Site-Directed Modification of the Ligands to the Bacteriochlorophylls of the Light-Harvesting LH1 and LH2 Complexes of *Rhodobacter sphaeroides*†

J. D. Olsen,‡ J. N. Sturgis,§ W. H. J. Westerhuis,‡ G. J. S. Fowler,‡ C. N. Hunter,‡ and B. Robert*,§

Robert Hill Institute for Photosynthesis and Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2TN, U.K., and Section de Biophysique des Protéines et des Membranes, DBCM/CEA et URA CNRS 2096, C. E. Saclay, 91191 Gifsur-Yvette Cedex, France

Received May 5, 1997; Revised Manuscript Received July 15, 1997⊗

ABSTRACT: The core light-harvesting LH1 complex of *Rhodobacter sphaeroides* consists of an assembly of membrane-spanning α and *β* polypeptides, each of which binds one bacteriochlorophyll molecule. In this study we have used site-directed mutagenesis to demonstrate that the B880 bacteriochlorophyll binding site of LH1 shows a high degree of specificity for the residue that provides the ligand to the Bchl Mg^{2+} ion. α His_0 (αH_0) was changed to Asn, Leu, and Tyr, and *β* His_0 (βH_0) to Asn, Gln, Leu, and Tyr; the mutated genes were expressed to yield both LH1-only and LH1 + RC strains, in two different carotenoid backgrounds. None of the αH_0 mutations formed an LH1 complex either in isolation or with RCs. Of the mutations of βH_0 those to Asn and Gln formed LH1 complexes but in the latter case the complex was very unstable and occurred at very low cellular levels. In a similar study, the αH_0 and βH_0 residues of the LH2 complex were changed to Asn. However, no complex was formed in either case. FT Raman spectroscopy of the $\beta H_0 N$ mutant LH1 shows perturbation of the interaction state of the keto carbonyl of one Bchl which sheds light on the possible H-bond partners for these keto oxygens. These data directly address the B880 binding site of the core LH1 complex and show that it is subtly different from the B850 binding site of the peripheral LH2 complex. A model of this binding site may be proposed from these results.

In the light-harvesting apparatus of purple photosynthetic bacteria energy is funneled to the reaction centers by a series of interconnecting antenna complexes. Most of these organisms synthesize two types of such proteins: the peripheral and core antennae (or LH2 and LH1, respectively).[1] Whereas LH1 is in contact with, and transfers excitation energy directly to, the reaction center, LH2 transfers energy only to LH1. Each of these complexes comprises repetitions of a basic unit, itself containing two small (∼5 kDa) polypeptides, α and *β*, which have been sequenced in a large number of species (Brunisholz & Zuber, 1992). The most consistent feature of these polypeptides is a histidine residue located in the hydrophobic phase, which is likely to coordinate the central Mg atom of a bacteriochlorophyll molecule (Bchl) in nearly all bacterial LH complexes. Recently there has been a dramatic increase in our understanding of bacterial LH complexes, which has resulted from the determination of the X-ray structures of the LH2 complex of *Rhodopseudomonas* (*Rps*) *acidophila* 10050 (McDermott *et al.*, 1995) and *Rhodospirillum* (*Rsp*) *molischianum* (Koepke *et al.*, 1996). These, together with the 8.5 Å projection map of the LH1 complex of *Rhodospirillum rubrum* (Karrasch *et al.*, 1995), have greatly enhanced our knowledge of the structure of these membrane protein complexes. The LH2 complex of *Rps acidophila* consists of two concentric cylinders of α-helical, membrane-spanning polypeptides. Nine Bchl molecules absorbing at 800 nm are located toward the cytoplasmic face of this molecule, and eighteen Bchls, which are responsible for the 850 nm transition of these complexes, are closely packed between the polypeptide rings. These latter pigments are coordinated to histidine residues, and sequence alignments for LH2 and LH1 polypeptides (Brunisholz & Zuber, 1992), as well as modeling (Olsen & Hunter, 1994) and spectroscopic studies (Robert & Lutz, 1985), suggest that the B880 binding sites of LH1 will show many similarities but also some differences to the B850 binding sites of LH2. The low-resolution structure of *Rsp rubrum* LH1 (Karrasch *et al.*, 1995) does not provide detailed information, although it appears that LH1 is composed of a larger ring-like structure of 16 $\alpha\beta$ pairs.

