A reaction center – light harvesting 1 complex from a \textit{Rhodospirillum rubrum} mutant with altered esterifying pigments: characterization by optical spectroscopy and cryo-electron microscopy

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Running title: Mutant RC-LH1 complex from \textit{Rs. rubrum} with phytolated Bchls
Abstract

Introduction of the \textit{bchP} gene from \textit{Rhodobacter sphaeroides} encoding geranylgeranyl reductase into \textit{Rhodospirillum rubrum} alters the esterification of the bacteriochlorophylls so that phytol is used instead of geranylgeraniol. The resulting transconjugant strain of \textit{Rs. rubrum} grows photosynthetically, showing that phytolated Bchla can substitute for the native pigment in both the reaction center and light harvesting LH1 complexes. This genetic manipulation perturbs the native carotenoid biosynthetic pathway; several biosynthetic intermediates are assembled into the core complex and are capable of energy transfer to the bacteriochlorophylls. RC-LH1 complexes containing phytolated Bchla, were analysed by low temperature absorption and fluorescence spectroscopy and by circular dichroism. These show that phytolated Bchls can assemble \textit{in vivo} into the photosynthetic apparatus of \textit{Rs. rubrum}, and that the newly introduced phytol tail provokes small perturbations to the Bchls within their binding sites in the LH1 complex. The RC-LH1 core complex was purified from membranes and reconstituted into well-ordered 2-D crystals with a $p4_{2}12$ space group. A projection map calculated to 9Å shows clearly that the LH1 ring from the mutant is composed of 16 subunits which surround the reaction center, and that the diameter of this complex is in close agreement with that of the wild-type LH1 complex.
Introduction

Most photosynthetic organisms use chlorophylls for the capture of light energy and its conversion into a useful form of cellular energy. This molecule consist of two main components, a magnesium-porphyrin macrocycle and a 'tail', usually phytol, a C-20 isoprenoid (1). The combination of these components constitutes the terminal step of the bacteriochlorophyll biosynthetic pathway, which is catalysed by the BchG enzyme in the photosynthetic bacteria Rhodobacter capsulatus and Rb. sphaeroides (2,3). The phytol tail is approximately 30% of the total molecular mass of (Bacterio)chlorophyll, and apart from being an important determinant of the hydrophobicity of the (B)chl molecule, it plays an essential role in the assembly and stability of photosynthetic complexes. This was apparent from the early discovery that mutations in bchG abolish the assembly of the bacterial photosynthetic apparatus (4). However, the roles of the various esterifying alcohols used in bacterial photosynthesis, which include phytol, geranylgeraniol and farnesol (1) are not understood. In this study a molecular genetic approach has been used to alter the esterification of the Bchls in Rs. rubrum so that Bchl\textsubscript{a}P is used instead of Bchl\textsubscript{a}GG.

High-resolution crystallographic data of photosynthetic reaction centers have revealed detailed information on the conformation of phytol tails of bacteriochlorophyll and bacteriopheophytin molecules within this complex (5, 6). The conformations of the phytol tails differ between the active and inactive branches of the electron-transferring pigment system in the reaction center, although the significance of this is unclear. In the bacterial light harvesting LH2 complex attention has been drawn to the way in which the phytol tails of the B800 and B850 bchls intertwine; such interactions have been proposed to play an important role in establishing fast energy transfer within these complexes, by controlling the orientation of the transition dipoles of the Bchl molecule (7).

Phytol is the most common esterifying alcohol of chlorophylls and Bchls (8-10). In this respect, Rhodospirillum rubrum differs from other purple bacteria such as Rb. sphaeroides and Rb. capsulatus, by employing the less saturated C\textsubscript{20} isoprenoid alcohol geranylgeraniol (GG) instead of phytol (11). Bchl\textsubscript{a}GG is a biosynthetic precursor of Bchl\textsubscript{a}P, and the product of a single gene is responsible for the three steps (Fig 1A)
necessary for reducing Bchl\textsubscript{a}GG to Bchl\textsubscript{a}P (10, 12, 13). This gene, \textit{bchP}, encodes the enzyme Bchl\textsubscript{a}GG reductase, and resides amongst other photosynthesis-related genes in a photosynthesis gene cluster (PGC) (12, 14, 15). Homologs of \textit{bchP} have been found in cyanobacteria and plants, where the gene product has been identified as the catalyst for the reduction of the isoprenoid moiety of chlorophyll (Chl) (16, 17).

