Membrane-Associated *c*-type Cytochromes from the Green Sulfur Bacterium *Chlorobium limicola* forma *thiosulfatophilum*: Purification and Characterization of Cytochrome c_{553}[†]

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ABSTRACT: Tetraheme cytochromes involved in photosynthetic electron transport have previously been described associated with the reaction centers of purple photosynthetic bacteria; however, similar heme proteins have not until now been characterized in the phylogenetically distinct green sulfur bacteria. In this paper we describe the first isolation and characterization of a multiheme, membrane-associated cytochrome from a green sulfur bacterium, *Chlorobium limicola* forma *thiosulfatophilum*. We show that this cytochrome contains a single polypeptide of 32 kDa apparent molecular mass on SDS−PAGE and has a characteristic broad α-band absorption at 553 nm. By both low-temperature absorption and electron paramagnetic resonance spectroscopy, we demonstrate that there are at least four distinct heme groups.

In contrast to the photosynthetic reaction centers (RC)[1] of purple bacteria and Chloroflexaceae, which are closely related to the photosystem II (PS2) of oxygen-evolving organisms, RC of green sulfur bacteria share many common features with the other type of reaction center present in cyanobacteria and chloroplasts, i.e., photosystem I (PS1). In particular, they are capable of photoreducing $NADP^+$ *via* ferredoxin in the absence of an electrochemically produced proton gradient (Buchanan & Evans, 1968) and they contain iron−sulfur (FeS) centers as terminal electron acceptors (Knaff & Malkin, 1976; Jennings & Evans, 1977; Swarthoff *et al*., 1981), which share similarities with those of PS1 (Nitschke *et al*., 1987, 1990). It was also shown that, as in PS1 (Golbeck & Bryant, 1991), the primary electron acceptor in these RCs is a Chl *a*-like pigment (van de Meent *et al*., 1990; Feiler *et al*., 1994). By contrast, the primary electron donor of these RC, P_{840}, is a bacteriochlorophyll (BChl) *a* dimer (Swarthoff *et al*., 1981; Nitschke *et al*., 1990; Olson *et al*., 1976; Feiler, 1991), as is found in the RCs from purple bacteria.

Although our knowledge of the RCs from green sulfur bacteria has significantly improved during the last 5 years, the nature of the electron donor to the photooxidized P_{840} remains unclear. A membrane-bound cytochrome, called cytochrome c_{553} in reference to the absorption maximum of its reduced α-band, was first identified as the electron donor to P_{840}^+ (Fowler *et. al*., 1971). Prince and Olson (1976) studied the oxidation kinetics of this cytochrome in membrane fractions, and observed that it was rapidly, biphasically, reducing P_{840}^+ ($\tau < 7$ and 70 μs). Furthermore, these authors measured different apparent midpoint potentials for the hemes oxidized after the first and the second flash. These observations were reminiscent of those made previously on many purple bacteria, where the tetraheme cytochrome, which is bound, either loosely or tightly, to the reaction center, acts as the electron donor to P^+. These reports point toward the involvement of a multiheme cytochrome in the reduction of the green sulfur bacterial primary donor. However, none of these experiments demonstrates the existence of such a cytochrome, nor do they clearly show its role in the reduction of the green bacterial primary donor.

A 32-kDa cytochrome with an α band at 553 nm was shown to copurify with the RC of *Chlorobium* (*C.*) *limicola* after mild solubilization of the membranes (Feiler *et al*., 1992). Okkels *et al*. (1992) reported that an 18-kDa cytochrome c_{551} copurifies with the RC from *Chlorobium vibrioforme* and that no other cytochrome was found associated with the RC. From the gene sequence of this cytochrome, it could be concluded that it possesses only one heme binding site, and no homologies to other known cytochromes, in particular to the RC-associated tetraheme cytochromes from purple bacteria, could be found. Furthermore, optical time-resolved experiments suggested that this cytochrome might act as immediate electron donor to P_{840}^+. Biphasic kinetics were measured for both the reduction of P_{840}^+ and photooxidation of this cytochrome, in agreement

