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Biochimica et Biophysica Acta 1506 (2001) 23–30

BIOCHIMICA ET BIOPHYSICA ACTA
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In vitro and in vivo electron transfer to the triheme cytochrome subunit bound to the photosynthetic reaction center complex in the purple bacterium *Rhodovulum sulfidophilum*

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Received 6 November 2000; received in revised form 16 March 2001; accepted 27 March 2001

Abstract

The cytochrome subunit bound to the photosynthetic reaction center (RC) complex in *Rhodovulum sulfidophilum* lacks one heme-binding motif (CXXCH) out of four motifs found in other purple bacteria resulting in the absence of the most distal heme from the RC-core complex (S. Masuda et al., J. Biol. Chem. 274 (1999) 10795). Cytochrome c_2, which acts as the electron donor to the RC was purified, and its gene was cloned and sequenced. The redox midpoint potential of cytochrome c_2 was determined to be $E_m = 357$ mV. The photo-oxidation and re-reduction of purified cytochrome c_2 were observed in the presence of membrane preparations. Flash-induced photo-oxidation and re-reduction of the RC-bound cytochrome were also observed in intact cells. Despite the unusual nature of the RC-bound cytochrome subunit, the cyclic electron transfer system in *Rdv. sulfidophilum* was shown to be similar to those in other purple bacteria.

Keywords: Photosynthesis; Purple bacteria; Electron transfer; Tetraheme cytochrome; Cytochrome c_2; *Rhodovulum sulfidophilum*

1. Introduction

The photosynthetic cyclic electron transfer in purple bacteria involves four components. Two of the components are multi-subunit complexes integrated in the cytoplasmic membrane, the photosynthetic reaction center (RC) complex and the cytochrome bc_1 complex. The other two, quinones and periplasmic soluble electron carrier proteins, mediate the electron transfer between these two complexes. Quinones, which are located within the hydrophobic domain of the membrane, are reduced by the photo-activated RC and are oxidized by the cytochrome bc_1 complex. Soluble electron-carrier proteins, e.g., cytochrome c_2,

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; E_m, redox midpoint potential; MES, 2-morpholinoethanesulfonic acid; MOPS, 3-morpholinopropanesufonic acid; P, special pair of bacteriochlorophylls; RC, photosynthetic reaction center; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Triton X-100, polyoxyethylene(10)octylphenyl ether

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0005-2728/01/$ – see front matter
PII: S0005-2728(01)00177-3

which are located in the periplasmic space of the cells, are reduced by the cytochrome bc_1 complex and oxidized by the photo-oxidized RC. The cyclic electron transfer generates the proton gradient across the membrane that provides the potential energy for synthesizing ATP [1].

There are two groups in purple bacteria in terms of the subunit composition of the RC complex. While the majority of purple bacteria have the cytochrome *c* subunit bound to the RC, this subunit is absent in some species. Other three subunits, L, M and H, are common among all purple bacteria. The physiological significance of the RC-bound cytochrome has not been clarified. At least, phylogenetic analyses have suggested that the RC-bound cytochrome was lost in some species during the course of evolution from the ancestral species possessing this subunit [2–4].

The cytochrome subunit is located at the periplasmic side of the RC and serves as the immediate electron donor to the photo-oxidized special pair of bacteriochlorophylls (P^+). RC-bound cytochromes have been shown to contain four *c*-type hemes per subunit and are often referred as tetraheme cytochromes. The four *c*-type hemes in the RC-bound cytochromes in purple bacteria are numbered according to the order of their heme-binding motifs (CXXCH) in the primary structure. Hemes in the tetraheme cytochromes are divided into two groups in terms of redox midpoint potentials (E_ms); two out of four are high-potential hemes (hemes 2 and 3) and the other two are low-potential hemes (hemes 1 and 4) [4].

The X-ray structure of the RC with the cytochrome subunit has been solved in *Blastochloris* (transferred from *Rhodopseudomonas* [5]) *viridis* [6]. The three-dimensional structure revealed that the four hemes are aligned approximately linearly along with the long axis of the subunit. A low-high-low-high ordering in terms of E_ms, from the distal end to the proximal end with regard to the RC-core complex, has been determined by mapping four optically distinguishable hemes on the structure [7–11].

