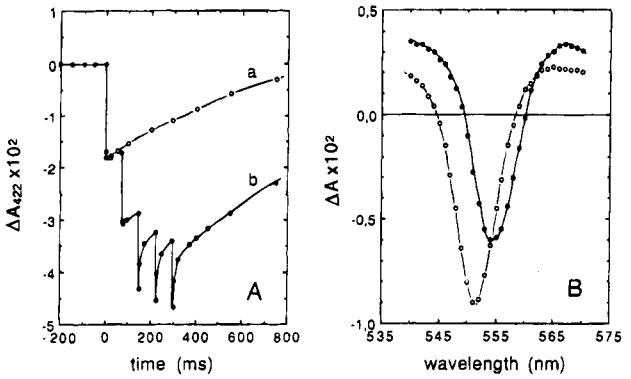

FIGURE 4: (Part A) Light-induced absorption changes measured at 422 nm for a suspension of intact cells of *Ru. gelatinosus* TG-9 Cad (RC concentration, 600 nM) placed under anaerobic conditions following one (curve a) or a series of five saturating actinic flashes (curve b). The flashes were spaced by 75 ms. (Part B) Light-induced difference spectra recorded 50 μs after actinic flashes number 1 (○) and number 3 (●).

recorded at 10 K for a dark-adapted anaerobic suspension of whole cells of *Ru. gelatinosus* TG-9 Cad. No signal with significant amplitude was detectable, except a small one centered around $g = 2$ which probably corresponds to an organic radical of undetermined nature. The excitation by continuous illumination at room temperature of the cell suspension before freezing induces the appearance of EPR signals characteristic of both oxidized HiPIP ($g = 2.11$ and 2.03) and photooxidized RC primary donor ($g = 2$) (Figure 3, curve b).

Absorbance changes induced by a series of flashes have been detected for an anaerobic suspension of whole cells of *Ru. gelatinosus* TG-9 Cad at several wavelengths characteristic of cyt redox changes. Figure 4A, curve b, shows typical kinetics observed at 422 nm following a series of five actinic flashes spaced by 75 ms. Photooxidation of the LP hemes is observed after the first two exciting flashes. Subsequent flashes induce the photooxidation of the HP hemes. This is clearly shown by the shape of the light-induced absorption changes observed 50 μs after the first and third actinic flash in the α-band of the cyt (Figure 4B). The re-reduction of the LP heme occurring after one excitation flash is very slow with an half-time around 400 ms (Figure 4A, curve a). On the other hand, a rapid re-reduction of the HP heme is observed on subsequent flashes (Figure 4A, curve b). The fast re-reduction of the HP hemes compared to the slow re-reduction of the LP hemes suggests that, under anaerobic conditions, an efficient cyclic electron transfer is only sustained by the HP hemes. A detailed study of the flash-induced HP heme kinetics reveals that about 50% of this heme is re-reduced in less than 2 ms (Figure 5, left part, curve a). The half-time of this fast re-reduction phase ($t_{1/2} = 300$ μs) is of the same order of magnitude as those measured for the electron transfer between soluble cyt *c* and RC-bound cyt in *C. vinosum* (Coremans et al., 1985), *Rp. viridis* (Garcia et al., 1993) or *Roseobacter denitrificans* (Garcia et al., 1994). However, in the case of *Ru. gelatinosus* TG-9 Cad, this fast re-reduction phase is not accompanied by the oxidation of a soluble cyt *c*. This is clearly shown by the light-induced difference spectra which are characteristic of HP heme irrespective of the detection time after excitation (Figure 5, right part). This result is in agreement with our finding that the periplasmic fraction of this species does not contain any high potential cyt *c* and with the fact that HiPIP is an efficient electron donor to the HP heme (see previous paragraph).

To provide further evidence that HiPIP is the physiological electron donor to the HP heme in *Ru. gelatinosus* TG-9 intact cells, we have measured light-induced absorbance changes at 490 nm. At this wavelength, the oxidation of HiPIP induces a small absorption *increase* while cyt re-reduction induces an absorption *decrease*. Kinetics recorded at 555 and 490 nm are compared in Figure 5, left part, curves a and c, respectively. The re-reduction phase of the HP heme in the 50−2000 μs range is synchronous with an absorption *increase* at 490 nm indicative of the oxidation of HiPIP during this phase. Attenuating the laser intensity, in order to decrease the concentration of the photooxidized RC by a factor of 5, does not affect the kinetics of re-reduction of the HP heme and slightly decreases the ratio between the slow and the fast phases of this re-reduction (Figure 5, left part, curves a and b). This experiment demonstrates that HiPIP and the RC-bound cyt is not diffusional and suggests that they form a complex *in vivo*.

