Stable Photobleaching of P840 in *Chlorobium* Reaction Center Preparations: Presence of the 42-kDa Bacteriochlorophyll *a* Protein and a 17-kDa Polypeptide[†]

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ABSTRACT: Simple procedures for the anaerobic preparation of photoactive and stable P840 reaction centers from *Chlorobium tepidum* and *Chlorobium limicola* in good yield are presented and quantitated. The subunit composition was tested by cosedimentation in sucrose density gradients. For *C. limicola*, it minimally comprises four subunits: the P840 reaction center protein PscA, the BChl*a* antenna protein FMO, the FeS protein PscB with centers A and B, and a positively charged 17-kDa protein denoted PscD. The preparation from *Chlorobium tepidum* additionally contained PscC, a cytochrome *c*-551. The BChl*a* absorption peak of the purified complexes was at 810 nm, with a shoulder at 835 nm. The ratio of the shoulder to the peak was 0.25, which corresponds to 1 reaction center per 70 BChl*a* molecules if a uniform extinction coefficient of BChl*a* is assumed. However, bleaching at 610 nm in continuous light corresponded up to 1 photoactive reaction center per 50 BChl*a* molecules. Therefore, either the extinction coefficient of BChl*a* in the reaction center is overestimated or the one for photobleaching is underestimated. In any case, the major portion of the reaction center was photoactive in the preparations. A P840 reaction center subcomplex, lacking PscD and deficient in FMO and PscB, but retaining the cytochrome *c* subunit, was obtained as a side product. It was photoinactive and had an absorption peak at 814 nm and a 835/814 absorbance ratio of 0.42. FMO and PscB show the tendency to form a complementary subcomplex. FMO and PscD are apparently required to stabilize the photoactive reaction center, while the cytochrome *c* subunit is not.

Photosynthetic reaction centers (RCs)[1] are classified according to the nature of their terminal electron acceptors: Q-type RCs (type II) reduce a mobile quinone, and are represented by the RC of purple bacteria and by the PSII of cyanobacteria and chloroplasts. FeS-type RCs (type I) reduce FeS centers, and occur in PSI, in green sulfur bacteria like *Chlorobia*, and in *Heliobacteria* (Golbeck, 1993).

The structure of the Q-type RCs in purple bacteria has been elucidated to atomic resolution by X-ray crystallography (Deisenhofer et al., 1985; Allen et al., 1986), and its pertinence to PSII has greatly stimulated photosynthetic research (Barber & Andersson, 1994). The core structure is composed of two different but related transmembrane subunits, which sandwich the primary electron donor—a BChl*a* dimer—and two sets of the electron acceptor sequence—chlorophyll, pheophytin, quinone—arranged in pseudo-*C2* symmetry. One set is preferred, and reduces the firmly bound, primary quinone Q_A. This in turn reduces the loosely bound quinone, Q_B, which belongs to the second set and which exchanges with the quinone pool. Q_A and Q_B interact via an Fe atom. The reason for this double, pseudosymmetric arrangement remains unclear up to now.

Knowledge on FeS-type RCs is lagging behind, but the PSI RC has been crystallized, and structural resolution is in progress (Krauss et al., 1993). Also in this case two mutually related and transmembrane subunits, designated PsaA and PsaB, are considered to hold the chlorophyll dimer of P700 and the electron acceptors A_0 (chlorophyll *a*) and A_1 (phylloquinone), probably again in two sets. A_1 is followed by electron acceptor X, an FeS center which occurs only once and is bound between the two core proteins by two cysteines on each of them. Two further FeS centers, FeS-A and -B, are located on the extra subunit PsaC and function as terminal electron acceptors (Golbeck & Bryant, 1991).

The similarity of the RCs in PSI and in *Chlorobia* and *Heliobacteria* is amply documented, and has been reviewed repeatedly (Nitschke & Rutherford, 1991; Nitschke & Lockau, 1993; Golbeck, 1993; Feiler & Hauska, 1995). Interestingly, the core of the FeS-type RCs in these strictly anaerobic organisms seems to be composed of two identical transmembrane subunits (Büttner et al., 1992; Liebl et al., 1993), and this homodimeric arrangement may provide clues to the better understanding of RCs, including evolution and the selection pressure for formation of heterodimeric cores in aerotolerant organisms (Blankenship, 1992). In particular,

[†] The nucleotide sequences for the *Chlorobium limicola* fmoA and pscD genes have been submitted to the EMBL data library under Accession Numbers X83529 and X83528, respectively.

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[1] Abbreviations: BChl, bacteriochlorophyll; FMO, Fenna/Mathews/Olson protein (=42-kDa BChl*a* protein); PMSF, phenylmethanesulfonyl fluoride; PS, photosystem; PscA, PscB, PscC, and PscD, P840 protein, FeS subunit, cytochrome *c*-551, and 17-kDa subunit of the *Chlorobium* RC, respectively (Bryant, 1994); RC, reaction center; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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the reason for two sets of electron acceptors may be clarified. Therefore, our efforts aim at the structural elucidation of the P840 RC of *Chlorobium.*

A highly purified preparation of the P840 RC from *Chlorobium* with only three subunits was obtained with the help of octyl glucoside, but was labile in photoactivity (Hurt & Hauska, 1984). Recently, several reports on the isolation of more efficient RCs from *Chlorobia* have been published (Feiler et al., 1992; Oh-Oka et al., 1993; Kusomoto et al., 1994; Kjaer et al., 1994). The results are conflicting, however, and quantitation has not been attempted. By changing octyl glucoside to Triton X-100 as the solubilizing detergent, we arrived at very stable preparations of the P840 RC from *C. tepidum* and *C. limicola*, which lend themselves to more detailed studies. These preparations retain two additional subunits, the 42-kDa BChl*a* protein FMO and a protein of about 17 kDa, designated PscD. Here we describe the isolation procedures, which are based on the early protocol to isolate the P700 RC of PSI (Bengis & Nelson, 1975), report the primary structures of the two additional polypeptides, and describe the stability in photobleaching of P840. An attempt to quantitate the RC content is also presented. Charge transfer to the FeS centers will be published in a subsequent EPR study.

