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A New Membrane-bound Cytochrome *c* Works as an Electron Donor to the Photosynthetic Reaction Center Complex in the Purple Bacterium, *Rhodovulum sulfidophilum*[*][S]

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A new type of membrane-bound cytochrome *c* was found in a marine purple photosynthetic bacterium, *Rhodovulum sulfidophilum*. This cytochrome *c* was significantly accumulated in cells growing under anaerobic photosynthetic conditions and showed an apparent molecular mass of ~100 kDa when purified and analyzed by SDS-PAGE. The midpoint potential of this cytochrome *c* was 369 mV. Flash-induced kinetic measurements showed that this new cytochrome *c* can work as an electron donor to the photosynthetic reaction center. The gene coding for this cytochrome *c* was cloned and analyzed. The deduced molecular mass was nearly equal to 50 kDa. Its C-terminal heme-containing region showed the highest sequence identity to the water-soluble cytochrome c_2, although its predicted secondary structure resembles that of cytochrome c_y. Phylogenetic analyses suggested that this new cytochrome *c* has evolved from cytochrome c_2. We, thus, propose its designation as cytochrome c_{2m}. Mutants lacking this cytochrome or cytochrome c_2 showed the same growth rate as the wild type. However, a double mutant lacking both cytochrome c_2 and c_{2m} showed no growth under photosynthetic conditions. It was concluded that either the membrane-bound cytochrome c_{2m} or the water-soluble cytochrome c_2 work as a physiological electron carrier in the photosynthetic electron transfer pathway of *Rvu. sulfidophilum*.

The pathway of photosynthetic electron transfer in purple non-sulfur bacteria is cyclic and composed of four components. Two are membrane-bound complexes, the reaction center (RC)[2] and the cytochrome bc_1 complexes. The other two are the membranous quinones and soluble electron carrier proteins in the periplasmic space. The process of electron transfer begins with the absorption of light energy by the special pair of bacteriochlorophylls in the RC, from which an electron is released and transferred to a loosely bound quinone molecule. The reduced quinones diffuse into the membrane matrix and transfer electrons to the cytochrome bc_1 complex. The reduced cytochrome bc_1 complex transfers electrons to the soluble electron carriers, such as cytochrome c_2, and translocates protons across the membrane. The reduced soluble electron carriers donate an electron back to the photooxidized RC (1, 2).

The RC complex of purple bacteria consists of L, M, and H subunits and, in most species, a cytochrome subunit (3). The L and M subunits form a heterodimer in the membrane and contain a special pair of bacteriochlorophylls, monomeric bacteriochlorophylls, bacteriopheophytins, an iron atom, and two quinones (3). These cofactors are roughly aligned from the periplasmic side to the cytoplasmic side, in this order. The RC-bound cytochrome subunit protrudes into the periplasmic space and serves as the immediate electron donor to the photooxidized special pair.

The three-dimensional structures of RCs determined in *Blastochloris viridis* (4) and *Thermochromatium tepidum* (5) have shown that the cytochrome subunit contains four *c*-type hemes aligned along the long axis of this subunit. The amino acid sequences of the RC-bound cytochrome subunit contain four heme-binding motifs (Cys-X-X-Cys-His) together with a methionine or a histidine residue as a possible 6th axial ligand to the heme iron (6). Such molecular building blocks seem widely conserved, because these four heme-binding motifs have been reported even in green filamentous bacteria, *Chloroflexus aurantiacus* and *Roseiflexus castenholzii*, which are rather distant from purple bacteria (7, 8). On the other hand, among purple bacteria themselves, in *Rhodovulum sulfidophilum*, the amino acid sequence of the RC-bound cytochrome subunit lacks one of the heme-binding motifs (9). Moreover, the methionine 6th-axial-ligand to one of the hemes is replaced by a cysteine residue (9, 10). The missing heme in *Rvu. sulfidophilum* corresponds to the most distal heme from the special

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB257852.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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[2] The abbreviations used are: RC, reaction center; E_m, redox midpoint potential; E_h, ambient redox potential; *Rvu.*, *Rhodovulum*; *Rba.*, *Rhodobacter*; LH2, light-harvesting 2; MOPS, 3-morpholinopropanesulfonic acid; DAD, diaminodurene (2,3,5,6-tetramethylphenylene diamine); TMBZ, 3,3′,5,5′-tetramethylbenzidine; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.

pair in tetraheme cytochromes, which has been shown to be the direct electron acceptor from the soluble electron carriers (11–15). These unusual features observed for the cytochrome subunit in *Rvu. sulfidophilum* are conserved in all other *Rhodovulum* species (16). In this respect, *Rhodovulum* species may have a unique pathway of photosynthetic electron transfer through the unusual triheme cytochrome. The oxidized hemes in these tetra- or triheme subunits are re-reduced by soluble electron carriers (1). Some purple bacteria, *i.e. Rhodobacter sphaeroides* and *Rhodospirillum rubrum*, do not possess the RC-bound cytochrome subunit. In this case, the cytochrome c_2 donates an electron directly to the photooxidized special pair (2, 3).

Most photosynthetic species belonging to the α-subclass of purple bacteria use soluble cytochrome c_2 as an electron carrier regardless of the presence or absence of the RC-bound cytochrome subunit. Indeed, a mutant of *Rba. sphaeroides* devoid of the cytochrome c_2 cannot grow photosynthetically, which demonstrates that cytochrome c_2 is an essential electron donor to the RC in this species (17). On the other hand, a mutant of *Rhodobacter capsulatus* lacking the cytochrome c_2 is capable of photosynthetic growth (18). It has been shown that *Rba. capsulatus* synthesizes, in addition to the cytochrome c_2, a membrane-bound cytochrome designated cytochrome c_y that can serve as an electron donor to the RC (19). Interestingly, *Rba. sphaeroides* also synthesizes the membrane-bound cytochrome c_y, although it does not work as a physiological electron donor to the RC (20). Proteins homologous to cytochrome c_y have been reported in non-photosynthetic species of purple bacteria, *Paracoccus denitrificans* (21) and *Bradyrhizobium japonicum* (22), which work as an electron carrier between cytochrome bc_1 and terminal oxidases.

Rvu. sulfidophilum synthesizes cytochrome c_2. Reconstitution experiments using the purified cytochrome c_2 and the membranes of this bacterium showed that this cytochrome is a good electron donor to the RC-bound triheme cytochrome in *Rvu. sulfidophilum* (23). However, a mutant lacking cytochrome c_2 was able to grow photosynthetically with a doubling time nearly equal to that of the wild type, showing that another electron carrier participates in the photosynthetic electron transfer in *Rvu. sulfidophilum* (24). A water-soluble *c*-type cytochrome, cytochrome *c*-549, was recently purified from this mutant as a candidate for an alternative electron transfer pathway (24). Reconstitution experiments showed that the second-order rate constant for the electron transfer reaction from the cytochrome *c*-549 to the RC-bound cytochrome is about 7 times larger than that from the cytochrome c_2, suggesting that the major electron donor to the RC in *Rvu. sulfidophilum* is this cytochrome, *c*-549 (24).

Here, we report that a new membrane-bound cytochrome *c*, with a molecular mass of 50 kDa, is found in *Rvu. sulfidophilum*. This cytochrome serves as a physiological electron donor to the RC. The gene coding this cytochrome was cloned and sequenced. The analysis of the amino acid sequence showed that the soluble cytochrome *c*-549 originates from this membrane-bound cytochrome *c*. Phylogenetic analyses suggest that the region containing a heme in the membrane-bound cytochrome *c* newly found in *Rvu. sulfidophilum* is closer to cytochrome c_2 rather than cytochrome c_y.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*Rvu. sulfidophilum* strains DSM 1374 (wild type) and C21 (a mutant in which the gene for the cytochrome c_2 was disrupted (24)) were used in this study. The cells were photosynthetically grown at 30 °C in screw-capped bottles filled with a PYS medium (0.5% polypeptone, 0.1% yeast extract, 0.5% sodium succinate, 1% basal salt solution (25)) supplemented with 2% NaCl. Illumination was provided by 60 watt tungsten lamps (50 watt/m^2).

