Isolation and Characterization of Homodimeric Type-I Reaction Center Complex from Candidatus Chloracidobacterium thermophilum, an Aerobic Chlorophototroph

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Background: Candidatus Chloracidobacterium thermophilum is the only aerobic chlorophototroph with type-I homodimeric reaction centers (RCs).

Results: An RC carotenoid-binding protein (CBP) complex was isolated from Ca. C. thermophilum.

Conclusion: Ca. C. thermophilum RCs contain bacteriochlorophyll a, chlorophyll a, and Zn-bacteriochlorophyll a'.

Significance: This is the first description of aerotolerant type-I homodimeric RCs from the only known chlorophototrophic member of the phylum Acidobacteria.

The recently discovered therophilic acidobacterium Candidatus Chloracidobacterium thermophilum is the first aerobic chlorophototroph that has a type-I, homodimeric reaction center (RC). This organism and its type-I RCs were initially detected by the occurrence of pscA gene sequences, which encode the core subunit of the RC complex, in metagenomic sequence data derived from hot spring microbial mats. Here, we report the isolation and initial biochemical characterization of the type-I RC from Ca. C. thermophilum. After removal of chlorosomes, crude membranes were solubilized with 0.1% (w/v) n-dodecyl β-D-maltoside, and the RC complex was purified by ion-exchange chromatography. The RC complex comprised only two polypeptides: the reaction center core protein PscA and a 22-kDa carotenoid-binding protein denoted CbpC. The absorption spectrum showed a large, broad absorbance band centered at ~848 nm from carotenoids as well as smaller Qs absorption bands at 672 and 812 nm from chlorophyll a and bacteriochlorophyll a, respectively. The light-induced difference spectra of whole cells, membranes, and the isolated RC showed maximal bleaching at 840 nm, which is attributed to the special pair and which we denote as P840. Making it unique among homodimeric type-I RCs, the isolated RC was photoactive in the presence of oxygen. Analyses by optical spectroscopy, chromatography, and mass spectrometry revealed that the RC complex contained 10.3 bacteriochlorophyll aP, 6.4 chlorophyll aPP, and 1.6 Zn-bacteriochlorophyll aP' molecules per P840 (12.8:8.0:2.0). The possible functions of the Zn-bacteriochlorophyll aP' molecules and the carotenoid-binding protein are discussed.

Reaction center (RC)4 complexes are the central components of (bacterio)chlorophyll (BChl)-based phototrophy and are responsible for the conversion of light energy into chemical energy. After absorbing a photon, a BChl dimer bound to the RC near the periplasmic surface of the membrane achieves a long lived, charge-separated state by transferring an electron through a series of bound cofactors to a terminal acceptor, which is bound to the RC near the cytoplasmic surface of the membrane. Based on their terminal electron acceptors, RC complexes are classified into two types (1). Type-I RCs utilize Fe-S clusters as terminal electron acceptors, whereas type-II RCs use quinones as terminal electron acceptors. Green sulfur bacteria (GSB; Chlorobi and Chlorobiales) and heliobacteria (Firmicutes and Heliobacteriaceae) possess type-I RCs; purple bacteria (Proteobacteria) and filamentous anoxygenic phototrophs (Chloroflexi) possess type-II RCs; and cyanobacteria (Cyanobacteria), similar to plants and algae, possess both type-I and type-II reaction centers, photosystems I and II, respectively.

All characterized GSB and heliobacteria are strict anaerobes, a trait once thought to be a consequence of the vulnerability of their RC-bound Fe-S clusters to oxygen (2, 3). However, Chlorobaculum tepidum is extremely tolerant to oxygen so long as cells are not illuminated. This observation suggests that reactive oxygen species are the true problem, and consistent with this hypothesis, mutants lacking enzymes for protection against

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§4 The abbreviations used are: RC, reaction center; Ca., Candidatus; CBP, carotenoid-binding protein; Chl, chlorophyll; BChl, bacteriochlorophyll; (B)Chl, either bacteriochlorophyll or chlorophyll; DDM, n-dodecyl β-D-maltoside; FMO, Fenna-Matthews-Olson; GSB, green sulfur bacteria; OG, n-octyl β-D-glucoside; P, phytol as esterifying alcohol; PD, peridinin-chlorophyll a

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reactive oxygen species are more sensitive to oxygen (4). The type-I RCs of GSB and heliobacteria uniquely have homodimeric core complexes, whereas all other RCs, including photosystems I and II, have heterodimeric core complexes (5). Despite their simpler composition, few detailed structural studies have been reported for homodimeric RCs, and some aspects of their biochemical and biophysical properties remain controversial (6).

Until recently, only five of the currently recognized phyla of the domain Bacteria contain species capable of chlorophototrophic growth (7). The discovery of Candidatus Chloracidobacterium thermophilum (hereafter Ca. C. thermophilum) extended this distinction to a sixth phylum, Acidobacteria (8). Metagenomic sequence data from the hot spring microbial mats in which Ca. C. thermophilum was discovered (8, 9) as well as the complete genome sequence of Ca. C. thermophilum (10) revealed the presence of pscA and pscB genes, which encode the homodimeric core subunit and the F_{A/F_{B}}-harboring subunit of a type-I RC, respectively. The Ca. C. thermophilum genome does not encode PscC, the c-type cytochrome that donates electrons to the primary donor (11, 12), or PscD, a protein that may enhance electron transfer from the F_{A/F_{B}} clusters of PscB to ferredoxin (13) in the RCs of GSB. Time course metatranscriptome profiling studies over a diel cycle have demonstrated that the transcripts for the pscA gene are least abundant during the day when the microbial mats areoxic, but pscA transcripts are highest during the late afternoon and evening when the mats are anoxic (14). Ca. C. thermophilum can be cultivated in the laboratory as an aerobe, and thus, its RCs can also be synthesized underoxic conditions as well. These properties make these RCs a unique system for investigating electron transport in homodimeric type-I RCs, and information gained from these studies may contribute new insights into the evolutionary events that led from anoxygenic to oxygenic photosynthesis.

We have previously reported the purification and characterization of chlorosomes (8, 15, 16) and the BChl a-binding, Fenna-Matthews-Olson (FMO) protein from Ca. C. thermophilum (17, 18), components of the photosynthetic apparatus whose roles in light harvesting have been extensively characterized in GSB (19–22). Chlorosomes are large light-harvesting organelles, which attach to the inner surface of the cytoplasmic membrane and which contain >200,000 self-aggregating BChl molecules. The suprastructure of the BChl d and c molecules in chlorosomes of Ca. tepidum were recently described (23). The FMO protein, which forms a layer between the chlorosomes and RCs (24), functions both as a light-harvesting complex and as a conduit for excitation energy transfer between the chlorosome baseplate and the RC (20–22). Although its genome predicts that Ca. C. thermophilum has a photosynthetic apparatus very similar to that of GSB (i.e. chlorosomes, FMO, and type-I RCs) (8, 10), the aerobic lifestyle of Ca. C. thermophilum suggests that its photosynthetic apparatus has unique modifications that allow it to remain functional in the presence of oxygen. We recently reported that the chlorosomes of Ca. C. thermophilum contain several novel proteins that are not known to occur in the chlorosomes of GSB or Chloroflexi (15). We have additionally reported that FMO from Ca. C. thermophilum has distinctive spectroscopic properties compared with FMO from GSB (17, 18). These new features of the light-harvesting complexes of Ca. C. thermophilum seem to be related to the ability of this organism to grow phototrophically under oxic conditions.