MATERIALS AND METHODS

Organisms. Chlorobium limicola f.sp. *thiosulfatophilum* (Deutsche Sammlung von Mikroorganismen, Göttingen/ Germany, DSM 249) was grown at room temperature as described before (Büttner et al., 1992). *Chlorobium tepidum* was obtained from Dr. M. Miller/Odense and from Dr. M. D. Madigan/Carbondale and was grown at 40−48 °C according to Wahlund et al. (1991). Both species were cultured first in inocula of 50 mL, followed by 0.8 L, and finally in 19 L of medium, with the following specifications. The inocula were gassed with nitrogen for 10 and 30 min, respectively, and were autoclaved for 20 min. For the final culture, the components of the medium, except vitamin B_{12} and Na_2S, were dissolved in 19 L in a 20 L bottle and were autoclaved for 45 min. After cooling, vitamin B_{12} (20 μg/L) was added as a sterile solution, and the pH was adjusted to 7.7 with 30% sulfuric acid. The medium was then gassed with nitrogen through a sterile filter for at least 2 h to obtain anaerobiosis. Subsequently, 12 g of $Na_2S{\cdot}9H_2O$, which had been autoclaved in 50 mL of water, was added, and the pH was adjusted to 6.70 with 30% sulfuric acid. The cultures were grown to an OD_{750nm} of 3.5−4.0 cm^{-1}. Cells were harvested from the final 19 L cultures by centrifugation, and were stored frozen at −75 °C if not immediately used.

Preparation of Membranes. All the following steps were carried out at 4 °C and under anaerobic conditions. The latter was achieved by working in an anaerobic tent (Kjaer et al., 1994), or more conveniently by including 20 μM FMN in the buffers and illuminating them with strong white light before use (Nelson et al., 1972). The time required to reach anaerobiosis was about 5 min, as tested with an oxygen electrode. It is also indicated by a fading of the yellow color.

Chlorobium cells from a 19 L culture were suspended in 100 mL of preilluminated 20 mM Tricine−Tris, pH 7.3 for *C. tepidum* and pH 7.0 for *C. limicola*, 20 μM FMN, and were passed 3 times through a French press cooled with ice, at 18 000 psi (1.4 × 10^8 Pa). This resulted in about 80% of cell breakage. Unbroken cells and debris were sedimented at 20000*g* for 20 min, and chlorosome-containing membranes were subsequently sedimented at 120000*g* for 1 h. For removal of electrostatically attached surface proteins, the membrane pellets were resuspended in the buffer above containing 150 mM NaCl plus 1 mM EDTA, and were sedimented as before. The washed membrane pellets were then homogenized in Tricine−Tris/FMN buffer, which additionally contained 2 mM ascorbate, 1 mM EDTA, 5 mM cysteine, 1 mM PMSF, and 2 mM *p*-aminobenzamidine (Sigma), to an OD_{750nm} of 200 cm^{-1} (2 mg of BChl*c*/mL), and were stored at −70 °C until further use. From a 19 L culture about 150 mL of this membrane suspension was obtained, which had a protein content of about 5 mg/mL.

Chlorosome-free membrane fractions of both *Chlorobium* species were obtained from the loosely packed layer above the membrane pellet of the first sedimentation at 120000*g*, as detailed elsewhere (Klughammer et al., 1995). These fractions probably correspond to the early reports on P840 RC preparations with the French press called "complex I" (Fowler et al., 1971; Olson et al., 1973), and amounted to about 2% of total BChl*a*.

Isolation of P840 Reaction Centers. (A) Chlorobium tepidum. The thawed membrane suspension was brought to 3% Triton X-100, was stirred for 30 min on ice, and was then centrifuged for 30 min at 120000*g*. The resulting pellet, which contained the majority of chlorosomes and residual membrane fragments, was resuspended, extracted, and centrifuged once again. The combined supernatants had an OD_{810nm} of 1.5−2.0 cm^{-1}, were titrated to pH 8.0, and were loaded onto a DEAE-cellulose column of about 50 mL bed volume, which had been equilibrated in the membrane suspension puffer, pH 8.0, containing 0.2% Triton X-100. Fractionation was started as soon as pigments flew through. After the column was loaded, the elution was continued with a linear gradient from 0 to 150 mM NaCl in the same buffer, 100 mL total volume. Fractions of the flow through and the early part of the gradient, containing the reaction center, as estimated from the shoulder at 835 nm on the long-wavelength side of the BChl*a* absorption peak, were combined and concentrated to a few milliliters by sedimentation overnight at 180000*g*. The concentrate was loaded onto linear sucrose density gradients from 10 to 50% sucrose, in the buffer used for equilibrating the DEAE-cellulose column, and was centrifuged for 14 h at 150000*g* (fixed-angle rotor Beckman 60Ti, without brake during deceleration). The sucrose density gradient was fractionated, and the P840 RC containing fractions were stored frozen at −20 °C until further use.

(B) Chlorobium limicola. The isolation protocol is largely identical to the one for *C. tepidum*, with two modifications. Because Triton X-100 dissolved the chlorosomes of *C. limicola* to a large degree, a pH shift after solubilization was introduced for the efficient separation of BChl*c* from the RC, and the dimension of the DEAE-cellulose column was increased, because the RC bound more strongly. The thawed membranes were solubilized as for *C. tepidum*, but at pH 7.0 first. After centrifugation for 30 min at 120000*g*, a pellet was obtained which contained over 80% of the RC. The supernatant containing at least 90% of the BChl*c* in monomeric form, as judged from the absorption peak of 670 nm, was discarded. The pellet was resuspended in the same volume of solubilization buffer, but at pH 8.0 and with 0.2%

Triton X-100 only. Renewed centrifugation left most of the RC in the supernatant. The resulting pellet was extracted in the same buffer and centrifuged once more. The combined supernatants were loaded onto a DEAE-cellulose column of 200 mL bed volume, which had been equilibrated in the same buffer at pH 8.0. After residual BChl*c* was washed out, a gradient from 0 to 0.5 M NaCl in the same buffer was applied. Fractions containing the P840 RC, eluting between 70 and 100 mM NaCl, were collected, concentrated by centrifugation, applied to sucrose density gradients, and further handled as for *C. tepidum*.