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[1] Abbreviations: (B)Chl, (bacterio)chlorophyll; *C*., *Chlorobium*; DEAE, diethylaminoethyl; DFP, diisopropyl fluorophosphate; DM, dodecyl maltoside; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; FMO, Fenna−Matthews−Olson protein; FWHM, full width at half-maximum; PMSF, phenylmethanesulfonyl fluoride; PS1/2, photosystem I/II; P_{840}, primary electron donor in green sulfur bacterial reaction centers; RC, reaction center; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TMBZ, 3,3′,5,5′-tetramethylbenzidine; Tris, tris(hydroxymethyl)aminomethane.

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with those previously reported by Prince and Olson (1976) on cytochrome c_{553} from *C. limicola*. The biphasic kinetics were interpreted by Okkels *et al*. (1992) as resulting from distinct conformations of the cytochrome/RC complex. *c*-type cytochromes with α bands between 550.5 and 551 nm and apparent molecular masses from 21 to 24 kDa have also been observed in different RC preparations from *C. limicola* and *Chlorobium tepidum* (Hurt & Hauska, 1984; Oh-oka *et al*., 1993, 1995; Kusumuto *et al*., 1994). The position of the α band of all these low molecular weight cytochromes differs significantly from those originally reported by Fowler *et al*. (1971). Okumura *et al*. (1994) observed different apparent redox potentials for the cytochrome c oxidized after the first and second flash in *C. tepidum* and proposed, in interpreting their result, the association of two identical monoheme cytochromes (c_{553} in this case) with each RC.

In summary, the nature of the electron donor to P_{840} is far from settled. Is it a multiheme, 553-nm-absorbing, 32-kDa cytochrome, or is it rather a 551-nm-absorbing, low molecular weight, monoheme cytochrome? The nature of this protein is of particular importance, since tetraheme cytochromes have previously been found as electron donors to the RCs of purple bacteria and of *Chloroflexus* [for review, see Nitschke and Dracheva (1995)]. The existence of such an electron carrier associated with the RC of a green sulfur bacterium would significantly extend the similarity between PS1-type RCs of green sulfur bacteria and the PS2-type RCs of purple bacteria and *Chloroflexus* on the electron donor side.

In this paper we report the isolation and characterization of a cytochrome c_{553} from *C. limicola*. We show that this 32-kDa cytochrome is a multiheme cytochrome, most likely a tetraheme. The interactions we observe between this cytochrome and the reaction center are at best very weak, since these proteins do not copurify during the final stages of the purification procedure. The role of this protein in *C. limicola* thus remains to be established; however, because of its absorption properties, the involvement of this multiheme cytochrome in the photosynthetic electron transfer chain as electron donor to the reaction center would be consistent with flash experiments previously performed on *C. limicola* (Fowler *et al*., 1971; Prince & Olson, 1976).

EXPERIMENTAL PROCEDURES

Protein Purification. *Chlorobium limicola* forma *thiosulfatophilum*, strain tassajara, was obtained from Professor N. Pfennig, Konstanz, Germany. Cells were grown as previously described (Feiler *et al*., 1992) and harvested in late log phase after 6−7 days of growth.

The chlorosome-depleted membranes were prepared according to Schmidt (1980). N-Dodecyl β-maltoside (DM) was obtained from Calbiochem (San Diego). Ion-exchange chromatography was performed on Fractogel TSK DEAE-650 (S) (Merck, Darmstadt) in a column (6 cm × 2 cm) equilibrated with 20 mM Tris-HCl, pH 9.0, 0.05% (w/v) and 10 mM NaCl. Gel filtration was performed on Fractogel TSK HW 55(S) (Merck, Darmstadt) in a column (72 cm × 2 cm), equilibrated with 20 mM Tris-HCl, pH 8.0, 0.03% (w/v) DM, 80 mM NaCl, and 5 mM β-mercaptoethanol. The hydroxyapatite chromatography was performed on Bio-Gel HTP (Bio-Rad, Hercules) in a column (2 cm × 0.8 cm) equilibrated with 20 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.5, 0.03% (w/v) DM, and 5 mM β-mercaptoethanol. All purification steps were carried out at 4 °C in dim light and all buffers were degassed immediately before use.