Previous work has indicated that Bchl\textsubscript{a}GG permits assembly of both LH1 and LH2 in \textit{bchP} mutants of \textit{Rb. capsulatus} and \textit{Rb. sphaeroides} (10, 13). However, LH2 is severely affected by this change, while LH1 is not. As already noted, \textit{Rs. rubrum} uses Bchl\textsubscript{a}GG, and can be thought of as a naturally-occurring \textit{BchP} mutant (18). Recently it has been shown that normal BchP function can be restored to \textit{Rs. rubrum}, creating a new transconjugant strain possessing Bchl esterified with phytol (18). In this new strain of \textit{Rs. rubrum} the new phytolated pigments have been assembled into an RC-LH1 complex, but there are no structural or spectroscopic data available for this new reaction center-antenna complex.

The LH1 light harvesting complex of \textit{Rs. rubrum} is the best characterized of any such complex, with a projection map at 8.5 Å resolution generated from cryo-electron microscopy studies (19). More recently, cryo-electron microscopy has also revealed the projection structure of the reaction-centre/light-harvesting complex I (RC-LHI) from \textit{Rs. rubrum} at 8.5 Å which shows a single reaction centre surrounded by 16 LH1 subunits in a ring of approximately 115 Å diameter (20). The availability of a transconjugant strain of \textit{Rs. rubrum} containing phytolated Bchls (18) therefore provides the first opportunity to see how the provision of a different C20 alcohol moiety influences the \textit{in vivo} assembly, structure and function of this light-harvesting LH1 complex.

**Experimental Procedures**

\textit{Growth of Rs. rubrum and preparation of membranes---}Cells of \textit{Rs. rubrum} containing the \textit{bchP} expression plasmid pSK1\textit{bchP} (13) were grown photosynthetically at low light intensity (3 W/m²) at 20°C in M22+ medium in the presence of 250 ng/ml doxycycline (18). Cells were disrupted and cell-free ICM fractions isolated as described previously (21). Absorbance spectra were recorded on a Cary 500 spectrophotometer.
**HPLC analysis of pigments**—Carotenoid pigments were initially extracted into acetone/methanol 2:1 (v/v) and subsequently transferred into ether by the method of Britton and Riesen (22). The samples were then evaporated to dryness under N₂, and the pigments dissolved in ethyl acetate/acetonitrile/water 10:9:1 (v/v/v). Carotenoids were separated by HPLC on a Spherisorb ODS2 column (4.6φ × 250 mm) using a protocol provided by Professor A. Young (Liverpool John Moores University, UK). The flow rate was 1 ml/min, with an initial solvent composition of 20% ethyl acetate, 72% acetonitrile, 8% water, changing over the course of 6 min to 60% ethyl acetate, 36% acetonitrile, 4% water. This solvent composition was maintained for 15 min, during which time the carotenoids eluted. Elution of carotenoids was monitored using a Waters 996 photodiode array detector, scanning from 270 to 600 nm every 2 seconds. Chromatograms at 447 nm were derived from the accumulated absorbance scans using the Millenium software (Waters). Carotenoids were identified by their retention times on the column, and by their absorbance spectra.

**Purification of the RC-LH1 complex**—Cells of Rs. rubrum containing pSK1bchP were disrupted using a French Press at a pressure of 1450 kg / cm². The cell extract was applied to the top of a sucrose gradient (15-40% sucrose in Tris-HCl buffer, pH 7.9) and spun at 100,000 g for 4h at 4 ºC. The clear membrane band just above the 40% sucrose layer was collected and its protein concentration was determined by the BCA method (Pierce and Warriner Ltd. Chester, England). The detergent DHPC (23) was added to membranes drop-wise over 15 min with gentle stirring, with the ratio of 3 mmoles DHPC per microgram protein in membrane. The solution, after equilibrium for another 30 min, was centrifuged for one hour at 150,000 g to remove undissolved material, and then was purified with a DEAE anion exchange column (20φ × 70 mm). The column was eluted with a gradient of buffer A (10 mM Tris, 1 mM EDTA, 3 mM DHPC, pH 7.9) and buffer B (300 mM NaCl in buffer A). The main peak, which contains the RC-LH1 complex, corresponds to 250 mM NaCl. The best fractions judged from the 880:280nm absorbance ratio (>2.0) were concentrated and loaded on a gel filtration column (Hiload 16/60, Superdex 200 preparative grade). The column was washed by buffer C (50 mM NaCl in Buffer A). Fractions with an 880:280 absorbance ratio of more than 2.2 were pooled and used for crystallization.
**2-D crystallization**---The purified RC-LH1 sample was concentrated to approximately 1 mg/ml protein, and was then mixed with one of the lipids DOPC, POPC or POPC-DMPC (4:1 w/w) in a lipid/protein ratio (LPR) from 0.6 to 1.6 (w/w). The final sample, which was used for dialysis, was adjusted to 100 µl with a protein concentration of 0.5 mg/ml. The detergent was removed slowly by dialysis against a continuously-flowing buffer (10 mM HEPES, 100 mM NaCl, 0.01% NaN₃, pH 7.5). The temperature was controlled as described in Walz et al. (24). After 64 hours dialysis, samples were collected and stored at 4°C before use.