Analytical Methods. SDS–PAGE was performed after Laemmli (1970). The gels were stained for heme (Thomas et al., 1976), and for protein with Coomassie-blue (Cabral & Schatz, 1979).

Optical spectra were recorded with a Kontron Uvikon 860 spectrophotometer. BChl*a* and BChl*c* contents were estimated according to Olson et al. (1976) and Stanier and Smith (1960), respectively.

Photobleaching by continuous far red light in the range between 540 and 640 nm was measured in an Aminco dual-wavelength DW2 spectrophotometer, modified for side illumination, with the reference wavelength at 540 nm. The actinic light was filtered through a Schott RG 715 cutoff filter, and the photomultiplier was protected by 1 mm Schott BG18 plus 1 cm $CuSO_4$ solution.

N-Terminal sequencing of proteins, electroblotted from SDS–PAGE (Matsudaira, 1987) onto Immobilon membranes (Millipore), was carried out with a gas-phase sequenator, type 477A (Applied Biosystems). Cloning and sequencing of the 42-kDa BChl*a* protein and the 17-kDa subunit in *C. limicola* followed the strategy published before (Büttner et al., 1992; Schütz et al., 1994), and are specified under Results. Techniques in molecular biology followed Sambrook et al. (1989).

Protein concentration was determined after Bensadoun and Weinstein (1976).

Calculation of P840 RC Content. In a first approximation, we assume that all BChl*a* is bound either to FMO or to the P840 RC, that its absorption does not change during purification, and that its extinction coefficient is uniform. With this, the content of total RC can be calculated from the ratio of the absorption shoulder at 835 nm to the BChl*a* absorption peak at 810–814 nm (r_{OD}):

$$\%\ \mathrm{RC} = 100[(r_{OD} - {}^1/_{13})/({}^2/_3 - {}^1/_{13})]$$

The values $^2/_3$ and $^1/_{13}$ represent the r_{OD} values for pure RC (Vasmel et al., 1983; Hurt & Hauska, 1984) and for pure FMO [see Olson (1980)], respectively.

The number of BChl*a* molecules per RC was calculated from % RC by assuming that pure RC carries 20 BChl*a* molecules (Vasmel et al., 1983).

The content of photoactive P840 RC was estimated from the ratio of the light-induced absorption change at 610 minus 540 nm to the absorption peak at 810–814 nm, with ϵ_{mM} values of 30 and 100 cm^{-1}, respectively (Olson et al., 1976).

RESULTS

Optical Spectra and Yield. The final sucrose density gradients of the P840 RC preparations of both *Chlorobium* species exhibited three colored bands: a bluish green band

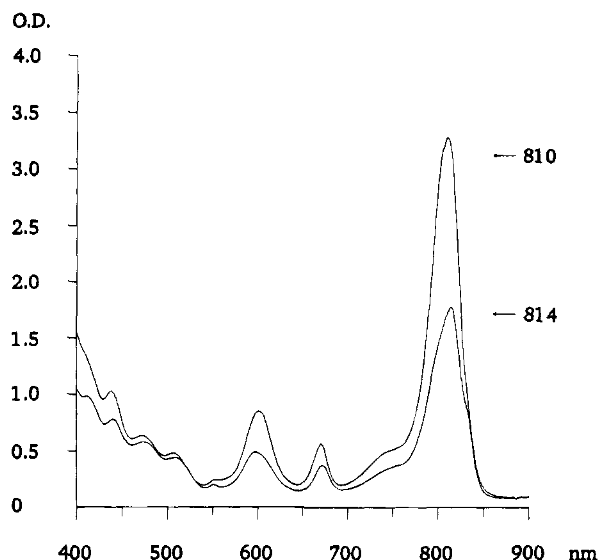


FIGURE 1: Absorption spectra of the two P840 RC bands from *Chlorobium tepidum* in the sucrose density gradient. The spectrum with the peak at 810 nm represents the bluish green band (fraction 7 in Figure 2A); the one with the peak at 814 nm represents the brownish green band of the sucrose density gradient (fraction 11 in Figure 2A). The latter is the one with the higher absorption at 500 nm and below.

at about 30% sucrose, a brownish green band at about 25% sucrose, and a brownish to green band on the top of the gradient. The later contained residual BChl*c* (green) from the chlorosomes, which had decomposed to bacteriopheophytin *c* (brownish) to varying extents. The spectra of the lower two pigmented bands of *C. tepidum* are depicted in Figure 1. The bluish green, lowest band has an almost symmetrical BChl*a* absorption peak at 810 nm. The shoulder at 835 nm is not pronounced. Also, the BChl*a* absorption peak at 603 nm is symmetrical. The brownish green, middle band exhibits a more structured BChl*a* absorption with the peak at 814 nm, and a clearly visible shoulder at 835 nm. The absorption around 600 nm is asymmetric, with the peak at a significantly shorter wavelength (597 nm). It contained relatively more carotenoid, as indicated by the absorption peaks around 500 nm and below. Both fractions contained ascorbate, and therefore exhibited ascorbate-reducible cytochrome *c*. For *C. limicola*, the spectra looked very similar, except that cytochrome *c*-551 was missing in the lower, bluish green band (see below).

