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Pigment Composition in the Reaction Center Complex from the Thermophilic Green Sulfur Bacterium, *Chlorobium tepidum*: Carotenoid Glucoside Esters, Menaquinone and Chlorophylls

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Distribution of pigments in the reaction center (RC) complex, chlorosomes and chlorosome-free membranes prepared from the green sulfur bacterium, *Chlorobium tepidum*, was analyzed. The RC complex contained approximately 40 molecules of bacteriochlorophyll (BChl) *a* per P840, half of which are estimated to be in the Fenna-Matthews-Olson (FMO) protein. Carotenes (2 molecules per P840) occupied only one third of the total carotenoids. The remaining carotenoids (4 to 5 molecules per P840) were OH-chlorobactene glucoside ester and OH-γ-carotene glucoside ester. Dihydrochlorobactene was decreased compared with cellular carotenes. Furthermore, approximately 9 C670 and 1 menaquinone-7 per P840 were also found. BChl *c* and carotenes, whose major component is chlorobactene, were found mainly in chlorosomes, and two kinds of carotenoid glucoside esters were observed in chlorosome-free membranes.

Key words: C670 — Carotenoid glucoside ester — Chlorobactene — *Chlorobium tepidum* — Menaquinone — Reaction center.

Chlorobium tepidum is a thermophilic green sulfur bacterium that inhabits acidic, high-sulfide hot springs (Wahlund et al. 1991). Although the major carotenoids of mesophilic green sulfur bacteria are chlorobactene and isorenieratene (Takaichi 1999), a recent study has revealed the presence of unusual carotenoids in *C. tepidum*; 1′,2′-dihydro-γ-carotene, 1′,2′-dihydrochlorobactene, OH-γ-carotene glucoside ester and OH-chlorobactene glucoside ester (Takaichi et al. 1997). Carotenoids serve as light-harvesting accessory pigments as well as for protection from damage by radical species produced by the photochemical reaction. The distribution of carotenoids, especially such unusual carotenoids, in the photosynthetic membranes is therefore of interest in order to understand their structural and functional relationship within the photochemical reaction systems.

The reaction centers (RCs) of photosynthetic organisms can be grouped into two types according to the nature of their terminal electron acceptors; that is, a quinone (Q)-type and an iron-sulfur (FeS)-type RCs (Blankenship 1992). The Q-type RCs, which are represented by the RC of purple bacteria and by the PSII RC of plants and cyanobacteria, contain one and two carotenoid molecules, respectively, as accessory pigments (Ermler et al. 1994, Satoh 1996). On the other hand, the PSI RC, which belongs to the FeS-type RC, has more carotenoids, 12 to 16 molecules of β-carotene (Nechushtai et al. 1996). However, the carotenoid composition in the RC complex of the green sulfur bacteria, which is also classified into the FeS-type RC (Feiler and Hauska 1995, Sakurai et al. 1996), is not yet known. Although carotenoids in the crude RC preparation from *Prosthecochloris aestuarii* were reported (Braumann et al. 1986), their identification was insufficient. The recent success in isolating the photoactive RC complex from several species (Kusumoto et al. 1994, Kjær et al. 1994, Oh-oka et al. 1995b, Hager-Braun et al. 1995) enables study of the detailed molecular structure, including pigment composition, as well as electron transfer and energy transfer mechanism within the RC complex.

In this study, the pigment distribution in the purified RC complex, chlorosomes and chlorosome-free membranes was analyzed using HPLC after fractionation of *C. tepidum* cells. The RC complex used here is highly photoactive and made up of five different subunits, that is, the core protein (PscA), the Fenna-Matthews-Olson (FMO) protein, the F_A/F_B protein (PscB), cytochrome *c* (PscC) and the 18-kDa protein (PscD) (Oh-oka et al. 1995b). Carotenoid glucoside esters, carotenes, menaquinone (MK)-7, bacteriochlorophyll (BChl) *a* and C670 were observed in this RC complex. BChl *c* and carotenes were found mainly in chlorosomes, and carotenoid glucoside esters were also found in chlorosome-free membranes.