Recently, the immediate electron acceptor from the soluble electron donors in the RC-bound cytochrome was determined by means of genetic manipulations and kinetic analyses using *Rubrivivax gelatinosus* [12–14]. The heme-1, one of the low-potential hemes that is located at the most distal position from the special pair, acts as the immediate electron acceptor.

The RC-bound cytochrome from *Rhodovulum sulfidophilum* was recently shown to lack the heme-binding motif for heme-1 [15,16]; this is the first exception to the conserved tetraheme structure of RC-bound cytochromes. In addition, the axial ligand methionine of heme-2 was also missing. This unusual RC-bound cytochrome was able to donate electrons to the RC-core complex [16]. The discovery of this unusual RC-bound cytochrome gave rise to the question of how this cytochrome subunit lacking the heme-1 accepts electrons from soluble electron carriers.

In this study, the photosynthetic electron transfer system in *Rdv. sulfidophilum* including the triheme RC-bound cytochrome was investigated. The electron transfer from cytochrome c_2 to the triheme RC-bound cytochrome was demonstrated in a reconstituted system.

2. Materials and methods

2.1. Cell growth and membrane preparation

Cells and membranes of *Rhodovulum sulfidophilum* were prepared as described previously [16].

2.2. Purification and characterization of the soluble cytochrome

The supernatant after the ultra-centrifugation of disrupted cells was subjected to ammonium sulfate precipitation. The fraction precipitated between 40% and 100% saturation of ammonium sulfate was collected, dialyzed against 10 mM MES-NaOH (pH 6.0) and applied to a CM Sepharose Fast Flow (Pharmacia) column equilibrated with the same buffer. A stepwise gradient of sodium chloride was applied for elution. Cytochrome c_2, bound to the column as a major band, was eluted around 150 mM sodium chloride. The concentration of cytochrome c_2 was estimated using the reduced-*minus*-oxidized extinction coefficient difference of 19 $mM^{-1}\,cm^{-1}$ at 551 nm-*minus*-540 nm [17]. Redox titration of the cytochrome c_2 was carried out according to [18].

2.3. Screening, cloning and sequencing of the gene encoding the soluble cytochrome

N-Terminal amino-acid sequencing of cytochrome c_2 was performed using an automatic sequencer (Applied BioSystems) with the sample blotted on a PVDF membrane (Atto Clear Blot Membrane-p). A mixture of the following oligonucleotides, which corresponded to the N-terminal amino acid sequence of the cytochrome c_2, was synthesized taking codon usage into account: 5′-GAYGCSGCIGCIGGS-GARAARGTSTTYAAYAAR-3′. The oligonucleotide mixture was labeled at the 5′ end with digoxigenin-dUTP, as instructed by the manufacturer (Boehringer Mannheim). The oligonucleotide mixture was then used as the probe for colony hybridization to screen the possible gene of the cytochrome c_2. Hybridization was carried out with a DIG Easy Hyb (Boehringer Mannheim) at 50°C for over 12 h. Two positive clones could be selected from the cosmid library, which was constructed in our previous study [19]. Inserted DNA fragments in one of the two cosmid vectors were digested with *Pst*I and screened by Southern blot hybridization using the same probe as described in the cosmid screening. An approximately 2.3 kb DNA fragment giving a positive signal was identified and subcloned into a plasmid pUC118; the resulting plasmid was named pCYCA101. Sequencing of the inserted DNA was performed using a Dye Terminator Cycle Sequencing kit and a 310A Genetic Analyzer (Applied Biosystems). Oligonucleotides designed to generate overlapping DNA sequences to complete the DNA sequence analysis (primer walking) were obtained from Pharmacia Biotech. The DNA sequences were analyzed using the DNASIS program (Hitachi). The nucleotide sequence data of the inserted DNA containing *cycA* gene encoding the cytochrome c_2 are available in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AB050579.