In the absence of crystallographic structure, resonance Raman spectroscopy is one of the only methods that yields precise, structural information about the Bchl binding sites in photosynthetic proteins. In particular, this method predicted that both tyrosines at positions +13 and +14 on the α subunit interact with the acetyl carbonyl groups of those Bchls responsible for the 850 nm absorption (B850) in LH2 of *Rb sphaeroides* (Fowler *et al.*, 1994), and these predictions have been confirmed by the three-dimensional structure subsequently obtained (McDermott *et al.*, 1995). In the case of LH1, this method indicates that the acetyl carbonyl groups of the B875 molecules interact with the indole side chains

† This work was supported in part by funds from the BBSRC, the EC Human Capital and Mobility Programme (Contract No. ERB CHRCT 930278), and a grant from the Human Science Frontier Program.

* Corresponding author.
‡ University of Sheffield.
§ C. E. Saclay.

⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

[1] Abbreviations: Bchl, bacteriochlorophyll; FT, Fourier transform; FWHM, full width at half-maximum; LH, light harvesting; LT, low temperature; OD, optical density; P, primary electron donor; *Rb*, *Rhodobacter*; RC, reaction center; *Rps*, *Rhodopseudomonas*; *Rsp*, *Rhodospirillum*; RT, room temperature; WT, wild type; λ_{max}, wavelength of maximum absorption.

S0006-2960(97)01048-9 CCC: $14.00

of α Trp_{+11} and β Trp_{+9} (Olsen *et al.*, 1994; Sturgis *et al.*, 1997). In the X-ray crystallographic structure of LH2 from *Rps molischianum*, these residues (α Trp_{+11} and β Trp_{+9}) are indeed involved in such interactions. It is thus highly likely that the B850 binding sites in the LH2 from this bacterium provide a good model for LH1 Bchls, as predicted by earlier studies (Germeroth *et al.*, 1993). However, it should be noted that while resonance Raman predicted that both keto carbonyls of the Bchl molecules present in LH1 interact with the surrounding protein, no amino acid in the crystallographic structure seems to be sufficiently close to these keto carbonyls to be involved in such interactions (Koepke *et al.*, 1996).

The tolerance of the B880 Bchl binding site of the LH1 α subunit of *Rhodobacter capsulatus* to site-selected mutations was examined by Bylina *et al.* (1988), who found that LH1 complexes could not accept any of the designed changes at this H_0 position, and that any residue larger than Val was not tolerated at the −4 position on the same subunit, a position normally occupied by an alanine residue. There is no report of a similar exercise being conducted upon the β subunit of any purple bacterium or upon the B850 Bchl binding site of LH2. Our findings agree with those of Bylina *et al.* (1988) and also indicate that the only other residue that can provide a ligand to the B880 Bchl in the β subunit is Asn. However, this residue cannot substitute for either His_0 residue in LH2. The work reported here provides new insights into the structure of the B880 Bchl binding site, which appears to be different from the B850 Bchl binding site of the LH2 of *Rps acidophila* 10050.

MATERIALS AND METHODS

Mutagenesis

Mutagenesis of the LH1 and LH2 genes was conducted according to the protocol of Kunkel (Kunkel, 1985, Kunkel *et al.*, 1987) with oligonucleotides designed to change the α His_0 residue to Asn, Leu, and Tyr and the β His_0 residue to Asn, Gln, Leu, and Tyr by introducing the preferred codons for these residues.