Material and Methods

Preparation of chlorosomes, chlorosome-free membranes

Abbreviations: BChl, bacteriochlorophyll; C670, chlorophyll *a*-like pigment with the Q_y band at 670 nm in vivo; FeS-type, iron-sulfur-type; FMO protein, Fenna-Matthews-Olson protein; MK, menaquinone; P840, primary electron donor in green sulfur bacterial reaction center; Q-type, quinone-type; RC, reaction center.

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and RC complex—The *Chlorobium tepidum* strain TLS (ATCC 49652^T) was grown as described by Wahlund et al. (1991). Chlorosomes and chlorosome-free membranes were prepared according to the sucrose density gradient method by Schmidt (1980) with some modifications (Oh-oka et al. 1998). The RC complex was isolated as described previously (Oh-oka et al. 1995b). The chemically oxidized-minus-reduced difference absorption spectrum for P840 was measured with a Shimadzu spectrophotometer (Model UV-3101PC, Japan) according to the method reported previously (Oh-oka et al. 1995a). The P840 content was estimated using the different extinction coefficient of $\Delta\varepsilon_{830}=100\ mM^{-1}\ cm^{-1}$ for $P840^+$/P840 (Olson et al. 1973).

Analysis of pigments—Pigments were extracted from the wet fractions with acetone : methanol (7 : 2, v/v). The crude extract was analyzed using HPLC equipped with a μBondapak C_{18} column (Waters, U.S.A.) and eluted with methanol (2.0 ml min^{-1}) as described previously (Takaichi and Ishidsu 1992). Next, the crude extract was subjected to silica gel 60 (Merck, Germany) column chromatography, and carotenes were eluted with *n*-hexane; then xanthophylls and BChls *a* and *c* were eluted with chloroform : methanol (3 : 1, v/v). Except for carotenes, the pigments were analyzed again using HPLC. The carotene fraction was analyzed using HPLC on a Novapak C_{18} column (Waters) eluted with acetonitrile : methanol : tetrahydrofuran (58 : 35 : 7, by vol.; 2.0 ml min^{-1}) as described previously (Takaichi et al. 1997).

Absorption spectra were recorded with a photodiode array detector (200–800 nm, MCPD-3600; Otsuka Electronics, Japan) attached to the HPLC apparatus (Takaichi and Shimada 1992). The molar absorption coefficients in the HPLC eluent were 132 $mM^{-1}\ cm^{-1}$ at 489 nm for carotenoids, 68 $mM^{-1}\ cm^{-1}$ at 770 nm for BChl *a*, 69 $mM^{-1}\ cm^{-1}$ at 669 nm for BChl *c* and at 665 nm for C670 (Takaichi et al. 1995), and 18.9 $mM^{-1}\ cm^{-1}$ at 247 nm for MK (Dunphy and Brodie 1971). The relative molecular weights were determined by field-desorption mass spectrometry using a double-focusing gas chromatograph mass spectrometer equipped with a field-desorption apparatus (M-2500; Hitachi, Japan) (Takaichi 1993).

Results and Discussion

Pigments in cells, chlorosomes and chlorosome-free membranes—Pigments extracted from whole cells, chlorosomes and chlorosome-free membranes were analyzed using HPLC, and identified as described previously (Takaichi et al. 1997). The pigment compositions are summarized on Table 1.

All of BChl *c* was distributed to chlorosomes. Major carotenoids in chlorosomes were carotenes, whose composition was exactly the same as that of cells. The molar ratio of BChl *c* to carotenes was approximately 13, while the molar ratio was approximately 4 in chlorosomes of the green filamentous bacterium, *Chloroflexus aurantiacus* (Tsuji et al. 1995). Because the carotene contents in chlorosomes are quite different between Chlorobiaceae and Chloroflexaceae, the functions and the location in chlorosomes as well as the interactions with BChl *c* might be somewhat specialized in each.