Preparation of Chromatophore Membranes Free from Soluble Proteins—Cells in a 1-liter culture of *Rvu. sulfidophilum* wild type were harvested by centrifugation at 6,800 × *g* for 20 min and washed with 400 mM Tris-HCl, pH 7.8. The washed cells were sedimented by centrifugation and suspended in 60 ml of a solution containing 200 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 500 mM sucrose followed by an addition of 10 μM lysozyme. The cell suspension was incubated at 30 °C for 30 min to digest the cell wall and then mixed with 600 ml of 20 mM MOPS-NaOH (pH 7.0), 2.4 M KCl, and 250 mM sucrose to remove periplasmic proteins. Spheroplasts were collected by centrifugation at 6,800 × *g* for 20 min and suspended in 50 ml of a buffer containing 20 mM MOPS-NaOH (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, and a trace amount of DNase I. Spheroplasts were disrupted using a glass homogenizer. Membranes from spheroplasts were collected by the method of differential centrifugation as a sedimented fraction between 27,000 × *g* for 15 min and 193,000 × *g* for 60 min and suspended in a buffer containing 20 mM MOPS-NaOH (pH 7.0) and 100 mM KCl.

Purification of Membrane-bound Cytochrome c—Cells of *Rvu. sulfidophilum* strain C21 were harvested by centrifugation at 6,800 × *g* and washed with a buffer containing 20 mM MOPS-NaOH (pH 7.0) and 350 mM NaCl. Harvested cells were suspended in the same buffer and disrupted using a French pressure cell (1100 kg/cm^2) after the addition of a trace amount of DNase I. The membranes were collected by the method of differential centrifugation as a sedimented fraction between 27,000 × *g* for 15 min and 193,000 × *g* for 60 min. The membranes were suspended in a buffer containing 20 mM MOPS-NaOH (pH 7.0) and 100 mM KCl to reach OD_{854} = 50 and incubated with 30 mM octylthioglucoside on ice for 20 min to solubilize the membrane-bound cytochrome *c*. The solubilized sample was dialyzed against the same buffer to remove octylthioglucoside and centrifuged at 19,300 × *g* for 60 min to remove aggregated proteins. The supernatant was applied to preparative PAGE. The buffer system was according to Davis (26). A pink-colored band was excised from the gel and crushed by a glass homogenizer. Cytochrome *c* was extracted from the crushed gels using a buffer containing 20 mM MOPS-NaOH (pH 7.0) and 100 mM KCl.

SDS-PAGE—SDS-PAGE was carried out according to Laemmli (27) with 15% polyacrylamide gel. Samples (membranes; 300 μg, purified proteins; 10 μg) were solubilized by the addition of 2% SDS and 5% β-mercaptoethanol followed by incubation at 37 °C for 5 min for membrane samples or at 100 °C for 3 min for purified proteins. A small amount of sodium ascorbate was added to the membrane samples to reduce cytochrome *c*.

Heme staining was performed by the method of Thomas *et al.* (28).

Cloning and Sequencing of the Gene Encoding the Membrane-bound Cytochrome c—The membrane-bound cytochrome *c* polypeptide separated by SDS-PAGE was transferred to a polyvinylidene difluoride membrane. The N-terminal amino acid sequence of the polypeptide was determined by the method of automated Edman degradation. Based on the N-terminal amino acid sequence, an oligonucleotide, which has a sequence of 5′-TCSAACAACTCSCAGAACCTNATCCGSCTN-3′, was designed and used as a probe to detect a gene for the membrane-bound cytochrome *c*. The oligonucleotide was labeled at the 5′-end with digoxigenin-dUTP, as instructed by the manufacturer (Roche Applied Science GmbH, Germany). The cosmid library constructed in our previous study (29) was screened against the probe. Cosmids showing positive signals were digested with EcoRI and PstI restriction enzymes and cloned into pUC119 plasmids. A plasmid containing a ~4.5-kb DNA fragment giving a positive signal was, thus, obtained. 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 were obtained from Amersham Biosciences. The DNA sequences were analyzed using a DNASIS software package (Hitachi Soft, Yokohama, Japan).

Mutant Construction—The 4.5-kb insert DNA described above was shortened into 1.8-kb DNA containing a full-length of the membrane-bound cytochrome gene by treatments with ApaI and HindIII endonucleases and self-ligation. A region containing the sequence for the heme-binding motif was then removed by digestions with SalI and SphI and ligation. The insert DNA was transferred into a suicide vector, pJPCm, a derivative of the plasmid pJP5603 (30), in which the kanamycin-resistance gene is replaced by a chloramphenicol-resistance gene derived from plasmid pHSG396. A DNA fragment containing a Km^r-marker gene and *sacB-sacR* genes was then inserted at the EcoRI restriction site in the multicloning site. The expression of the *sacB-sacR* genes is lethal for cells grown in the presence of sucrose (31). This plasmid was introduced into the cells of *Rvu. sulfidophilum* wild type and a mutant lacking cytochrome c_2, strain C21 (24), by conjugal transfer from *Escherichia coli* S17-1 λpir host cells. First, *Rvu. sulfidophilum* cells growing on agar plates containing kanamycin were picked up as transformants keeping the plasmid in their genomic DNAs via a single crossover homologous recombination. After cultivation in a medium without kanamycin to allow a second homologous recombination, the cells were grown in a medium containing 15% sucrose. Cells tolerant to sucrose were selected and the deletion of the gene for the membrane-bound cytochrome was confirmed by Southern hybridization, PCR, and DNA sequencing.

Spectroscopy—The flash-induced absorbance changes were recorded with a single beam (32) and a double beam (33) spectrophotometers assembled in our laboratories. The absorption spectra were recorded with a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan).

Mass Spectrometry—Laser desorption/ionization mass spectrometric analysis was performed with a Perseptive Biosystems (Framingham, MA) Voyager Elite XL time-of-flight mass spectrometer with delayed extraction, operating with a pulsed nitrogen laser at 337 nm. Positive ion mass spectra were acquired using a linear, delayed extraction mode with an accelerating potential of 25 kV, a 90% grid potential, a 0.2% guide wire voltage, and a delay time of 200 ns for the recombinant p2.

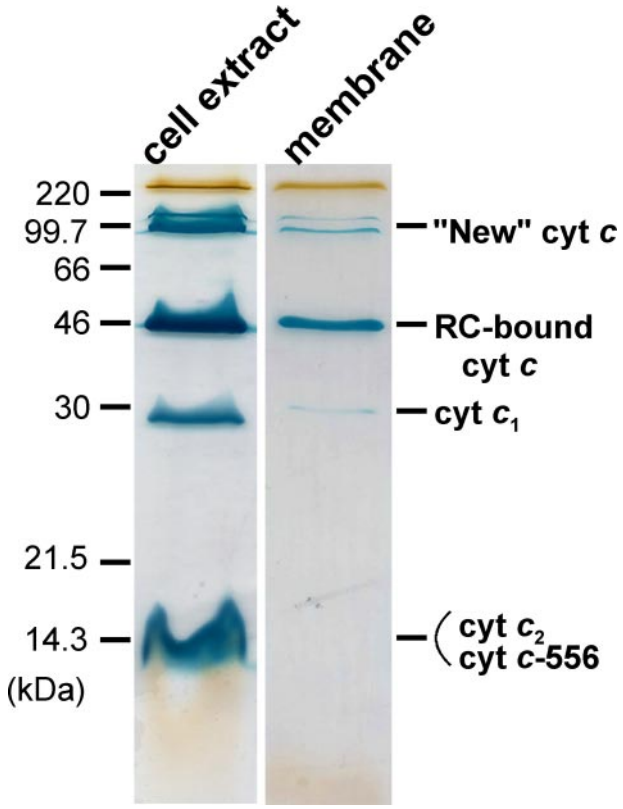

FIGURE 1. **SDS-PAGE analysis of cytochrome *c* in the membranes and cells of *Rvu. sulfidophilum*.** *Left*, wild type cells grown to mid-exponential phase; *right*, membranes free from soluble proteins. The samples were treated with 2% SDS and 5% β-mercaptoethanol at 37 °C for 5 min before loading. The gel was stained for hemes by TMBZ/H_2O_2.