In this report, we describe the isolation, spectroscopic properties, and pigment composition of the Ca. C. thermophilum RCs. These oxygen-tolerant RCs are complexes formed from a PscA homodimer and a novel carotenoid-binding protein (CBP; denotes the complex formed by the CbpA apoprotein and carotenoids). Unexpectedly, these RC-CBP complexes contain two molecules of Zn-BChl a’ (the C-13° epimer of Zn-BChl a), which may act as the primary electron donor (P840) or an electron acceptor. The properties of these RCs are discussed and compared with those of other chlorophototrophs.

**EXPERIMENTAL PROCEDURES**

*Purification of RC Complex from Ca. C. thermophilum—Ca. C. thermophilum cells were cultured phototrophically at 53 °C under oxic conditions in an orbital shaking incubator (85 rpm) as described previously (8). Cells (9 g, wet weight) were harvested by centrifugation; resuspended in 10 ml Tris-HCl, pH 7.5 containing 2 mM NaSCN, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol (DTT), and 3 mg of lysozyme ml^{-1}; and incubated for 30 min. The cells were disrupted by sonication for 5 min and then passed three times through a French pressure cell at 138 megapascals at 4 °C. Unbroken cells and large cell debris were removed by centrifugation (8,000 × g) for 10 min, and the resulting supernatant was subjected to centrifugation at 220,000 × g for 1.5 h. The resulting pellet containing total membranes and chlorosomes was suspended in the same buffer and loaded onto sucrose density gradients (20–50%), which were centrifuged for 18 h at 4 °C (220,000 × g). The membrane layer that formed below the chlorosome layer was collected, diluted with buffer C (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM DTT), and the suspension was centrifuged again at 220,000 × g for 1.5 h. The resulting membrane pellets were suspended in ~8 ml of buffer C (~70 μg of pigments (BChl a and BChl c) ml^{-1}) and solubilized with 0.1% (w/v) n-dodecyl β-D-maltoside (DDM). After ultracentrifugation (220,000 × g for 1.5 h), the supernatant was decanted and subjected to anion-exchange chromatography on a DEAE-Sepharose column (2.5 × 8 cm) equilibrated with buffer C containing 0.02% (w/v) DDM. The orange-colored RC preparation was eluted with buffer C containing 150 mM NaCl. The fractions were pooled and concentrated by ultrafiltration (10-kDa molecular mass cutoff; Millipore, Billerica, MA).

*Isolation of CBP—Ca. C. thermophilum cells were suspended in 20 mM Tris-HCl buffer, pH 7.6; disrupted by sonication for 5 min; and passed three times through a French pressure cell at 138 megapascals at 4 °C. After unbroken cells and large cell debris were removed by centrifugation (8,000 × g for 10 min), the supernatant was centrifuged at 220,000 × g for 1.5 h. The resulting pellet containing total membranes and chlorosomes...*
was suspended in 20 mM Tris-HCl buffer, pH 7.6 containing 0.6 M sodium carbonate and incubated overnight at 4 °C. The suspension was clarified by centrifugation (220,000 × g for 1.5 h), and the resulting blue supernatant enriched in FMO was stored at −80 °C until required for other studies. The resulting pellet was suspended in 20 mM Tris-HCl buffer, pH 7.6 containing 18 or 34 mM n-octyl β-D-glucoside (OG) and incubated for 2 h. After centrifugation (220,000 × g for 1 h), the resulting supernatant was decanted, taking care to avoid the soft pellet containing the chlorosomes (although this supernatant usually exhibited a minor absorbance peak at ~740 nm due to residual contaminating chlorosomes). This supernatant was loaded onto sucrose density gradients (10−50% (w/v) sucrose prepared in 20 mM Tris-HCl buffer, pH 7.6 containing 20 mM OG). After centrifugation at 220,000 × g for 18 h, an orange-colored layer containing the CBP was collected, diluted with the same buffer, and concentrated by ultrafiltration (Ultragel 10,000, Millipore).

Protein Identification—Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS) was performed by the method of Schägger and von Jagow (25). Non-denaturing (native) PAGE was performed according to Allen and Staehelin (26) with minor modifications: SDS was replaced with 0.02% (w/v) DDM and 0.05% (w/v) sodium deoxycholate. The separating gel and the stacking gel contained 8 (w/v) and 4% (w/v) acrylamide, respectively (the ratio of acrylamide to N,N′-methylenebisacrylamide was 29.1 (w/w)). After electrophoresis, proteins were stained with Coomassie Brilliant Blue.

Tryptic peptide mass fingerprinting analyses were performed using protein bands directly excised from the gel. Poly-peptides in the gel slices were digested with trypsin as follows. Gel slices that had been stained with Coomassie Brilliant Blue were destained with 25 mM ammonium bicarbonate in 50% (v/v) acetonitrile. After vortexing for 10 min, gel slices were pelleted, and the liquid was removed. If the gel pieces were still blue, this process was repeated. Destained gel slices were dried by vacuum centrifugation. The gel pieces were then incubated with 10 mM DTT in 25 mM ammonium bicarbonate at 56 °C for 1 h. Samples were centrifuged, and the liquid was removed. Iodoacetamide solution (10 mg ml⁻¹ in 25 mM ammonium bicarbonate) was added, and the samples were incubated at room temperature for 45 min in the dark. The gel samples were washed with 25 mM ammonium bicarbonate, dehydrated with 25 mM ammonium bicarbonate in 50% acetonitrile, and dried by vacuum centrifugation. The gel samples were incubated with trypsin solution (12.5 µg of trypsin ml⁻¹ in 25 mM ammonium bicarbonate; Promega) at 37 °C for 16 h after which the liquid was collected into a clean vial. After adding 5% (v/v) formic acid solution (in 50% acetonitrile), the gel pieces were vortexed for 20 min and sonicated for 15 min, and the liquid was collected into the same vial. This step was repeated to increase the peptide yield. The solution containing the peptides from the digested protein was dried by vacuum centrifugation to reduce the volume and analyzed by LC-MS/MS, which was performed by the Mass Spectrometry Facility at the Huck Institutes for the Life Sciences at The Pennsylvania State University (University Park, PA). Peptides produced by trypptic digestion were identified using the search engine Mascot (Matrix Science, Boston, MA), and amino acid sequence data were deduced from the genome of Ca. C. thermophilum (10).

