Hydrogen Bonding and Circular Dichroism of Bacteriochlorophylls in the *Rhodobacter capsulatus* Light-Harvesting 2 Complex Altered by Combinatorial Mutagenesis†

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ABSTRACT: We have investigated the spectroscopic properties of two classes of light-harvesting 2 (LH2, B800−850) mutants of *Rhodobacter capsulatus* obtained by combinatorial mutagenesis to the C-terminal half of the β-apoprotein: a pseudoLH2 (pLH2) class, in which the 800-nm absorption was normal but the 850-nm peak was blue-shifted by up to 14 nm, and the other a pseudoLH1 (pLH1) class, which lacked the 800-nm absorption band and showed 850-nm absorption red-shifts of up to 30 nm. In several of the pLH1 antennae, carotenoid depletion contributed to the phenotype, while in the pLH2 complexes there was some carotenoid enrichment. A number of mutants from each class have also been characterized by low-temperature absorption and fluorescence spectroscopy, resonance Raman spectroscopy, and circular dichroism. In all of the mutants investigated, the B850 bacteriochlorophyll *a* binding site remained intact, conserving both the hydrogen bonding environment of the chromophores and their conformation and liganding. In contrast, the intensity of the CD spectra of pLH1 complexes was considerably reduced, relative to that of wild-type or pLH2 complexes, consistent with alterations in the interactions between pigments and in their relative orientation. Elevated fluorescence polarization over the red wing of the B850 band in the pLH2 complexes indicated a reduction of exciton mobility within the ring of BChl molecules. Possible structural alterations governing the spectral properties of the different mutants are discussed.

The photosynthetic units of *Rhodobacter capsulatus* consist of three bacteriochlorophyll *a* (BChl)[1] binding integral membrane proteins: the peripheral antenna LH2 (B800−850), the core antenna LH1 (B880), and the photochemical reaction center. Radiant energy collected by LH2 is rapidly transferred to LH1, which directs excitations to the reaction center, where they are transduced into a transmembrane charge separation *(1)*. LH2, the most abundant pigment−protein complex in cells grown at low light intensity, is composed of two apoprotein subunits (α- and β-), containing 60 and 49 amino acid residues, respectively *(2, 3)*, which bind two B850 BChls, one B800 BChl, and one to two carotenoids, as well as a 14-kDa γ-subunit *(4)*. The LH2 and LH1 complexes are highly conserved among a large number of photosynthetic bacteria *(5)*, exhibiting a common tripartite structure with a single transmembrane α-helix separating N- and C-terminal domains at the cytoplasmic and periplasmic surfaces of the intracytoplasmic membrane, respectively.

The three-dimensional structure of the LH2 complex from *Rhodopseudomonas acidophila*, homologous to the one studied here, has been determined by X-ray crystallography *(6)* and shown to consist of two concentric cylinders of transmembrane helices with the β- and α-apoproteins, comprising the respective outer and inner walls. A ring of 18 B850 BChls with overlapping bacteriochlorin molecules is sandwiched between the polypeptide subunits, while the nine B800 BChls are positioned between the outer helices. One of the two carotenoid molecules is intertwined with the phytol chains of the BChls and almost completely spans the membrane. More recently, a similar structure, but containing eight rather than nine $\alpha\beta$-units, has been determined for the crystallized LH2 complex of *Rhodospirillum molischianum (7)*.

Prior to elucidation of the LH2 crystal structure, pigment−protein interactions had been identified by site-directed

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[1] Abbreviations: BChl, bacteriochlorophyll *a*; CD, circular dichroism; FT, Fourier transform; ICM, intracytoplasmic membrane; LDAO, lauryl *N*,*N*-dimethylamine-*N*-oxide; LDS, lithium dodecyl sulfate; LH1, core light-harvesting complex with near-IR absorption maximum at ~880 nm; LH2, peripheral light-harvesting complex with near-IR absorption maxima at ~800 and 850 nm; β-OG, *n*-octyl-β-D-glucopyranoside; pLH1, pseudoLH1 complex lacking B800 with red-shifted B850 band, arising from LH2; pLH2, pseudoLH2 complex retaining B800 with blue-shifted B850 band; RC, reaction center.

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Table 1: *Rhodobacter capsulatus* Combinatorial Mutants Used in This Study

strain[a]	phenotype	sequence[b]	reference
Wild−type	LH2+	RVFGAMALVAHILSAIATPWLG	*(2)*
S2	pLH1[c]car-[d]	RVFLAMGLVAHILAAIATPWLG	*(25)*
S4	pLH1	RVFVAMGVVAHILAGIATPWLG	
S5	pLH1 car-	RVFLAMAAVAHILAGIATPWLG	
1.4.1	pLH1	ILFLWVALVAHILSAIATPWLG	*(28)*
1.4.2	pLH1 car-	RVWAAGALVAHILSAIATPWLG	
1.4.4	pLH1	YQWLLWALVAHILSAIATPWLG	
1.4.8	pLH1	LMYAWLALVAHILSAIATPWLG	
REM1.8	pLH2[e]	RVFGAMALVAHILSAILIWPVL	*(27)*
REM1.9	pLH2	RVFGAMALVAHILSAIALWPLV	

[a] Isolated as described in Materials and Methods. [b] Mutations in portions of *puc*B gene encoding the C-terminal half of the LH2-β-polypeptide (residues 28−49); altered residues underlined. Residues 34−39 have also been mutagenized *(26)*, but the resulting combinatorial mutants were not examined here. [c] Pseudo-LH1 antenna lacking B800 with red-shifted B850 band. [d] Carotenoid-depleted. [e] Pseudo-LH2 antenna with blue-shifted B850 band.

mutagenesis of amino acid residues in both the LH1 *(8−11)* and LH2 complexes *(12−15)*. However, alteration of one, or at most two residues at a time, samples protein sequence space insufficiently to permit the isolation of many mutants with highly interesting and informative phenotypes. Indeed, even in the case of the photosynthetic reaction center, where the high-resolution structure has been known for some time *(16−18)*, important functional residues or combinations of residues may not be obvious, which is illustrated by the difficulties that have been encountered in symmetrizing electron flow *(19, 20)*.