RESULTS

In a previous study, we showed that *Rvu. sulfidophilum,* grown under photoheterotrophic conditions, synthesizes at least three different water-soluble cytochrome *c* (24). One of these cytochromes was identified as cytochrome c_2, based on its redox property (E_m = 358 mV) and amino acid sequence. Its apparent molecular mass resolved in SDS-PAGE analysis was 15 kDa. The other two cytochromes were named cytochrome *c*-549 (E_m = 238 mV) and *c*-556 (E_m = 73 mV) based on the wavelength maxima of their α-bands. Their apparent molecular masses were 25 kDa and 14 kDa, respectively (24).

We have suggested that cytochrome *c*-549 is the major electron donor to the RC in *Rvu. sulfidophilum* on the basis of *in vitro* reconstitution experiments (24). However, we have since noticed that the relative yield of the soluble cytochrome *c*-549 varies with the culture batch used for the preparation. Especially, cells grown to the mid-exponential phase contain a much smaller amount of cytochrome *c*-549 than other soluble cytochromes. This is illustrated in Fig. 1, where the heme-protein content of such intact cells was examined by SDS-PAGE and

specific staining. Bands with apparent molecular masses around 30 and 46 kDa were assigned to the cytochrome c_1 of the cytochrome bc_1 complex and to the RC-bound cytochrome subunit, respectively, in agreement with a previous study (9). An intense band attributable to both cytochrome c_2 and cytochrome *c*-556 was detected at around 14 kDa. This band was very weak in mutant cells lacking cytochrome c_2, reflecting the fact that the band is mainly derived from cytochrome c_2 (data not shown). On the other hand, no heme staining was observed at around 25 kDa, where the contribution of cytochrome *c*-549 is expected. Even in experiments using cells grown over a stationary phase, we could not detect clear signals derived from cytochrome *c*-549 (data not shown). Instead, a large amount of unknown cytochrome *c* was detected at around 100 kDa.

The absence of a significant amount of cytochrome *c*-549 led us to examine the possibility that a membrane-bound electron donor to the RC is involved in the *Rvu. sulfidophilum* photo-induced cyclic electron transfer. In a first set of experiments, membranes free from water-soluble proteins were prepared from the *Rvu. sulfidophilum* wild-type cells grown to the mid-exponential phase. The content in heme *c* was then analyzed by SDS-PAGE and specific staining. As shown in Fig. 1, several bands with apparent molecular masses of around 30, 46, and 100 kDa were detected. A brown band with a high molecular mass (around 220 kDa) was probably derived from protein aggregates containing carotenoids. Presence of the cytochrome c_1 and the RC-bound cytochrome was confirmed as 30- and 46-kDa bands, respectively. The difference of staining between the bands corresponding to cytochrome c_1 and RC-bound cytochrome is caused by the presence of three hemes in the latter compared with one in the former. In addition, the purification procedure of the membranes may have induced a partial loss of bc_1 complex (34, 35). The lack of the 14-kDa band in the membrane prepared in this study demonstrates the total absence of periplasmic proteins. Two other bands with molecular masses of ~100 kDa, which were also observed in the total cell extract, were still detected in this preparation, showing that these are membrane-bound cytochrome *c*.

To examine whether or not the light-induced cyclic electron transfer in *Rvu. sulfidophilum* is maintained by solely membrane-bound components, we performed measurements of flash-induced absorbance changes on membranes prepared from the *Rvu. sulfidophilum* strain C21, a mutant lacking the cytochrome c_2. As shown in Fig. 2, at an ambient redox potential (E_h) of 85 mV, where the high potential heme in the RC-bound cytochrome subunit and the cytochrome c_1 in the bc_1 complex are reduced prior to flash activation, the photo-oxidized cytochrome was rapidly re-reduced. Upon addition of myxothiazol, an inhibitor of the electron transfer from the quinol to the cytochrome bc_1 complex, a large amount of cytochrome was rapidly photooxidized after the flash and remained in that state over several hundred milliseconds. The light-induced difference spectrum measured under this condition presented a peak at 552 nm. The strong effect of the addition of myxothiazol on the rate of re-reduction of the photooxidized cytochrome(s) demonstrates the occurrence of a photo-induced cyclic electron transfer between the RC and the cytochrome bc_1 even in the absence of soluble cytochrome c_2 and

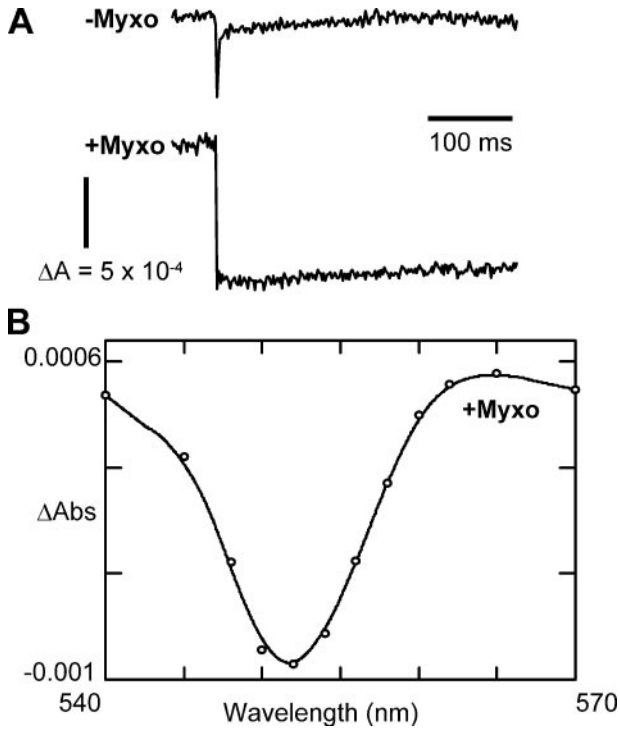

FIGURE 2. **Flash-induced absorbance changes of cytochrome *c* in the membranes prepared from *Rvu. sulfidophilum* mutant lacking cytochrome c_2.** *A*, kinetic traces shown were measured at 552 nm. *B*, spectra were obtained by plotting the absorbance changes several ms after the flash-activation. Membranes were suspended in a buffer containing 50 mM potassium phosphate and 50 mM KCl, pH 7.0, with OD_{850} = 1.0. To stabilize redox potential, 50 μM DAD, 5 μM ferric-EDTA, and 100 μM ferrocyanide were added. Redox potential of the sample was poised at 85 mV by addition of ascorbate. Myxothiazol was added at 5 μM.

c-549. This is a strong argument for the connection by a membrane bound electron carrier between these two membranous components.

We thought that the electron transfer from the bc_1 complex to the RC resolved in the flash-induced kinetic measurements was mediated by membrane-bound cytochrome *c* with apparent molecular masses of ~100 kDa in SDS-PAGE analysis. To examine this hypothesis, these cytochrome *c* were purified and characterized. Membranes were treated with a low concentration (30 mM) of octylthioglucoside. Then, the solubilized proteins were separated by preparative PAGE. A pink-colored band in the high molecular mass region, containing possible *c*-type cytochromes, was excised and extracted. Isolated proteins showed a single band at about 100 kDa in SDS-PAGE analysis when the protein was denatured at 100 °C for 3 min prior to loading (Fig. 3*A*, *lane 1*). However, when the treatment was carried out at 37 °C for 5 min, a band showing a mobility of 120 kDa was observed (Fig. 3*A*, *lane 2*), and, sometimes, two bands of 100 and 120 kDa were observed (data not shown). Although the reason that this cytochrome shows different mobility in SDS-PAGE according to the denaturing conditions was not further addressed in this study, we concluded that the two bands that resolved at around 100 kDa in the SDS-PAGE analysis of the membranes and cells (Fig. 1) are derived from the same cytochrome *c*. This cytochrome *c* was further analyzed by MALDI-TOF mass spectroscopy (see Supplemental Data). The molecular mass was determined to be 50,429 Da, which is about