Spectroscopic and High Performance Liquid Chromatography (HPLC) Analyses—Absorption spectra were recorded with a Cary-14 spectrophotometer modified for computerized data acquisition (Olis, Inc., Bogart, GA) and a Genesys 10 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Light-induced difference spectra were recorded using a JTS-10 spectrophotometer (Bio-Logic, Claix, France) and a series of interference filters (full-width half-maximum ≤10 nm) to monitor absorption changes at specific wavelengths. Actinic light was provided by light-emitting diodes that emitted maximally at 630 or 740 nm. Samples were subjected to continuous illumination until maximum bleaching was achieved (as judged by absorbance changes at 840 nm), and the magnitude of the absorbance change was plotted against wavelength. The pigment ratio of BCHl a per special pair was estimated using the known extinction coefficients for the type-I RC of GSB: ε₆₆₅ nm = 100 mM⁻¹ cm⁻¹ for antenna BCHl a in the RC (27) and Δε₇₃₀ nm = 90 mM⁻¹ cm⁻¹ for the special pair (28).

Electron paramagnetic resonance (EPR) spectroscopy was performed using a Bruker ECS-106 X-band spectrometer equipped with an Oxford liquid helium cryostat and temperature controller. Spectra were the average of eight scans recorded with the following conditions: frequency, 9.487 GHz; gain, 20,000; modulation amplitude, 5 gauss at 100 kHz. Power and temperature are specified in the legend for Fig. 4. A Spectra-Physics Millenia CW laser operating at 2.2 watts provided actinic light, and dark-adapted samples were illuminated directly in the cavity. Light-induced spectra were obtained by subtracting the spectrum of a dark-adapted sample from that of the illuminated sample.

The pigment compositions of the RC preparations were analyzed by reversed-phase (RP) HPLC on C₁₈ columns (Supelco, Bellefonte, PA) as described by Frigaard et al. (29). RP-HPLC analyses of carotenoids on a C₃₀ column (Bischoff Chromatography, Leonberg, Germany) were performed as follows. The gradient was composed of Solvent A (30% methyl t-butyl ether, 66% methanol, 4% water (v/v/v)) and Solvent B (50% methyl t-butyl ether, 30% methanol, 20% acetonitrile (v/v/v)). At the time of injection, the mobile phase was 30% Solvent B at a flow rate of 1 ml min⁻¹. Solvent B was linearly increased to 100% over 40 min followed by a constant flow of 100% Solvent B for 8 min after which Solvent B was returned to 30% in 1 min. Pigment ratios were determined using the following molar extinction coefficients: ε₆₆₅ nm = 71.43 mM⁻¹ cm⁻¹ for Chl a (30), ε₇₇₀ nm = 54.8 mM⁻¹ cm⁻¹ for BCHl a (31), and ε₄₉₁ nm = 141 mM⁻¹ cm⁻¹ for carotenoids (32).

The Zn-BChl aₐ was synthesized as follows. BCHl aₐ was extracted from a purple bacterium, Roseobacter sp., with acetone-methanol (7:2, v/v) and purified by RP-HPLC. The purified BCHl aₐ was treated with 1% (v/v) HCl to produce bacteriopheophytin aₐ, and the bacteriopheophytin aₐ was incubated with zinc acetate to produce zinc-chelated BCHl a (hereafter Zn-BChl aₐ). Diethyl ether and then water were added to the solution, and the ether phase containing Zn-BChl aₐ and residual bacteriopheophytin aₐ was collected and evaporated.
orated to dryness by a stream of nitrogen. The dried pigments were dissolved in acetone:methanol (7:2, v/v) for further analyses by RP-HPLC.

Carotenoids were extracted from CBP with acetone:methanol (1:1, v/v), purified by RP-HPLC, and dried under a stream of nitrogen. To test for the presence of keto group(s), the purified carotenoids were dissolved in isopropanol and incubated with NaBH₄ as described (33). Absorption spectra were recorded before and after the NaBH₄ reduction. To test for the presence of glycosyl and/or acyl esters, carotenoids extracted from CBP were dissolved in methanol and saponified using 5% (w/v) KOH. An equal volume of ether and then water was added to the solution, and the carotenoid-containing ether phase was collected. The carotenoid solution was dried under a stream of nitrogen, dissolved in methanol, and analyzed by RP-HPLC using the C₁₈ column system described above.

RESULTS

Purification and Identification of RC Complex from Ca. C. thermophilum—To isolate RCs from Ca. C. thermophilum, a chlorosome-depleted membrane fraction was first obtained by sucrose density gradient ultracentrifugation using a buffer containing 2.0 M sodium thiocyanate. Sodium thiocyanate is a chaotropic agent that has been used to detach chlorosomes from cytoplasmic membranes in GSB and Ca. C. thermophilum (8, 15, 34). Although the membrane preparations obtained were not completely free of chlorosome contamination as indicated by a chlorosome-specific absorbance peak at ~740 nm (data not shown), a large portion of the chlorosomes was removed by this method. Other chaotropes (e.g. sodium iodide) were tested, and they were also effective in completely detaching the chlorosomes and produced results similar to those with sodium thiocyanate. The chlorosome-depleted membranes were solubilized using 0.1% (w/v) DDM. After ultracentrifugation, the pellet contained the residual contaminating chlorosomes, and the supernatant no longer exhibited an absorption peak at ~740 nm. The supernatant fraction was subjected to anion-exchange column chromatography, and orange-colored, RC-containing fractions were collected.

SDS-PAGE analysis of the RC-containing fractions showed two polypeptides with apparent masses of 110 and 22 kDa (Fig. 1A). These bands were directly excised from the gel and subjected to tryptic peptide mass fingerprinting analysis (supplemental Fig. S1). The results showed that the 110-kDa band was PscA (Cabther_A2188; predicted mass, 99.2 kDa), and the 22-kDa band was a hypothetical protein (Cabther_A1191; predicted mass, 17.2 kDa), which was annotated as containing a pre policing-type N-terminal cleavage/methylonylation domain. The coverage percentages for the peptides detected in this analysis were 19.7% for PscA and 42.4% for the product of Cabther_A1191 to which we have assigned the gene locus designation cebp (carotenoid-binding protein; see below). The PscB protein, which has a predicted molecular mass of 19.2 kDa and is predicted to ligate the two terminal electron-accepting [4Fe-4S] clusters (Fₐ and Fₜ) of the RC, was not observed. PscB may have been lost because of the use of chaotropic agents to remove chlorosomes during membrane isolation. PscB in the RCs of C. tepidum and PshB of RCs of Helio bacterium modes-
cidoes with the presence of PscA (as measured by SDS-PAGE), we attribute the absorbance change at 840 nm to the special pair, which we denote as P840. Using extinction coefficients for complexes of GSB (\(\epsilon_{840 \text{ nm}} = 100 \text{ mm}^{-1} \text{ cm}^{-1}\) for antenna BChl \(a\) (25) and \(\Delta\epsilon_{830 \text{ nm}} = 90 \text{ mm}^{-1} \text{ cm}^{-1}\) for P840*/P840 (26)) and freshly isolated RC complexes, the ratio of BChl \(a\) per special pair in Ca. C. thermophilum was estimated to be 10.3 ± 0.96.

Consistent with the absence of absorbance features around 740 nm in the UV-visible spectrum, the RC complexes showed no measurable activity when illuminated with 740-nm actinic light. As expected, samples containing chlorosomes were active when illuminated with 740-nm actinic light (Fig. 3B). Note that all of the samples exhibited similar photobleaching behavior even in the presence of oxygen. It was not necessary to use a sealed, anoxic cuvette, which must be used to measure absorbance changes for oxygen-sensitive RCs (i.e. GSB and heliobacterial RCs; see below). These data demonstrated that the RCs retained photocactivity even after prolonged exposure to air and illumination.