Fortunately, through the simultaneous introduction of multiple mutations at different positions in a protein sequence by combinatorial mutagenesis, mutants with desired phenotypes can be obtained from a large library of expressed proteins *(21)*. Protein diversity, useful in creating improved catalytic capabilities or binding sites, can be maximally exploited, as in the engineering of new antibodies for expression in phage display libraries *(22)*. Moreover, compensatory residue changes can be introduced that permit expression of proteins with mutations that would normally give rise to unstable structures, and multiple mutations can produce phenotypes that differ from the sum of the characteristics of individual changes. Hydropathy and molar volume constraints can also be systematically studied, as has been demonstrated for bacterial reaction centers *(23)*.

The *R. capsulatus* LH2 complex, together with automated colony screening by digital imaging spectroscopy *(24)*, has provided a useful model for developing combinatorial mutagenesis strategies *(25−28)*, since this protein is readily amenable to molecular genetic analysis *(2, 29)*, and both B800 and B850 BChls can serve as colorimetric indicators of protein expression and assembly. Mutagenesis in the C-terminal half of the LH2-β-apoprotein gave rise to two spectroscopic classes with altered LH2 complexes: a pseudo-LH1 (pLH1) phenotypic group characterized by the absence of B800 and a red-shift in the B850 band; and a pLH2 group that retained an LH2-type spectrum, albeit with a B850 blue-shift in some strains. The low-temperature optical properties, resonance Raman spectra, circular dichroism, and stability of several modified LH2 proteins produced by these strains are described here. Despite the multiple mutational changes, the observed alterations could be largely attributed to residues homologous to those found to be of structural and functional importance in the crystal structure *(6)*, demonstrating the utility of combinatorial mutagenesis in revealing crucial residues in an integral membrane protein (for a preliminary report on some of these studies, see ref *30*).

MATERIALS AND METHODS

The *R. capsulatus* strains used in this study are listed in Table 1. In constructing strains S2, S4, and S5, phylogenetic constraints *(5)* were used to limit the size of codon target sets for simultaneous mutagenesis of seven amino acids, including the β-BChl liganding βHis38, modeled to lie on the same face of the transmembrane α-helix as the B850 BChl binding site *(25)*. Random mutagenesis of residues 28−33 (RVFGAM) gave rise to strains 1.4.1, 1.4.2, 1.4.4, and 1.4.8 *(28)*. Optimizing algorithms, using information from previous rounds of mutagenesis of the six C-terminal residues, were used to generate strains REM1.8 and REM1.9 *(27)*.

All combinatorial mutants were expressed in *R. capsulatus* strain U71 (LH2 chromosomal deletion background with LH1 and reaction centers expression inactivated by a point mutation)*(29)*, which produced sufficient levels of the modified LH2 complexes for these studies. To ensure that the unique pLH1 spectral properties did not arise solely from the expression of residual LH1 or by complementation of LH2-α by LH1-*β*, the pLH1 mutant libraries in ref *25* were also expressed in *Udd4*, an LH1$^-$ LH2$^-$ chromosomal deletion background. Spectra of these pLH1 strains were unchanged in this double deletion background, demonstrating that their pLH1 proteins were indeed encoded by the modified, plasmid-borne LH2 genes *(25)*. Control strains were U15g(pU2922)(RC$^+$ LH1$^+$ LH2$^+$) pseudo-wild-type and U43(pU2922)(RC$^+$ LH1$^+$ LH2$^-$), constructed by complementation of background strains (U15g and U43) *in trans* with pU2922, carrying the *puf* operon encoding the LH1-α- and -*β*-polypeptides and the L and M subunits of the reaction center *(31)*.

The various strains were grown semiaerobically in the dark at 32 °C for 2−3 days in a mixture of RCV and MPYE media (2:1 v/v)*(32)* supplemented with tetracycline (2.5 *μ*g/mL). Cells were disrupted in a French press at 20 000 lb/in^2, cell-free extracts were applied to sucrose gradients (20−60% wt/wt), and ICM vesicles (chromatophores) were isolated by isopycnic centrifugation *(33)*. Chromatophore bands were pooled and sedimented by centrifugation 1 h at 70 000 rpm in a Beckman 75Ti rotor at 4 °C.

The pLH1 complexes were extracted from chromatophores by treatment with 1% (wt/v) *β*-OG for 20 min in an ice bath in the dark and purified on a step gradient consisting of 0.3, 0.6, and 1.2 M sucrose prepared in 50 mM Tris-HCl, pH 7.5, containing 0.2% *β*-OG. Gradients were centrifuged in a Beckman SW40Ti rotor at 30 000 rpm for 18 h at 4 °C, and antenna bands were concentrated by further centrifugation at 70 000 rpm in the 75Ti rotor for 1.5 h at 4 °C, after dialysis against 0.5% *β*-OG in the above Tris buffer. The pLH2 complexes were extracted with 1% (wt/v) LDAO for 30 min at room temperature in the dark and isolated by centrifugation on a sucrose step gradient as described above with sucrose solutions and dialysis buffers containing 0.2% LDAO. Purified chromatophores and pLH1 and pLH2 complexes were stored in 25% (v/v) glycerol at −20 °C. Denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis *(4)* showed that while the isolated complexes were not pure on a protein basis, they remained intact with the LH2-α-, and -*β*-apoproteins, and usually the LH2-*γ*-polypeptide, clearly evident within these antenna-enriched detergent extracts (not shown). A faint band running at ∼12 kDa, apparently representing residual LH1-α, was also observed, especially in preparations from the pLH2 strains, but the LH2-α and -*β* polypeptides were major bands in the low-molecular-weight region of the gel in all purified detergent extracts from strains with both pLH1 and pLH2 phenotypes.

For pigment analysis, chromatophores were extracted with acetone:methanol:water (7:2:1, v/v). BChl was estimated from the absorbance at 770 nm using the extinction coefficient of Clayton *(34)* and spheroidenone levels were determined at 484 nm using the extinction coefficient of Cohen-Bazire et al. *(35)*.

Absorption, fluorescence excitation, and emission spectra at 77 K were obtained as described previously *(36)* with a Johnson Research Foundation DBS-3 dual-wavelength spectrophotometer (University of Pennsylvania, Philadelphia, PA), modified for fluorometry and equipped with an Oxford DN1704 variable-temperature liquid-nitrogen cryostat (Oxford Instruments, Osney Mead, U.K.). Samples were suspended in 60% (v/v) glycerol; control chromatophores were frozen under illumination with red light to accumulate reaction centers in the photooxidized state. Fluorescence polarization measurements were made with HR sheet polarizers (Polaroid Corporation, Cambridge, MA), with correction for residual transmission resulting from incomplete blockage of the perpendicular component of the electric vector, as described (W.H.J. Westerhuis and R.A. Niederman, in preparation).