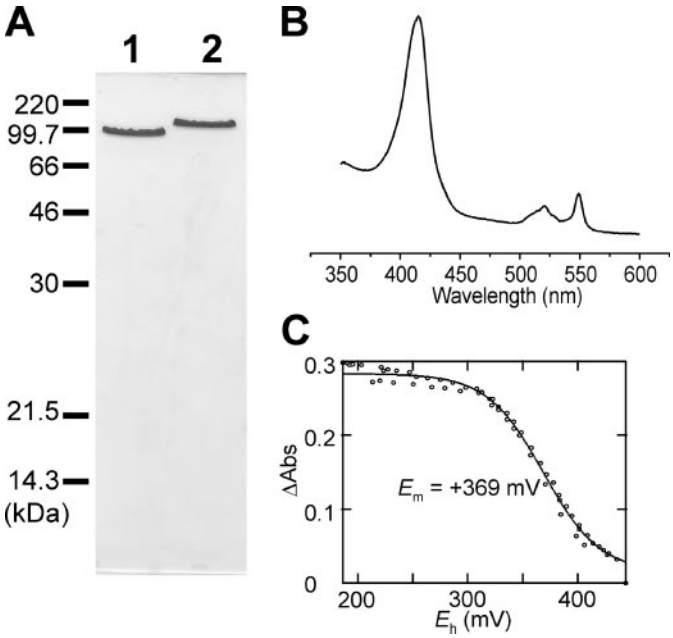

FIGURE 3. **Properties of membrane-bound cytochrome *c* purified from *Rvu. sulfidophilum*.** *A*, SDS-PAGE analysis; *B*, measurement of absorption spectrum; and *C*, redox titration were carried out. *Lane 1*, cytochrome was denatured at 100 °C for 3 min or *lane 2*, 37 °C for 5 min in the SDS-PAGE analysis. The absorption spectrum was recorded after addition of ascorbate to reduce the cytochrome. The redox titration was carried out as described previously (23). Redox poise in the titration cuvette (E_h) was changed oxidatively by addition of ferricyanide and reductively by dithionite. All the data obtained by both the oxidative and reductive titrations were plotted.

half of the value estimated from the SDS-PAGE analysis. This large discrepancy will be discussed later, and this membrane-bound cytochrome *c* newly isolated from *Rvu. sulfidophilum* will be referred to as the 50-kDa cytochrome *c* in this article.

The absorption spectrum of the membrane-bound 50-kDa cytochrome *c* reduced by ascorbate showed α, β and Soret absorption peaks at 550, 521, and 416 nm, respectively (Fig. 3*B*), as typically observed for *c*-type cytochromes. Fig. 3*C* shows the result of the redox titration of this cytochrome *c* obtained by plotting the height of the α-absorption band against E_h. The data were well fitted with a one-electron Nernst curve with E_m of 369 mV.

To confirm the electron transfer ability of the 50-kDa cytochrome *c* to the RC, reconstitution experiments with the membrane and the purified cytochrome were performed. The native membrane-bound cytochrome *c* was detached from the membrane by treatment with 0.04% Triton X-100. Fig. 4 shows the flash-induced absorbance changes of cytochrome *c* with or without the addition of the extra cytochrome *c*. The measurements were carried out at an E_h poised by the addition of 1 mM sodium ascorbate, where both the 50-kDa cytochrome *c* and the highest potential heme (*c*-553) in the RC-bound cytochrome were reduced prior to the flash. The kinetic trace obtained at 550 nm from the membrane free from the 50-kDa cytochrome *c* showed a small but rapid absorption decrease immediately after the flash, consecutive to the photooxidation of the highest potential heme in the RC-bound cytochrome. Consistently, the flash-induced spectra recorded 2 ms after the flash activation showed a downward peak at 553 nm. In the absence of an extra 50-kDa cytochrome *c*, the heme remained oxidized over several hundred milliseconds. On the other hand, in the presence of the 2 μM extra 50-kDa cytochrome *c*, the flash-induced kinetics showed a large long lived absorption decrease because of the oxidation of this new cytochrome *c*. In this case, the flash-induced spectrum at 200 ms showed a clear shift of the α-absorption band from 553 nm toward the shorter wavelength (Fig. 4). Such a spectral shift was not observed in the sample without the extra 50-kDa cytochrome *c*. These results indicate that this 50-kDa cytochrome *c* can work as an electron donor to the photooxidized RC-bound cytochrome subunit.

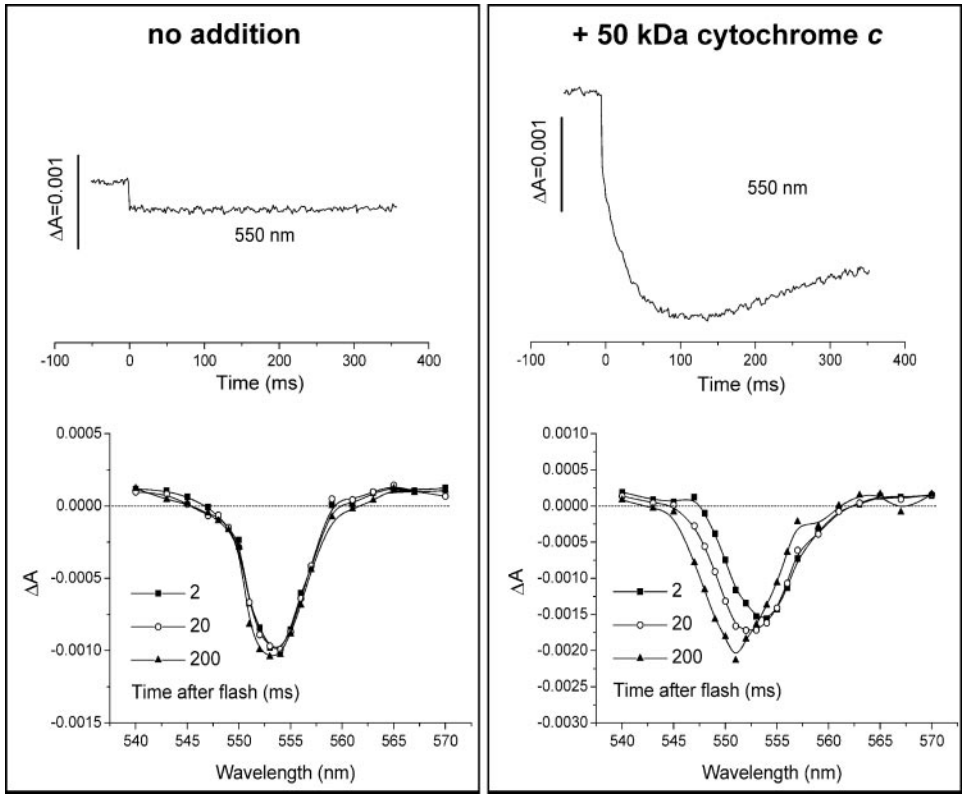

FIGURE 4. **Flash-induced absorbance changes of cytochrome *c* in the membranes in the presence or absence of the extra 2 μM 50-kDa cytochrome *c*.** Membranes were suspended to OD_{854} = 1.0 in a buffer containing 20 mM MOPS-NaOH (pH7.0) and 100 mM KCl. The membranes were treated with 0.04% Triton X-100 to remove native membrane-bound 50 kDa cytochrome *c*.