**Electron microscopy and image processing**---The quality of the 2-D crystals was checked by negative staining with 0.75% (w/v) uranyl formate. Electron micrographs were taken with 1024 × 1024 CCD camera (Gatan) attached to a Philips CM100 transmission electron microscopy operated at 100 kV with a nominal magnification of 28500 ×. For cryo-EM, 2-D crystals were adsorbed onto a 16 nm-thick carbon film mounted on molybdenum grid. The grid was washed twice with distilled water followed by 1% glucose. Electron micrographs were taken with a Philips CM200 FEG microscope operated at 200 kV acceleration voltage with the sample holder (Oxford Instruments) cooled by a liquid nitrogen. Low-dose images, about 5-10 e⁻/Å² at the specimen, were recorded at a nominal magnification of 50000 × on Kodak SO163 film and developed with Kodak D19 for 12 min. Crystalline areas on the film were checked roughly by optical diffraction and the well-ordered areas were digitized with a Zeiss SCAI scanner using a step size of 7 µm per pixel. The MRC image processing program package version 2000 was used for data processing (25,26).

**Low temperature absorption**---The purified RC-LH1 sample was mixed with 80% glycerol solution in 10 mM Tris-HCl, pH 8.0 to a final concentration of OD₈₈₀ = 0.4 - 0.6 and 60% glycerol. Sample was cooled down slowly under nitrogen gas using an Optistat™ LN-2 cooled bath cryostat (Oxford Instruments) coupled with a temperature detector. After the temperature had stabilized, the cryostat was placed in a Cary 500 UV-Vis-NIR spectrophotometer. Spectra were recorded with a resolution of 0.2 nm. Room temperature spectra of samples were recorded with the same instrument.
Fluorescence and CD spectroscopy---Fluorescence emission and excitation spectra of RC-LH1 complexes were measured using a SPEX Fluorolog FL3-22 spectrophotometer (Jobin-Yvon) equipped with xenon excitation and a CCD detector. The sample preparation and cooling procedure were the same as for the absorbance measurements. For excitation spectra the 425-625 nm excitation range covered the carotenoid and Bchl Q_{x} absorption region; detection of fluorescence emission was at 925 and 917 nm for wild-type and mutant complexes of *Rs. rubrum*, respectively.

CD spectra were recorded on a Jasco J810 spectropolarimeter at room temperature using a 5mm path length cell with resolution of 0.5 nm. The absorbance of each sample was adjusted to 1.0 in a 1 cm pathlength at the maximum in the near infrared region.
Results

Verification of BchlaP in the transconjugant strain of Rs. rubrum containing bchP---
HPLC traces of acetone-methanol extracts from whole cells are shown in Fig. 1B. Under the conditions used BchlαGG (Fig. 1Bi) had a retention time of approximately 18.4 min compared to 20.7 min for the BchlαP control (Fig. 1Bii). The HPLC profile of Bchls from the transconjugant Rs. rubrum (Fig. 1Biii) clearly indicates that introducing the bchP gene from Rb. sphaeroides into Rs. rubrum replaces the native BchlαGG with the phytolated pigment.

Alteration of BchlαGG to BchlaP has a marked effect on the carotenoid composition---The carotenoids in the wild type Rs. rubrum follow the spirilloxanthin branch of the biosynthetic pathway (Fig 2A). HPLC analysis of the carotenoids present in the strain of Rs. rubrum which contained the 'empty' pRKSK1 expression plasmid as a control, showed that spirilloxanthin is the major component as expected, along with minor amounts of 3,4-didehydrodorhodopin, rhodovibrin and anhydrorhodovibrin (Fig 2Bi). It was noticed that under photosynthetic conditions, cultures of Rs. rubrum [pSK1bchP] were distinctly red/brown in appearance, in contrast to the normal bright red colour found in wild-type or pRKSK1-containing Rs. rubrum. Consistent with this change in colour, Rs. rubrum [pSK1bchP] had a significantly altered carotenoid content (Figure 2B (ii)). Spirilloxanthin was present, but did not constitute a significantly greater proportion of the total carotenoid content than many of the other pigments present. The most abundant carotenoid was one of the previously mentioned spirilloxanthin precursors, putatively identified as rhodovibrin; the other carotenoids present appear to include rhodopin, 3,4-didehydrodorhodopin and anhydrorhodovibrin.