Nearly half of the carotenoids in chlorosome-free membranes were carotenoid glucoside esters, and the remainder were carotenes. The composition of carotenes in membranes was almost the same as that in cells. Although small amounts of BChl *c* were found in membranes, this was probably due to contamination of chlorosomes, where an extremely large amount of BChl *c* exists. It can be esti-

Table 1 Pigment compositions of cells, chlorosomes, chlorosome-free membranes and RC from *Chlorobium tepidum*

	Cells	Chlorosomes	Membranes		RC	
Pigment composition (molar ratio to BChl *a*)						
BChl *a*	1	1	1	(55)[a]	1	(41)[a]
BChl *c*	40	46	0.3	(15)	0.01	(0.3)
P840	ND[b]	ND	0.02	(1)	0.02	(1)
C670	ND	ND	0.13	(7.1)	0.2	(9.0)
Carotenoids	3.2	3.5	0.5	(25)	0.2	(7.1)
MK-7	2.5	0.9	0.3	(17)	0.02	(0.9)
Carotenoid composition (% of total)						
Chlorobactene	64	63	33	(8.3)[a]	29	(2.1)[a]
1′,2′-Dihydrochlorobactene	21	21	17	(4.3)	2	(0.1)
γ-Carotene	11	11	5	(1.0)	4	(0.3)
1′,2′-Dihydro-γ-carotene	1	1	0	(0.0)	0	(0.0)
Lycopene	0	0	0	(0.0)	1	(0.1)
OH-Chlorobactene	1	1	5	(1.3)	0	(0.0)
OH-γ-Carotene	0	0	1	(0.2)	0	(0.0)
OH-Chlorobactene glucoside ester	1	1	15	(3.8)	36	(2.6)
OH-γ-Carotene glucoside ester	2	2	24	(5.8)	27	(1.9)

[a] molar ratio to P840.
[b] ND: not determined.

mated that approximately 13% of the total BChl *a* in cells was distributed in membrane from the comparison of the ratio of BChl *c*/*a* in cells and chlorosomes. At present, carotenoid glucoside esters have been found only in *C. tepidum* among Chlorobiaceae (Takaichi 1999). Although some species of *Chloroflexus* and *Halorhodospira*, Ectothiorhodospiraceae of haloalkaliphilic purple sulfur bacteria, have recently been shown to contain carotenoid glycoside esters (Takaichi 1999), the location in chlorosome-free membranes and the functions in photosynthesis are not yet known.

Pigments in RC complex—Pigments extracted from the RC complex of *C. tepidum* were analyzed using HPLC on the μBondapak C_{18} column (Fig. 1). The elution profile revealed peaks of both BChls and carotenoids. A major peak with a retention time of approximately 5 min was identified as BChl *a* according to its absorption spectrum and the specific retention time. The peak with a Chl *a*-like spectrum was C670, whose absorption maxima were 261, 338, 431, 618 and 665 nm in methanol, while the most red absorption maximum in the isolated RC complex is 670 nm in vivo (Oh-oka et al. 1995a). Carotenoids, including carotenes and carotenoid glucoside esters, were eluted at 12 to 16 min. The RC complex was estimated to contain 41 BChl *a*, 9.0 C670 and 7.1 carotenoid molecules per P840 (Table 1). The RC complex used in this study contains the FMO protein (Oh-oka et al. 1995b), which forms the trimeric structure with 7 BChl *a* molecules in each monomer (Olson 1978, Li et al. 1997). If the P840 RC contains three FMO proteins, the RC core complex contains approximately 20 molecules of BChl *a*. This value is almost comparable to