The amino acid sequence of the N-terminal 10 residues of the 50-kDa cytochrome *c* was directly determined for the purified protein. Using an oligonucleotide, corresponding to this amino acid sequence, a gene coding this cytochrome was screened from the cos-

A

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   1 GGACGCCGGAATGCCCTTTGGCGCTCTGGCGGTTCGTCTGGCCGATGCGGGAGGCATCAGGGGCCCCCGTCGGAACCCGCAGGATGACGTCCTGCCGCGGACGGGGTGGTCCCGGCCGTC  120
 121 GGCAGCGGAACGAAAAAAGGATGAACCATGTCCAATAATTCGCAGAACCTGATCCGGCTGATCGTCGCCGCGCCGGCGCTTGGCATCGCGATCGCGGCCGTCGGCTTCGGCGGCGGCAAG  240
   1                          M  S  N  N  S  Q  N  L  I  R  L  I  V  A  A  P  A  L  G  I  A  I  A  A  V  G  F  G  G  G  K   31

 241 GAAACCAAGAACGAGCCCGTGATCGAGACGCCGGTCGCCGAGCCTTCCGCTGCCGAAGCCGCCGCGCCGGCCGAAGAGGCCGTTCGGAAGAGGCCGTTCGGAAGAGGCTGCGGCCGAG  360
  32 E  T  K  N  E  P  V  I  E  T  P  V  A  E  P  S  A  A  E  A  A  A  P  A  E  E  A  V  S  E  E  A  V  S  E  E  A  A  A  E   71

 361 GCGCCTGCCACTGACGCCACCGAAGCTGCCGACGCGCCGGCCGCCGAGGAAGCCGCTGCCGAGGAACCGGCCGCCGAAGCTGCCGACGCGCCCGCTGCCGAAGATGCCGTCGAGGATGCC  480
  72 A  P  A  T  D  A  T  E  A  A  D  A  P  A  A  E  E  A  A  A  E  E  P  A  A  E  A  A  D  A  P  A  A  E  D  A  V  E  D  A  111

 481 GCCGCGACCCAGGAAGCTGTCGCCGCCGCGCCGCCGCTGCCGAGGAAGCTGCCGCCGAGGAACCGGCCGCCGAGGAACCTGCTGCCGAAGCCGCTGAAGCGCCCGCTGCCGAGGCTGCCGCC  600
 112 A  A  T  Q  E  A  V  A  A  A  P  A  A  E  E  A  A  A  E  E  P  A  A  E  E  P  A  A  E  E  A  A  E  A  P  A  A  E  A  A  A  151

 601 CCGACCGAGGAGGCTGCTGCCGCCGCCCCCGCCGCCGAAGAGGACACCGCCGAAGAGCCGGCTGCTGCCGAGGAACCGGCCGCCGAAGACGCCGCCGCTGCGGATGACGCCGTCGAGGCG  720
 152 P  T  E  E  A  A  A  A  A  P  A  A  E  E  D  T  A  E  E  P  A  A  A  E  E  P  A  A  E  D  A  A  A  A  D  D  A  V  E  A  191

 721 CCTGCGGTCGACACCCCCGAGGCGGTTGCCTCGGCGATGACCCTGGCCGAGCAGGCCTCTGCGCTTGCCGCCGAATATGCCGATCTCGCCGAGCGCGCCGCTGCCCTGGCCGGCAAGTCC  840
 192 P  A  V  D  T  P  E  A  V  A  S  A  M  T  L  A  E  Q  A  S  A  L  A  A  E  Y  A  D  L  A  E  R  A  A  A  L  A  G  K  S  231

 841 TCGGCCCTCACCGCGGCGACCGAAGCGGCCGCCGAAGCGCCGGAAAGCGTCGAGGAGCTGTTCGGCAACATCGCCGCCGCCGCGACCGAGGAAACCACCGAGGAAACCGAGGAACCGGCT  960
 232 S  A  L  T  A  A  T  E  A  A  A  E  A  P  E  S  V  E  E  L  F  G  N  I  A  A  A  A  T  E  E  T  T  E  E  T  E  E  P  A  271

 961 GCCGAGGCACCGGCCGCTGAAGAGCCTGCTGCCGAAGAGCCGACTATCGCCGAGAAGGCCGCCGATGCCGCGACCGCTGCCGTCGAGACTGCCGAGGACGCGGCCGACAGCGCGGGCGAG 1080
 272 A  E  A  P  A  A  E  E  P  A  A  E  E  P  T  I  A  E  K  A  A  D  A  A  T  A  A  V  E  T  A  E  D  A  A  D  S  A  G  E  311

1081 GCCGTGACCGAGGCGGTGCAGGACACCCGCGAGGCGATCACCGGCAGCGAAGAGCCCGTGATCGGTGGCGATGCGCCCGAGAAAGCCGCCGCCGAACCGGCCGCGACCGAGGAAGCTGCC 1200
 312 A  V  T  E  A  V  Q  D  T  R  E  A  I  T  G  S  E  E  P  V  I  G  G  D  A  P  E  K  A  A  A  E  P  A  A  T  E  E  A  A  351

1201 GCCGAACCTGCTGCGGAAGAACCGGCCGCCGAGGAGCCTGCCGCTGAAGAACCGGCTGCTGCCGAGGAACCTGCCGCGGCCCCGGCCGCCGTGGTTACCGGCGATGCCGCCAAGGGCGAA 1320
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1321 AAGGCCTTCATCCAGTGCGCCACCTGCCACAGCGTCGTCAGCCCTTCGGGCGAGACGCTGGCCGGTCGCGGTCTGAAGGTCGGCCCGAACCTCTATGGCATGCCGGGCCGCACCGCCGGA 1440
 392 K  A  F  I  Q  C  A  T  C  H  S  V  V  S  P  S  G  E  T  L  A  G  R  G  L  K  V  G  P  N  L  Y  G  M  P  G  R  T  A  G  431

1441 ACGCTGGAAGGCTTCCGCTATTCCAAGTCGATGAAGGCCGCCGGTGAAGCCGGTCTGGTCTGGGACGAGGACAAGGTCTCGATCTATGCCGAAGATCCGACCGCCTACCTGCGTGACTAT 1560
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1561 CTCGACGATCCCCGGGCGCGCGGTCTGATGACCTACAACGCCCGCGACCCGCAAGAGGTCAAGGACGTCTACGCCTATATCTCGAAGCTCTCCGAAGAGTAATCGTCGTCCGGGGGGGCTG 1680
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1681 TCCTCCGCGACACGCGCCAAAGCCCCGTCCCGGCCGCCTGCCGGGGCGGGGTTTCTCTTTCGGGCATGGCTCAGGCCTGAAGCAGGGGCGCGAGCGCCTCCAATAGCGGCCCGGGTGAAA 1800

1801 CCGGCCTGCCGCCCTCTTCCCAGGCCGCCTGGAATTC                                                                                    1837
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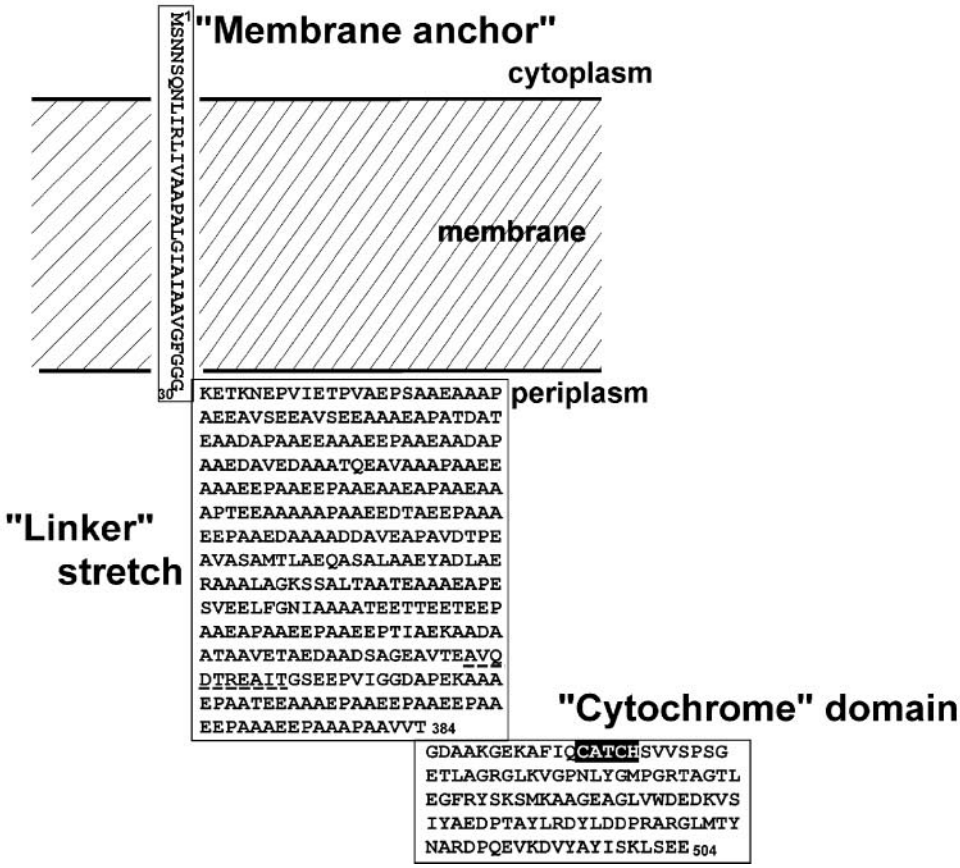
B