Spectroscopic studies of membranes containing the BchlaP LH1-RC mutant complex from Rs. rubrum---Absorbance spectra of the bacteriochlorophylls within bacterial LH1 complexes are sensitive to the aggregation state and environment of these pigments (21, 27), and therefore provide essential information on the effects of altering the esterifying pigments within the LH1-RC complexes studied. Low temperature absorbance spectroscopy revealed differences between RC-LH1 core complexes purified from wild-type and transconjugant strains; the Qy absorption maximum of the newly-phytolated
Bchlα molecules within LH1 is slightly blue-shifted in comparison with the native complex containing BchlαGG. At room temperature, this shift is about 2 nm (876 nm to 874 nm; data not shown) and it extends to approximately 4 nm (from 894 nm to 888 nm, see Fig. 3) at 77K, although the Qx maximum is maintained at these different temperatures. In the visible region, the different carotenoid composition of the mutant apparent from the HPLC analysis in Fig 2 is manifested as an altered series of absorbance bands. For the purified wild-type complex the bands at 457 (shoulder), 485, 517, 547 and 568 (shoulder) nm mainly reflect the presence of spirilloxanthin, whereas the increased number of well-resolved bands in this region for the mutant at 433, 457 (shoulder), 487, 505, 529, and 549 nm arises from the spirilloxanthin precursor rhodovibrin together with rhodopin, 3,4-didehyrdorhodopin and anhydrorhodovibrin. The absorbance spectrum of the membranes containing the mutant complex is also shown in Fig 3 for comparison; it demonstrates that the purified complex has retained the features of the membranes from which it was prepared, with regard to both the carotenoid and bacteriochlorophyll absorbance bands in the visible and near infra-red regions of the spectrum, respectively.

The blue shift in absorbance for the mutant is reflected in the fluorescence emission spectra. Fluorescence emission spectra of the purified RC-LH1 core complexes recorded at 77K showed a clear difference in emission maximum, with a blue shift of 8 nm in the BchlαP mutant relative to the control (Fig 4A). To observe any energy transfer from carotenoids to Bchls in the RC-LH1 core complex containing BchlαP, an excitation spectrum of the carotenoid region was recorded (Fig. 4B), with detection of emission from the Bchls. The excitation spectra were normalised to the Bchl Qx band, in order to provide an indication of the relative efficiencies of the energy transfer process. For the wild-type control three main excitation peaks are observed, at 481, 515 and 549 nm, in approximate agreement with the absorbance spectra and therefore reflecting the dominance of spirilloxanthin in terms of carotenoid composition. An efficiency of approximately 36% can be estimated, which is in reasonable agreement with the figure of ~35% obtained by Duysens (28), cited in (29). In contrast the excitation spectrum of the BchlαP mutant shows six well-defined peaks, at 432, 457, 487, 503, 529 and 549 nm reflecting the presence of several carotenoids in this mutant, such as rhodovibrin, rhodopin, 3,4-didehyrdorhodopin and anhydrorhodovibrin.
CD spectroscopy can provide a very sensitive indicator of alterations in the aggregation state and orientation of bacterial LH complexes (for example, 30-33). Near-IR CD spectra of intact membranes containing wild-type and mutant complexes were therefore recorded at room temperature and are presented in Fig 5B. The corresponding absorbance spectra are shown in Fig 5A, in which the shift to the blue noted for the purified Bchl\(\text{a}\) complex in Fig 3 relative to the WT is also seen for the membrane sample; this time the shift is 4 nm. Similarly, the CD spectrum of membranes containing the Bchl\(\text{a}\) RC-LH1 complex was blue-shifted in comparison with the WT. The differences between the CD spectra are the zero crossing points and the peak intensities. The zero crossing point for the WT complex corresponds exactly to the absorbance maximum, whereas in the case of the Bchl\(\text{a}\) mutant this zero crossing point is blue shifted by 2 nm with respect to absorbance maximum. The peak intensity ratios at 870/897 nm for the mutant and 865/892 for the wild type are similar, and overall, the shapes of the two spectra differ only slightly.