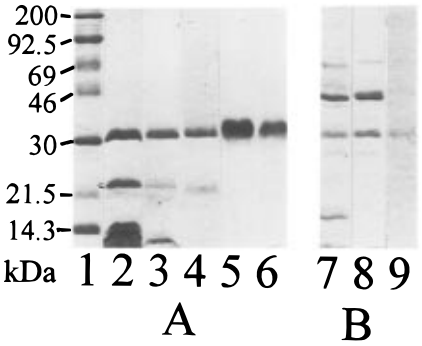

FIGURE 1: SDS−PAGE of fractions containing the 32-kDa cytochrome c_{553}. Lane 1, molecular mass markers. Lanes A (2−6) were stained with TMBZ; lanes B (7−9) were stained with Coomassie brilliant blue. The following samples were applied: (2) membrane; (3) solubilized membrane; (4, 7) eluate from the DEAE column; (5, 8) eluate from the moleular sieve; (6, 9) eluate from the hydroxyapaptite.

Electrophoresis. SDS−PAGE was performed on 12% acrylamide gels according to Laemmli (1970) as modified by Ikeuchi and Inoue (1988). The gels were stained with TMBZ as described by Thomas *et al*. (1976) or with Coomassie brilliant blue (Cabral & Schatz, 1979). Apparent molecular masses were determined by comparison with Buchler rainbow markers (Amersham, Little Chalfont).

Optical Spectroscopy. Room-temperature and low-temperature absorption spectra were recorded on a Cary 5 spectrophotometer (Varian plc, Sidney). Low-temperature absorption spectroscopy was carried out in a liquid helium-cooled flow cryostat. The samples were diluted with glycerol to a final glycerol concentration of 60% (v/v) prior to mounting in the cryostat.

EPR Spectroscopy. EPR spectroscopy was performed on fractions containing only the 32-kDa cytochrome eluted from the DEAE column. EPR spectra were recorded on a Bruker 200 X-band spectrometer fitted with an Oxford Instrument helium cryostat and temperature control system.

RESULTS

Cytochrome Purification. Chlorosome-depleted membranes (typically 40 mL at 30 μM BChl *a*) from *C. limicola* were solubilized with 0.4% (w/v) dodecyl maltoside (DM) for 40 min according to Feiler *et al*. (1992) and centrifuged for 1 h at 100000*g*. SDS−polyacrylamide gels of the resulting supernatant contain three TMBZ-staining proteins, with apparent molecular masses of 12, 24, and 32 kDa (Figure 1, lane 3), the latter being the most intense. By comparing this result to gels of membranes before solubilization (Figure 1, lane 2), it can be seen that a 14-kDa TMBZ-staining protein is not extracted by DM treatment. Addition of DFP, PMSF, and EDTA as protease inhibitors during membrane preparation (including cell breakage), and solubilization, resulted in no change in this gel pattern, indicating the absence of any significant proteolytic activity against these four proteins. As proteins other than *c*-type cytochromes have been reported to stain in the presence of TMBZ (Miller & Nicholas, 1984), the 32-kDa protein should not be referred to as cytochrome until further characterization.