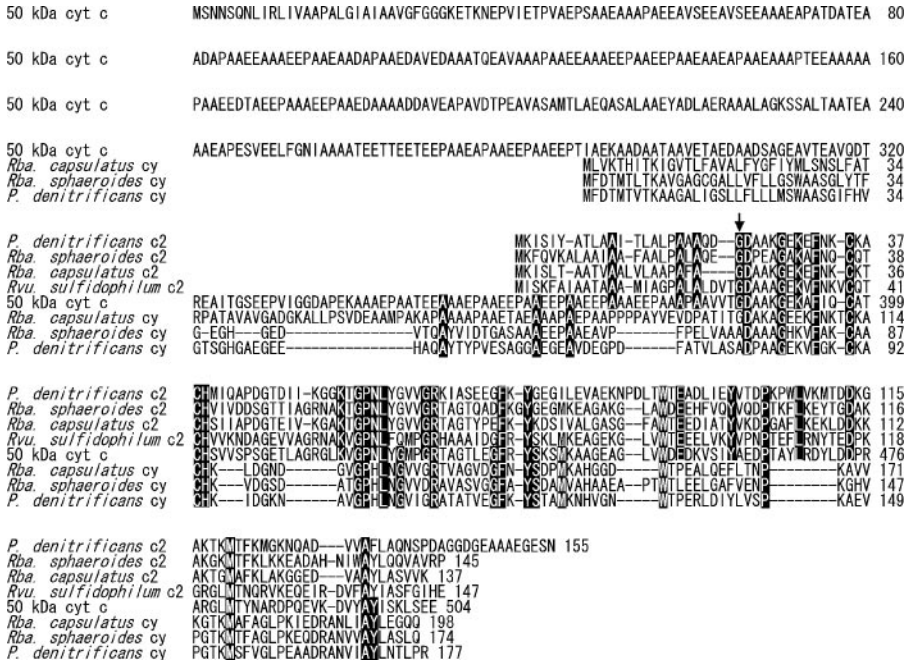


FIGURE 6. **Amino acid sequence alignment of the 50-kDa cytochrome *c* and cytochrome c_2 and c_y.** Amino acid sequences of cytochrome c_2 and c_y from species having both cytochromes were used for comparison. The cytochrome c_2 of *Rvu. sulfidophilum* was also included. Conserved amino acid residues among the 50-kDa cytochrome *c* and 4 cytochrome c_2 (*upper*) and among the new cytochrome *c* and 3 cytochrome c_y (*lower*) were shown by *reverse contrast*. An *arrow* marks a position of the N-terminal first residue of mature cytochrome c_2 of *Rba. capsulatus*.

mid-based DNA library of the *Rvu. sulfidophilum* genome. The nucleotide sequence for the 1837-base DNA region containing the gene for this cytochrome was determined as shown in Fig. 5. The N-terminal 10 residues in the deduced amino acid sequence were completely matched with those determined for the purified protein, which suggests that the N-terminal region is not processed during the maturation. The deduced amino acid sequence consisted of 504 residues and contained one heme-binding motif, showing that this cytochrome *c* is a monoheme cytochrome *c*. A molecular weight of 50,528 was calculated for this cytochrome when the presence of a *c*-type heme was taken into account. This value is quite consistent with the molecular mass obtained by MALDI-TOF mass spectrometry, 50,429 Da, when considering the protonation of more than one hundred acidic amino acids contained in this protein. However, the value is lower than the apparent molecular mass of about 100 kDa obtained in the SDS-PAGE analysis. It is possible that these cytochromes form dimers via disulfide bonds. However, no cysteines were found in the whole sequence except for the ones involved in the heme-binding motif. Another explanation for this discrepancy between the predicted molecular weight and apparent molecular mass is the highly acidic property of this protein (see below).

A hydrophobic segment consisting of about 20 amino acids was found in the N-terminal region (8th to 30th from the initial Met), which was predicted to form an α-helix structure, suggesting that the 50-kDa cytochrome is anchored to the membrane by the hydrophobic segment. This hydrophobic segment was followed by strange repeats of short sequences mainly consisting of Pro, Ala, and Glu (or Asp) residues, which occupy more than half of the whole sequence and make the protein very acidic (calculated pI, 3.62). This acidic sequence might be one of the reasons why the SDS-PAGE analysis overestimates the molecular mass. This might be caused by poor interaction with SDS and/or extreme elongation of the peptide because of intramolecular electrostatic repulsion by the large negative charge, as have been discussed in studies on other acidic natively unfolded proteins (36). The C-terminal region of the polypeptide contained a heme binding motif (-Cys-X-X-Cys-His-) and showed a high sequence identity to cytochrome c_2. From the structural viewpoint this cytochrome *c* can be divided into three parts, the N-terminal membrane-anchor (1st to 30th), the significantly long and acidic linker stretch (31st to 384th), and the C-terminal cytochrome domain (385th to the end, 504th).

An important finding in the deduced amino acid sequence of the 50-kDa membrane-bound cytochrome *c* was that the N-terminal amino acid sequence determined for the soluble cytochrome *c*-549 is included in this sequence, showing that the soluble cytochrome *c*-549 is generated from the 50-kDa cytochrome *c* by a specific cleavage of the peptide. Based on the deduced amino acid sequence, a molecular weight of 20,217 was calculated for the soluble cytochrome *c*-549, which is not so different from the apparent molecular mass resolved in SDS-PAGE, 25 kDa.

When the amino acid sequence of the 50-kDa cytochrome *c* was compared with the known sequences of other cytochrome *c*, the C-terminal cytochrome domain showed strong sequence identities to cytochrome c_2, as shown in Fig. 6. The highest sequence identity of 54% was obtained in comparison with *Rvu. sulfidophilum* cytochrome c_2 and was higher than the identities among the group of cytochrome c_2 (35–53%, see Fig. 6). The sequence identities between the cytochrome domain of the 50-kDa cytochrome *c* and cytochrome c_2 of three other species belonging to the α-3 subgroup, *P. denitrificans*, *Rba. sphaeroides*, and *Rba. capsulatus*, were 34–46%. No insertions and deletions specific to the 50-kDa cytochrome *c* were observed in the cytochrome domain when compared with

FIGURE 5. *A*, nucleotide sequence of the gene coding the 50-kDa cytochrome *c* and *B*, a proposed structure of the product based on the deduced amino acid sequence. The heme-binding motif is *boxed*. The region presumed to bind to the membrane is *hatched* in *A*. The amino acid sequences directly determined from the purified proteins of the 50-kDa cytochrome *c* and the soluble cytochrome *c*-549 are *underlined* by a *continuous line* (*A*) and a *thick-broken line* (*A* and *B*), respectively. *Head-to-head arrows* show a possible hairpin-loop structure for the transcription termination.