2.4. Flash-induced absorbance change spectrophotometry

The flash-induced absorbance changes were recorded either with a double-beam spectrophotometer [20] or a single-beam spectrophotometer [21]. Membrane preparations were treated with sodium chloride and Triton X-100 to exclude residual cytochrome c_2 as described previously [16].

3. Results

3.1. Purification and characterization of the soluble cytochrome

The absorption spectrum of a soluble cytochrome purified from *Rhodovulum sulfidophilum* showed α, β and Soret peaks at 551, 522 and 416 nm, respectively (data not shown). The redox midpoint potential (E_m) of this cytochrome was 357 mV, based on redox titration in the α-band region (Fig. 1). The amino-acid sequence of this cytochrome was aligned with those of cytochrome c_2 of *Rhodobacter sphaeroides* and *Rba. capsulatus*, showing 46.5% and 40.9% identities, respectively (Fig. 2). A possible Shine-Dalgarno sequence (GGGAG) was located 11 to 7 bases

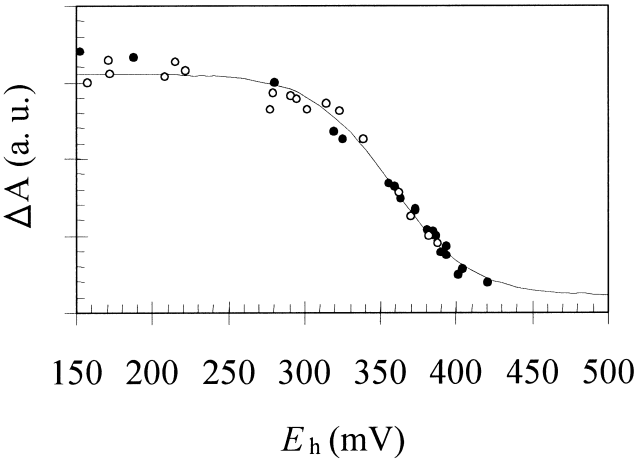

Fig. 1. Redox titration of cytochrome c_2 from *Rdv. sulfidophilum*. The extent of the absorbance changes at the α-band peak of cytochrome c_2 was plotted against the ambient redox potential. Closed and open symbols indicate oxidative and reductive titrations, respectively. The extent of the absorbance change at 551 nm-*minus*-540 nm was fitted to a Nernst equation with $n = 1$, and the redox midpoint potential was determined to be $E_m = 357$ mV. Cytochrome c_2 was suspended in 10 mM MOPS (pH 7.0) supplemented with 100 mM potassium chloride. One hundred μM Fe–EDTA, 1 mM 2Na–EDTA, and 10 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine were present in the solution. The suspension was continuously purged by nitrogen gas. Potassium ferricyanide and sodium dithionite were used as oxidative and reductive titrants, respectively.

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                10        20        30        40        50        60
Rdv. sul  MISKFAIAATAAAMIAGPALALDVTGDAAAGEKVFNKVCQTCHVVKNDAGEVVAGRNAKV
Rba. sph  MKFQVKALAAIAAFAALPALAQ--EGDPEAGAKAFNQ-CQTCHVIVDESGTTIAGRNAKT
Rba. cap  MKISLTAATVAALVLAAPAFA-----GDAAKGEKEFNK-CKTCHSIIAPDGTEIV-KGAKT
          *         *  * ** *   **   * * **  * ***      *       **

                70        80        90       100       110       120
Rdv. sul  GPNLFQMPGRHAAAIDGFR-YSKLMKEAGEKGLVWTEEELVKYVPNPTEFLRNYTEDPKG
Rba. sph  GPNLYGVVGRTAGTQADFKGYGEGMKEAGAKGLAWDEEHFVQYVQDPTKFLKEYTGDAKA
Rba. cap  GPNLYGVVGRTAGTYPEFK-YKDSIVALGASGFAWTEEDIATYVKDPGAFLKEKLDDKKA
          ****    ** *    *  *      *  *  * **    **  *  **     * *