Near-IR CD spectra were obtained at room temperature on a Jasco J-500C spectropolarimeter, provided by Dr. Thomas Strekas of the City University of New York. FT Raman spectra were recorded at 4 cm^{-1} resolution using a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module equipped with a continuous Nd:YAG laser, which ensured preresonance with the long-wavelength BChl Q_y transition. The apparatus, laser powers, and sample behavior have been extensively described *(37)*. Depending on the samples, spectra were the result of 3000 to 10000 co-added interferograms.

RESULTS

The mutants used in this study (Table 1), fall broadly into two categories based on their spectral properties: pLH2, containing the normal double maximum in their near-IR absorption spectrum composed of a peak near 800 nm and a second peak at longer wavelengths near 845 nm; and pLH1, in which the peak near 800 nm characteristic of LH2 complexes is absent. These mutants were derived from three different combinatorial mutagenesis procedures, targeting different regions of the LH2-*β*-polypeptide. Mutations at positions lying one and two helical turns either side of the *β*His38-liganded B850 BChl allowed selection of pLH1 (S2, S4 and S5) phenotypes. Strains 1.4.1, 1.4.2, 1.4.4, and 1.4.8, that were mutated in residues 28−33 of the *β*-polypeptide (RVFGAM), also gave rise to pLH1 phenotypes. Mutagenesis of the six C-terminal residues of LH2-*β* yielded mutants REM1.8 and REM1.9 in which the penultimate and strongly conserved residue pair (PW) was inverted to WP; both of these mutants formed pLH2 complexes.

Low-Temperature Optical Properties and Stability of pLH1 and pLH2 Complexes. Low temperature (77 K) near-IR absorption spectra of chromatophores from the various strains examined in this article are shown in Figure 1. The long-wavelength shoulder or peak, near 890 nm, visible especially in panel C, is attributed to a residual LH1-like contaminant that arose from the background strain, and has been discussed previously *(29)*.

The spectra of the pLH1 strains at 77 K (S2, S4, and S5 in Figure 1A, and 1.4.1, 1.4.2, 1.4.4, and 1.4.8 in Figure 1B) showed that both B800 and free BChl bands were absent and that the B850 bands were red-shifted, resulting in spectra nearly identical to the LH1 control. The spectra of the pLH2 mutants (REM1.8 and 1.9) which have ∼14-nm blue-shifts

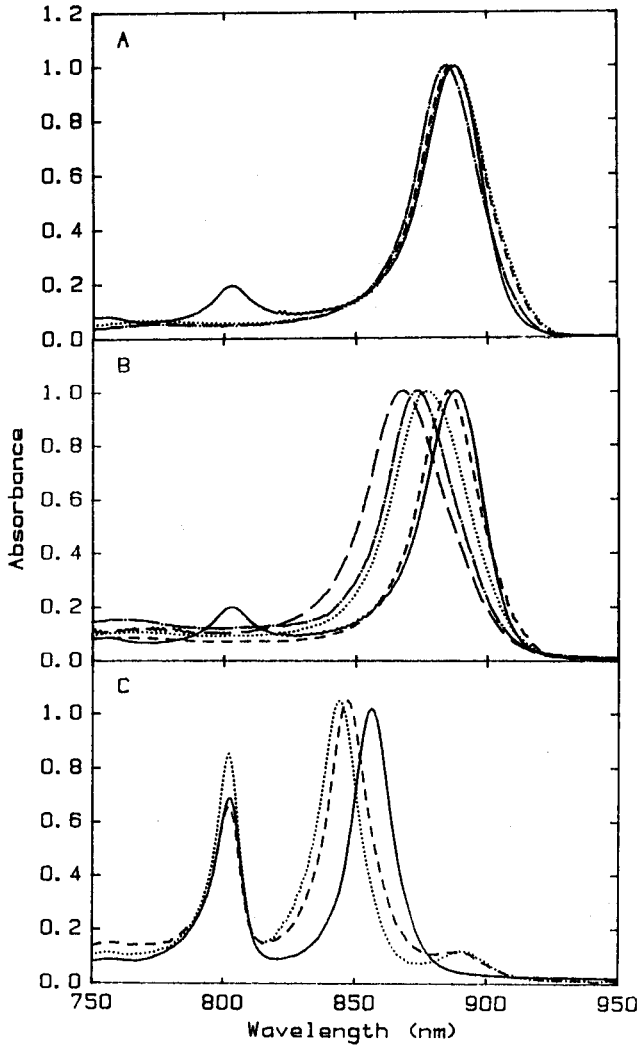

FIGURE 1: Near-IR absorption spectra of chromatophores from mutant and control strains at 77 K. Spectra were normalized at absorption maxima of Q_y band. (A) (—), control strain U43(pU2922)(RC^+ $LH1^+$ $LH2^-$); (- - -), strain S2; (···), S4; and (—·—), S5. (B) (—), control strain U43(pU2922); (- - -), strain 1.4.1; (···), 1.4.2; (—·—), 1.4.4; and (— —) 1.4.8. (C) (—), LH2 complex from pseudo-wild-type strain U15g(pU2922); (- - -), strain REM1.8; and (···), REM1.9.

in their B850 band at 77 K are shown in Figure 1C. The precise position of the absorption maxima and the pigment content of the various mutants are given in Table 2.

Among the pLH1 mutants, pigment analysis revealed that in strains S2, S5, and 1.4.2, a decrease in the carotenoid content was observed while the three pLH1 mutants 1.4.1, 1.4.4, and 1.4.8 retained nearly normal carotenoid levels.

Figure 2 shows absorption and fluorescence spectra of complexes isolated from membranes of the different mutant strains. Isolation of pLH2 complexes with the detergent LDAO allowed a partial purification of these proteins, as evidenced by the lack of contaminating band near 890 nm in the low-temperature absorption spectra. In these partially purified preparations, the marked hyperchromicity of the B850 bands in chromatophores of REM1.8 and REM1.9 (Table 2) was retained (ϵ_{mM}^{-1} cm^{-1} of ~252 and 236 at 300 K, respectively). Fluorescence emission spectra of these samples showed respective Stokes shifts of 19 and 15 nm, and the absence of the 908-nm emission band observed in chromatophores confirmed the removal of the ~890-nm contaminant. In addition, the rise of fluorescence polarization, over the red wing of the B850 band was greatly enhanced for these complexes as compared to wild-type. A much smaller fluorescence polarization increase was observed for the B850 band in the REM1.8 and REM1.9 chromatophores at 77 K (not shown); however, this apparently reflected depolarization of fluorescence upon energy transfer to the ~890-nm component.