2-D crystallization of the RC-LH1 complex containing Bchl\(\text{a}\)---Recent progress in the preparation of RC-LH1 crystals from \textit{Rs. rubrum} has allowed the projection structure of this complex to be determined at 8.5 Å resolution (20). This shows a single reaction centre surrounded by 16 LH1 subunits in a ring of approximately 115 Å diameter. Within each LH1 subunit, densities for the \(\alpha\)- and \(\beta\)-polypeptide chains are clearly resolved. The availability of a mutant of \textit{Rs. rubrum} provided the opportunity to see if alteration of the Bchl tail influences the structure and organization of this complex.

The RC-LH1 complex was purified from membranes prepared from the Bchl\(\text{a}\)-synthesising strain of \textit{Rs. rubrum} and was used in crystallisation trials. The best quality 2-D crystals were formed with DOPC/protein (w/w) ratio of 1.0 after 64 hours dialysis; the typical size of a vesicular crystal was about 1600 x 1600 nm (Fig 6). The quality of crystal was judged by negative stain EM, with more than 4 orders of calculated diffraction. All individual images showed that the mutant RC-LH1 core complex forms a 2-D crystal with a square lattice and \(p42_12\) space group symmetry.

The crystals were embedded in glucose and were found to be of sufficient quality to use for cryo-EM; the contrast transfer function (CTF) plot of a Fourier transform of a typical
image is shown in Fig. 7. The phase residuals in resolution shells are summarized in Table 1.

Fig 8A shows a projection map displayed with $p1$ symmetry, within which densities forming two concentric rings can be seen, representing membrane-spanning $\alpha$ and $\beta$-helices. The densities in the middle are contributed by helices in the reaction center. Three individual unbent, CTF-corrected lattices were merged and $p42_12$ symmetry applied, giving the image in Fig 8B. A high-resolution map (Fig 8C) has been calculated with a resolution of 9 Å, and it is clear enough to show that the RC sits in the middle of the ring, surrounded by a closed ring of LH1. Rotational power spectrum analysis of the density from Fig 8B, shows a strong 16-fold component far above the noise level (Fig 8D). The lattice parameters $a$ and $b$ calculated from high-resolution images are $168 \pm 0.5$ Å, which corresponds to a molecule diameter of 116 Å. This diameter of the BchlaP LH1 ring is in close agreement with the figure of 115 Å for the wild-type LH1 complex, also determined by cryo-EM (20).
Discussion

Alteration of pigment synthesis in a transconjugant strain of Rs. rubrum containing the bchP gene from Rb. sphaeroides: Bchls and carotenoids---We recently demonstrated that BchP enzyme of Rb. sphaeroides converts BpheaGG to BpheaP, as well as BchlaGG to BchlaP, whereas BchP of Rs. rubrum is restricted to the former of these activities (18). As such, Rs. rubrum is a naturally-occurring bchP mutant. In addition, it was shown that introducing the Rb. sphaeroides bchP gene into Rs. rubrum generates a novel transconjugant strain in which the Bchls are esterified with phytol. Given that the LH1 antenna complex of Rs. rubrum has been heavily studied using structural, spectroscopic and reconstitution techniques (for example 19, 20, 29, 34-36), we decided to investigate this new Rs. rubrum core complex containing BchlaP.

It was a surprise to find that an altered carotenoid composition had accompanied the change from BchlaGG to BchlaP. The transconjugant strain had fallen short of completing the carotenoid biosynthetic pathway to spirilloxanthin (see Fig 2A). The close match between the absorbance spectra of the membrane and the purified complex for the mutant in the 400-600 nm region (Fig 3) suggests that the carotenoid composition of the membranes (Fig 2B) is also reflected in the purified complex. The BchlaP-synthesising transconjugant strain accumulated several biosynthetic intermediates, among them rhodovibrin and rhodopin. The reason for this is unclear; one possibility is a linkage between the carotenoid and (bacterio)chlorophyll biosynthetic pathways at the level of GGPP. In the former case two molecules of GGPP can condense form the C-40 carotenoid phytoene (37), and, in the second case, GGPP (either 'free' GGPP or attached to the (B)chlide macrocycle as (B)chlGG) can be reduced to form phytol pyrophosphate (PPP) or (B)chlaP (13, 17). The Rb. sphaeroides GGPP reductase, when introduced into Rs. rubrum, could therefore deplete a common pool of GGPP, thus depriving the cell of the GGPP molecules necessary to maintain normal functioning of its carotenoid biosynthetic pathway. A second possibility is that the linkage between the type of Bchl tail and the carotenoid reflects structural factors imposed by the presence of a less rigid phytol tail, which in turn influences the type of carotenoid that can be efficiently assembled within the mutant complex.
The assembly of the carotenoid biosynthetic intermediates rhodovibrin, rhodopin, 3,4-didehydrorhodopin, and anhydrorhodovibrin into the RC-LH1 complex did take place, as judged by the appearance of a series of new peaks in both the absorbance and excitation spectra of purified complexes in Figs 3 and 4B. The excitation spectra also reveal that these new carotenoids are at least partially efficient in transferring energy to the Bchls within LH1. In fact the native carotenoid, spirilloxanthin, is itself only 36% efficient in this respect; such inefficiency is the consequence of the relatively high number (13) of conjugated C=C bonds in spirilloxanthin (38) which lowers the S1 state of the carotenoid relative to the S1 state of the Bchl Qy transition (39).