The yield of RC during purification can be estimated from the total BChl*a* content and the ratio of the shoulder at 835 nm to the absorption peak at 810–814 nm (see Materials and Methods). It is listed for *C. tepidum* in Table 1. The 100% value refers to the cell homogenate, which had a volume of 90 mL, a chlorosome absorption of 200 cm^{-1} at 750 nm (see Materials and Methods), and a 750/810 nm absorption ratio of 30. Thus, it corresponded to a total absorption of 600 at 810 nm. Only about 40% were found in the combined two Triton X-100 extracts. Further extractions of the chlorosome-rich residue did not increase the yield enough to justify the increase in volume. The yield of photoactive P840 RC in the bluish green zone of the sucrose density gradients was 10% in the particular preparation of Table 1. It varied between 8 and 16%. Some of the RC, together with relatively more FMO, was bound more tightly to the DEAE column and eluted later in the salt gradient (70–150 mM NaCl). In these fractions, FMO had dissoci-

Table 1: Yield of the P840 RC in a Preparation from *Chlorobium tepidum*[a]

fraction	OD_{peak}	r_{OD}	% yield
cell homogenate	600		100
detergent extract	240	0.20	40
DEAE flow through	100	0.29	29
DEAE fractions 1−7	26	0.29	7
DEAE fractions 8−16	58	0.14	5
concentrate for SDG	101	0.29	29
brownish green zone	18	0.42	8
bluish green zone	42	0.25	10

[a] The isolation of the P840 RC is described under Materials and Methods. Percent yield on RC during purification was estimated from the total BChl*a* absorption peak at 810−814 nm (OD_{peak}), i.e., OD × volume, and the absorption of the shoulder at 835 nm (r_{OD}; see Materials and Methods). For the 90 mL homogenate (OD_{750} = 200), a ratio of the absorption at 750 to 810 nm of 30 was measured. DEAE fractions 1−7 and 8−16 represent the eluates at 0−70 and at 70−150 mM NaCl of the salt gradient, respectively. SDG stands for sucrose density gradient.

ated from the RC (see Discussion). Another part of FMO remained on the DEAE column as a blue zone at the top.

The yield for *C. limicola* was somewhat lower, because of the more complicated removal of chlorosomes/BChl*c* which made the extraction of the RC less efficient.

Polypeptide Patterns. Figure 2 shows the SDS−PAGE patterns of the fractionated sucrose density gradients for both *Chlorobium* species.

For *C. tepidum* in Figure 2A, fractions 7 and 8 represent the bluish green band, and fractions 11 and 12 the brownish green band. Five major polypeptides are present in the bluish green zone with relative masses on SDS−PAGE of 65, 42, 32, 24, and 17 kDa. The 65-kDa polypeptide staining as a diffuse band corresponds to the hydrophobic P840 RC core polypeptide (Hurt & Hauska, 1984), and according to the gene of *C. limicola* actually represents a 82.2-kDa protein (Büttner et al., 1992). It is denoted PscA (Bryant, 1994). The 42-kDa subunit represents FMO, a water-soluble BChl*a* antenna, which was the first chlorophyll protein to be resolved by X-ray crystallography (Mathews et al., 1979; Olson, 1980). The 32-kDa subunit represents a protein with two FeS centers (Illinger et al., 1993), has been denoted PscB, and resembles PsaC of PSI which binds the FeS centers A and B. It actually is a 23.9-kDa protein (Büttner et al., 1992). The subunit migrating at 24 kDa on SDS−PAGE is a heme-protein and represents a special type of cytochrome *c*, whose gene has been sequenced for *C. vibrioforme* (Okkels et al., 1992). This codes for the protein PscC with an mass of 22.9 kDa. The fifth subunit of apparent mass 17 kDa, consequently designated PscD, has not been assigned to a function yet.

The brownish green zone further up in the sucrose density gradient for *C. tepidum* is devoid of the 32- and 17-kDa subunits, and is deficient in FMO, but the concentration of the cytochrome *c* subunit PscC is peaking a second time in this fraction (Figure 2A). Another prominent polypeptide of about 39 kDa peaks just above the brownish green zone, and yet another protein of 42 kDa is found still further up in the sucrose density gradient. The latter does not contain BChl*a* and is different from FMO. It also does not resemble cytochrome *b* (Hurt & Hauska, 1984; Schütz et al., 1994). The cytochrome *c* of 24 kDa is present also in the top part of the gradient.

The SDS−PAGE patterns of the sucrose density gradient fractions for *C. limicola* are shown in Figure 2B. The centers of the bluish green and brownish green bands correspond to fractions 10 and 14, respectively. The polypeptide patterns observed largely resemble the ones for *C. tepidum*, with one interesting difference. The heme-containing 24-kDa polypeptide PscC is lost from the bluish green band, as also indicated by the absorption spectra (not shown), and by the spectrum of photobleaching (Figure 5). It is present only in the brownish green band and in the top region of the gradient. As for *C. tepidum*, the brownish green band is deficient in FMO and lacks PscD, which again are both enriched in the bluish green band at higher sucrose density. The deficiency of the brownish green band in FMO is somewhat obscured in the SDS−PAGE pattern of Figure 2B, because, as for *C. tepidum*, another 42-kDa protein which does not carry BChl*a* is found in the top of the gradient and contaminates the brownish green zone. Its N-terminus was determined as AGGSKKHVVV, which corresponds neither to FMO (Dracheva et al., 1992) nor to cytochrome *b* (Schütz et al., 1994).