Table 2: Pigment Content and Near-IR Absorption Maxima and Extinction Coefficients of Chromatophores from Mutant Strains

strain	near-IR maxima at 77K (nm)	BChl/carotenoid (mol/mol)	ϵ_{mM}^{-1} $_{cm}-1$ (300 K) (red-most peak)
U43(pU2922)[a]	889	0.8	135
U15 g(pU2922)[b]	801, 858	2.4	110
S2	886	>4.8	155
S4	889	2.6	157
S5	886	>5.7	174
REM1.8	802, 846[c]	1.2	231
REM1.9	802, 844[c]	1.4	199
1.4.1	884	2.7	198
1.4.2	876	3.5	182
1.4.4	874	1.6	213
1.4.8	868	2.0	169

[a] Control strain ($RC^+LH1^+LH2^-$), with LH1 (B880) core complex as sole antenna *(31)*. [b] Pseudo-wild-type control strain ($RC^+LH1^+LH2^+$)*(31)*. [c] The low level of residual LH1-like absorbance at ~890 nm, attributed to the strain U71 background, was eliminated by extraction of the pLH2 complexes with LDAO and purification as described in Materials and Methods.

The 77 K near-IR absorption spectra of complexes isolated from several pLH1 producing strains are also shown in Figure 2. These complexes are unstable in LDAO, and they could be extracted from chromatophores with, and isolated in the presence of β-OG. During the isolation procedure, small absorption blue-shifts in the Q_y band were observed (3−8 nm) relative to their absorption maxima in chromatophores, and the long-wavelength shoulder indicated a retention of the ~890-nm contaminant, the presence of which prevented accurate fluorescence measurements on the altered B850 bands.

Resonance Raman Spectra. FT Raman spectroscopy in preresonance with the long-wavelength BChl Q_y transition provides a sensitive technique for probing the BChl binding site, and in particular the hydrogen-bonding interactions of the conjugated carbonyl groups of the BChl macrocycle. This technique has been used to study both the LH2 *(14, 38−41)* and LH1 *(11, 42−45)* apoproteins, and it was shown to be extremely sensitive to perturbations of the BChl binding sites. To assess changes in the binding sites and in particular the hydrogen bonding environments of the pigments associated with alterations in the Q_y-absorption bands of the combinatorial mutants, preresonance FT-Raman spectra were thus measured.

Figure 3 shows the carbonyl stretching region of the FT-Raman spectra of the different pLH1 and pLH2 mutants, as compared to wild-type LH2. FT-Raman spectra of LH2 from *R. capsulatus* are characterized by four clearly resolved bands between 1600 and 1700 cm^{-1} *(39)*. The band at 1695 cm^{-1} was attributed to the free 9-keto carbonyl stretching mode of B800 BChl *(46)* and, consistent with this attribution, it is not observed in any Raman spectra of the pLH1 mutants (Figure 3B). Its intensity is slightly enhanced in the pLH2

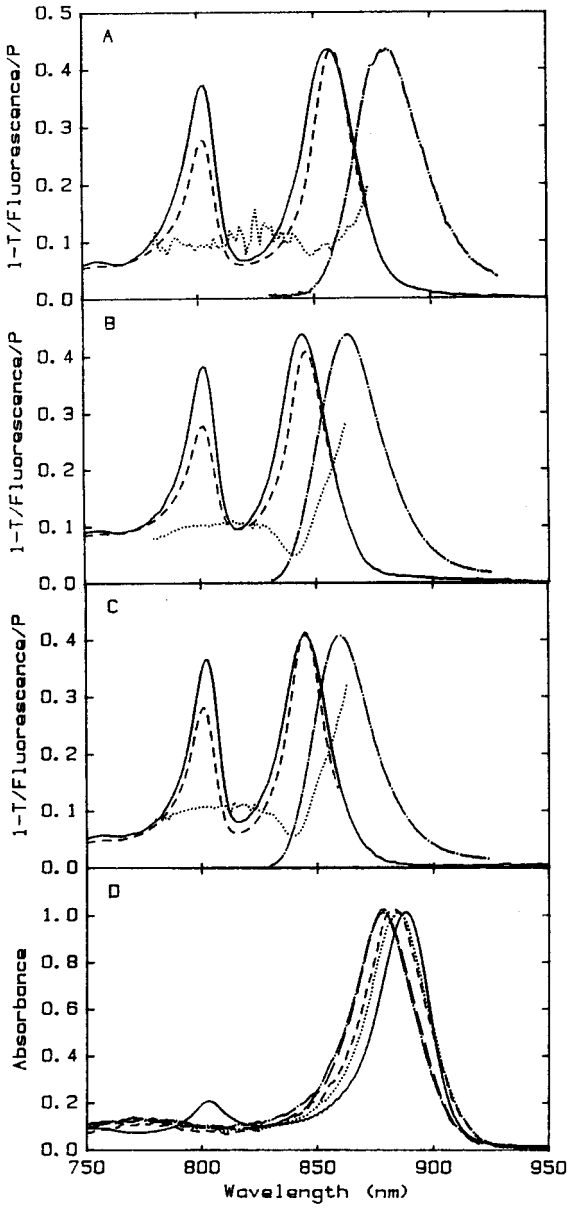

FIGURE 2: Optical properties of isolated antenna complexes at 77 K. The pLH2 and pLH1 complexes were detergent-extracted and purified as described in Materials and Methods. (A) LH2 complex from pseudo-wild-type strain U15g(pU2922): (—), fractional absorption (1-T) spectrum; (- - -), excitation spectrum; (···), fluorescence polarization; (—·—), emission spectrum (excitation at 800 nm, corrected for response of measuring system). Absorption maximum at 856 nm. Fluorescence excitation and polarized excitation spectra were detected through an 890-nm band-pass filter. Polarization values (corrected as described in the text) are given by $p = (I_{||} - I_{\perp})/(I_{||} + I_{\perp})$, where $I_{||}$ and $I_{\perp}$ are the relative intensities of fluorescence with polarization either parallel or perpendicular, respectively, to the polarization direction of the excitation light. (B) pLH2 complex from strain REM1.8; same measurements and line types as in A. Absorption maximum at 845 nm. Excitation and polarization spectra were detected through an 880-nm band-pass filter in this panel and in panel C. (C) pLH2 complex from strain REM1.9; same measurements and line types as in A. Absorption maximum at 845 nm. (D) Absorption spectra of isolated pLH1 complexes: (—), membrane control (strain U43(pU2922)); (- - -), pLH1 complex from strain S2; (···), S4; (—·—), S5; and (— —) 1.4.1. Absorption maxima were at 888, 883, 885, 878, and 878 nm, respectively. Fluorescence emission spectra (not shown) were broadened and anomalously red-shifted (emission maxima at 922, 921, 914, and 913 nm for pLH1 complexes isolated from strains S2, S4, S5, and 1.4.1, respectively).