                130       140
Rdv. sul  RGLMTNQRVKEQEIRDVFAYIASFGIHE
Rba. sph  KGKMTFKLKKEADAHNIWAYLQQVAVRP
Rba. cap  KTGMAFKLAKGGE--DVAAYLASVVK--
             *     *        **
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Fig. 2. Alignment of amino acid sequences of cytochrome c_2 encoded by *cyc*A gene of *Rdv. sulfidophilum* (Rdv. sul), *Rba. sphaeroides* (Rba. sph), and *Rba. capsulatus* (Rba. cap). Asterisks indicate identical amino acids. Possible heme-binding sites are boxed. Putative signal peptide regions that did not appear in the mature protein are indicated by italicized letters.

before the start codon, ATG. The amino-acid sequence of this cytochrome, as well as the physicochemical properties described above, resembled those of cytochrome c_2 commonly found in purple bacteria [22]. This cytochrome was assigned to cytochrome c_2.

3.2. Electron transfer mediated by soluble cytochrome in membrane preparations

The flash-induced oxidation and re-reduction of cytochromes in the membrane preparations in the absence and presence of cytochrome c_2 are shown in Fig. 3. Flash-induced kinetic traces taken at 549.2 nm in the presence and absence of cytochrome c_2 are shown in Fig. 3A. The kinetic trace taken in the absence of cytochrome c_2 (Fig. 3A, open circles) shows initial absorbance increase, which is not resolved by the apparatus used, followed by gradual absorbance decrease. These changes are mostly due to carotenoid band shift [23] that indicates photo-induced changes in the membrane potential. The stable negative absorbance level remained after these changes corresponds to the photo-oxidation of the RC-bound cytochrome. The wavelength used