A relatively large absorbance increase at 676 nm was a second feature that was common to the light-induced difference spectra of whole cells, chlorosome-containing membranes, and RC preparations. A similar feature has been observed in RCs from GSB, and in that case, it has been attributed to an electrochromic shift that occurs for Chl molecules bound near the special pair (5, 39, 40). Similar to the RCs of GSB (see below), the RC complexes of Ca. C. thermophilum bind Chl \(a\). Furthermore, the lifetime of the absorbance increase at 676 nm is highly similar to that at 840 nm. Thus, we tentatively assign the absorbance increase at 676 nm to an electrochromic shift of a Chl \(a\) molecule near the special pair.

The light-induced difference spectrum of whole cells also showed a relatively large bleaching at 553 nm, but no similar bleaching was observed in the difference spectrum of chlorosome-containing membranes (Fig. 3B). Furthermore, the lifetime for the recovery of oxidized P840 + as measured by the increase in absorbance at 840 nm was much longer in membranes than in whole cells. The addition of a soluble protein fraction back to membranes resulted in shorter recovery lifetimes for the absorption at 840 nm and the reappearance of the bleaching at 553 nm. Given the wavelength of this change, its absence in membrane fractions, and its effect upon the recovery of 840 nm photobleaching, we ascribe the feature at 553 nm to one or more soluble \(c\)-type cytochromes that act as electron donors to the oxidized special pair.

The light-induced EPR spectrum of chaotrope-treated membranes recorded at 84 K showed a derivative-shaped signal with a crossover at \(g = 2.002\) (Fig. 4). This signal could only be generated using intense illumination. Plots of the signal intensity versus microwave power or temperature suggested that this signal originated from an organic radical; its line width of 8.8 gauss was consistent with that of a (B)Chl dimer. After the actinic illumination was turned off, the signal decayed to undetectable levels within minutes; hence, the light-induced EPR signal was completely reversible (data not shown). Based on the \(g\)-value, power and temperature dependences, and line width, this light-induced signal was assigned to the oxidized primary donor (P840+).

**Pigment Composition of Ca. C. thermophilum RC Complex—** Pigments extracted from the RC complexes were analyzed by RP-HPLC (Fig. 5). The elution profiles of pigment extracts were monitored at 770 nm for BChl \(a\), 667 nm for Chl \(a\) and BChl \(c\), 491 nm for carotenoids, and 270 nm for quinones. As shown in Fig. 5, the HPLC analyses verified the presence of BChl \(a\) (35 min), Chl \(a\) (39.5 min), and two major elution peaks corresponding to carotenoids (42 and 43 min). No BChl \(c\) was detected. When monitoring was performed at 270 nm (data not shown), a compound with an absorption spectrum like that of menaquinone was sometimes but not always observed at 59 min (data not shown). Cells and chlorosomes of Ca. C. thermophilum contain menaquinone-8(H\(_2\)), which is menaquinone-8 with one reduced double bond in the isoprenoid tail (16, 41). The molar ratio of BChl \(a\) to Chl \(a\) was found to be 1.60 ± 0.05. Combined with the ratio of BChl \(a\) to P840 calculated above, the molar ratio of BChl \(a\):Chl \(a\):P840 was estimated to be 10.3:6.44:1.00.

The absorption spectrum of the pigment eluting at 35 min (Fig. 5A, black line) was typical of BChl \(a\); this pigment had the same elution time as authentic BChl \(a\)\(\text{pr}\) derived from *C. tepidum* (29). Thus, the BChl \(a\) in Ca. C. thermophilum RCs is esterified with phytol (supplemental Fig. 2B). To determine the identity of the esterifying alcohol of the Chl \(a\) in the purified RC complexes (Fig. 5A, gray line), we used Chl \(a\) esterified with phytol (Chl \(a\)\(\text{a}\)) from *Synechococcus* sp. PCC 7002 and Chl \(a\) esterified with \(\Delta2,6\)-phytadienol (Chl \(a\)\(\text{a}\)) from *C. tepidum* as HPLC standards (40). The Chl \(a\) derived from the *Ca. C. ther-*

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In addition to the major peak for BChl \(a\)\(\text{pr}\) eluting at 35 min, a smaller peak eluting at ~40 min with a spectrum similar to that of a BChl was always observed in six different RC complex preparations. The absorption spectrum of this component had a maximum at 763 nm (supplemental Fig. S2C) and was very similar to that of Zn-BChl \(a\). To verify its identity, a Zn-BChl \(a\) standard was chemically prepared (see “Experimental Procedures”), and the absorption spectrum of the resulting Zn-BChl \(a\) standard was measured (shown in supplemental Fig. S2D). Although the 500–700-nm region of the absorption spectrum of the component eluting at 40 min was somewhat distorted by
the overlapping absorbance of Chl $a_{\text{pp}}$ eluting at 39.5 min, the spectrum of this component was clearly similar to that of the Zn-BChl $a$ standard.

To investigate this component further, the putative Zn-BChl $a$ fraction was collected and analyzed by mass spectrometry. The putative Zn-BChl $a$ eluting at 40 min had a mass of 951.7 Da and also had the isotopic mass pattern that is typical for Zn-containing molecules (Fig. 6). These results establish that the RCs of *Ca. C. thermophilum* contain Zn-BChl $a$.

No Zn-BChl $a$ was observed in pigment extracts of the purified FMO protein (16) or chlorosomes (8, 15, 16), but this component was always observed in whole cells and RC preparations of *Ca. C. thermophilum*, which suggests that Zn-BChl $a$ is an RC-specific pigment. The ratio of the major BChl $a$ (at 35 min) to Zn-BChl $a$ (at 40 min) was 6.41/1.58. Given that 10.3 BChl $a$ molecules are bound to one RC complex, this suggests that 1.61 molecules of Zn-BChl $a$ are present per RC. Alternatively, if one assumes that there are actually 2.0 molecules of Zn-BChl $a$, per RC (per P840), then these RCs contain 12.8 BChl $a$; 8.0 Chl $a_{\text{pp}}$; 2.0 Zn-BChl $a$ per RC.

The Zn-BChl $a_p$ in the RC complex had the same mass (Fig. 6) and absorption spectrum (supplemental Fig. S2) as the Zn-BChl $a_p$ standard, but it eluted about 1 min later during RP-HPLC analysis (supplemental Fig. S4). Because of this difference, we propose that the Zn-BChl $a_p$ in the *Ca. C. thermophilum* RC is the C-132 epimer, i.e. Zn-BChl $a_{p}'$. It has previously been reported that BChl $a'$ and Chl $a'$, the C-13$^2$ epimeric forms of BChl $a$ and Chl $a$, are slightly more hydrophobic than the latter and thus elute earlier upon normal-phase HPLC (40, 42).