This supernatant was loaded onto a DEAE column and washed with buffer containing 10 mM NaCl until no absorbance at 280 nm was detected in the eluate. During this washing procedure, the breakthrough fraction contained the 12-kDa TMBZ-staining protein. Elution was then performed with a 10−600 mM NaCl gradient. At 50 mM the 24-kDa TMBZ-staining protein was eluted, together with a large amount of Fenna−Matthews−Olson (FMO) protein. The 32-kDa TMBZ-staining protein is eluted at about 80 mM NaCl, in the presence of large amounts of both the FMO protein and carotenoids. Often traces of the 24-kDa TMBZ-staining protein were present in this fraction (Figure 1, lane 4). Reaction centers (RC) from *C. limicola* are eluted only slightly after this fraction, at NaCl concentrations of 120−150 mM. A certain degree of cross-contamination exists between these two fractions, but the 32-kDa TMBZ-staining protein seems to be only weakly associated with the RC under the buffer conditions employed here. Fractions containing this protein were concentrated by ultrafiltration (Amicon, Centriprep 30) to a final volume of 600 μL. Coomassie-stained gels reveal that it contains four major polypeptides, with apparent molecular masses of 68, 42, 32, and 18 kDa, together with traces of higher molecular mass polypeptides (Figure 1, lane 7). The 68-kDa protein corresponds to the large subunit of *Chlorobium* RCs. The 42-kDa polypeptide is the FMO apoprotein, and it is one of the major contaminants in this fraction. The identity of the 18-kDa polypeptide is unknown. In attempts to avoid the presence of such high levels of contamination by the FMO protein at this stage, various alternative solubilization procedures were investigated, using octyl glucoside, sodium cholate, and LDAO in buffers of several different ionic strengths, as well as selective ammonium sulfate precipitation after solubilization. However, all of these procedures resulted in difficulties during the purification of the different TMBZ-staining proteins which we were unable to circumvent (see below).

After concentration, the fraction containing the 32-kDa TMBZ-staining protein was loaded onto the gel-filtration column. At low flow rates (0.2 mL/min), three different-colored bands were separated on the column. First, a greenish-blue band containing some FMO and RC was eluted from the column, then, a red one containing the 32-kDa TMBZ-staining protein, and finally, an orange one containing carotenoids with residual amounts of FMO. This step thus results in a partial purification of the 32-kDa protein from the carotenoid molecules, the remaining RCs, part of the FMO protein, and other polypeptides around 60 and 18 kDa. At this stage in the purification, the FMO protein is the major, if not the only, contaminant of the 32-kDa TMBZ-staining protein (Figure 1, lanes 5 and 8).

The sample was then loaded on a hydroxyapatite column preequilibrated with 20 mM phosphate buffer. Under these conditions the 32-kDa TMBZ-staining protein is released from the column, while the FMO protein binds to it and is eluted at 250 mM phosphate buffer only. After this step, a reasonably well-purified fraction of the 32-kDa TMBZ-staining protein was obtained (Figure 1, lanes 6 and 9). This fraction exhibits optical properties characteristic of *c*-type cytochromes (see below).

Optical Properties of the 32-kDa Cytochrome from C. limicola. Figure 2 shows the room-temperature absorption spectra of the purified 32-kDa TMBZ-staining protein poised at three different redox potentials. These spectra show the typical features of *c*-type cytochromes: a Soret transition at 411 nm, which shifts to *ca*. 417 nm in the presence of reductants, together with prominent α and β bands under reducing conditions present near 550 and 520 nm, respectively. The ratio of the Soret band (under reducing conditions) to the 280-nm band arising from the protein tryptophan and aromatic residues is 2.0, which is rather low relative to the values that have been observed in other *c*-type cytochromes (Freeman & Blankenship, 1990; Meyer & Kamen, 1982). This may be at least partially due to traces of contaminant proteins which can be distinguished in the gels (Figure 1, lane 9). Under reducing conditions the β and α bands are observed at 522 and 553 nm. Closer examination of the latter transition (Figure 3) reveals that it is particularly broad (11 nm FWHM) as compared to the α band of horse heart cytochrome c, a typical monoheme, *c*-type cytochrome (8 nm FWHM). Moreover, it is asymmetrical (Figure 3), and its shape varies depending on the redox potential. These observations suggest that more than one heme could be bound to the 32-kDa cytochrome from *C. limicola*.