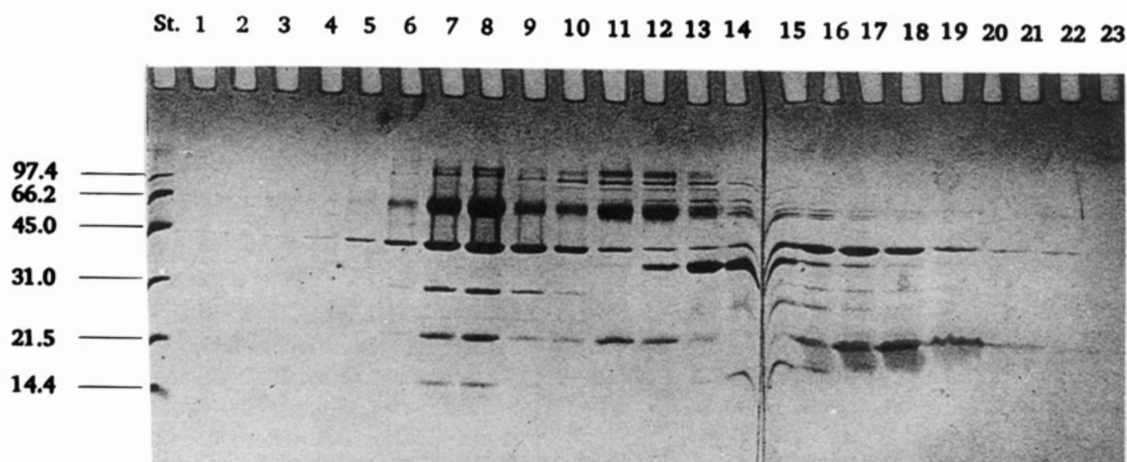
Sucrose density gradients of fractions eluting at higher salt concentrations from the DEAE column (fractions 8−16 in Table 1) had a dominant brownish green zone, with a blue zone below. SDS−PAGE of these gradients showed a polypeptide pattern for the brownish green zone as above, but the blue zone contained FMO and the FeS protein PscB only (not shown).

Primary Structures. The amino acid sequence for the P840 binding protein PscA and the FeS protein PscB, which migrate at 65 and 32 kDa on SDS−PAGE, respectively, has been deduced for *C. limicola* from a transcription unit (Büttner et al., 1992). For FMO, it has been determined for *Prostecochloris aestuarii* (Daurat-Larroque et al., 1986) and *C. tepidum* (Dracheva et al., 1992). We have now sequenced the corresponding gene from *C. limicola* (EMBL data library, Accession No. X83529 *C. limicola* fmoA gene). The 365 amino acids add up to a mass of 40.3 kDa. Only 12 residues are different from *C. tepidum*, which are mostly conservative changes (R for S13, V/A37, N/D49, G/D107, S/T111, M/L121, R/M139, R/K167, T/A174, F/S244, V/I322 and V/I361). To the protein from *Prostecochloris* 81 amino acids are different which indicates a larger phylogenetic distance. The residues considered to bind BChl*a* are all conserved.

The primary structure of PscC has been deduced from the gene for *C. vibrioforme*, and we have identified this component for *C. limicola* with the heme-containing subunit of the brownish green band migrating at 24 kDa in SDS−PAGE (Figure 2B) by microsequencing the N-terminus. It reads MDNKSNGKLI, which completely matches the *C. vibrioforme* sequence.

For *C. limicola*, the 17-kDa subunit of the bluish green zone was electroblotted, and the N-terminal amino acid sequence MQPQLSRPQTATNQVRNSV was obtained. Positive colonies were identified in the *Sau*3AI library of *Chlorobium limicola* DNA (Büttner et al., 1992) with an oligonucleotide corresponding to the peptide. The 0.4-kb insert of one of these positives reacted with the oligonucleotide on Southern blots, and its sequence coded for the N-terminus. Southern hybridization of the labeled *Sau*3AI insert to genomic *Chlorobium* DNA cut with various combinations of restriction enzymes was carried out subsequently. A positive fragment of 1.6 kb, cut with *Nco*I and

A



B

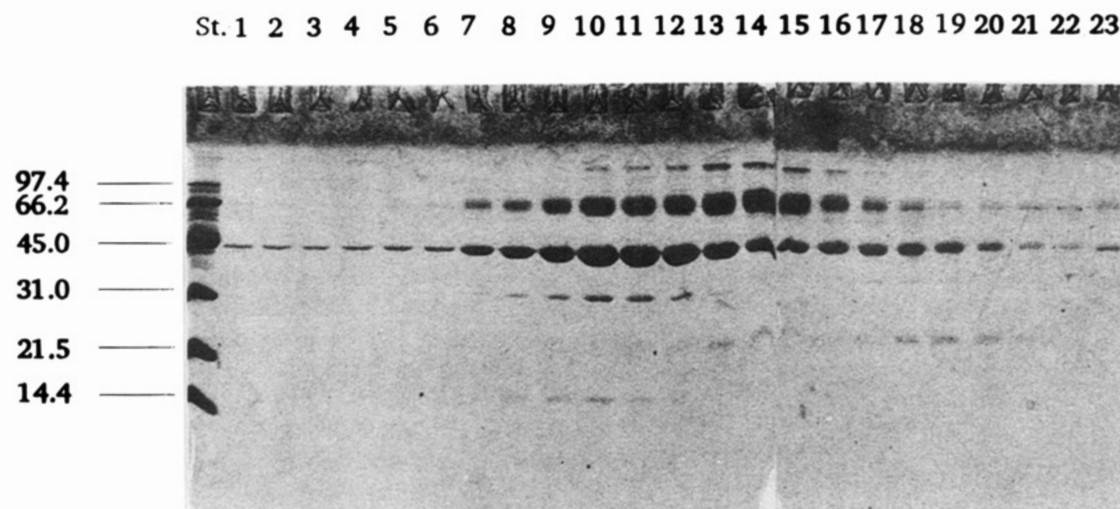


FIGURE 2: Polypeptide patterns of the fractionated sucrose density gradients. The fractions of *C. tepidum*, numbered 1 −23 from bottom to top of the gradient, are shown in (A); the ones of *C. limicola* are in (B). The values on the left represent the molecular weights ($\times 10^3$) of standard proteins (St.).

*Bam*HI, was identified, and a special library was constructed in the vector pTrc99A from Pharmacia in the corresponding restriction sites. This library was screened with the *Sau*3AI fragment, and the 1.6-kb insert of a positive clone was sequenced using oligonucleotide primers for both DNA strands as before (Büttner et al., 1992; Schütz et al., 1994). Figure 3 gives the nucleotide sequence and the derived amino acid sequence for the 143 codon open reading frame, which corresponds to a highly charged, basic protein of actual mass 16.52 kDa, with an isoelectric point at pH 9.0. Its hydropathy plot is shown in Figure 4. The N-terminus fully confirms the amino acid sequencing. A terminating stem−loop structure is evident after the C-terminus, but the gene lacks the usual ribosome binding motifs upstream of the N-terminus (Figure 3). No significant identity to any known protein was found during search through the data bases.