Because the RP-HPLC profiles of carotenoids extracted from the RC complex and CBP complex were nearly identical (Figs. 5B and 7D), most of the extracted carotenoids from the RC complex, especially the two major carotenoid species eluted at 42 and 43 min, are probably derived from CBP. However, a carotenoid that eluted at 50 min was not observed in the carotenoids extracted from the CBP alone, and this carotenoid also increased in membrane fractions enriched in PscA (see supplemental Fig. S5). This carotenoid had the same retention time and absorption spectrum as an authentic lycopene standard. Based on these results, lycopene appears to bind specifically to the RC core complex (the PscA homodimer), although it is possible that other carotenoids might also be components of this complex.

**Characterization of Carotenoid-binding Protein**—When membranes were solubilized with OG instead of DDM, fractions containing only the CBP could be isolated. Sucrose density gradient centrifugation of membranes solubilized with OG resolved three fractions: a thick orange-colored fraction, a brownish green fraction, and a greenish brown fraction (see Fig. 7A). As judged from absorption properties, the middle green layer was a chlorosome-containing fraction, and the lower
greenish-brown layer was a CBP-depleted, RC-enriched fraction that still contained some contaminating chlorosomes. The upper orange layer that contained the CBP was collected, diluted, and concentrated by ultrafiltration.

SDS-PAGE analyses showed that the upper orange layer contained a single polypeptide, CbpC, with an apparent mass of 22 kDa (Fig. 7B). The absorption spectrum of the fraction containing only the CBP complex exhibited a large absorbance peak at 485 nm and a small peak at 672 nm (Fig. 7C). The ratio of the 672 nm peak to the 485 nm peak depended on the concentration of detergent used in the isolation. When the concentration of OG was increased from 18 to 34 mM, the peak at 672 nm became nearly undetectable (Fig. 7C, gray line). RP-HPLC analysis of pigments extracted from the CBP demonstrated the presence of the same two carotenoid species as in the RC-CBP complex (Fig. 7D). This observation suggested that the two carotenoids detected in the RC-CBP complex were mostly derived from the CBP complex. BChl a and BChl c were not detected in the purified CBP complex (data not shown). Chl a was detected in the CBP sample that was isolated using 18 mM OG, and this suggested that the absorption peak at 672 nm was probably due to the presence of a small amount of Chl a. Ultrafiltration experiments showed that the pigments absorbing at 485 nm were bound to the protein and were unlikely to represent carotenoid pigments in detergent micelles (data not shown). When the CBP was electrophoresed at 4 °C by PAGE containing 0.1% (w/v) LDS instead of SDS, the unstained protein retained its yellow-orange color and had an apparent mass of ~22 kDa. Thus, it is proposed that the CbpC polypeptide
binds carotenoids (see results from the RP-HPLC analysis described below).

When the pigment extract from the CBP complex was analyzed by RP-HPLC on a C18 column, two major carotenoid peaks (denoted peaks 1 and 2) were detected (Fig. 7D). These peaks were collected and reanalyzed by RP-HPLC on a C30 column as described under “Experimental Procedures.” The elution profile of peak 1 on the C30 column showed that peak 1 contained two carotenoid species (denoted as peaks 1A and 1B) (supplemental Fig. S6, left panel), whereas the compound in peak 2 still eluted as a single compound (data not shown). To test whether these carotenoids contained keto groups, peaks 1A, 1B, and 2 were reduced with NaBH4. After NaBH4 reduction, the absorption spectra of all three carotenoid fractions changed and showed enhanced fine structure features (supplemental Fig. S6, A, B, and C, gray lines). These results indicated that all three carotenoid species contained at least one keto group. The mass \([\text{MH}^+]/\text{H}11001\] of peak 1A was determined to be 551.4 Da. Based upon the absorption spectra before and after the NaBH4 treatment, the elution times from RP-HPLC, and its mass, peak 1A was identified as echinenone, which is known to be one of the major carotenoids in chlorosomes of \(\text{Ca. C. thermophilum}\) (16, 41, 43).

To test whether these carotenoid species contained glycosyl moieties, carotenoids extracted from the CBP complex were saponified by treatment with KOH, and the saponified carotenoids were analyzed by RP-HPLC (supplemental Fig. S7, red line). After saponification, peak 2 and about half of the material eluting in peak 1 disappeared, and a single new carotenoid (peak 4) appeared. This indicated that peak 2 and half of the material eluting as peak 1 contained glycosyl and/or acyl moieties. The mass of the non-saponified portion of peak 1 (denoted as peak 3) (supplemental Fig. S7, left panel, red line) also had an \([\text{MH}^+]/\text{H}11001\] mass of 551.4 Da. The absorption spectrum of peak 3 was similar to that of echinenone and peak 1A (see supplemental Fig. S6A), and the elution time of peak 3 upon RP-HPLC was the same as that of the echinenone standard purified from chlorosomes of \(\text{Ca. C. thermophilum}\). Based on these results, peak 3 is assigned as echinenone (see supplemental Fig. S7F). Therefore, the saponified portion of peak 1 must have given rise to peak 1B. The appearance of the single large peak 4 and its absorption spectrum suggested that the chromophore portions of peak 2 and the saponified material eluting in peak 1 are the same compound. The absorption spectrum of peak 4 was similar to the spectra of peaks 1B and 2 in supplemental Fig. S6. The \([\text{MH}^+]/\text{H}11001\] mass of peak 4 was 567.4 Da. Based on the mass data, the absorption spectra, an analysis of the carotenoid biosynthesis genes in \(\text{Ca. C. thermophilum}\) (discussed below), and the fact that the carotenoids in CBP complex have keto groups, the chromophore portion of the two major carotenoids in the CBP complex is probably deoxyflexixanthin (supplemental Fig. S7E). The difference in elution times for the non-echinenone portion of peak 1 (peak 1B) and peak 2 likely arises from differences in the glycosyl and/or acyl moieties attached to the deoxyflexixanthin chromophore. No further attempts were made to identify the nature of these modifying groups that must occur at the 1’-OH of the ψ-end of these molecules.

**Oxygen Tolerance of Reaction Center Complex**—To study the oxygen tolerance of the RC complexes that had been purified on the benchtop under oxic conditions, RCs were exposed to
repeated illumination under oxic or anoxic conditions, and photobleaching of P840 was measured optically at 840 nm. When the RC complexes were diluted in anoxic buffer and sealed in a cuvette under anoxic conditions, the RC retained nearly 100% activity after eight rounds of P840 photobleaching and recovery (Fig. 8, diamonds). When the RCs were assayed under oxic conditions, the complexes still retained 99% activity after eight rounds of illumination and recovery (Fig. 8, squares). For comparison, RC core complexes, which had been isolated from the strict anaerobe H. modesticaldum and were devoid of the PshB1 and PshBII proteins (36), lost nearly 40% activity after only six photobleaching cycles when assayed under similar oxic conditions (Fig. 8, circles). These results indicate that when PscB is dissociated from the RC core homodimer the RC-CBP complex isolated from Ca. C. thermophilum is much more oxygen-tolerant than the homodimeric RCs of heliobacteria.