To clarify whether the 32-kDa cytochrome from *C. limicola* contains more than one heme, low-temperature (30 K) absorption spectra of the isolated protein were recorded at several redox potentials. Although lowering the temperature may induce a splitting of, as well as a shift in the

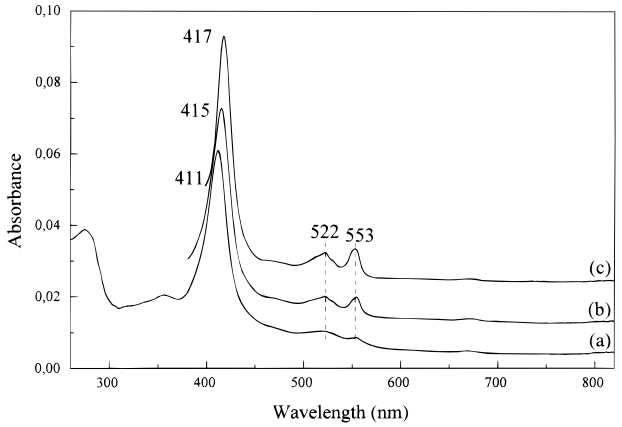

FIGURE 2: Room-temperature absorption spectra of the purified cytochrome c_{553} from *C. limicola*. (a) Spectrum of the cytochrome (with no addition), (b) in the presence of ascorbate, and (c) in the presence of dithionite.

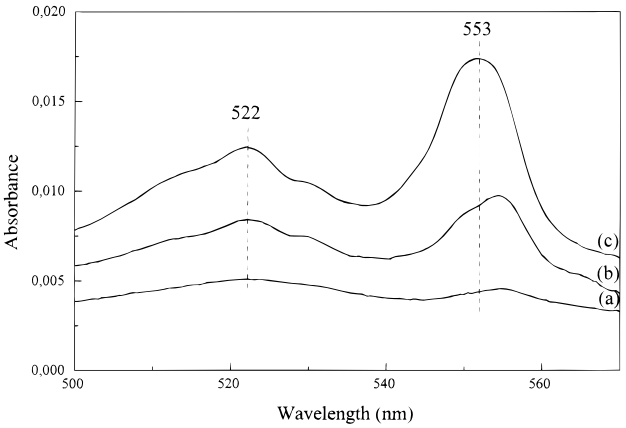

FIGURE 3: Room-temperature absorption spectra of the purified cytochrome c_{553} from *C. limicola*: enlarged view of the α and the β bands. (a) Spectrum of the cytochrome with no addition, (b) in the presence of ascorbate, and (c) in the presence of dithionite.

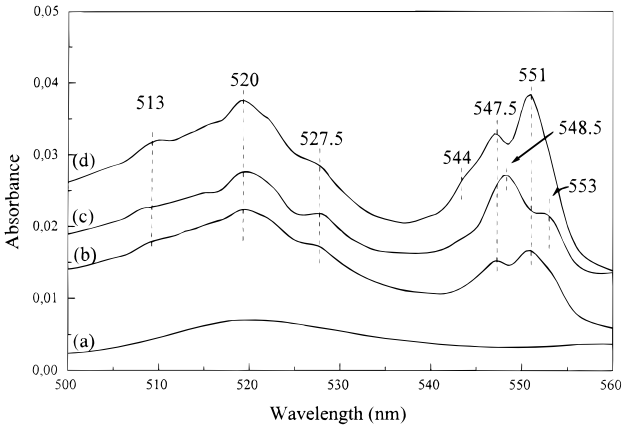

FIGURE 4: α and β bands of the purified cytochrome c_{553} at 30 K (a) with 1 mM ferricyanide, (b) with 550 μM ascorbate, (c) with 200 μM dithionite, and (d) with 2 mM dithionite.