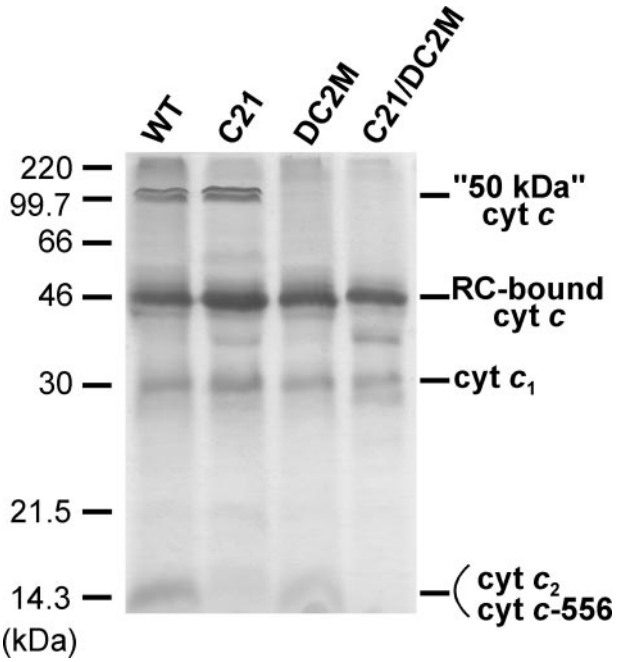


FIGURE 7. **SDS-PAGE analysis of cytochrome *c* in the cells of *Rvu. sulfidophilum* wild type and mutants.** The cells of wild type and the C21 and DC2M mutants were grown under the photosynthetic conditions. The C21/DC2M double mutant was grown under the aerobic respiratory conditions. Treatments for samples and conditions of PAGE were the same as those described for Fig. 1. The gel was stained for hemes by TMBZ/H_2O_2.

the sequences of cytochrome c_2, as shown in Fig. 6. Cytochrome c_y also showed significant sequence identities to the 50-kDa cytochrome *c* at the C-terminal region. The sequence identities were 30–34% in this region, *i.e.* lower than those among the cytochrome c_y family, 61–62%. Moreover, many insertions were observed in the sequence of the 50-kDa cytochrome *c* when aligned with the sequences of the cytochrome c_y.

To clarify the physiological function of the 50-kDa membrane-bound cytochrome *c*, a mutant in which the gene coding this cytochrome was disrupted, DC2M, was constructed. By applying the same mutagenesis procedure to the C21 cytochrome c_2-less mutant, a double mutant lacking both the cytochrome c_2 and the 50-kDa cytochrome, the C21/DC2M mutant strain, was also obtained. Fig. 7 shows a result of SDS-PAGE and heme-staining for cytochrome *c* in intact cells of the mutants. Bands with apparent molecular masses around 100 kDa were missing in the DC2M and C21/DC2M mutants. When grown in a PYS medium under aerobic conditions, these mutants showed no differences in growth rates from the wild type and the C21 mutant strain (data not shown). However, under photosynthetic conditions, the double mutant C21/DC2M showed no growth, as shown in Fig. 8, while mutants lacking only the 50-kDa cytochrome *c*, DC2M, or the cytochrome c_2, C21, showed nearly the same growth rate as the wild type. It is clear that the physiological function of the 50-kDa cytochrome *c* is the same as that of cytochrome c_2 under photosynthetic conditions. These cytochromes compensate for each other.

Fig. 9 shows light-induced absorbance changes linked to cytochrome photooxidation (Fig. 9*A*), and membrane potential-sensitive carotenoid bandshift (Fig. 9*B*) for intact cells of the wild type and mutants of *Rvu. sulfidophilum* placed under

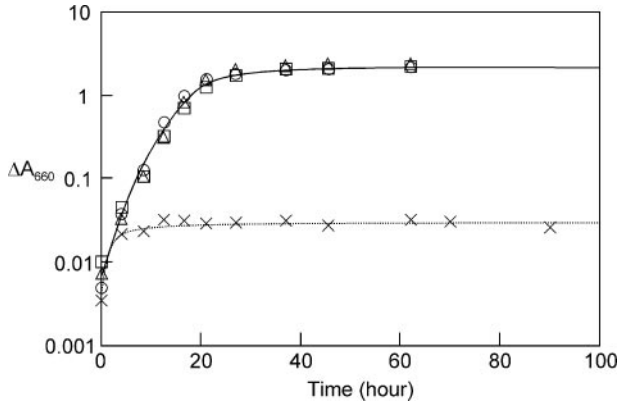


FIGURE 8. **Growth curves of *Rvu. sulfidophilum* wild type and mutants lacking cytochrome *c* under photosynthetic growth conditions.** The measurements were started by 1/100 volume of the cells in late exponential phase grown aerobically in PYS medium. The cells were grown in screw-capped tubes filled with the medium in the light supplemented with 60-watt tungsten lamp placed 20-cm apart from the culture. The absorbance at 660 nm was measured in the tube, the diameter of which was 18 mm. The average of three out of five independent measurements omitting the minimum and maximum was plotted against the time. Symbols used are *open circles* (○) for wild type, *open squares* (□) for C21 mutant, *open triangles* (△) for DC2M mutant, and *crosses* (×) for C21/DC2M double mutant.

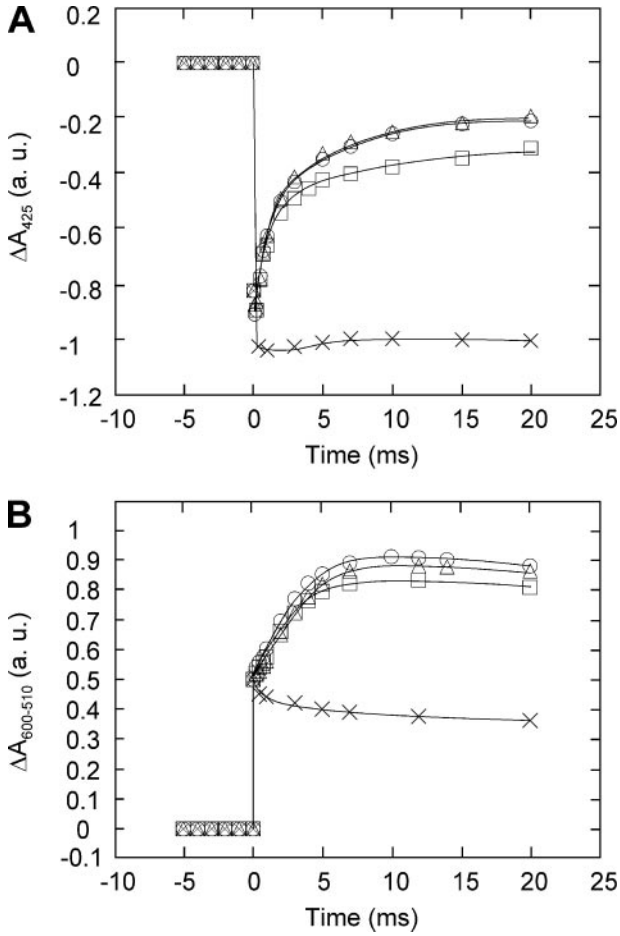


FIGURE 9. **Kinetics of re-reduction of photo-oxidized cytochrome *c* (*A*) and photo-induced electrochromic band shifts of carotenoids (*B*) in intact cells of *Rvu. sulfidophilum* wild type and mutants lacking cytochrome *c*.** The redox change of cytochrome *c* was monitored at 425 nm. Carotenoid band shift was measured as the absorption difference change between 600 and 510 nm. The vertical scale is arbitrary unit (a. u.). Symbols used are *open circles* (○) for wild type, *open squares* (□) for C21 mutant, *open triangles* (△) for DC2M mutant, and *crosses* (×) for C21/DC2M double mutant.

anaerobic conditions. Cells were grown under dark aerobic conditions because of the PS^- phenotype of the double-mutant C21/DC2M. We determined in both the wild type and the PS^+ mutants that these different growth conditions (dark aerobic and light anaerobic) do not affect the kinetics of the light-induced electron transfer (data not shown). The only difference between these growth conditions is that growth in the presence of oxygen induces the formation of oxidized carotenoids absorbing at longer wavelengths. The maximum absorption changes linked to the carotenoid bandshift were observed at 600-minus-510 nm under these conditions. The photooxidation of cytochromes was measured at 425 nm. At this wavelength, cytochrome photooxidation induces a negative absorption change. As already reported (23), a very fast photooxidation of the RC-bound cytochrome was followed by its rapid re-reduction ($t_{½}$ = 2 ms) in the wild-type cells. This re-reduction was concomitant with the slow phase of the carotenoid bandshift, which is indicative of an efficient photoinduced cyclic electron transfer. The kinetics of cytochrome photooxidation and carotenoid bandshift observed for the mutant cells lacking cytochrome c_2 (C21) or the 50-kDa cytochrome *c* (DC2M) were very similar to those measured for the wild-type cells. This indicates that these mutations do not significantly affect the light-induced cyclic electron transfer in agreement with their capability of photosynthetic growth. As expected, the situation was radically different for the double mutant C21/DC2M. In this mutant, the RC-bound cytochrome remained photooxidized after flash excitation ($t_{½}$ = 300 ms, data not shown), and no slow phase of the carotenoid bandshift was observed. This implies a lack of cyclic electron transfer in accordance with the PS^- phenotype of this mutant.