Whole cells and chlorosome-containing membranes from Ca. C. thermophilum showed nearly no decrease in photactivity even after dozens of actinic exposures (data not shown). Given that the PscB and PshB proteins that harbor the F₈ and F₉ [4Fe-4S] clusters in other homodimeric RCs are lost after treatment with chaotropes or high ionic strength buffer washes (36), it is highly likely that PscB is retained in whole cells and chlorosome-containing membranes, which were prepared under low ionic strength conditions. Combined with the results that indicated that the RC core complexes were relatively oxygen-tolerant, these observations suggest that the RC-CBP complex of Ca. C. thermophilum is much more oxygen-tolerant than the homodimeric RCs of heliobacteria and GSB both in the presence and the absence of PscB. This tentative conclusion will be tested more rigorously in future studies involving RC-CBP complexes containing PscB.

Carotenoids are known to function in photoprotection by quenching Chl triplet states and by quenching singlet oxygen (44). To test for a possible role of the CBP complex in oxygen tolerance, RC preparations that were depleted of the CBP complex were also assayed under oxic conditions. After eight illumination and recovery periods, the CBP-depleted, PscA-enriched RC fractions retained 94% activity (Fig. 8, triangles), but after 12 illumination periods, only 87% activity remained. These results suggest that the CBP complex might play a role in the oxygen tolerance of the RC-CBP complex. Attempts to reconstitute CBP-depleted RCs with the isolated CBP did not restore oxygen tolerance (data not shown).

DISCUSSION

Table 1 summarizes and compares properties of the RCs of Ca. C. thermophilum, C. tepidum, and H. modesticaldum. In combination with RP-HPLC analyses, spectroscopic measurements suggested that BChl a₁, Chl a₀, and Zn-BChl a₃ molecules are bound to the RC complex of Ca. C. thermophilum in the ratio 12.8:8.0:2.0 (per P840). The total (B)Chl content (~23 (B)Chl molecules) of these RCs is similar to those of other organisms with homodimeric type-I RCs (31, 45). The PscA core subunit may bind the entire complement of (B)Chl pigments, and the deduced amino acid sequence of PscA from Ca. C. thermophilum includes 22 histidine residues that might serve as (B)Chl ligands. However, CbpC may also bind some Chl a (see below). The PscA homodimer in GSB is estimated to bind 16 BChl a and four to six Chl a molecules (5, 31, 45). C. tepidum PscA contains 19 histidine residues per monomer as potential ligands for binding these (B)Chl molecules.

The BChl-like component eluting at ~40 min in the RP-HPLC profile (Fig. 5A) was confirmed to be Zn-BChl a₁ by its mass, the isotopic pattern of the mass spectrum (Fig. 6), its absorption spectrum with a wavelength maximum at 763 nm (supplemental Fig. 52C), and the similarity of its retention time upon RP-HPLC to that of Zn-BChl a₁ (supplemental Fig. 54). However, because of the small difference in the retention times of chemically produced Zn-BChl a₁ and the compound detected in the Ca. C. thermophilum RCs, we propose that the latter is actually the C-13° epimer, i.e. Zn-BChl a₁. This is the first time that a wild-type phototrophic bacterium has been shown to synthesize both Mg-BChl a₁ and Zn-BChl a₁. Some species of the genus Acidiphilium have Zn-BChl a₁ as their sole BChl (46). In the case of Acidiphilium rubrum, cells synthesize but do not accumulate Mg-BChl a; the substitution of magnesium by zinc apparently occurs non-enzymatically postsynthesis. A. rubrum uses Zn-BChl a not only for electron transfer reactions but also as an antenna pigment in the RC and light-harvesting 1 complexes. A recent study showed that Rhodobacter capsulatus produces small amounts of Zn-BChl a when the magnesium chelatase subunit ChlD is eliminated by mutation (47). In this case, ferrochelatase is responsible for the insertion of zinc into protoporphyrin IX. It is noteworthy that Ca. C. thermophilum is found in neutral to slightly alkaline environments (pH 7–9), and hence, it seems unlikely that the magnesium release and zinc insertion naturally occurs in the environment after the synthesis of Mg-BChl a. The insertion of zinc may therefore occur enzymatically in Ca. C. thermophilum.

Based upon the analyses conducted in this study, the RCs of Ca. C. thermophilum most likely contain two molecules of Zn-
BChl $a'$ per homodimer or P840 (Table 1). These two Zn-BChl $a'$ molecules could function as the special pair, the $A_o$ acceptor, or even as secondary electron transfer components functioning between $A_o$ and the Fe-S cluster $F_x$. Whereas the $\lambda_{\text{max}}$ of Zn-BChl $a$ (763 nm) occurs at a shorter wavelength than that of Mg-BChl $a$ (770 nm), the Q$_\alpha$ absorption band of the special pair in the Ca. C. thermophilum RC occurs at a longer wavelength (840 nm) than in GSB RCs (830 nm), although the difference spectrum has a very different shape. In the light induced difference spectra for cells, membranes, and RC-CBP difference spectrum has a very different shape. In the light-induced photobleaching at 840 nm and the observation of light-induced bleaching at 553 nm in whole cells suggested that soluble c-type cytochrome(s) donate electrons to P840$^+$. The cbpC gene (Cabther_A1191), which encodes the apoprotein of the CBP complex, is annotated as having a prepilin-type N-terminal cleavage/methylation domain. Cabther_A1191 is not co-localized with any other pilus-related genes, which often occur in operons (50). The cbpC gene product obviously binds carotenoids (Fig. 7), and it seems highly unlikely that the Cabther_A1191 product is actually a pilus-related protein.

In the native PAGE experiments, the purified RC complex migrated as a single band at $\sim$480 kDa, whereas SDS-PAGE and mass spectrometry only showed the presence of 99-kDa PscA (apparent mass, $\sim$110 kDa) and 22-kDa CbpC polypeptides. By RP-HPLC analysis, we estimated the ratio of carotenoids to Chl $a$ in the purified RC complex to be 5.23 $\pm$ 1.04 (Table 1). Assuming 6.44 Chl $a$ molecules are present in each RC, there are about 34 carotenoid molecules in the RC complex. It is highly unlikely that a 22-kDa CbpC apoprotein could bind 34 carotenoids, and thus, there are probably multiple CbpC subunits in an RC complex. Assuming that the PscA core homodimer accounts for $\sim$220 kDa of a 480-kDa complex, then 11.8 CbpC subunits would be required. Based on this stoichiometry, about 2.8 carotenoids are probably bound to one CbpC subunit. Future studies will be required to establish whether Chl $a$ and/or carotenoids are being removed from the CbpC during solubilization and isolation and whether the CbpC-bound pigments can transfer energy to the (B)Chls of the RC core complex.