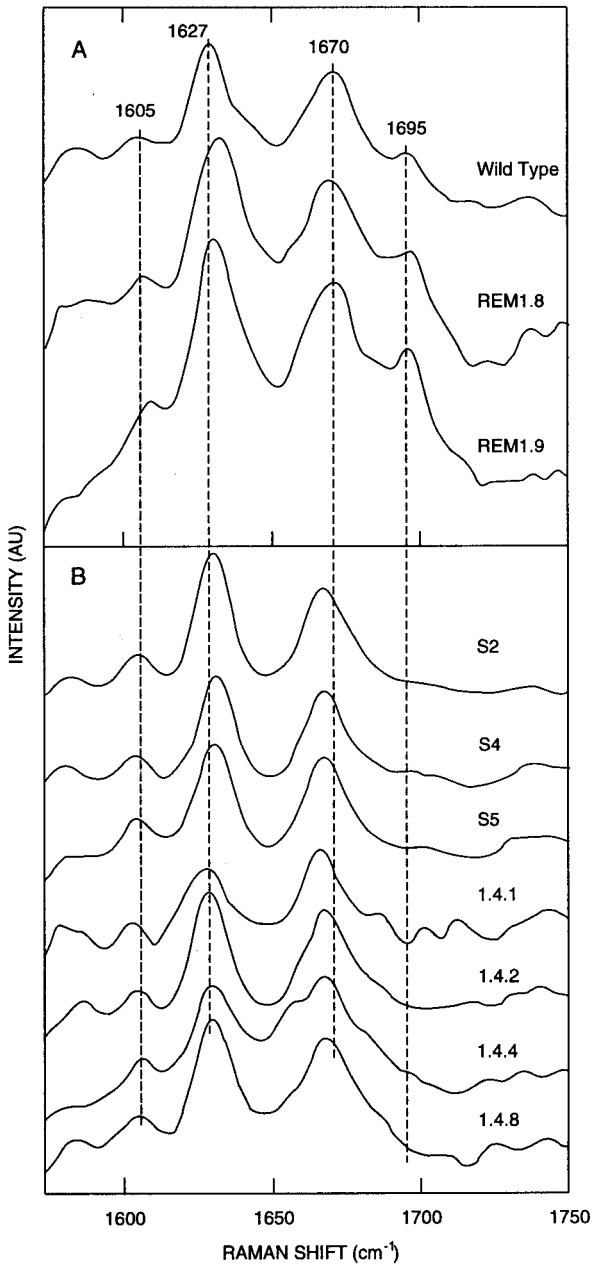

FIGURE 3: FT resonance Raman spectra in the carbonyl stretching region of LH2 complexes from *R. capsulatus* mutants. Spectra were obtained as described in Materials and Methods and normalized at the intense 730-cm^{-1} BChl skeletal mode. (A) pLH2 complexes from strains REM1.8 and REM1.9 isolated as described in Materials and Methods; wild-type spectrum *(39)* was obtained with the LH2 complex isolated from strain 2.3.1. It was necessary to use purified pLH2 complexes because of interference from the contaminating 890-nm peak in chromatophores. (B) Chromatophores from pLH1 mutants strains.

complexes (Figure 3A), relative to the wild-type complex. This is probably due to the blue-shifts of the B850 absorption transition, which result in a decrease of the Raman contribution of the B850 BChl, and thus, in turn, in an apparent increase of the B800 BChl contributions.

The more intense, broad, band at 1670 cm^{-1} arises from the stretching mode of the 9-keto carbonyl groups *(39)*. These modes are observed at ca. 1685 cm^{-1} when these groups are free from interactions. It was thus concluded that both the keto carbonyl groups from the BChl responsible for the 850-nm absorption of the LH2 complexes are involved in