Mutants were screened for by sequencing using the Sequenase system (Tabor & Richardson, 1987, 1989a,b). Mutant RF M13 DNA was digested with *Bam*HI and *Xba*I to release the LH1 genes as a 480 bp fragment which was cloned into the expression vector pRKEK1 encoding *pufQBA* for LH1-only strains, and pRKEH1 encoding *pufQBALMX* for LH1 + RC strains. Mutagenesis of the *pucBA* genes was carried out on a 420 bp *Kpn*I−*Bam*HI fragment as described in Crielaard *et al.* (1994). Following mutagenesis this fragment was subcloned into pRKCBC1 (TcR plasmid containing a 4.4 kb fragment encmpassing *pucBAC*). These were then used to transform *Escherichia coli* S17-1 cells (Simon *et al.*, 1983) for conjugative transfer of the genes into the $LH2^-$ $LH1^-$ RC^- double-deletion strains DD13 (Jones *et al.*, 1992) and DD13/DG2, a spontaneous green mutant of DD13. Colonies harboring the mutant genes were grown chemoheterotrophically in the dark at 34 °C on M22+ agar plates in the presence of neomycin and tetracycline at 20 and 5 μg mL^{-1}, respectively. Screening for expression of the mutant genes was conducted directly on the colonies using a Guided Wave model 260 spectrophotometer (Guided Wave Inc., El Dorado Hills, CA) with a home-built agar plate holder. A selection of positives were grown up in M22+ liquid culture containing 0.1% casamino acids (antibiotics, temperature as before), and intracytoplasmic membranes were prepared (Olsen *et al.*, 1994) and stored at −20 °C.

Strains and Plasmids

Strains and plasmids used in this study were as follows: *E. coli* S17-1 (Simon *et al.* 1983); *Rb sphaeroides* DD13 (Jones *et al.* 1992a); pRKEK1 (Jones *et al.* 1992a); pRKEH1 (Jones *et al.* 1992a; pRKCBC1 (Jones *et al.* 1992a).

Spectroscopy

RT spectra were taken of the membrane samples using quartz cuvettes in the Guided Wave spectrophotometer fitted with a 1 cm cell holder. The membranes were diluted with 15% w/w sucrose solution to keep the maximum absorption below 0.8 OD. Protein concentrations of the membrane samples were determined according to the method of Bradford (1976) using the Bio-Rad assay system, with 0.22% SDS (final concentration) to solubilize the membranes.

Low-temperature (77K) absorption spectra were obtained using an Oxford Instruments (DN1704) cryostat in a variable path length adapter for the Guided Wave spectrophotometer. The samples (OD 0.1−0.2 at 77 K, 1 cm path length) were illuminated with red light, using an RG 630 filtered white light source, during cooling to accumulate the primary electron donor (P) in the photooxidized state. Fluorescence emission and excitation spectroscopy were conducted on the same sample, immediately after the LT absorption reading, in a Spex Fluorolog 2 equipped with a 100 W tungsten lamp and a cooled photomultiplier detector (R406, Hamamatsu). The fluorescence emission spectra were recorded over the range 860−960 nm with a spectral resolution of 9 nm, exciting into the Q_x band at 590 nm. The resolution of the fluorescence excitation spectra (detection wavelength 920 nm) was 4.5 nm.

Efficiencies of energy transfer from carotenoid to Bchl were determined as the ratio of the carotenoid bands in excitation and fractional absorption spectra after applying a linear base line correction to the latter and normalization of both spectra at the Q_y absorption maximum. The LH1-only mutant membrane samples exhibited considerable light scattering; in this case fractional absorption spectra of the corresponding WT membranes were used instead, assuming that the carotenoid to Bchl ratios in mutant and WT membranes were the same.