position of, the α band of cytochromes *c*, it is often easier to distinguish the different hemes in these experimental conditions. Figure 4 displays the α band observed in these conditions. In the presence of potassium ferricyanide, only a weak absorbance is seen, indicating that all the hemes in this protein are oxidized (Figure 4a). When sodium ascorbate is added to the preparation as a mild reductant, an α band appears, which has two peaks at 551 and 547.5 nm and a long-wavelength shoulder (Figure 4b). Upon addition of a low concentration of dithionite (200 μM), the area of the whole α band increases (by about a factor of 2), and the major component is at 548.5 nm (Figure 4c). After further addition of dithionite, the area of the α band again increases considerably (again by a factor of about 2), and its major components shift to 551 and 547.5 nm with a distinct shoulder at 544 nm (Figure 4d). These spectra show unambiguously that the 32-kDa cytochrome from *C. limicola* contains at least three hemes, with distinct redox potentials. There is a high-potential heme and an α-band maximum at 551 nm at 30 K, which is reduced in the presence of ascorbate. A second heme, with an α-band maximum at 548.5 nm, has a slightly redox potential and is thus reduced only in the presence of low concentrations of dithionite. Finally, a third heme with a redox potential lower than the second heme ($c_{548.5}$), and with an α-band maximum at 551 nm is necessary to explain the low-potential spectrum. It can thus be inferred that the 32-kDa cytochrome from *C. limicola* is at least a triheme *c*-type cytochrome.

Magnetic Properties of the 32-kDa Cytochrome from C. limicola. EPR studies were performed on the cytochrome c_{553} in the oxidized state at low temperature (see above). Under these conditions the spectrum contains a complex heterogeneous g_z peak, due to the presence of a number of partially resolved signals, between $g = 3.3$ and 3.0, two g_y lines both at $g = 2.25$ but with very different linewidths of 8 and 30 mT, and a broad g_x band near $g = 1.46$ (Figure 5). The two different g_y lines are well-evidenced when EPR spectra of the cytochrome c_{553} oxidized and partially reduced (Figure 5b) are compared. The signals in the range of 320−350 mT are due to manganese, and the derivative line at $g = 2$ reflects free radicals both being typical paramagnetic contaminants in biological samples.

In the g_z region (Figure 6a), a peak at $g_z = 3.02$ with two partially resolved shoulders at $g_z = 3.13$ and 3.28 is present. When the redox potential is lowered, the intensity of the EPR

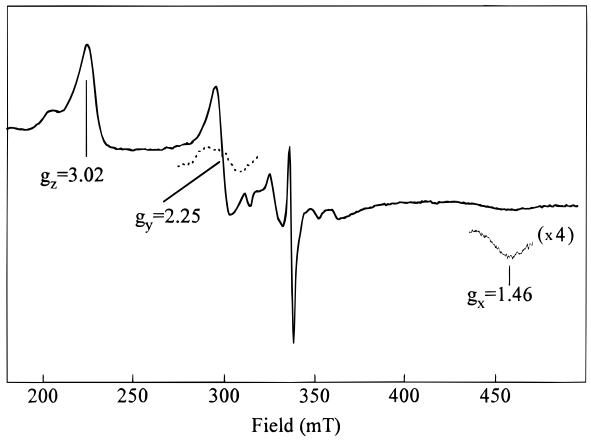

FIGURE 5: EPR spectrum of cytochrome c_{553} from *C. limicola* as isolated without further additions, i.e., in a rather oxidized state. Instruments settings: temperature 15 K; microwave power 6.3 mW; microwave frequency 9.44 GHz; modulation amplitude 2.2 mT. Dotted line: g_y peaks region after ascorbate treatment.