Photoactivity. Illumination of the bluish green fractions in P840 RC preparations from both *Chlorobia* with continuous far-red light shows a broad photobleaching peaking at 610 nm, which reflects $P840^+$ (Swarthoff & Amesz, 1979). Photooxidation of cytochrome *c* with the peak at 551 nm (Figure 5) is only observed in the preparation from *C. tepidum*. This corroborates the lack of the 24-kDa heme-protein from the bluish green zone of *C. limicola* (fractions 10/11 in Figure 2B).

From the absorption at 810 nm of 0.95 and the photobleaching at 610 nm of 0.0057 (Figure 5), a ratio of 1 photoactive P840 per 50 BChl*a* molecules can be estimated for *C. tepidum* (see Materials and Methods). Furthermore, with a differential ϵ_{mM} of 20 cm^{-1} for cytochrome *c*, 2.3 hemes *c* are photooxidized per P840 in this fraction.

Similarly, from the OD_{810nm} of 2.0 and the corresponding absorption change at 610 nm of 0.0068 in Figure 6 of the bluish green fraction 10 of *C. limicola* (Figure 2B), a ratio of 90 BChl*a* molecules per P840 can be calculated. This higher ratio may reflect higher damage of the RC and/or higher amounts of FMO (see Figure 2). As reported above, cytochrome *c* is absent in this preparation from *C. limicola*.

Figure 6 documents for *C. tepidum* that photobleaching of P840 is confined to the lower, bluish green zone, while the brownish green zone is inactive. A very similar pattern was obtained for *C. limicola* (not shown). Photobleaching

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               10                  30                  50
AAGGTTTACACCAGCCACGAGGTGCGCCAGTCAGCCAATCAAACCGAGCAGAACCATGCA
                                                         M  Q

               70                  90                 110
GCCTCAGCTAAGCCGTCCGCAAACCGCGACCAACCAGGTTCGCAACTCTGTTTCTGGCCC
 P  Q  L  S  R  P  Q  T  A  T  N  Q  V  R  N  S  V  S  G  P

              130                 150                 170
CTGGTCAGGAAATGCGGCACACAAAGCCGAAAAGTATTTCATCACATCAGCAAAACGCGA
 W  S  G  N  A  A  H  K  A  E  K  Y  F  I  T  S  A  K  R  D

              190                 210                 230
TCGCAACGACAAGCTCCAGATCGAGATTATGCCTTCATCTGGCCGTCGGAAGCTGTCGCC
 R  N  D  K  L  Q  I  E  I  M  P  S  S  G  R  R  K  L  S  P

              250                 270                 290
AACCCCGGAGATGATCCCCAAGCTCATCGACGGCGAGATCGAAATTTACGTTCTCACCAC
 T  P  E  M  I  P  K  L  I  D  G  E  I  E  I  Y  V  L  T  T

              310                 330                 350
CCAGCCTGACATCGCCATCGATCTTGGCAAGCAGGTGATCGACATGGAAAACCGCTACGT
 Q  P  D  I  A  I  D  L  G  K  Q  V  I  D  M  E  N  R  Y  V

              370                 390                 410
CATCGATTTCGACAAGCGCGGCGTCAAGTGGACGATGAGGGATATTCCCGTGTTCTACCG
 I  D  F  D  K  R  G  V  K  W  T  M  R  D  I  P  V  F  Y  R

              430                 450                 470
CGAAGGCAAGGGCCTGTGCGTTGAACTCGACAACAGGATTTACACCCTCAACGAATTCTT
 E  G  K  G  L  C  V  E  L  D  N  R  I  Y  T  L  N  E  F  F