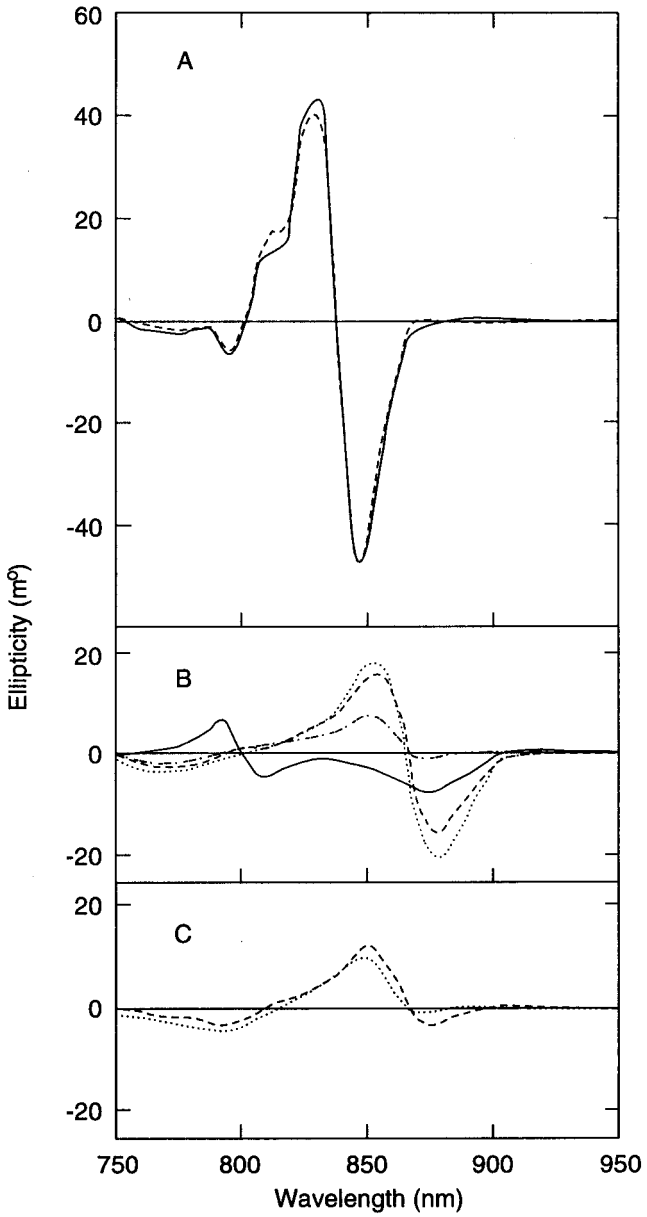

FIGURE 4: Near-IR CD spectra of isolated antenna complexes. The pLH2 and pLH1 complexes were isolated as described in Materials and Methods. Spectra were obtained at room temperature and normalized to 1.0 A at their Q_y-absorption maxima. (A) pLH2 complexes: (—), strain REM1.8; (- - -), REM 1.9. (B) pLH1 complexes: (—), membrane control (strain U43(pU2922)); (- - -), strain S2; (···), S4; (—·—), S5. (C) pLH1 complexes: (- - -), strain 1.4.1; (···), 1.4.2. CD spectra measured with chromatophores from the mutant strains were similar (not shown). The CD in the control strain (panel B) was essentially identical to that obtained with membranes from the LH2$^-$ *R. sphaeroides* M21 mutant on the same apparatus *(48)*.

hydrogen bonding with the surrounding protein. However, no evidence that these carbonyl groups accept hydrogen bonds has yet been found in the LH2 crystal structure from *Rp. acidophila (47)*, and crystallographic studies have thus been unable to help in determining their putative hydrogen-bond partners *(45)*. In Figure 3, it clearly appears that this 1670 cm^{-1} band is essentially unchanged in both the pLH2 and pLH1 mutants.

The narrower peak at 1627 cm^{-1} is degenerate, with contributions from the stretching modes of hydrogen-bonded 2-acetyl carbonyls of both B850 BChls, downshifted from ~1660 cm^{-1}, together with a small additional resonance from a similar hydrogen bond to B800 BChl *(39)*. αTyr44 and αTrp45 have been shown to interact with the 2-acetyl carbonyl of the B850 BChls by a combination of resonance Raman spectroscopy and site-directed mutagenesis *(14)*. The *Rp. acidophila* LH2 crystal structure fully confirmed this result, as these residues appeared to be in positions to form hydrogen bonds with the respective 2-acetyl carbonyls of *β*- and α-B850 BChls, respectively. Again, this band is largely unaffected in the different mutants, indicating that these H-bonds are largely conserved upon the different mutations. Last, the band at 1605 cm^{-1}, which arises from skeletal stretching modes in the methine bridges of the chlorins, was evident in the mutants, showing that all BChls remained pentacoordinate with a single axial ligand to the central Mg atom.

Circular Dichroism Spectra. Possible alterations in the optical activity of the B850 BChls in the mutant LH2 complexes were assessed by CD spectroscopy (Figure 4). The strong conservative near-IR CD signal of the B850 band has been attributed to coupling of excitations among several BChl molecules *(49, 50)* and is strongly dependent on the relative orientations of the interacting chromophores. Aside from the expected blue-shift in zero crossing, the CD of the pLH2 complexes was similar to that of the native antenna *(51, 52)*, showing that the extent of coupling between the B850 BChls was unaltered and indicating only minimal alterations in the relative orientations of the different B850 pigments (Figure 4A). In addition, the optical activity of B800 BChl, seen as a negative trough near 800 nm, was retained in the pLH2 complexes (Figure 4A).

The CD signal of pLH1 is, in general, largely perturbed. In some mutants (S2 and S4) it is attenuated, but still conservative, i.e., retaining most of the characteristics of the LH2 CD signal. This is not the case for the mutants S5, 1.4.1 and 1.4.2. for which the CD signal largely consists of a positive lobe at about 850 nm (Figure 4B,C). It must, however, be noted that none of the pLH1 mutants exhibits a CD signal comparable to that of wild-type LH1 complexes (Figure 4B), where the broad negative band at ~875 nm in the control membranes is attributed to LH1 (48). For all pLH1 mutants, the intensity of the CD signal arising from the 800-nm-absorbing BChl has nearly disappeared, suggesting that the altered B850 pigment coupling in the pLH1 complexes arises from global structural changes that also affect occupancy of the B800 BChl site.[2]

DISCUSSION

It is now generally accepted that two different types of mechanisms underlie the red-shift of the BChl absorption in the light-harvesting proteins of purple bacteria: (i) pigment—pigment interactions; and (ii) the tuning of the individual

[2] Koolhaas et al. *(53)* have recently reported that in two *Rhodobacter sphaeroides* LH2-only mutants lacking a B800 band, only a 3—4 nm red shift in the B850 band was observed, while the long-wavelength conservative CD signal of the B850 BChls was retained. The absence of the contribution of B800 in their CD spectra revealed a small negative signal in the 780-nm region, also visible in Figure 4A, that was attributed to the upper exciton component of the B850 BChl ring. These strains were constructed by alterations in N-terminal portions of the α-polypeptide that provide part of the B800 BChl binding site, resulting in only minimal effects on the B850 BChl pocket.

monomer absorption by interactions with the surrounding protein. The second of these mechanisms has been well documented experimentally by the demonstration that hydrogen-bonds form between the 2-acetyl carbonyl of the BChl molecules and that the local dielectric properties of the protein could modify the absorption of both interacting BChl molecules and BChl monomers *(11, 14, 41, 44, 47)*. In these cases, perturbation of the BChl binding site could be described from their effect on the resonance Raman spectra of the LH complexes. The FT-Raman spectra of the mutants reported here all showed very similar resonances attributed to the B850 BChls and their binding sites must thus be nearly identical at a molecular level. Similarly, alterations in the conformation of the bacteriochlorin cycle(s) of one or both BChl responsible for the red-most transition can also be observed in the FT-Raman spectra, but since no such perturbations were seen in any of the various mutants, we may conclude that the observed differences in red-most absorption do not result from a change in the absorption of the individual monomers, but rather from perturbations at the level of the pigment−pigment interactions.