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. The setup, laser powers, and sample behavior are extensively described in Mattioli *et al.* (1993). All spectra were recorded at room temperature with back-scattering geometry from pellets of membrane held in standard aluminum cups. Depending on the samples, spectra were the result of 1000−10 000 co-added interferograms. Resonance Raman spectroscopy was carried out on the LH1 complexes using 1064 nm excitation, which corresponds to pre-resonance of the Q_y transition of Bchl *a*. The Raman signal thus obtained arises mainly from these molecules. All the resonance Raman spectra shown are in the "red" DD13 double-deletion strain (main carotenoid spheroidenone), which has the same carotenoid content as the WT *Rb*

Table 1: Phenotypes and RT Absorption Maxima of Membranes of LH1-Only, LH1 + RC, and LH2-Only Strains in the Two Carotenoid Backgrounds of the Deletion Mutant DD13

			λ_{max} of LH1 (nm)	
	mutant	LH phenotype	DD13	DD13/DG2
LH1-only	WT	+	877	875
	αH_0N	−		
	αH_0L	−		
	αH_0Y	−		
	βH_0N	+	871	867
	βH_0Q	±		
	βH_0L	−		
	βH_0Y	−		
	αH_0N, βH_0N	−		
LH1 + RC	WT	+	876	877
	αH_0N	−		
	αH_0L	−		
	αH_0Y	-		
	βH_0N	+	873	875
	βH_0Q	±		
	βH_0L	−		
	βH_0Y			
LH2-only	WT	+	800, 850	800, 850
	αH_0N	−		
	βH_0N	−		

sphaeroides, but the results were the same when membranes were used from the "green" deletion strain DD13/DG2 which contains neurosporene as the main carotenoid (data not shown).

RESULTS

The α and β His_0 residues of LH1 were exchanged for Asn, Gln, Tyr, and Leu, both in the presence and absence of the RC. A list of all of the mutations made, in the two deletion strains, appears in Table 1. Most of the changes at the positions α or β His_0 were deleterious for LH1 formation, as was the double-mutant α Asn_0, β Asn_0. The β Gln_0 mutant was not stable enough to withstand the processing of the cells to produce membranes, and analysis by FT Raman and LT electronic spectroscopy could thus be performed on the β Asn_0 mutant only. When these experiments were reproduced on LH2 complexes, it was observed that both the α and the β Asn_0 mutations were deleterious for the peripheral antenna (Table 1).

In comparison with the WT equivalent, the LH1-only β Asn_0 mutant showed 6 and 8 nm blue shifts in the absorption maximum to 871 and 867 nm in the red and green backgrounds, respectively (Table 1), as well as a greatly reduced level of complex (Figure 1). The level of protein in the membrane increased when the mutant was co-expressed with RCs, although this was still only about 50% of the WT equivalent (Figure 1); in addition, the blue shifts were less pronounced (3 and 2 nm, respectively, see Table 1). The effects of replacement of β His_0 by Asn on the LT absorption maxima were similar to those seen at RT, although at 77 K the blue shift in the presence of RCs (6 nm) was somewhat greater (Table 2). Aside from these effects on the Q_y region, a blue shift of 4 nm also occurred in the Q_x band in the β Asn_0 mutant, both in the presence and absence of RCs, and in either carotenoid background. Callahan and Cotton (1987) noted that the Q_x band of monomeric Bchl *a*