              490                 510                 530
CAAATAAACAGGCTTGTTTCAGCACCTCTCTCAGGCAAATCTGTTCAGAAAGAGCCATGC
 K  *

              550                 570                 590
TCCAGCGTGGCTCTTTTTTTGTGCATAAAAAAGCCCGGATCATCGCCGGGCTTTTCAGAG
```

FIGURE 3: Nucleotide and amino acid sequence of the 17-kDa subunit in the bluish green P840 RC fraction of *Chlorobium limicola*. Amino acids are given in the single-letter code. The hairpin structure at the 3′-end is underlined. The nucleotide sequence of the entire 1.6-kb fragment can be obtained from the EMBL data library, Accession Number X83528 for the *C. limicola* pscD gene.

of the bluish green fractions from both species was very stable. When anaerobically kept at 4 °C or frozen at −20 °C, practically no decrease was observed after 1 week, and after 7 weeks about 70% of the photobleaching was still measured. At room temperature, 20% and 70% were lost after 1 and 7 weeks under anaerobiosis, respectively. Loss of photobleaching activity at room temperature and at 4 °C, but not in the frozen state, was considerably faster when the samples had been aerated.

A detailed EPR study of the P840 RC preparations from both *Chlorobium* species will be published elsewhere (Astrid Riedel et al., in preparation). In summary, it revealed that as in membranes (Nitschke et al., 1990) the three FeS centers corresponding to FeS-A, -B, and -X of PSI (Golbeck & Bryant, 1991) can be photoreduced, and that a major portion of the RCs in the preparations are capable of doing so.

DISCUSSION

Evaluation of the P840 Reaction Center Preparation. Several solubilized, photoactive P840 RC preparations from *Chlorobia* have been reported [Swarthoff & Amesz, 1979; Hurt & Hauska, 1984; Feiler et al., 1992; Okkels et al., 1992; Oh-Oka et al., 1993; Kusumoto et al., 1994; see Feiler and Hauska (1995) for a review]. Comprehensive data on stability and yield are lacking, however. The procedures for *C. tepidum* and *C. limicola* presented here are more quantitatively documented and have the following conveniences: (1) The common detergent Triton X-100 was used. It leads to preparations with very stable photobleaching activity. (2) Inclusion of FMN and preillumination of the isolation media to maintain anaerobiosis avoid the use of an anaerobic tent. (3) No special equipment for cell breakage (Kusumoto et al., 1994) is required; a French press or sonifier (Hurt & Hauska, 1984) suffices. (4) Working with chlorosome-deficient cell cultures (Okkels et al., 1992) is not necessary. (5) The isolation of chlorosome-depleted membranes before solubilization was omitted (Feiler et al., 1992; Okkels et al., 1992). Starting from total membranes increases the yield, which can be 10% or more on a BChl*a* basis (Table 1).

In Table 2, the percentage of BChl*a* representing the RC and the ratio of BChl*a*/RC are estimated (see Materials and Methods) for the two RC bands of the sucrose density gradient in comparison to chlorosome-free membranes. The photoactive bluish green band retains about 70 BChl*a*/RC. This is most of the BChl*a* per RC present in the membranes, while the inactive brownish green band has lost a good deal. Surprisingly, from the extent of photobleaching, a ratio of 50 BChl*a* per photoactive RC is obtained for *C. tepidum* (Figures 5 and 6), which corresponds to an impossible value of 140%. Therefore, either the average extinction coefficient for BChl*a* in the RC is lower than in FMO or the extinction coefficient for photobleaching at 610−540 nm is higher than assumed (Olson et al., 1976; Feiler et al., 1992). In the preparation from *C. limicola*, only about half the amount of RC per total BChl*a* compared to *C. tepidum* was active, as can be estimated from Figure 5. This reflects relatively more FMO present (see Figure 2) and/or higher damage during purification.

Swarthoff and Amesz (1979) also used Triton X-100 to solubilize the P840 RC from chlorosome-free membranes of *Prostecochloris aestuarii* in photoactive form. Sucrose density gradient centrifugation yielded a fraction with a pigmentation similar to our bluish green band. Further purification by a second centrifugation through 1 M guanidine hydrochloride removed over half of the BChl*a*, yielding a fraction pigmented like our brownish green fraction. Both fractions remained highly photoactive, however. Light minus dark difference spectra from 400−900 nm were presented and show that practically all the RCs remained active during the purification. The numbers of BChl*a* per photoactive RC in chlorosome-free membranes denoted "complex I" and of the two isolates "PP" and "RCPP" were reported to be 80, 75, and 35, respectively, which correspond well to our values in Table 2. Unfortunately, stability has not been tested, and the polypeptide patterns have not been documented by Swarthoff and Amesz (1979).