Regarding other examples of LH complexes from purple bacteria in which perturbations of pigment−pigment interactions induce a change in their main Q_y absorption, with strain R26.1, a partial revertant *(54)* of the carotenoidless strain R26 of *R. sphaeroides*, the LH2 complex is devoid of both colored carotenoid and 800-nm absorption and absorbs at slightly lower energy (860 nm) than the wild-type protein (850 nm). FT-Raman studies have shown that the binding site of the 860 nm-absorbing BChl molecules is hardly, if at all, perturbed by the mutation (A. Gall and B. Robert, unpublished data). Likewise, selective carotenoid extraction from the LH2 antenna in *R. capsulatus* membranes gave rise to a pLH1-like complex in which B800 BChl was dissociated and the B850 absorption band was red-shifted by up to 10 nm *(55)*. It must, however, be noted that none of these results demonstrates that the alteration in the red-most absorption transition is a direct consequence of the absence of carotenoid molecules (see below).

Since the LH2 complex from *Rs. molischianum* exhibits the binding site properties typical of LH1 *(7, 38, 42, 44)*, while its absorption in the near-infrared shows peaks at 800 and 850 nm, it also represents an antenna protein in which the absorption properties are altered in the absence of changes in the BChl binding site. While no clear explanation has yet been proposed to explain these properties, it is worth noting that the size of the polypeptide ring of the LH2 complex of *Rs. molischianum* is comprised of only 16 polypeptides, while a ring structure containing 32 polypeptides was deduced for LH1 from a cryo-electron microscopy analysis of two-dimensional crystals *(56)*. It is likely that this change in ring size influences, either directly or indirectly, the absorption properties of this protein.

From these examples, it is clear that some of the main factors governing the absorption of LH complexes of purple bacteria are not well understood, in particular those defining the LH1-like or the LH2-like absorption. The set of mutants described in this work provide an opportunity to shed more light on this particular phenomenon.

PseudoLH2 Complexes. Among the mutants examined, two exhibit LH2-like absorption properties; however, their lower energy transition was blue-shifted and the main maxima in the near-IR were at ~800 and 845 nm (Table 2). These two proteins have related mutations near the C-terminal end of the polypeptide, and as common features exhibit an inversion of the strongly conserved PW motif to a WP sequence, together with the presence of more bulky amino acids at positions +7 and +11 (in relation to the position of the B850 BChl liganding βHis38). Tryptophan residues are commonly observed in transmembrane helices at the interface between the membrane and the water phase, though their exact role at this position is not completely understood. However, it is possible that their presence at the interface helps in positioning the α-helices in the membrane. According to such an hypothesis, the shift of the Trp+9 by one position, together with the appearance of more hydrophobic amino acids at position +7 and +11, could drastically modify the C-terminal conformation of the β-polypeptide.

The physicochemical mechanisms underlying the blue-shift of the absorption of these pLH2 proteins are unclear. As previously stated, neither the protein−BChl interactions nor the BChl conformations are perturbed by the mutations present in these complexes. Moreover, the CD signals arising from their 845-nm BChls look very similar to that of the wild-type, suggesting that the coupling between these molecules is not much perturbed either. While such a conclusion strongly indicates that the pairwise organization of the BChl molecules in these mutants is nearly identical to that of the wild-type, this does not necessarily imply that the supramolecular organization of the BChl in these complexes is similar. As an example, the LH2 from *Rs. molischianum* exhibits an LH1-like CD spectrum *(57)*, although it arises from an octamer of subunits. The change in the absorption properties of pLH2 mutants could thus result from a change of the supramolecular organization of the BChl molecules. The increase in fluorescence polarization of the 850 band is in good agreement with this hypothesis, as it likely indicates a decrease in the B850 BChl cluster size. However, up to now, we have been unable to find evidence for changes in the antenna ring size, since the mutant proteins exhibit sedimentation properties essentially identical to wild-type LH2 complexes in sucrose density gradients and are indistinguishable in gel exclusion chromatography (not shown).

PseudoLH1 Complexes. Several lines of evidence establish that the main spectral properties observed for pLH1 complexes in this study arose from modified forms of the LH2 complex expressed from plasmid borne genes, rather than residual LH1 produced by strain U71 *(29)*, in which modified LH2 was not assembled. First, the resonance Raman spectra in the carbonyl stretching region of all pLH1 mutants were clearly those of an LH2 complex (albeit lacking the free 9-keto carbonyl stretching mode of B800 BChl), rather than the distinct spectrum seen with LH1 complexes *(11, 42, 44, 45)*. Likewise, none of the antenna complexes isolated from the pLH1 mutants exhibited the CD spectra of LH1 complexes. In addition, the major low-molecular-weight polypeptides seen in the antenna-enriched preparations isolated from these strains were those of the LH2 rather than the LH1 complex, with only a small contamination from the latter. Last, in low-temperature absorption spectra, the distinct Q_y bands of several pLH1 complexes showed minor,

Table 3: Comparison of Amino Acid Residues Altered in *R. capsulatus* Combinatorial Mutants with Pigment-Contacting Residues in *Rp. acidophila* LH2 Crystal Structure[a]

R. capsulatus residue	equivalent position in *Rp. acidophila*	pigment(s) contacted	interaction type
βArg28	βArg20	B800 BChl	hydrogen bond
βVal29	βVal21	B800 BChl	van der Waals
βPhe30	βPhe22	αB850 BChl	van der Waals
		βB850 BChl	van der Waals
		carotenoid	van der Waals
βGly31	βLeu23	βB850 BChl	van der Waals
βMet33	βLeu25	αB850 BChl	van der Waals
βAla34	βAla26	αB850 BChl	van der Waals
		βB850 BChl	van der Waals
βSer41	βAla33	αB850 BChl	van der Waals
		βB850 BChl	van der Waals
βThr45	βThr37	βB850 BChl	van der Waals
βTrp47	βTrp39[b]	βB850 BChl	van der Waals

[a] *(47)*. [b] Also involved in protein−protein interactions.

long-wavelength shoulders near 890 nm, arising from residual LH1 formed by this strain.