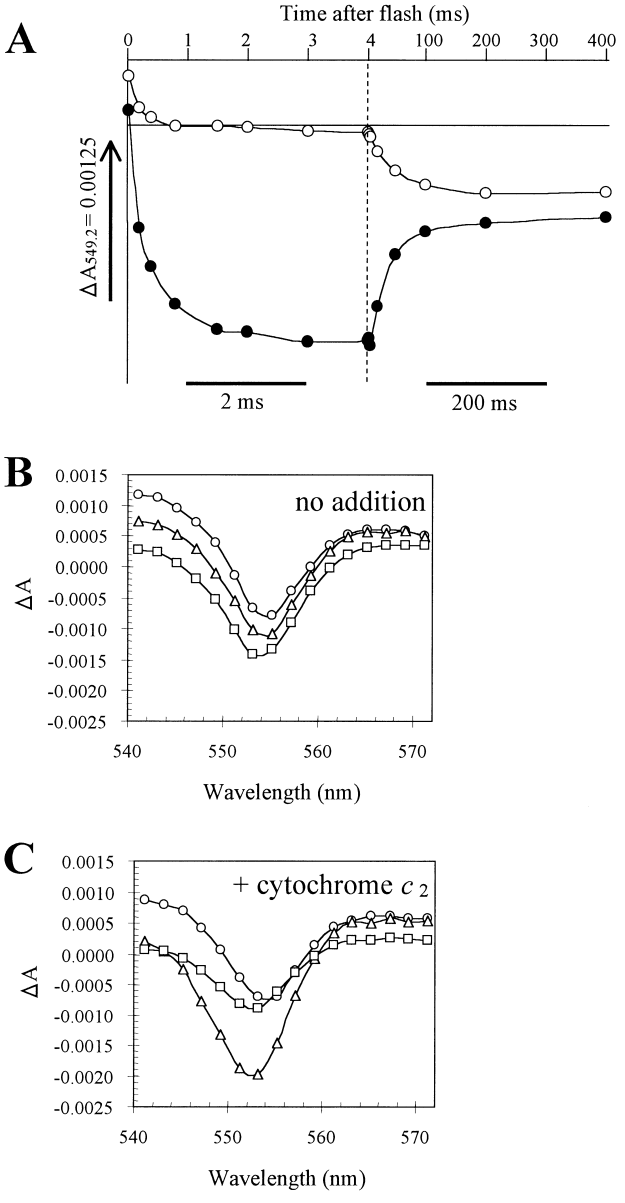

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Fig. 3. Electron transfer mediated by cytochrome c_2 in membrane preparations of *Rdv. sulfidophilum*. Flash-induced absorbance-change kinetic traces taken at 549.2 nm in the absence (○) and in the presence (●) of 10 μM cytochrome c_2 are shown (A). The horizontal line indicates the zero level of absorbance change. Flash-induced absorbance-change spectra of membrane preparations of *Rdv. sulfidophilum* in the absence (B) and presence (C) of 10 μM cytochrome c_2 are shown. Spectra at 20 μs (○), 7 ms (△) and 200 ms (□) after the actinic flash are shown. Membrane preparations free of soluble components were suspended as to be $A_{855} = 2.0$ (corresponding to 14 μM bacteriochlorophyll) in 10 mM Tris–HCl (pH 7.5) supplemented with 0.01% Triton X-114. One hundred μM sodium ascorbate, 100 μM potassium ferricyanide and 10 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine were present in the suspension. Membrane preparations were treated through washing procedure with 10 mM Tris–HCl (pH 7.5) supplemented with 200 mM sodium chloride and 0.01% Triton X-114.

(549.2 nm) was chosen to minimize the sum of the effects of carotenoid band shift and the photo-oxidation of the RC-bound cytochrome in the wavelength region to observe the changes in cytochrome c_2. The kinetic trace taken in the presence of 10 μM cytochrome c_2 (Fig. 3A, closed circles), on the other hand, shows rapid absorbance decrease and subsequent increase indicating the photo-oxidation and re-reduction of cytochrome c_2, respectively. Flash-induced absorbance-difference spectra (Fig. 3B,C) were depicted from a set of kinetic measurements similar to those shown in Fig. 3A. The spectra at 20 μs after the flash excitation, both in the absence (Fig. 3B, circles) and in the presence (Fig. 3C, circles) of 10 μM cytochrome c_2, showed a peak at around 554 nm due to the photo-oxidation of the RC-bound cytochrome. The absorbance increase in the shorter wavelength regions of the spectra is mostly due to the effect of the carotenoid band shift [23]. The spectrum at 7 ms after the flash excitation in the presence of cytochrome c_2 (Fig. 3C, triangles) showed a shift of the peak wavelength to around 552 nm, suggesting the oxidation of the soluble cytochrome, while that in the absence of cytochrome c_2 (Fig. 3B, triangles) showed no such shift. The spectrum at 200 ms after the flash excitation in the presence of cytochrome c_2 (Fig. 3C, squares) showed a smaller extent of cytochrome photo-oxidation than that at 7 ms indicating the partial re-reduction of the photo-oxidized cytochrome. The re-reduction was probably due to the electron transfer from the cytochrome bc_1 complex. No re-reduction was observed at this time range in the absence of cytochrome c_2 (Fig. 3B, squares).

3.3. Flash-induced carotenoid band shift in whole cells

Fig. 4 shows the carotenoid band shift taken at 536 nm -*minus*- 517 nm in whole cells of *Rdv. sulfidophilum*. In the cells without the uncoupler (Fig. 4a), after the initial absorbance increase, a relatively slow absorbance increase ($t_{1/2}$ = 2.3 ms) and a subsequent slow absorbance decrease ($t_{1/2}$ = 100 ms) was observed. The initial absorbance increase was ascribed to electrogenesis due to the electron transfer within the RC; the relatively slow absorbance increase was ascribed to electrogenesis due to the electron transfer within the cytochrome bc_1 complex (see below); and the absorbance decrease was assigned to