We conclude from this series of experiments that HiPIP is the physiological electron donor to the RC-bound HP hemes in intact cells of *Ru. gelatinosus* TG-9. These two components form a tight complex and transfer electron in less than 2 ms.

Mechanism of Re-Reduction of the HiPIP. Since the absorption changes linked to the redox changes of HiPIP are small and partially masked by those of the cyt, the turnover of HiPIP has been determined by measuring the amplitude of the fast (<1 ms) re-reduction phase of the HP heme as a function of dark time between flashes. The recovery of this fast phase corresponds to the availability of reduced HiPIP to rebind to the tetrahemic cyt. The results, plotted in Figure 6, show that this recovery presents two distinct phases. The first phase (33%) occurs in less than 10 ms. It can be ascribed to a rapid binding of the small part of HiPIP which is present in excess compared to the tetrahemic cyt (1.3 HiPIP per 1 tetrahemic cyt). The half-time of the second phase (35 ms) is similar to the one measured for the slow phase of the HP heme re-reduction

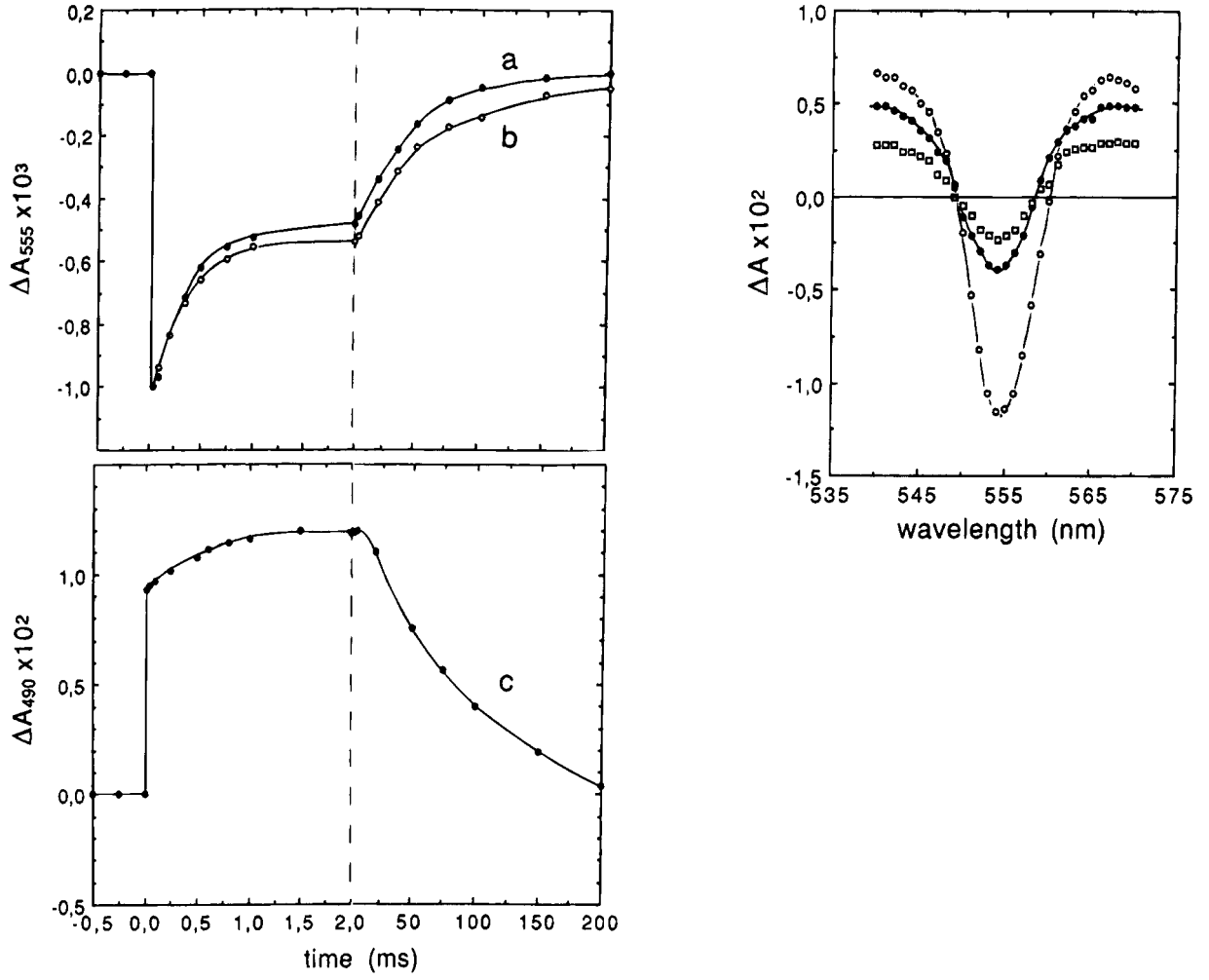

FIGURE 5: (Left part) Kinetics recorded on intact cells of *Ru. gelatinosus* TG-9 Cad (RC concentration, 1.2 μM) under anaerobic conditions after actinic flash number 3. Curve a represents the kinetics recorded at 555 nm following a low intensity flash which hits only 20% of the RC, and curve b represents the kinetics after excitation with a saturating flash. The two kinetics have been normalized at 50 μs. Kinetics recorded at 490 nm under saturating excitation are depicted in curve c. (Right part) Light-induced difference spectra recorded at different times after flash excitation [50 μs (○), 2 ms (●), and 50 ms (□) after actinic flash number 3].

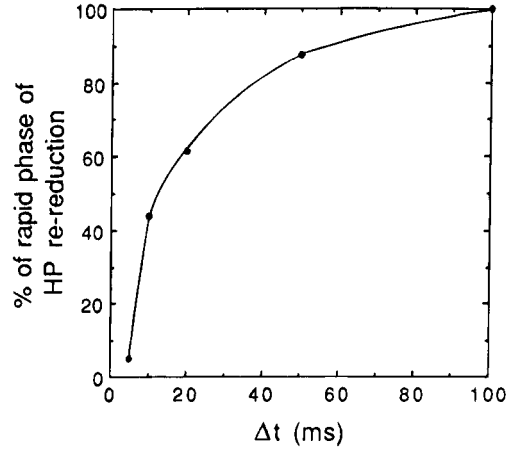