The spectroscopic properties of the CBP complex with its intense absorbance from carotenoids and very weak absorbance from Chl $a$ are reminiscent of peridinin-Chl $a$ protein (PCP), a 34-kDa light-harvesting antenna protein found in marine algae (51). PCP is unusual among light-harvesting complexes because of its high ratio of a carotenoid (peridinin) to Chl
The crystal structure of PCP from the dinoflagellate *Amphidinium carterae* revealed that eight peridinin molecules and two Chl a molecules are bound per PCP monomer (52). In PCP, peridinin harvests light energy and transfers the excited states to Chl a. In line with the high carotenoid to Chl a ratio, the absorption spectrum of PCP displays a dominant absorbance band from peridinin in the 400–550-nm region and a small Qy band from Chl a at 670 nm (51). These spectral features are similar to those of the CBP complex of *Ca. C. thermophilum*, although in the purified CBP fraction, the absorbance value for Chl a depended on the concentration of OG used during purification. This might imply that some Chl a molecules are located at the interface between the PscA core and the CbpC subunits and that these Chl a molecules can be displaced by detergent molecules during the purification. Whether the CBP complex functions as a light-harvesting complex like PCP is currently uncertain. However, the experiments shown here suggest that the CBP complex contributes to the photostability of the RC-CBP complex under oxic conditions (Fig. 8). It should also be noted that a ketocarotenoid, 3′-hydroxyechinonene, acts as a strong quencher in the orange carotenoid protein, which acts as a quencher of excess excitation in cyanobacteria (53).

The major carotenoid in chlorosomes of *Ca. C. thermophilum* was identified previously as echinenone, which is also the most abundant carotenoid in whole cells (16, 41, 43). The synthesis of echinenone from lycopene requires cyclase(s) capable of producing β-carotene and a 4-ketolase (54). The *Ca. C. thermophilum* genome contains both *cruA* and *crtYcYd* genes, representing two of the four families of lycopene cyclases (54, 55), and a *crtO* gene for the 4-ketolase (10). The genome also contains *crtC* (1′,2′-hydratase) and *crtD* (3′,4′-desaturase) genes (10). The two major carotenoids in the CBP complex were shown to have glycosyl moieties, and the chromophore portion of these carotenoids was identified as deoxyflexixanthin. The synthesis of deoxyflexixanthin from lycopene requires a lycopene monocyclase (either CruA or CrtYcYd), CrtC, CrtD, and a 4-ketolase (CrtO). The presence of genes for two lycopene cyclases suggests that one may act preferentially as a monocycle, whereas the other enzyme is either a bicyclic or preferentially adds a second ring to γ-carotene like CruB in BChl e-containing GSB strains (56). The complement of genes for carotenogenesis in *Ca. C. thermophilum* is completely consistent with the assignment of deoxyflexixanthin as the chromophore of these glycosylated (and/or acylated) carotenoids.

In summary, we have purified an RC-CBP complex from *Ca. C. thermophilum* and demonstrated that it retains light-induced photobleaching of P840 in the presence of oxygen. Overall, these RCs have properties that are intermediate between the more complex RCs of GSB and the simpler RCs of heliobacteria (Table 1). The purified RC-CBP complex contained only two polypropyroles, the homodimer core subunit PscA and a novel carotenoid-binding subunit, which may function in light harvesting, oxygen tolerance, and/or photoprotection. The CBP complex itself presents an interesting subject for future spectroscopic studies because of its high carotenoid to protein ratio and the possibility that it binds Chl a. Like other previously characterized homodimeric type-I RCs, *Ca. C. thermophilum* RC-CBP complex binds a relatively small number of BChl a and Chl a molecules, but this RC is unique because it contains both Mg-BChl a and Zn-BChl a. Because of its simple subunit composition, oxygen tolerance, and unique pigment complement, the RC of *Ca. C. thermophilum* may provide new insights into the structural, functional, and evolutionary relationships of RCs.