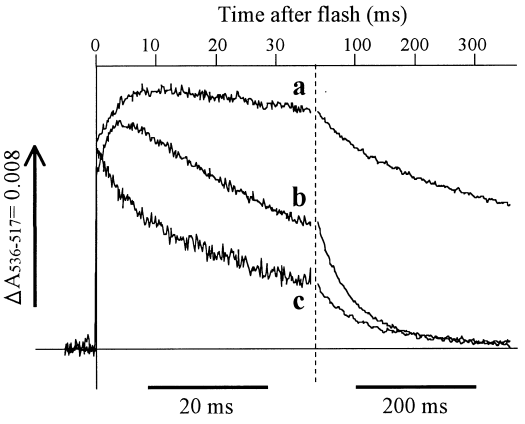

Fig. 4. Flash-induced absorbance change due to carotenoid band shift in whole cells of *Rdv. sulfidophilum*. Kinetic traces taken at 536 nm-*minus*-517 nm with no addition (a), with 10 μM CCCP (b) and with further addition of 10 μM myxothiazol (c) are shown. The cells were once suspended in 50 μM 1,4-benzoquinone, and re-suspended after centrifugation in a fresh medium used for cell growth supplemented with 2 mM potassium cyanide. Cells were suspended as to be 21 μM bacteriochlorophyll.

the relaxation of the membrane potential due mainly to the ion flux through ATPase. In the presence of 10 μM CCCP (Fig. 4b), the relatively slow absorbance increase became about twofold faster ($t_{1/2}$ = 1.2 ms), and the slow absorbance decrease became faster ($t_{1/2}$ = 35 ms). After the addition of 10 μM myxothiazol, an inhibitor of the electron transfer through the cytochrome bc_1 complex (Fig. 4c), the relatively slow absorbance increase completely disappeared, indicating that this phase was due to the electrogenic effect in the cytochrome bc_1 complex, without affecting the slow absorbance decrease ($t_{1/2}$ = 21 ms).

3.4. Cyclic electron transfer in whole cells

Flash-induced spectral changes at α-band region of cytochromes were recorded in whole cells of *Rdv. sulfidophilum* (Fig. 5). Fig. 5A shows a flash-induced absorbance-change taken at 555.2 nm-*minus*-561.2 nm in the presence and absence of myxothiazol. In the absence of myxothiazol (Fig. 5A, open circles), the kinetic trace shows initial absorbance decrease in the sub-ms-time range and subsequent absorbance increase in the ms-time range, mostly due to the photo-oxidation and re-reduction of cytochrome, respectively, judged from the spectral