Stability and Polypeptides. The photoactivity of the bluish green fractions from the sucrose density gradients was remarkably stable (see above). Following the SDS−PAGE patterns through the fractionating sucrose density gradients (Figure 2) allows us to identify the required subunits. The bluish green zone from *C. tepidum* (Figure 2A, fractions 7 and 8) contains the five subunits PscA to PscC and FMO (Bryant, 1994) plus PscD, but the one from *C. limicola* (Figure 2B, fractions 10 and 11) contains only four. It is devoid of PscC, indicating that the cytochrome *c* subunit (Okkels et al., 1992) does not contribute to stabilize the P840 RC. Our earlier P840 RC preparation from *C. limicola* using octyl glucoside was unstable (Hauska & Hurt, 1984; Büttner et al., 1992). This isolate only contained the 65-kDa P840 protein PscA (Bryant, 1994), the FeS protein PscB migrating at 32 kDa (Illinger et al., 1993), and the cytochrome *c* PscC, but was deficient in FMO and lacked PscD.

The spectrum of the inactive, brownish green P840 RC fractions in the sucrose density gradients (Figure 1) had the

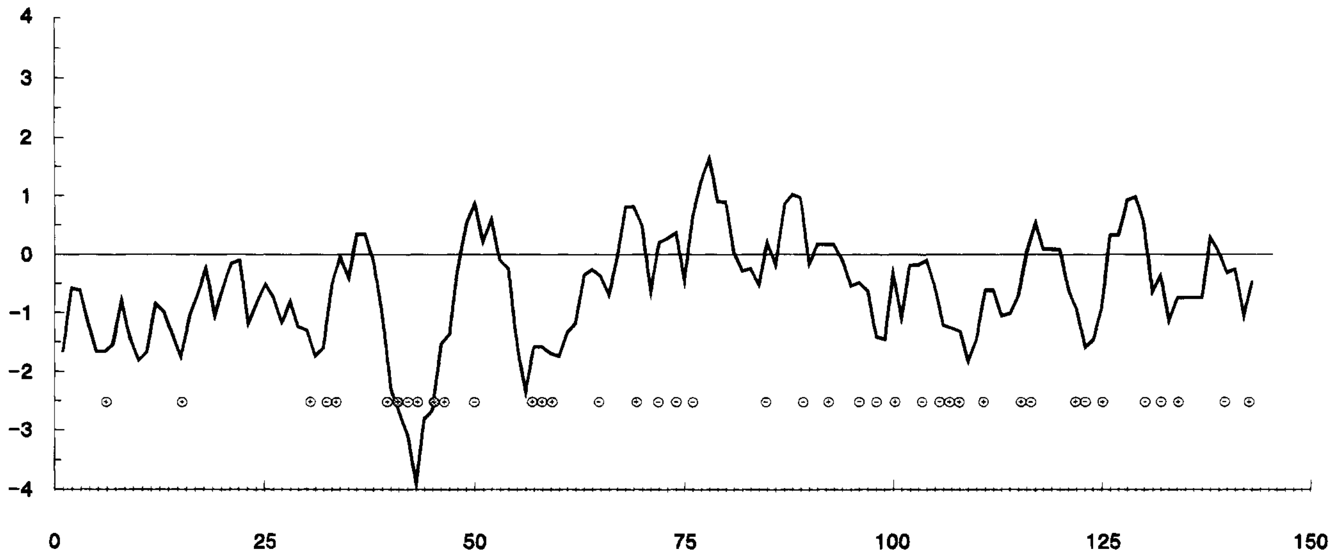

FIGURE 4: Hydropathy plot of the 17-kDa subunit. The algorithm of Kyte and Doolittle (1982) with a window of 9 was used. The charged residues along the sequence of the amino acids are indicated.

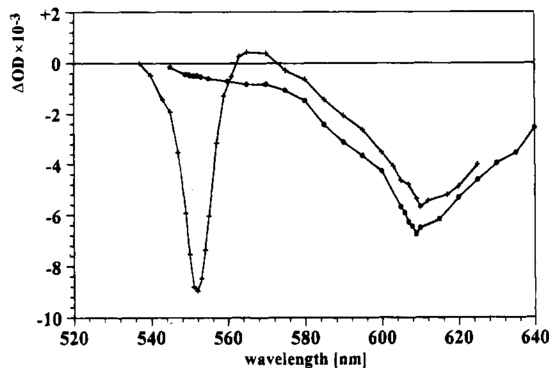

FIGURE 5: Spectra of light-induced absorption changes of the bluish green P840 RC fractions. The fraction of *C. limicola* (dots) had an OD_{810nm} of 2.0; the one of *C. tepidum* (crosses) had an OD of 0.95.

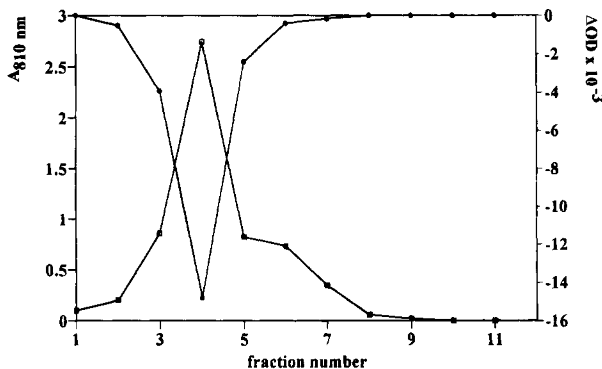

FIGURE 6: Distribution of photoactivity in the sucrose density gradient for *Chlorobium tepidum*. The figure compares the absorption at 810 nm (left ordinate scale, squares) with the photobleaching at 610−540 nm (right ordinate scale, circles). The preparation is different from the one used for Figure 5.

BChl*a* absorption peak at 814 nm, like the earlier preparation with octyl glucoside (Hurt & Hauska, 1984). However, in addition to FMO at 42 kDa and PscD at 17 kDa, also PscB at 32 kDa is lacking (Figure 2). Only the 65-kDa P840 protein PscA and the 24-kDa cytochrome *c* PscC peaked together in this part of the gradients (fractions 11 and 12 in Figure 2A, fractions 13−15 in Figure 2B), while in the preparation with octyl glucoside part of PscB was additionally present (Hurt & Hauska, 1984). However, the tendency

Table 2: Estimation of RC Contents and BChl*a*/RC Ratios in Fractions of the P840 RC Preparation from *Chlorobium tepidum*[a]

fraction	r_{OD}	% RC	BChl*a*/RC
chlorosome-free membranes	0.21	23	87
bluish green band	0.25	29	69
brownish green band	0.42	58	34

[a] Percent total RC on a BChl*a* basis and BChl*a*/total RC were calculated as described under Materials and Methods; r_{OD} represents the ratio of the OD at 835 nm to the peak at 810−814 nm.

of PscB and PscD to associate with FMO below PscA and PscC in the gradient has also been observed in preparations with octyl glucoside, and it is possible that in this case PscB actually is not bound at all to the RC [Figure 2 in Illinger et al. (1993)]. Thus, octyl glucoside seems to split the RC complex into FMO/PscB/PscD and PscA/PscC more efficiently than Triton X-100.

The related detergent dodecyl maltoside also seems to labilize the complex, removing part of the FeS subunit PscB (Feiler et al., 1992; Okkels et al., 1992; Kjaer et al., 1994; Miller et al., 1994).

The photoactive preparation of Kusumoto et al. (1994) from *C. tepidum* also using Triton X-100 probably resembles our preparation most closely. However, the absorption peak surprisingly is found at 814 nm, which indicates severe FMO deficiency.

The presence of FMO and PscD in our RC preparations coincides with the stability of photobleaching, but their stabilizing role is unclear and requires further investigation. The highly charged and basic nature of PscD (Figure 4) resembles subunit PsaD of the PSI RC [see Golbeck and Bryant (1991)]. We suggest that PscD may stabilize the binding of the FeS protein PscB to the P840 RC like PsaD stabilizes PsaC (Li et al., 1991).

Conclusion and Prospect. We have arrived at remarkably stable preparations of the P840 RC from *Chlorobia* in photoactive form and good yield.

FMO is present almost to the same extent as in the membranes. We are currently investigating whether FMO can be removed without loss of photoactivity, and whether that affects stability.

Although the PscC cytochrome *c* subunit adheres to certain RC fractions, a stabilizing effect can be excluded, because a stable preparation of the RC from *C. limicola* free of this cytochrome can be obtained (Figures 2 and 6). This observation is pertinent with regard to the nature of the primary donor cytochrome (Feiler et al., 1992; Okkels et al., 1992; Okumura et al., 1994).

PscD, the 17-kDa subunit, is a good candidate to stabilize the RC. It is polar, with a positive net charge, and may serve a similar function as PsaD in PSI (Golbeck & Bryant, 1991).

Subcomplexes of PscA and the cytochrome *c* PscC, and of FMO/PscB/PscD, can be isolated. This is consistent with the localization of the latter three subunits on the cytoplasmic surface, forming the baseplate between the chlorosomes and the membrane (Staehelin et al., 1980; Olson, 1980).

Further characterization of pigment composition and spectroscopic properties, resolution into the subunits and reconstitution, and 3d/2d-crystallization are projected now.

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