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REFERENCES

1. Golbeck, J. H. (1993) Shared thematic elements in photochemical reaction centers. Proc. Natl. Acad. Sci. U.S.A. 90, 1642–1646
2. Overmann, J. (2001) in Bergey’s Manual of Systematic Bacteriology (Boone, D. R., Castenholz, R. W., and Garrity, G. M., eds) 2nd Ed., Vol. 1, pp. 60–605, Springer-Verlag, New York
3. Madigan, M. T. (2001) in Bergey’s Manual of Systematic Bacteriology (Boone, D. R., Castenholz, R. W., and Garrity, G. M., eds), 2nd Ed., Vol. 1, pp. 625–630, Springer-Verlag, New York
4. Li, H., Jubeliger, S., Garcia Costas, A. M., Frigaard, N. U., and Bryant, D. A. (2009) Multiple antioxidant proteins protect *Chlorobaculum tepidum* against oxygen and reactive oxygen species. Arch. Microbiol. 191, 853–867
5. Hauska, G., Schoedl, T., Remigy, H., and Tsotsis, G. (2001) The reaction center of green sulfur bacteria. Biochim. Biophys. Acta 1507, 260–277
6. Oh-oka, H. (2007) Type 1 reaction center of photosynthetic heliobacteria. Photochem. Photobiol. 83, 177–186
7. Garrity, G. M., Lilburn, T. G., Cole, J. R., Harrison, S. H., Ezubejy, J., and Tindall, B. J. (2007) The Taxonomic Outline of Bacteria and Archaea, Release 7.7, Michigan State University, East Lansing, MI
8. Bryant, D. A., Costas, A. M., Maresca, J. A., Chew, A. G., Klatt, C. G., Bateson, M. M., Tallon, L. J., Hostetler, J., Nelson, W. C., Heidelberg, J. F., and Ward, D. M. (2007) *Candidatus Chloracidobacterium thermophilum*: an aerobic phototrophic acidobacterium. Science 317, 523–526
9. Klatt, C. G., Wood, J. M., Rusch, D. B., Bateson, M. M., Hamamura, N., Heidelberg, J. F., Grossman, A. R., Bhaya, D., Cohan, F. M., Kuhl, M., Bryant, D. A., and Ward, D. M. (2011) Community ecology of hot spring cyanobacterial mats: predominant populations and their functional potential. ISME J. 5, 1262–1278
10. Garcia Costas, A. M., Liu, Z., Tomsho, L. P., Schuster, S. C., Ward, D. M., and Bryant, D. A. (2012) Complete genome of *Candidatus Chloracidobacterium thermophilum*, a chlorophyll-based phototrophotroph belonging to the phylum Acidobacteria. Environ. Microbiol. 14, 177–190
11. Oh-oka, H., Iwaki, M., and Itoh, S. (1997) Viscosity dependence of the electron transfer rate from bound cytochrome c to P840 in the photosynthetic reaction center of the green sulfur bacterium *Chlorobium tepidum*. Biochemistry 36, 9267–9272
12. Tsukatani, Y., Miyamoto, R., Itoh, S., and Oh-oka, H. (2006) Soluble cytochrome c-554, CycA, is not essential for photosynthetic electron transfer in *Chlorobium tepidum*. FEBS Lett. 580, 2191–2194
13. Tsukatani, Y., Miyamoto, R., Itoh, S., and Oh-oka, H. (2004) Function of a PscD subunit in a homodimeric reaction center complex of the photosynthetic green sulfur bacterium *Chlorobium tepidum* studied by insertional gene inactivation. Regulation of energy transfer and ferredoxin-mediated NADP⁺ reduction on the cytoplasmic side. J. Biol. Chem. 279, 51122–51130
14. Liu, Z., Klatt, C. G., Wood, J. M., Rusch, D. B., Ludwig, M., Wittekindt, N., Tomsho, L. P., Schuster, S. C., Ward, D. M., and Bryant, D. A. (2011) Metatranscriptomic analyses of chlorophototrophs of a hot-spring microbial mat. ISME J. 5, 1279–1290
33. Britton, G. (1985) General carotenoid methods. Methods Enzymol. 111, 113–149
34. Vassilieva, E. V., Antonkine, M. L., Zybailev, B. L., Yang, F., Jakobs, C. U.,
Golbeck, J. H., and Bryant, D. A. (2001) Electron transfer may occur in the
chlorosome envelope: the Csm1 and Csm6 proteins of chlorosomes are
2Fe-25 ferredoxins. Biochemistry 40, 464–473
35. Jagannathan, B., and Golbeck, J. H. (2008) Unifying principles in homodimeric
type I photosynthetic reaction centers: properties of PscB and the
FA, FB and FX iron-sulfur clusters in green sulfur bacteria. Biochim. Bio-
phys. Acta 1777, 1535–1544
36. Romberger, S. P., Castro, C., Sun, Y., and Golbeck, J. H. (2010) Identification
and characterization of PhsBII, a second FA/FB-containing polypeptide
in the photosynthetic reaction center of Heliobacterium modesticaldum.
Photosynth. Res. 104, 293–303
37. Oh-oka, H., Kamei, S., Matsubara, H., Iwaki, M., and Itoh, S. (1995) Two
molecules of cytochrome c function as the electron donors to P840 in the
reaction center complex isolated from a green sulfur bacterium,
Chlorobium tepidum. FEBS Lett. 365, 30–34
38. Sakurai, H., Kusumoto, N., and Inoue, K. (1996) Function of the reaction
center of green sulfur bacteria. Photochem. Photobiol. 64, 5–13
39. Oh-oka, H., Kakutani, S., Kamei, S., Matsubara, H., Iwaki, M., and Itoh, S.
(1995) Highly purified photosynthetic reaction center (PscA/cytoceme
$c551$) complex of the green sulfur bacterium Chlorobium limicola. Bio-
chemistry 34, 13091–13097
40. Kobayashi, M., Oh-Oka, H., Akutsu, S., Akiyama, M., Tominaga, K., Kise,
H., Nishida, F., Watanabe, T., Amesz, J., Koizumi, M., Ishida, N., and Kano,
H. (2000) The primary electron acceptor of green sulfur bacterium, bacteri-
ochlorophyll 663, is chlorophyll a esterified with $\Delta$2,6-phytylendiol. Pho-
tosynth. Res. 63, 269–280
41. Garcia Costas, A. M. (2010) Isolation and Characterization of Candidatus
Chloracidobacterium thermophilum. Ph.D. thesis, The Pennsylvania
State University
42. Kobayashi, M. (1996) Study of precise pigment composition of photosys-
tem I-type reaction centers by means of normal-phase HPLC. J. Plant Res.
109, 223–230
43. Garcia Costas, A. M., and Bryant, D. A. (2008) in Photosynthesis. Energy
from the Sun. (Allen, J. F., Gianni, E., Golbeck, J., and Osmond, B., eds) Vol
1, pp. 1161–1164, Springer, New York
44. Frank, H. A., and Brudvig, G. W. (2004) Redox functions of carotenoids in
photosynthesis. Biochemistry 43, 8607–8615
45. Griesbeck, C., Hager-Braun, C., Roegl, H., and Hauska, G. (1998) Quantifi-
tion of P840 reaction center preparations from Chlorobium tepidum:
chlorophylls and EMMO protein. Biochim. Biophys. Acta 1365, 285–293
46. Wakao, N., Yokoi, N., Isayama, N., Hiroishi, A., Shimada, K., Kobayashi,
M., Kise, H., Iwaki, S., Itoh, S., Takaichi, S., and Sakurai, Y. (1996) Discovery
of natural photosynthesis using zinc-containing bacteriochlorophyll in an
aerobic bacterium Acidiphilium ruthenium. Plant Cell Physiol. 37, 889–893
47. Jaschke, P. R., Hardjas, A., Digby, E. L., Hunter, C. N., and Beatty, J. T.
(2011) A BchD (magnesium chelatase) mutant of Rhodobacter sphearoides
synthesizes zinc bacteriochlorophyll through novel zinc-containing inter-
mediates. J. Biol. Chem. 286, 20313–20322
48. Fowler, C. F., Nugent, N. A., and Fuller, R. C. (1971) The isolation and charac-
terization of a photochemically active complex from Chloropseudomonas
ethlica. Proc. Natl. Acad. Sci. U.S.A. 68, 2278–2282
49. Noy, D., Diederichs, K., Hartwig, G., Scheer, H., and Scherz, A. (1998) Metal-
substituted bacteriochlorophylls. 2. Changes in redox potentials and elec-
tronic transition energies are dominated by intramolecular electrostatic
interactions. J. Am. Chem. Soc. 120, 3694–3693
50. Mattick, J. S., Whitchurch, C. B., and Alm, R. A. (1996) The molecular
genetics of type-4 fimbriae in Pseudomonas aeruginosa—a review. Gene
179, 147–155
51. Polivka, T., Hiller, R. G., and Frank, H. A. (2007) Spectroscopy of the
peridinin-chlorophyll-a protein: insight into light-harvesting strategy of
marine algae. Arch. Biochem. Biophys. 458, 111–120
52. Hofmann, E., Wrench, P. M., Sharles, F. P., Hiller, R. G., Welte, W., and
Diederichs, K. (1996) Structural basis of light harvesting by carotenoids:
peridinin-chlorophyll-protein from Amphidinium carterae. Science 272,
1788–1791
53. Kirilovsky, D. (2010) The photoactive orange carotenoid protein and pho-
toprotection in cyanobacteria. Adv. Exp. Med. Biol. 675, 139–159
54. Maresca, J. A., Graham, J. E., and Bryant, D. A. (2008) The biochemical
basis for structural diversity in the carotenoids of chlorophototrophic bacteria. Photosynth. Res. 97, 121–140
55. Maresca, J. A., Graham, J. E., Wu, M., Eisen, J. A., and Bryant, D. A. (2007) Identification of a fourth family of lycopene cyclases in photosynthetic bacteria. Proc. Natl. Acad. Sci. U.S.A. 104, 11784–11789
56. Maresca, J. A., Romberger, S. P., and Bryant, D. A. (2008) Isorenieratene biosynthesis in green sulfur bacteria requires the cooperative actions of two carotenoid cyclases. J. Bacteriol. 190, 6384–6891
57. Azai, C., Tsukatani, Y., Itoh, S., and Oh-oka, H. (2010) c-type cytochromes in the photosynthetic electron transfer pathways in green sulfur bacteria and heliobacteria. Photosynth. Res. 104, 189–199
58. Heinnickel, M., and Golbeck, J. H. (2007) Heliobacterial photosynthesis. Photosynth. Res. 92, 35–53
59. Takaichi, S., and Oh-oka, H. (1999) Pigment composition in the reaction center complex from the thermophilic green sulfur bacterium, Chlorobium tepidum: carotenoid glucoside esters, menaquinone and chlorophylls. Plant Cell Physiol. 40, 691–694
60. Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., and Bairoch, A. (2003) ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 31, 3784–3788