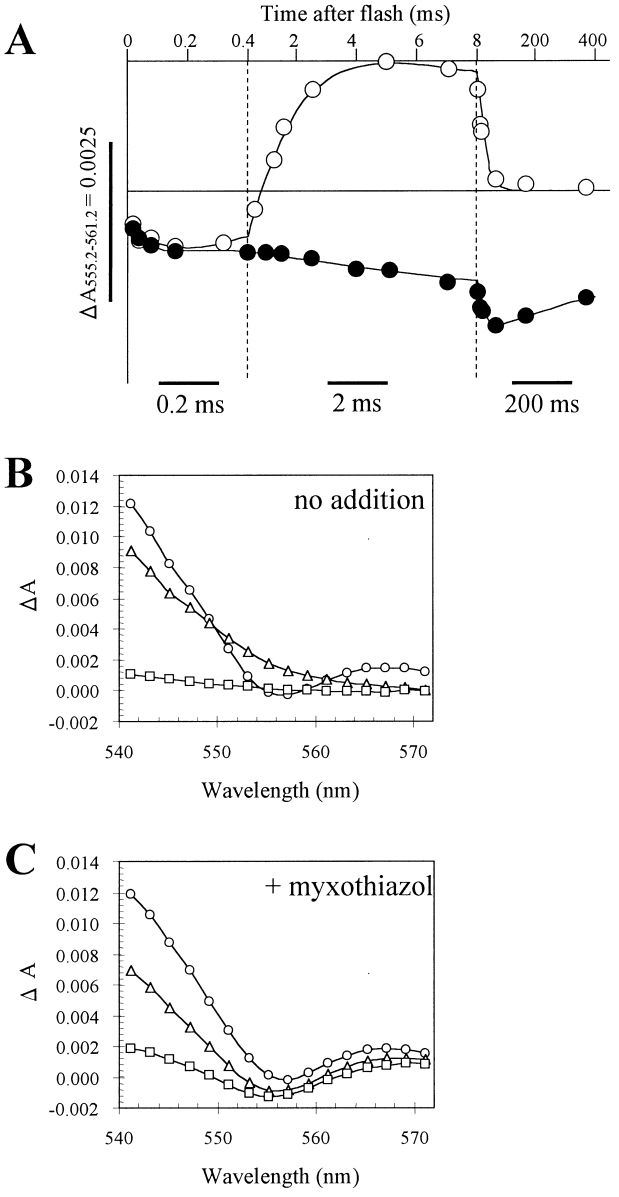

Fig. 5. Flash-induced absorbance change in whole cells of *Rdv. sulfidophilum*. Flash-induced absorbance-change kinetic traces are shown (A). Traces taken at 555.2 nm-*minus*-561.2 nm with no addition (○) and after addition of 10 μM myxothiazol (●) are shown. The horizontal line indicates zero-level of absorbance change. Spectra at 50 μs (○), 20 ms (△) and 70 ms (□) after the actinic flash, in the absence (B) and presence (C) of 10 μM myxothiazol are shown. Cells were suspended as to be 35 μM bacteriochlorophyll, and treated as in Fig. 4. Ten μM CCCP was present in the suspension.

changes and the effect of myxothiazol as described below. The re-reduction of the cytochrome was inhibited in the presence of 10 μM myxothiazol (Fig. 5A, closed circles). The effects of the carotenoid band shift are superimposed as very-fast absorbance increase and its decay in tens of ms on both of the kinetics. Flash-induced absorbance-difference spectra, in the absence (Fig. 5B) and in the presence (Fig. 5C) of myxothiazol were derived from kinetics traces similar to those shown in Fig. 5A. The spectra at 50 μs after the actinic flash peaked at around 556 nm both in the absence and the presence of myxothiazol (Fig. 5B,C, circles), showing photo-oxidation of the RC-bound cytochrome. The large increase of absorbance in the shorter wavelength region and the apparent red-shift of peak wavelength of the cytochrome photo-oxidation, compared to those observed in the membrane preparations (Fig. 3), are probably due to the carotenoid band shift [23], since these effects were larger in the absence of CCCP (data not shown). In the absence of myxothiazol, this cytochrome photo-oxidation almost disappeared at 20 ms after the flash (Fig. 5B, triangles) and the carotenoid band shift has disappeared by 70 ms after the flash (Fig. 5B, squares). On the other hand, in the presence of myxothiazol, the spectrum at 20 ms after the flash still showed a clear band resulted from the cytochrome photo-oxidation (Fig. 5C, triangles). The cytochrome remained oxidized even after the disappearance of the carotenoid band shift at 70 ms after the flash (Fig. 5C, squares). This spectrum of the cytochrome photo-oxidation, free of the effect from the carotenoid band shift, resembles the photo-oxidation spectrum of the RC-bound cytochrome observed in the membrane preparations (Fig. 3B). The effects of the inhibitor indicate that the RC-bound cytochrome is re-reduced by electrons from the cytochrome bc_1 complex in the cells of *Rdv. sulfidophilum*.

4. Discussion

The electron transfer to the triheme RC-bound cytochrome of *Rhodovulum sulfidophilum* was analyzed in isolated preparations and whole cells.

A major soluble cytochrome found in *Rdv. sulfidophilum* showed the typical properties of cytochrome c_2 (Figs. 1 and 2), and was shown to participate in the electron transfer to the RC in membrane preparations (Fig. 3). The amino acid sequence of cytochrome c_2 from *Rdv. sulfidophilum* was similar to those of closely related purple bacteria, which do

not have the RC-bound cytochrome such as *Rhodobacter sphaeroides* and *Rba. capsulatus*, supporting the phylogenetical closeness of *Rdv. sulfidophilum* to these bacteria [24] in spite of their major difference in terms of the subunit composition of their RC complexes.