Spectroscopic properties of the membrane-bound mutant RC-LH1 complex from Rs. rubrum containing BchlαP---Phytol tails are predicted to be less rigid than GG tails, so the conversion of BchlαGG to BchlαP in the RC-LH1 complex might be expected to change the ways in which these tails pack and interact with the LH polypeptides and carotenoids. For example, it has been observed that the phytol tails of the Bchls bound to the α polypeptides are nearly fully extended and make close contacts with the carotenoids in the LH2 complex of Rps acidophila (7). Consequently, the conversion of BchlαGG to BchlαP in the RC-LH1 complex could alter the mutual orientation of pairs of antenna-bound Bchl molecules in the LH1 ring, with attendant effects on the absorbance, fluorescence emission and CD spectra.

As noted previously (21) the absorbance spectra of membranes, which contain RC-LH1 complexes in an aggregated form, are shifted to the red in both mutant and wild-type, when compared to purified complexes (882/876 nm for the wild type; to 877/874 nm for the mutant). Upon re-aggregation to form 2-D crystals the spectrum of the purified RC-LH1 of Rs. rubrum complex shifts back to the red by a few nm (21). Thus, this reversible shift could provide a simple indication of the aggregation state of the complex. Similar shifts to the blue and back again to the red have been seen for membranes, purified monomeric complexes, and 2D crystals of the LH2 complex from Rb. sphaeroides (24), which suggests that this is a general property of bacterial LH complexes. In the present work the extent of the blue shift provoked by liberation of complexes from the membrane by detergent treatment is 6 nm for the wild-type and only 3 nm for the mutant, which
might indicate that in its native membrane environment the mutant RC-LH1 complex containing Bchl\(\alpha\)P is in a less aggregated form than the wild-type complex.

The CD spectra of the membrane-bound mutant and wild-type RC-LH1 complexes were measured in order to provide information on the complexes in their native environment (Fig 5B). The spectra are very similar, showing a non-conservative spectrum mainly arising from the LH1 bchls. The differences between the spectra are the zero crossing points and the peak intensities, with the zero crossing point of the Bchl\(\alpha\)P mutant shifted 2 nm to the blue, over and above the 5 nm shift in the absorbance spectra. The effects of these shifts are that the zero crossing point for the WT complex corresponds exactly to the absorbance maximum, whereas in the case of the Bchl\(\alpha\)P-containing mutant this zero crossing point is blue shifted by 2 nm with respect to absorbance maximum. The significance of CD zero crossing/absorption shifts has been discussed and modelled extensively by Koolhaas \textit{et al.} (30), in the context of the LH2 complex. They conclude that the CD/absorption red shift seen for LH2 arises from an energy mismatch between the \(\alpha\)- and \(\beta\)-bound B850 Bchls. It is also interesting that dimeric LH systems have been observed to exhibit a CD/absorption blue shift (31,32). We suggest that the appearance of a CD/absorption blue shift in the Bchl\(\alpha\)P-containing mutant is a consequence of the new phytol tail exerting an influence on the relative distances and orientations of the Bchls, simultaneously decreasing the energy mismatch between the \(\alpha\)- and \(\beta\)-bound B875 Bchls and increasing their dimeric character. We note however that the CD spectra of LH complexes are extremely sensitive to small changes in the angles made by Q\_y transition moments between paired bchls (31, 33), so our data indicate that there are no large-scale changes to the geometry of the Bchls in the mutant. One reason for expecting significant changes was the work of Parkes-Loach \textit{et al.} (35), who compared the CD spectra of LH1 complexes from \textit{Rs. rubrum} reconstituted from purified bchl and polypeptide components. This approach made it possible to compare the effects of using different pigments, in this case Bchl\(\alpha\)P and Bchl\(\alpha\)GG. Their CD spectra showed that Bchl\(\alpha\)P was incapable of restoring the normal CD spectrum seen using Bchl\(\alpha\)GG, and an inverted spectrum was obtained. Similarly, \textit{in vitro} experiments with RC complexes reconstituted with Bchl\(\alpha\)P showed that this pigment cannot substitute for Bchl\(\alpha\)GG in \textit{Rs. rubrum} (8). Our results, using a genetic approach to alter pigment
composition, show that Bchl\textsubscript{a}P does substitute for Bchl\textsubscript{a}GG \textit{in vivo}, in both the LH1 and RC complexes. Bollivar et al. (10) working in the other direction by substituting native Bchl\textsubscript{a}P with Bchl\textsubscript{a}GG in \textit{Rb. capsulatus}, concluded that both light harvesting and photochemical functions of LH1 and RC complexes were unaffected. It should be borne in mind that both carotenoids and reaction centers are absent from the reconstitution work in Parkes-Loach et al (35). Possibly one or both of these components, as well as LH1-RC-specific assembly factors (40-42) act as an additional constraints on the way in which Bchl geometries are assembled within LH1 \textit{in vivo}.