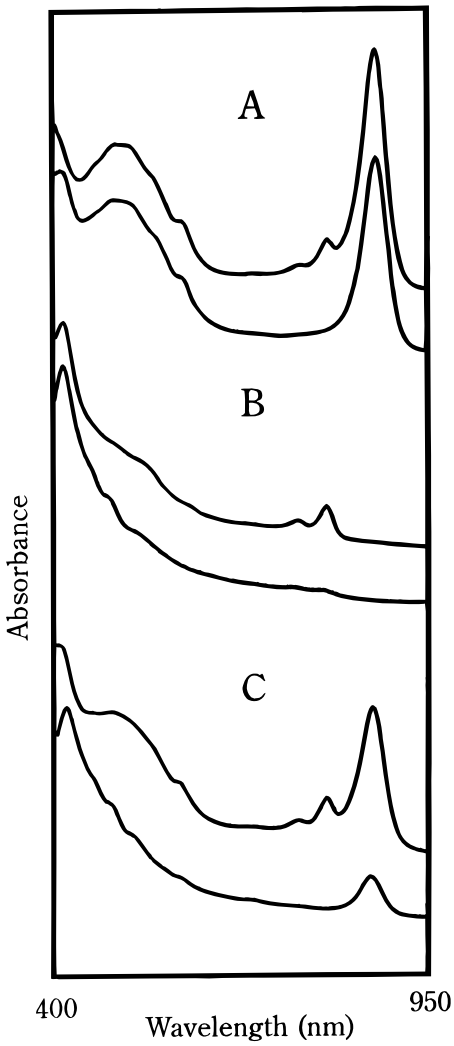

FIGURE 1: RT absorption spectra of membranes of LH1-only and LH1 + RC in DD13. The upper trace of each pair shows the LH1 + RC core complex and the lower the LH1-only complex. The spectra were normalized on the basis of their membrane protein concentrations. (A) WT. (B) α Asn_0 (Absorption peaks in the upper trace arise from the presence of RCs in these membranes). (C) β Asn_0. As none of the mutants except β Asn_0 had an LH1 complex in the membranes only the α Asn_0 mutant has been plotted for clarity.

shifted by up to 20 nm according to the coordination state of the Mg ion and the nature of the ligand, with nitrogen imparting a greater red shift than oxygen. This shift is thus likely to be directly related to the β Asn_0 mutation.

A comparison of the LT spectral maxima of membranes prepared from various control strains (Table 2) shows that the absence or presence of RCs and the type of carotenoid in the complex have a small but noticeable effect on the electronic properties of the Bchl molecules. In both carotenoid backgrounds the absence of RCs and PufX causes the LH1 λ_{max} to be red shifted by 2−3 nm compared to the respective LH1 + RC strain, similarly these were reflected in the 4−6 nm red shift of the corresponding fluorescence emission maxima (Table 2). The presence of neurosporene (DD13/DG2) rather than spheroidenone (DD13) appeared to result in red shifts of a similar magnitude. The Q_y bandwidths of the βH_0N mutant complex are broader in all cases than their WT equivalents (see Table 2). The greatest difference was observed between LH1-only strains containing WT or β Asn_0 complexes, which were 23 and 28 nm,

Table 2: Absorption (λ_{max}), Q_y Bandwidths (FWHH), Fluorescence Emission (F_{max}) Wavelength Maxima and Energy Transfer Efficiency of the LH1 Complex at 77 K

		DD13					DD13/DG2				
		λ_{max} (nm)[a]		FWHH[b]	ET[c]	F_{max}	λ_{max} (nm)[a]		FWHH[b]	ET[c]	F_{max}
	mutant	Q_x	Q_y	(nm)	(%)	(nm)	Q_x	Q_y	(nm)	(%)	(nm)
LH1-only	WT	588	889	23	71	908	590	890	26	66	912
	βH_0N	584	882	28	33	905	585	881	28	40	904
LH1 + RC	WT	588	886	26	76	902	591	888	26	57	908
	βH_0N	584	880	28	60	903	586	882	29	56	906

[a] Wavelengths of Q_x and Q_y absorption maxima. [b] Q_y bandwidths, full width at half-height. [c] Efficiency of energy transfer from carotenoid to Bchl, based on the relative size of the carotenoid bands in excitation and fractional absorption spectra, normalized at the Q_x absorption maxima. Absorption spectra of the LH1-only mutants could not be used for the energy transfer calculations due to extensive light scattering by the membranes. In these cases the fractional absorption spectra of the corresponding WT membranes were used instead.

respectively. The other combinations exhibited a 2 nm broader bandwidth on average.

Excitation spectra were recorded in the visible region of the spectrum in order to obtain a qualitative comparison of the WT and β Asn_0 strains (data not shown). The energy transfer efficiencies from carotenoid to Bchl in the WT LH1-only complexes were about 65−75% in the case of spheroidenone and 55−65% for neurosporene. Whereas in the β Asn_0 + RC mutants transfer from carotenoids appeared to proceed with a similar efficiency, the carotenoid bands in the excitation spectra of LH-only mutant membranes were 30−50% lower than in the corresponding spectra of WT membranes. Because of their relatively low levels of complex, membrane samples of these mutants were highly scattering, thus distorting the absorption spectra in the carotenoid region. However, acetone:methanol extraction of mutant membranes indicated that the carotenoid levels were comparable to those seen in the WT equivalent (data not shown), and thus it is concluded that the efficiency of energy transfer from carotenoid to Bchl is slightly reduced. Whether all these carotenoid molecules are indeed inserted in the mutant LH1 complexes, i.e., whether the reduction of excitation transfer efficiency from the carotenoid to the Bchl reflects a lower occupancy of carotenoid binding sites, is still unclear.