FIGURE 6: Relative amplitude of the fast phase of the HP heme re-reduction as a function of the dark time (Δt) between two actinic flashes. Other conditions are as in Figure 5.

(Figure 5 left, curve a). We ascribe this phase to the rebinding of HiPIP which has been re-reduced by a cyt bc_1 complex. Indeed, EPR spectroscopy (Nitschke and Rutherford, personal communication) and immunological recognition (data not shown) indicate the presence of the "Rieske" subunit in isolated membranes of *Ru. gelatinosus* TG-9. Moreover, a quinol oxidoreductase activity, with duroquinol as electron donor and HiPIP or horse heart mitochondria ferricytochrome *c* as electron acceptor, can be demonstrated on purified membranes (data not shown).

To provide evidence for the involvement of a cyt bc_1 complex in whole cells of *Ru. gelatinosus*, we have analyzed the light-induced carotenoid band-shift, an electrochromic indicator of the membrane potential. Electron flow through the cyt bc_1 complex in photosynthetic bacteria is accompanied by an increase of the membrane potential in the millisecond time scale. Figure 7A shows the kinetics of the light-induced carotenoid band-shift, measured at 502−518 nm for a suspension of whole cells of *Ru. gelatinosus* TG-9. The cells were placed under anaerobiosis and in the presence of 10 μM TNBT. The addition of TNBT, an inhibitor of the ATPase, allows a more precise measurement of the different phases of the carotenoid band-shift by slowing its decrease. On the first two flashes, a very fast increase of the carotenoid band-shift is observed (Figure 7A). This fast increase is due to the membrane potential linked to the photoinduced electron transfer between the LP heme and the primary acceptor. This fast change is not followed by a further increase of the carotenoid band-shift, indicating that there is no subsequent transmembrane electron transfer after the