\textit{The structure of the RC-LH1 complex containing Bchl\textsubscript{a}P}---The LH1 and RC-LH1 complexes of \textit{Rs rubrum} have repeatedly demonstrated a tendency to form well-ordered 2D crystals \textit{in vitro} (19), using carotenoid-less B820 dimers, or carotenoid-containing RC-LH1 complexes (20) as starting material. The present work on the Bchl\textsubscript{a}P complex further emphasizes the suitability of the \textit{Rs. rubrum} RC-LH1 complex for 2D structural studies. This complex was examined using purification and crystallization approaches similar to those used successfully for the wild-type RC-LH1 complex from \textit{Rs. rubrum} (20). This recent cryo-electron microscopy study revealed the projection structure of this complex at 8.5 Å resolution, and showed that it consists of a single reaction centre surrounded by 16 LH1 subunits in a ring of approximately 115 Å diameter. The availability of a transconjugant strain of \textit{Rs. rubrum} containing phytolated Bchls (18) provided an opportunity to see if the provision of a different C20 alcohol moiety had affected the \textit{in vivo} assembly, structure and function of this light-harvesting LH1 complex.

The data show that this mutant complex (diameter 116 Å) has an architecture very similar to that of the native complex (diameter 115 Å; 20). One striking finding for the wild-type complex was the occurrence of both tetragonal and orthorhombic crystal forms, comprising ordered arrays of circular and elliptical complexes, respectively. This reveals a degree of flexibility in the LH1 ring, which might be important for its function in terms of allowing reduced quinone to leave the reaction center. The fact that 20 individual images of the crystals from the Bchl\textsubscript{a}P mutant show only the tetragonal form does not allow us to draw any conclusions, in terms of any effects of Bchl\textsubscript{a}P on the flexibility of the LH1 ring. However, since the mutant grows photosynthetically, it can be assumed
that Bchl aP does not impair the assembly or function of RC complexes in *Rs. rubrum*, and that quinone transfer from the RC can still proceed.

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References

### Abbreviations

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<td>2-D</td>
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<td>3-D</td>
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<td>BChl</td>
<td>Bacteriochlorophyll</td>
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<td>Bchl&lt;sub&gt;aP&lt;/sub&gt;</td>
<td>Bacteriochlorophyll &lt;i&gt;a&lt;/i&gt; esterified with phytol</td>
</tr>
<tr>
<td>CTF</td>
<td>Contrast transfer function</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<td>CCD</td>
<td>Charge coupled device</td>
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<td>DHPC</td>
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<td>DMPC</td>
<td>1,2-Dimyristoyl-&lt;i&gt;sn&lt;/i&gt;-Glycero-3-phosphocholine</td>
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<td>Ethylene diaminetetra-acetic acid</td>
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<td>Electron microscopy</td>
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<tr>
<td>FFT</td>
<td>Fast Fourier transform</td>
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<tr>
<td>GGPP</td>
<td>Geranylgeranyl pyrophosphate</td>
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<tr>
<td>HEPES</td>
<td>(n-[2-Hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid])</td>
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<tr>
<td>ICM</td>
<td>Intracytoplasmic membrane</td>
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<td>LPR</td>
<td>Lipid to protein ratio</td>
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<td>PPP</td>
<td>Phytol pyrophosphate</td>
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<tr>
<td>RC-LH1</td>
<td>Reaction center - light-harvesting complex I</td>
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Figure legends

Figure 1. (A) Proposed biosynthetic pathway of the terminal hydrogenation of bacteriochlorophyll based on the chlorophyll biosynthesis in green plants (43). DHGG, dihydro-GG; THGG, tetrahydro-GG and BchlP. (B) HPLC profile of acetone-methanol extracts from whole cells of: (i) wild-type Rs. rubrum; (ii) wild-type Rb. sphaeroides and (iii) transconjugant Rs. rubrum containing the bchP gene from Rb. sphaeroides. The peaks labeled on the HPLC traces correspond to: 1, BchlGG; 2, BchlDHGG; 3, BchlTHGG and 4, BchlP. The detector was set at 375 nm.