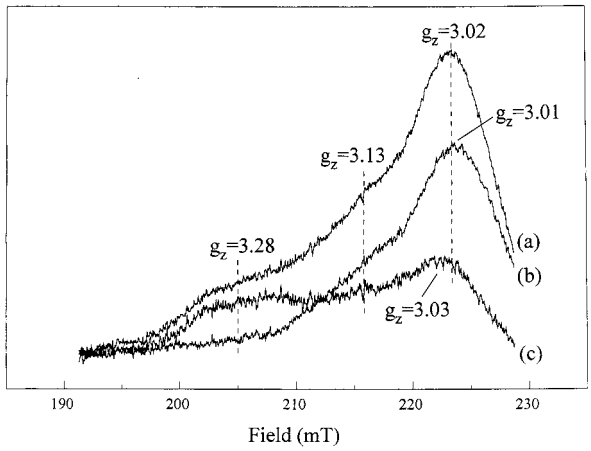

FIGURE 6: EPR spectrum of cytochrome c_{553} showing the g_z peaks of (a) untreated, (b) treated with ascorbate, and (c) difference spectrum untreated minus ascorbate-reduced. Instrument settings were as described in Figure 4.

signal decreases in intensity as the hemes become reduced. Reduction of the 32-kDa cytochrome with ascorbate resulted in (i) about 30% loss of the main signal at $g_z = 3.02$ together with a shift of this transition by 0.7 mT toward higher field, (ii) a loss of intensity of the $g_z = 3.13$ peak, and (iii) the disappearance of the low-field shoulder at $g_z = 3.28$, indicating that the heme responsible for the peak at $g_z = 3.28$ is fully reduced by ascorbate. This description of the spectral changes is facilitated by examining the untreated minus ascorbate difference spectrum (Figure 6c). All three of the distinguishable features in this region of the spectrum were partially ($g_z = 3.02$ and 3.13) or fully ($g_z = 3.28$) removed by reducing the sample with ascorbate. In addition, the shift of the peak at $g_z = 3.02$ upon reduction demonstrates that it arises from two distinct hemes. It may thus be safely concluded from these spectra that at least four distinguishable hemes contribute to the EPR spectrum of the cytochrome c_{553}.

DISCUSSION

In this work, we show the presence of four TMBZ-staining proteins associated with the cytoplasmic membrane of *C. limicola* forma *thiosulfatophilum*. The most abundant of these has a molecular mass of 32 kDa, and it is a *c*-type

cytochrome, the α-absorption band of which is located at 553 nm at room temperature. Both low-temperature absorption spectroscopy and EPR spectroscopy indicate that this cytochrome is a multiheme cytochrome, and our analysis of the EPR data indicates that this cytochrome c_{553} is probably a tetraheme protein.

Of the four TMBZ-staining proteins observed in the membrane, namely, the 12-, 14-, 24-, and 32-kDa proteins, all of the 14-kDa and a part of the 24-kDa protein are not solubilized. The remaining 24-kDa and the 12-kDa heme proteins are separated from the reaction centers during the DEAE chromatography, and the 32-kDa cytochrome is eluted slightly before the RC from this anion-exchange column. It is thus clear that none of the four TMBZ-staining bands is tightly bound to the RC. Though we did not quantify the amount of 32-kDa cytochrome relative to RC, this ratio did not appear to vary significantly between preparations.