The triheme RC-bound cytochrome was able to accept electrons from cytochrome c_2. In the tetraheme subunit, a cluster that is feasible for binding cytochrome c_2 is found in the vicinity of heme-1 in the *Blastochloris viridis* RC-bound cytochrome [25]. Our recent studies concluded that the docking site for soluble electron carrier proteins in *Rubrivivax gelatinosus* is located in the vicinity of heme-1 [12–14]. Since electron transfer from soluble cytochrome c_2 was observed in the triheme RC-bound cytochrome lacking this heme-1, there must be an alternative docking site for soluble electron carriers in *Rdv. sulfidophilum*. Kinetic analyses in combination with genetic manipulations would reveal the alternative docking site. The rate of the oxidation and rereduction of cytochrome c_2 became slow under high ionic strength conditions (data not shown), indicating the formation of transient complexes based on electrostatic interactions in this process.

The component of absorbance increase in ms-time range in carotenoid band shift shown in Fig. 4 indicate the electrogenesis due to the cytochrome bc_1 complex because it was clearly inhibited by myxothiazol. This component ($t_{1/2}$ = 2.3 ms and 1.2 ms in the absence and presence of CCCP, respectively) was considerably faster than in other bacteria containing the RC-bound cytochrome; *B. viridis* showed re-reduction of cytochrome c_2 in $t_{1/2}$ = 25 ms and 8 ms under aerobic and anaerobic conditions, respectively [26], and *Rvi. gelatinosus* showed membrane potential generation due to the cytochrome bc_1 complex in $t_{1/2}$ = 10–35 ms [27]. The observation of a fast electrogenesis phase in *Rdv. sulfidophilum* is certainly linked to a high ratio between the cytochrome bc_1 complex and the RC resulted from the high abundance of the cytochrome bc_1 complex [28], in contrast to the low ratio seen in other bacteria possessing RC-bound cytochromes (e.g., in *B. viridis*, there would appear to be no more than one cytochrome bc_1 complex per five RCs [29]). While bacteria without the RC-bound cytochrome often contain a high amount of the cytochrome bc_1 complexes and show fast electrogenesis, e.g., $t_{1/2}$ = ca. 1.2 ms for *Rba. sphaeroides* [30], the combination of the presence of the RC-bound cytochrome and a high amount of the cytochrome bc_1 complex is, to our knowledge, unique. To state that the loss of a heme-binding site on the RC-bound cytochrome has somehow led to the high content of cytochrome bc_1 complexes or vice versa is tempting but too speculative at this point.

The flash-induced absorbance changes at the α-band region of cytochromes in whole cells shown in Fig. 5 also indicated that cyclic electron transfer takes place in vivo in *Rdv. sulfidophilum* because they showed the photo-oxidation and myxothiazol-inhibitable re-reduction of the RC-bound cytochrome although the photo-oxidation of cytochrome c_2 was not observed under these conditions. Since the oxidation of cytochrome c_2 was not observed in whole cells, the cellular content of cytochrome c_2 may be small compared to that of RC.

Thus, even in the absence of the heme-1 in the RC-bound cytochrome, a sufficiently efficient electron transfer to the RC occurs both in vitro and in vivo. The natural deficiency of heme-1 in an RC-bound cytochrome did not seem to significantly affect the photosynthetic electron transfer in *Rdv. sulfidophilum*. This suggests that a complete set of four hemes is not essential to the function of this subunit. Studies introducing mutations to decrease the number of heme-binding sites in other purple bacteria possessing this subunit, e.g., *B. viridis* or *Rvi. gelatinosus*, can be conducted in the future in order to clarify the significance of the presence of the set of four hemes in this subunit. The physiological meaning of the presence of the RC-bound cytochrome subunit itself may be solved by these studies.

Acknowledgements

We thank Dr K. Fukushima-Haino for her assistance on amino acid sequencing. This work was supported by grants from the Ministry of Education, Science, and Culture of Japan and funds for Special Research Project at Tokyo Metropolitan University.

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