Figure 2. (A) Probable pathway of carotenoid biosynthesis in Rs. rubrum. This linear scheme does not depict a cryptic branch of the pathway in Rs. rubrum, which became apparent in a mutant lacking the rhodopin 3,4-desaturase (44). (B) Carotenoid analysis by HPLC. The traces correspond to extracts from (i) the wild type Rs. rubrum control containing the 'empty' pRKSK1 expression plasmid and (ii) the transconjugant Rs. rubrum mutant containing the bchP gene from Rb. sphaeroides. Labeled peaks are: (1) rhodovibrin, (2) a neurosporene-type pigment, (3) rhodopin, (4) spirilloxanthin, (5) 3,4-didehydrorhodopin, and (6) anhydrorhodovibrin. The detector was set at 447 nm.

Figure 3. Absorbance spectra at 77 K of RC-LH1 core complexes of mutant and wild type strains of Rs. rubrum. Dot-dash line, membranes used as the source of mutant complex containing BchlαP; Solid line, RC-LH1 complex containing BchlαP; dotted line, wild type Rs. rubrum.

Figure 4. Fluorescence spectroscopy at 77K of RC-LH1 core complexes purified from mutant and wild type strains of Rs. rubrum. (A) Normalised emission spectra from complexes excited in the visible region at 505 nm. Solid line, RC-LH1 complex containing BchlαP; dotted line, wild type Rs. rubrum. (B) Excitation spectrum with detection of emission at 926 nm for the wild type complex and at 918 nm for the mutant complex. (A). The excitation spectra were normalised at the Bchl Qx band at ~590 nm. Lines as for (A).

Figure 5. A comparison of CD spectra recorded on membranes from the WT and mutant strains of Rs. rubrum. Three scans were averaged for each spectrum; these spectra have
been normalized to the same near-infrared absorbance at approximately 880nm. Solid line, RC-LH1 complex containing BchlP; dotted line, wild type *Rs. rubrum*.

**Figure 6.** Electron micrograph of negatively stained 2-D crystal of the RC-LH1 complex containing BchlP from *Rs. rubrum*. The crystal was reconstituted with lipid DOPC at LPR of 1.0 by continuous flow dialysis for 64 hours. The scale bar represents 100 nm. The insert shows a computer-generated diffraction pattern from the electron micrograph.

**Figure 7.** The contrast transfer function (CTF) plot of a Fourier transform of a typical image of an RC-LH1 crystal embedded in glucose. The circles represent the zero transitions of the CTF and the boxed numbers indicate the IQ values of the individual reflections (25).

**Figure 8.** Electron cryo-microscopy of glucose-embedded 2-D crystals of RC-LH1 complexes containing BchlP. (A) Projection map displayed with *p1* symmetry. The densities forming two concentric rings represent membrane-spanning *α* and *β*-helices. The densities in the middle are contributed by helices in the reaction center. (B) Merged projection map from three individual unbent, CTF-corrected lattices and with *p4212* symmetry applied. (C) The 16-fold rotationally filtered image extracted from Fig 8B (D) The rotational power spectrum for one ring of density of merged images using the calculation method in Crowther and Amos (45).
Table 1. Mean phase residuals in resolution shells for the merged image with $p42_12$ symmetry

<table>
<thead>
<tr>
<th>Resolution range (Å)</th>
<th>Number of spots</th>
<th>Mean phase residuals (°) (random=45°)</th>
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<td>36.7</td>
<td>2.0</td>
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<tr>
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<td>54</td>
<td>39.8</td>
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<tr>
<td>12 – 9</td>
<td>59</td>
<td>35.3</td>
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Figure A: Fluorescence spectrum for purified wild type and purified mutant RC-LH1 complexes. The peaks at 918 and 926 nm indicate the excitation maxima for these complexes.

Figure B: Fluorescence spectrum showing multiple peaks at 432, 457, 481, 487, 503, 515, 520, 546, 549, and 590 nm. These peaks correspond to different wavelengths of fluorescence emission.
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Pu Qian, Hugh A. Addlesee, Alexander V. Ruban, Peiyi Wang, Per A. Bullough and C. Neil Hunter

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