Alterations to B800 BChl Binding Site. The first striking property of all the pLH1 mutants is that they are devoid of an 800-nm absorbing BChl molecule. In strains S2, S5, and 1.4.2, the reduced carotenoid content could partially account for this phenomenon, since selective carotenoid extraction from the LH2 antenna in *R. capsulatus* membranes has been shown to give rise to a complex in which the 800-nm absorbing BChl was dissociated *(55)*. Similarly, in *R. sphaeroides* R26.1, LH2 complexes devoid of colored carotenoids do not bind this molecule. In strains 1.4.1, 1.4.4, and 1.4.8, with essentially normal carotenoid levels, the B800 BChl environment is drastically perturbed, first by the absence of the βArg28, which interacts with this monomeric chromophore (Table 3). In the *R. sphaeroides* LH2 complex, an exchange of βArg28 to a glutamic acid results in a diminution of ∼3-fold in the B800 peak *(15)*, though many other residues may be accepted at this locus *(41)*, in particular leucine, with no drastic effect on the level of B800 absorbance. The fact that in mutant 1.4.8, a leucine is present at this locus suggests that, though mutations affecting the βArg28 may play a role, they alone cannot explain the absence of 800 nm-absorbing BChl. However, there are a number of other nonconservative mutations in these three strains in the environment of the B800 molecule, and most of them result in an increase of the volume of the amino acid side chains at positions 31−33 (Table 1). In the 1.4.1, 1.4.4, and 1.4.8 mutants, the wild-type sequence GAM is replaced by LWV, LLW, and AWL, respectively, which, in these three cases, results in an increase of the side chain volume at the three positions. Less easy to understand is the absence of B800 in the S4 strain, where the LH2 sequence is less perturbed, and which is still able to bind carotenoid. However, also in this strain, the βGly31 is replaced by a bulky valine, and it is tempting to suggest that within its sequence context *(28)*, this residue is particularly important for the organization of the N-terminal part of the polypeptide and for it to adopt its stable conformation.

The possibility that the B800 absorbance loss in the pLH1 strains is due to misfolding of the apoprotein is supported by the mutant frequency data obtained with the 1.4 group *(28)*. The greater propensity for emergence of pLH1 rather than pLH2 phenotypes was thought to reflect a less stringent requirement for assembly of an LH1-like structure. Residues mutated in this group were proposed to be involved in sensitive interactions necessary to achieve a B800-containing LH2 phenotype; if this region merely assumes a sufficiently stable conformation, a pLH1 phenotype appears instead. Because the B800 BChl binding pocket in these mutants appears to be empty, interactions between the N-terminal regions of the α- and β-polypeptides are likely to be important for the assembly of these pLH1 complexes. In this connection, the requirements of N-terminal residues in LH1 formation has been studied extensively by mutagenesis *(58, 59)* and by reconstitution of this complex from isolated components *(60−62)*. Once appropriate systems are available for such studies with the *R. capsulatus* LH2 complex, much more will be learned about structural requirements that are crucial for forming monomeric BChl binding pockets such as those that give rise to the B800 band.

Alterations of the Electronic Properties of the B850 BChl Molecules. As discussed above, either carotenoid absence or carotenoid extraction has been reported to result in both B800 disappearance and a red-shift of the 850-nm absorption transition of about 10 nm. Moreover, carotenoid extraction was accompanied by significant decreases in optical activity, resulting in attenuation of the conservative CD and the appearance of degenerate peaks *(52)*. This was ascribed to reorientation of electronically interacting B850 chromophore clusters and structural rearrangement of the associated apoprotein, that like the changes reported here, did not affect the hydrogen-bonding environment of the B850 BChls as assessed in resonance Raman spectra. Among the pLH1 mutants, the carotenoidless S5 and 1.4.2 indeed exhibit an attenuated, non-conservative CD, while S2 exhibits a CD spectrum very similar to that of the carotenoid-containing S4 mutant. On the other hand, the carotenoid-containing 1.4.1 mutant exhibits a CD spectrum similar to that of S5 and 1.4.2. We can thus conclude that the influence of the depletion of both carotenoid and B800 molecules indirectly perturbs the electronic properties of the B850 through some structural rearrangement of the protein polypeptides. It is also worth noting that carotenoid-depleted LH2 is normally unstable both in vitro *(55)* and in vivo *(63)*, and thus that compensatory residue changes are expected to be responsible for assembly of the carotenoid-depleted pLH1 complex. It must, however, be emphasized that the pLH1 complexes do not exhibit normal LH2 stability, as they cannot be purified by LDAO treatment, and that, thus, these compensatory mutations only partially restore protein stability.

The comparison of those pLH1 mutants that do and do not bind carotenoid at stoichiometries similar to that of the wild-type may give information on which residue(s) are important for carotenoid binding by these proteins. Indeed, comparing strain S2 to the carotenoid-containing S4 mutant shows that only three amino acids differ between the sequences of their β-polypeptides, namely at positions 31 (L31/V31, respectively), 35 (L35/V35), and 42 (A42/G42). A comparison of S5 and S4 shows only two differences (L31/V31 and A35/V35, respectively). This demonstrates that the mutants deficient in carotenoid binding have a residue at position 31 that is more bulky than the strain which is still able to bind carotenoids, suggesting that this may result in a steric hindrance that prevents assembly of the internal carotenoid molecule. Clearly, such a possibility is dependent

upon the sequence context of mutants of the S group, since strain 1.4.2 is also carotenoid-deficient but has an alanine inserted into position 31.

All the pLH1 complexes showed alterations at positions aligning with a homologous, chromophore-associated residue in the *Rp. acidophila* LH2-β apoprotein (Tables 1 and 3). Because of the several amino acid side chains in both the α- and β-apoproteins that are within van der Waals distance of the B850 BChls *(47)*, the causes of the large B850 red-shifts in these mutants are likely to be complex. It is worth noting that in the mutants of the 1.4 group, the generally conserved βPhe30, thought to have a role in tuning the absorption range of the B850 band *(64)*, is either retained or replaced by an aromatic residue (Y or W). In the sequences of all these mutants, a tryptophan residue also appears, either at position 30 or 32. In no case, does the sequence of the pLH1 polypeptides appear to acquire sequence determinants of LH1 polypeptides, and thus the pseudoLH1 designation is truly justified. This group of mutants clearly shows that some of the sequence determinants essential for the absorption properties of the LH complexes are far from BChl binding sites, and their full biophysical characterization will certainly lead to a better understanding of both the structure-sequence and structure−function relationships in LH complexes from purple bacteria. Furthermore, site-directed mutagenesis, using as a database the set of mutants characterized in this paper, will readily allow a more precise description of the role of each amino acid involved in the definition of the LH function in these organisms, especially when they are expressed in an appropriate LH1$^-$ background.

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