DISCUSSION

In this study, we present evidence for the presence of a 50-kDa membrane-bound cytochrome *c* working as a physiological electron donor to the photo-oxidized RC in *Rvu. sulfidophilum*. Growth tests and measurements of light-induced absorption changes for the mutants lacking both or either the cytochrome c_2, and this novel cytochrome *c* indicated that both cytochromes can accomplish an effective electron transfer between the RC-bound cytochrome and the cytochrome bc_1 complex. In addition, no other electron carriers are able to confer photosynthetic growth in the cells of *Rvu. sulfidophilum*.

Sequence analysis showed that the soluble cytochrome *c*-549 is directly generated from the 50-kDa cytochrome *c* (Fig. 5). However, no apparent accumulation of cytochrome *c*-549 was observed in cells grown to the mid-exponential growth phase, as shown in Fig. 1. Presumably, in growing cells of *Rvu. sulfidophilum* under photosynthetic conditions, the cytochrome *c*-549 does not contribute to electron transfer. After the cell growth reaches the stationary phase, a part of the 50-kDa cytochrome *c* might be processed by an unknown mechanism. Another possibility is that cytochrome *c*-549 is an artificial product generated by auto-cleavage during the purification procedure or upon aging of the cells.

The overall structure of the *Rvu. sulfidophilum* 50-kDa cytochrome *c* resembles that of cytochrome c_y, which have a similar hydrophobic membrane-anchor at the N-terminal region and a

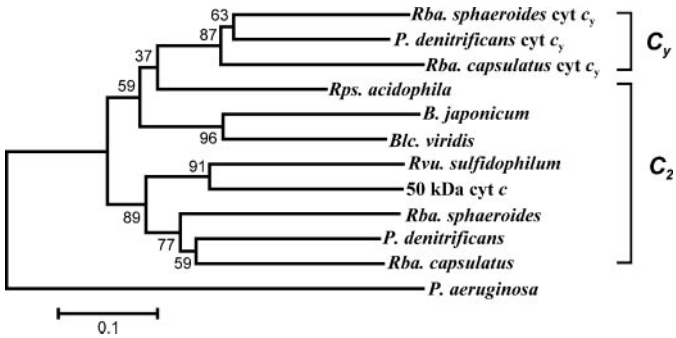


FIGURE 10. **Phylogenetic tree of cytochrome *c*.** Sequence alignment shown in Fig. 6 was basically used for the source of the analysis except for some other cytochrome c_2 of species belonging to the α-2 subgroup. Cytochrome *c*-550 of *Pseudomonas aeruginosa* was used as an outgroup. The tree was constructed by neighbor-joining method, applying the *p*-distance parameter as a distance estimator. Gaps were pairwise omitted for the calculation. Bootstrap values are shown in the figure.

C-terminal heme-binding domain with some sequence similarity to cytochrome c_2 (Fig. 6). In the cytochrome c_y family, the length of the linker stretch connecting these two domains is variable, depending upon the species, and ranges from 42 to 68 amino acids in length (20). The linker stretch in the 50-kDa cytochrome *c* of *Rvu. sulfidophilum* is very long (354 amino acids), potentially allowing quite random movement of the cytochrome domain. This linker stretch seemed to have no clear effects on the interactions between the cytochrome domain and its reaction partners. Indeed, the second-order rate constant roughly estimated from the kinetics data shown in Fig. 4 is 2–3 × 10^7 M^{-1} s^{-1}. This value corresponds to the value estimated for the reaction from its soluble form, cytochrome *c*-549, to the RC, 2.3 × 10^7 M^{-1} s^{-1} (24).

The redox midpoint potential (E_m) of the 50-kDa cytochrome *c*, 369 mV (Fig. 3), is nearly the same as that for cytochrome c_2, 357 mV, in this bacterium (23). On the other hand, the E_m of cytochrome *c*-549 is 238 mV (24). This downshift is the only effect provided by the membrane anchor and the linker stretch, which could be rationalized when taking into account the possible exclusion of water molecules in the vicinity of the heme. At present, it is difficult to determine the function of the acidic-long linker stretch. Such a mysterious structure has been found in some other proteins in other species. Cytochrome c_1 of the bc_1 complex in *P. denitrificans*, a non-photosynthetic bacterium closely related to *Rhodovulum*, also contains an extremely acidic sequence of about 150 amino acids consisting of tandem repeats including many glutamic acids (37). A recent analysis of the whole genome sequence of *Rba. sphaeroides* has shown that the α-subunit of the second light-harvesting 2 (LH2) complex has a long extension in its N-terminal region (38). This extension contains many repeats of similar sequences, mainly composed of Pro, Ala, and Glu residues. The function of these extensions is unclear but may be related to their locations. These extensions are exposed to a periplasmic space with an anchor in the membrane.

Although significant homologies were suspected between the structures of the *Rvu. sulfidophilum* 50-kDa cytochrome *c* and cytochrome c_y, the amino acid sequence of the cytochrome domain of the 50-kDa cytochrome *c* showed higher identities to those of cytochrome c_2 than those of cytochrome c_y. Fig. 10

shows a phylogenetic tree based on the sequence alignment shown in Fig. 6. Some other cytochrome c_2 from species belonging to the α-2 subgroup of purple bacteria were added for comparison. Cytochrome c_y formed a cluster distinct from that of the cytochrome c_2 of species belonging to the α-3 subgroup, showing that cytochrome c_y had evolved before the divergence of the α-3 subgroup. The *Rvu. sulfidophilum* membrane-bound cytochrome *c* was positioned within the cluster of the cytochrome c_2 of the α-3 subgroup and has the closest relationship to that of the *Rvu. sulfidophilum* cytochrome c_2, suggesting that the 50 kDa cytochrome *c* has recently evolved from cytochrome c_2, presumably after the divergence of the genus *Rhodovulum*. Based on its significant sequence identity to cytochrome c_2, we propose that the 50-kDa cytochrome *c* is an isoform of cytochrome c_2. However, all cytochrome c_2 described so far are water-soluble proteins with small molecular masses of about 10 to 15 kDa. Because of the membrane-bound nature of the 50-kDa cytochrome *c*, we designate this cytochrome as cytochrome c_{2m} and the gene coding for this as *cycAm*. Possibly, cytochrome c_{2m} evolved through a relatively recent duplication of the gene for cytochrome c_2 and might have independently acquired the membrane-anchor and the extensively long linker sequence, although the origins of such components are unknown. The *Rhodovulum* species have an unusual triheme cytochrome subunit in which the recognition site of the electron donor, the most distal heme in the tetraheme prototype, is missing. It can be speculated that cytochrome c_{2m} had evolved to adapt to a new binding site on the triheme subunit.

At this time, it seems clear that, whereas the core structure of the photosynthetic reaction center seems highly conserved among purple bacteria, its donor side seems much more variable. The periplasmic electron carriers that are capable of reducing the oxidized special pair are numerous: tri- or tetraheme cytochromes, cytochrome c_2, and cytochrome c_y, as discussed in this article, as well as cytochrome c_8 (39) and HiPIP (33). In this study, we have identified a new carrier and shown that, in this respect, the photosynthetic electron transfer in *Rvu. sulfidophilum* is quite unique since it associates with two unusual partners, an RC-bound triheme cytochrome and a membrane-anchored cytochrome. The multiplication of soluble electron carriers via gene duplication and rearrangements may contribute to the diversification and specialization of metabolic pathways, resulting in reinforcing the robustness of bioenergetic chains.

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