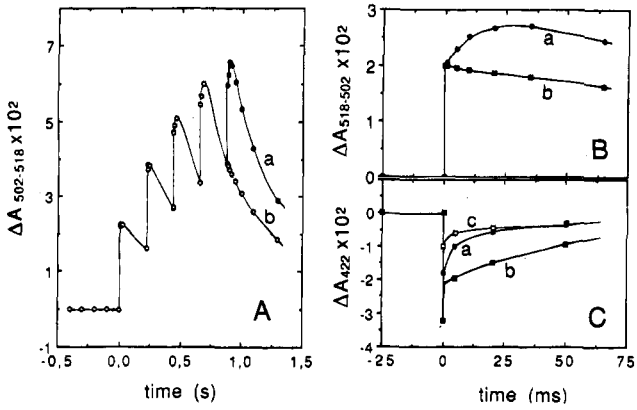

FIGURE 7: (Part A) Light-induced absorption changes linked to the carotenoid band-shift measured as the difference 502−518 nm in whole cells of *Ru. gelatinosus* TG-9 (RC concentration, 1.2 μM). Curves a and b correspond to the signals observed after four and five actinic flashes, respectively. The dark time between flashes was 200 ms. The intact cells were placed under anaerobiosis in the presence of 10 μM TNBT. Others conditions are similar to those of Figure 4. (Part B) Kinetics of the carotenoid bandshift changes induced by the fifth flash deduced from experiments similar of those of part A. (a) No addition and (b) in the presence of 5 μM stigmatellin. (Part C) Kinetics of light-induced cyt redox changes measured at 422 nm under conditions similar to part B. (a) No addition, (b) in the presence of 5 μM stigmatellin, and (c) in the presence of 5 μM myxothiazol.

charge separation. On the other hand, following flashes number 3, 4, and 5, a slow rise of the carotenoid band-shift can be observed after the first fast increase due to the light-induced charge separation. This is clearly shown in Figure 7B, curve a, where the kinetics of the carotenoid band-shift induced on the fifth flash are depicted. The half-time of the slow increase of the carotenoid band-shift occurring on the third and subsequent flashes is about 10 ms, in good agreement with the rate of re-reduction of the HP heme (Figure 7C). Note that the re-reduction of the HP heme observed in Figure 7 is much faster than that measured in Figure 6. This is a consequence of the addition of TNBT, which apparently induces a large reduction of the quinone pool. From this series of experiments, we ascribe the slow increase of the carotenoid band-shift to electron transfer within the cyt bc_1 complex.

We also studied the effect of classical inhibitors of the cyt bc_1 complex on the turnover of the HiPIP by following the kinetics of re-reduction of the HP heme. Myxothiazol induces a slight increase in the rate of the slow phase of the HP heme re-reduction (Figure 7C), and antimycin A has no observable effect (data not shown). On the other hand, addition of stigmatellin significantly affects the rate of re-reduction of the HP heme and the amplitude of the slow phase of the carotenoid bandshift (Figure 7B,C, curves b).

DISCUSSION

The presence of HiPIP is limited to proteobacteria (Bartsch, 1991; Meyer et al., 1993; Moulis et al., 1993). With the exception of a halophilic *Paracoccus* strain (Tedro et al., 1977), HiPIP is found in photosynthetic bacteria. All the photosynthetic bacteria containing HiPIP, except *Rhodomicrobium vannielii*, possess a RC-bound tetrahemic cyt (Bartsch, 1991). This author has therefore postulated that HiPIP may substitute for cyt c_2 as secondary electron donor to the RC-bound tetrahemic cyt. In this article we present evidence for the participation of HiPIP in the cyclic electron transfer of *Ru. gelatinosus* TG-9 and as the immediate electron donor to the RC-bound cyt. To our knowledge, this is the first demonstration for a precise *in vivo* function for this type of electron transfer component. Preliminary observations in our laboratory show that HiPIP is also the electron donor to HP heme in intact cells of *Ru. gelatinosus* strain S-1 and *Rc. tenuis*. HiPIP has also been shown to be an electron donor to the *Chromatium* RC in *in vitro* experiments (Kennel et al., 1972). During completion of this work, a similar conclusion was proposed by Hochkoeppler et al. (1995) in the case of *Rhodoferax fermentans* from reconstitution experiments.