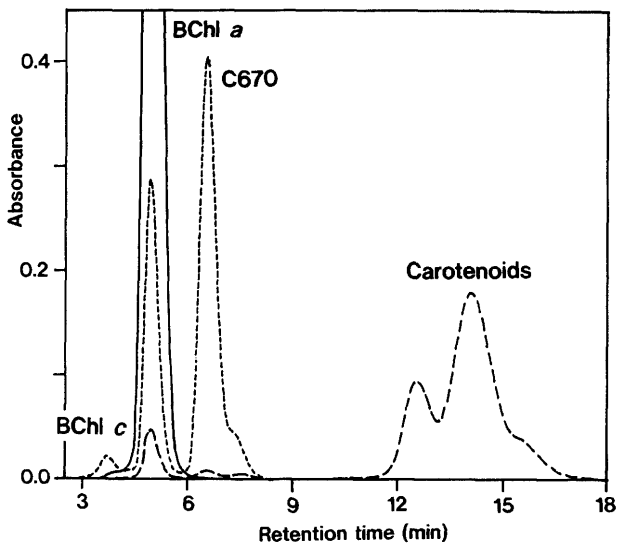

Fig. 1 HPLC elution profiles of the total pigments extracted from the RC complex of *Chlorobium tepidum*. Absorbances at 770 nm (—), 665 nm (---) and 489 nm (– – –) are shown. Absorbance at the BChl *a* peak was ca. 2.2.

that (~27) reported previously for the *Chlorobium* RC-2 complex, comprised of a core and cytochrome *c*, of another green sulfur bacterium, *Chlorobium limicola* (Oh-oka et al. 1995a). The content of C670 in the present RC preparation was rather smaller compared to that reported in the RC complex of *P. aestuarii* (Braumann et al. 1986), in which C670 was designated as BChl 663. One of C670 seems to serve as a primary electron acceptor (Van de Meent et al. 1992). Small amounts of BChl *c* were probably due to contamination of chlorosomes. Bacteriopheophytins *a* and *c* could not be detected under the present experimental conditions.

Carotenoids were further separated into carotenoid glucoside esters and carotenes by silica gel column chromatography, and they were analyzed using HPLC on the μBondapak C_{18} and the Novapak C_{18} columns, respectively (Takaichi et al. 1997). The major components were OH-γ-carotene glucoside ester, OH-chlorobactene glucoside ester and chlorobactene (Table 1). Composition of dihydrocarotenes, 1′,2′-dihydrochlorobactene and 1′,2′-dihydro-γ-carotene, was significantly decreased compared with that in cells. The contents of total carotenoid glucoside esters (OH-γ-carotene glucoside ester and OH-chlorobactene glucoside ester) were estimated to be 4 to 5 molecules per P840, and carotenes were 2 molecules per P840. In the PSI RC complex of plants, only β-carotene molecules exist and serve as light-harvesting and/or photoprotection, although the exact location of each molecule has not been identified by the recent X-ray crystallographic analysis (Schubert et al. 1997). The presence of the 15-*cis* form of carotenes has recently been shown not only in the cyanobacterial PSI RC complex but in the *Chlorobium* RC preparation used here, suggesting some peculiar physiologic and/or structural meaning for the photochemical reaction (Bialek-Bylka et al. 1998). However, it is not clear in our experimental conditions whether the carotenoid glucoside esters found in the *Chlorobium* RC complex also take the *cis* form. Because the chemical structure of carotenoid glucoside esters is quite different from that of carotenes, it will be of interest to investigate their roles as well as spatial distributions within the RC complex in the future studies.

MK-7 was detected using HPLC on the Novapak C_{18} column. Its absorption maxima, 207, 247, 263 and 330 nm in the eluent, and the specific retention time were compatible with those of the authentic MK-7, whose relative molecular weight was confirmed to be 648 with a mass spectrometry. Although there are at least three kinds of quinones in *C. tepidum*, MK-7, chlorobiumquinone and polar quinone (Quinone 1) (Frigaard et al. 1997), MK-7 was the sole quinone species contained in this photoactive RC complex. The content of MK-7 was estimated to be 0.9 molecule per P840. Frankenberg et al. (1996), however, have reported that no quinones were present beyond the detection limit (below 0.2 molecule per RC) in the pho-

toactive RC complex prepared from *C. tepidum*. In contrast, the RC complex of *Chlorobium vibrioforme* has recently been shown to contain 1.7 molecules of MK-7 per P840 and exhibit the photoaccumulation of a semiquinone-type EPR signal (Kjær et al. 1998). Further studies are necessary to resolve the issue concerning the quinone molecules on the electron transfer pathway within the *Chlorobium* RC complex.

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