At present, several RC preparations have been published in which the RC is reported to copurify with a cytochrome, often different from the 32-kDa one we have isolated. In 1984, Hurt and Hauska reported a RC fraction from *C. limicola* forma *thiosulfatophilum* containing a 24-kDa cytochrome $c_{550.5}$. After solubilization of the membranes with a mixture of octyl glucoside and cholate at moderate ionic strength, a 32-kDa cytochrome with an α band at 552.5 nm was separated from the RC and the 24-kDa cytochrome. RCs, together with cytochrome $c_{550.5}$ and cytochrome b_{562}, were solubilized by a second detergent treatment at high salt concentration and purified by an ammonium sulfate precipitation followed by chromatography of the precipitate on a hydroxyapatite column. Oh-oka *et al*. (1993), who have reported that cytochrome c_{551} copurifies with RC of *C. limicola*, similarly make use of an ammonium sulfate precipitation. However, no information was given about the whereabouts of the cytochrome c_{553}, which was probably lost during the earlier steps of their purification procedure. We have repeated the ammonium sulfate precipitation treatment and have observed the aggregation of the various different cytochromes. We also observe the RCs copurifying with both the 24-kDa and the 32-kDa cytochrome, but it seems very likely that the cytochrome−RC association is induced by high concentrations of ammonium sulfate. The remaining reports of cytochromes copurifying with green sulfur bacterial reaction centers all avoided treatment with ammonium sulfate. Feiler *et al*. (1992) described that the RC of *C. limicola* copurified with the 32-kDa cytochrome c_{553}, but from our results, it seems that this apparent copurification was probably due to limited chromatographic resolution rather than tight interactions between this cytochrome and the RCs. Okkels *et al*. (1992), using a procedure very similar to that of Feiler *et al*. (1992), have reported the copurification of an 18-kDa monoheme cytochrome c_{551} with the RCs of *C. vibrioforme*. Kusumoto *et al*. (1994) developed a protocol for purifying RCs of *C. tepidum* in which they use Triton X-100 to extract the membrane proteins, followed by ultracentrifugation on a sucrose density gradient and a hydroxapatite column; they described a cytochrome c_{551} as copurifying with the RCs. More recently, Hager-Braun *et al*. (1995), after membrane solubilization with Triton X-100, reported that the photoactive RC from *C. limicola* forma *thiosulfatophilum* is purified without a trace of cytochrome, while that of *C. tepidum* copurifies with a 24-kDa cytochrome c_{551}.

It is worth noting that the aim of all these purification procedures was to obtain a photoactive RC complex rather than to specifically study RC/cytochrome copurification. It is in general a difficult problem to address, from biochemical work, existence of weak protein−protein interactions *in vivo*. From our previous experience of the separation of the membrane-associated cytochromes of *C. limicola*, it seems probable that some of the reported copurifications are due either to nonspecific interactions or to residual contamination. Sample inhomogeneity must also be considered. For example, Hager-Braun *et al*. (1995) report that they obtained from *C. limicola*, prior to a fraction of cytochrome-devoid, fully photoactive RCs, a fraction of RCs lacking the PscB subunit but retaining a low molecular mass cytochrome subunit. Although that particular case is not fully documented yet, one may wonder whether the presence of the cytochrome is indeed correlated with the absence of intrinsic subunits of the RCs, i.e., whether the absence of those could induce interactions between the RCs and this cytochrome, for example, by exposing a partially hydrophobic protein surface. Furthermore, the last two reports mentioned above involve green bacteria other than *C. limicola*. It is well-known that in purple bacteria the nature of the electron donor depends on the bacterial species studied (e.g., a RC-bound tetraheme in *Rhodopseudomonas viridis* and a soluble c_2 cytochrome in *Rhodobacter sphaeroides*).

The 32-kDa cytochrome that we have isolated exhibits an α band at 553 nm. This position corresponds well to that originally measured by Fowler *et al*. (1971). The observation of different E_m values for the hemes involved in the reduction of P^+ after the first and the second flash (Prince & Olson, 1976) can be rationalized in two different ways: (i) two identical hemes are simultaneously able to donate electrons to the photooxidized pigment (Case & Parson, 1971) or (ii) donation is performed by a cytochrome containing at least two (electrochemically inequivalent) hemes, only one of which is correctly positioned to directly donate electrons to P^+ (Nitschke & Dracheva, 1995). Of course, the involvement of the tetraheme cytochrome characterized in this paper at this level in the electron transfer chain would fit with the second interpretation. We are currently trying to precisely determine the role of c_{553} in *C. limicola*.

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