Our *in vivo* experiments demonstrate an electron transfer reaction between HiPIP and the HP heme in the submillisecond range. The value of k_{et} determined from the reconstitution experiments (Figure 2) is too low to account for this rate of electron transfer. Several explanations can account for this discrepancy. First of all the preparation of isolated membranes and the purification of HiPIP may have altered these components. Moreover, one has to consider that, due to the small volume of the periplasmic space, the concentration of HiPIP *in vivo* is in the millimolar range, i.e., two orders of magnitude higher than in the *in vitro* experiment. The rate we have determined for the electron transfer between HiPIP and the HP heme is of the same order of magnitude as that measured between RC-bound tetrahemic cyt and soluble cyt c_2 for several other species (Coremans et al., 1985; Garcia et al., 1993, 1994). Moreover, our experiments show that the HiPIP forms a complex with the RC-bound tetrahemic cyt. Similar observations have been made for the *in vivo* and *in vitro* interaction of cyt c_2 and RC in *Rp. viridis* (Knaff et al., 1991; Meyer et al., 1993; Garcia et al., 1993). The re-reduction of the HP heme follows two phases of about equal amplitude. *In vivo* the first phase is complete in 1.5 ms and is due to electron transfer between HiPIP and HP heme. The half-time of the second phase lies between 10 and 35 ms depending upon the redox conditions. This phase is concomitant with electron transfer through the cyt bc_1 complex, as shown by the experiments presented in Figures 6 and 7. The first phase of HP re-reduction can be explained by a fast equilibration of positive equivalents between the HP hemes and the HiPIP. Due to the small difference in E_m between these carriers, the positive charge is shared about equally between these compounds. This equilibrium is slowly displaced during the re-reduction of the oxidized HiPIP by the cyt bc_1 complex and its re-association to the RC-bound tetrahemic cyt. Since the cyt bc_1 complex is present in low amounts in comparison to the RC, several turnovers of this complex are necessary to allow a total re-reduction of the positive equivalent created at the RC level. This explains the rather long half-time measured for both the slow phase of the carotenoid band-shift and the second phase of re-reduction of the HP heme (10−35 ms). This half-time is of the same order of magnitude as that measured for the cyclic electron transfer in whole cells of *Rp. viridis* (Garcia et al., 1993), which is known to contain a low ratio between cyt bc_1 complex and RC (Wynn et al., 1985).

The participation of HiPIP in the photosynthetic cyclic electron transfer of *Ru. gelatinosus* TG-9 raises several interesting questions. The structure of the RC-bound tetrahemic cyt may present some particular features to allow a

good interaction with the HiPIP. For example, the HiPIP isolated from *Ru. gelatinosus* cannot act as an electron donor to the HP heme of RC purified from *Rp. viridis* (Meyer et al., 1993), a species which does not possess HiPIP. The cyt bc_1 complex of *Ru. gelatinosus* may also present some peculiarities to allow good electron transfer to the HiPIP. Since we have no evidence for a soluble high-potential cyt, HiPIP may also be involved in the respiratory electron transfer chain. We have indeed observed, by EPR spectroscopy, that oxygenation of a suspension of whole cells of *Ru. gelatinosus* induces a large oxidation of the HiPIP (unpublished results). This suggests that a cyt *c* type oxidase is able to interact with the HiPIP of *Ru. gelatinosus* TG-9. In this context, the participation of HiPIP in the respiratory electron transfer chain of the chemoorganotrophic bacterium *Rhodothermus marinus* has recently been proposed (Pereira et al., 1994). Therefore the involvement of the HiPIP in respiratory and photosynthetic electron transfer may imply specific features to allow a good interaction between this electron transfer component and the membranous complexes, RC, cyt bc_1 complex, and oxidase, involved in these activities. On the other hand, there are examples where two different types of soluble electron carriers can interact with the same membrane protein. This situation is found for the photosynthetic electron transfer chain in aquatic microalgae and cyanobacteria. In these organisms, plastocyanin, the soluble electron carrier between the cyt b_6f complex and the PS I reaction center, is replaced by a *c*-type soluble cyt under Cu-deficiency (Wood, 1978; Bohner et al., 1980). It will be interesting, therefore, to isolate the cyt bc_1 and the oxidase complexes from *Ru. gelatinosus* TG-9 to characterize their site of fixation to the HiPIP and the possible competition with soluble cyt *c* isolated from related species. Such experiments are in progress.

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