FT Raman spectroscopy was conducted on the β Asn_0 mutant expressed as both LH1-only and LH1 + RC strains, in the presence of spheroidenone (DD13), as previous work had shown that the different carotenoids had no effect on the Raman spectrum (Olsen *et al.*, 1994). In the pre-resonance conditions used it is expected that the resonance Raman spectra only contain contributions from the Bchl and carotenoid molecules (Mattioli *et al.*, 1993). In the higher frequency region of the Raman spectrum (1580−1710 cm^{-1}) a band at ca. 1600−1610 cm^{-1} is visible, arising from the methine bridge stretching mode of the Bchl *a*, the frequency of which is sensitive to the co-ordination state of the central Mg ion of these molecules (Cotton & Van Duyne, 1981) or more precisely to the degree of distortion experienced by the dihydrophorbin macrocycle (Näveke *et al.*, 1997). In the 1620−1710 cm^{-1} range there are contributions from the stretching modes of the carbonyl groups conjugated with the dihydrophorbin macrocycle, that is the C_2 acetyl and C_9 keto carbonyl groups (Lutz & Robert, 1988). Using excitation at 1064 nm, these modes are intense, dominating the band arising from the methine bridge stretching mode (Mattioli *et al.*, 1991). The stretching mode frequencies of both the acetyl and keto carbonyl groups are sensitive to the involvement of these groups in intermolecular interactions, and these bands are located at ca. 1660 and 1695 cm^{-1}, respectively, when they are free from any intermolecular interactions. Upon formation of hydrogen bonds they shift down to ca. 1620 and 1660 cm^{-1}, respectively, depending on the strength of the H-bond formed (Lutz & Robert, 1988).

As published previously (Olsen *et al.*, 1994), in the WT LH1-only spectrum (Figure 2) bands are present at 1612, 1642, and 1661 cm^{-1}. The presence of a 1612 cm^{-1} band indicates that both the Mg atoms of the two Bchl responsible for the 880 nm absorption are five-coordinate. The presence of only two bands in the carbonyl stretching frequency region (1642−1662 cm^{-1}), where up to four different bands could be expected, has been interpreted as arising from contributions from the C_2 acetyl carbonyls of each of the two Bchl *a* molecules in the minimal unit (an $\alpha\beta$ dimer), both being involved in similar intermolecular interactions. The 1642 cm^{-1} band was thus attributed to both the C_2 acetyl carbonyl of the two B880 Bchls, each H-bonded with neighboring amino acid side chains. This was substantiated by mutagenesis of each of the amino acid H-bond partners of the C_2 acetyl carbonyls , α Trp_{+11} (Olsen *et al.*, 1994) and β Trp_{+9} (Sturgis *et al.* 1997). In the spectral range corresponding to the stretching modes of the C_9 keto carbonyl groups (1660−1705 cm^{-1}), only the 1661 cm^{-1} band is observed, and thus it must be concluded that the C_9 keto stretching contributions of both the 880 nm-absorbing Bchl molecules are degenerate, each of these groups being involved in similar intermolecular interactions (Olsen *et al.*, 1994). To date, however, neither the combination of site-selected mutagenesis with Raman spectroscopy nor X-ray crystallography had revealed any good candidates for these interactions (Olsen, Sturgis, Robert, and Hunter, unpublished results, Koepke *et al.*, 1996).

Resonance Raman spectra of the membrane of the LH1-only β Asn_0 mutant, contain additional, non-resonant, contributions, which result, in particular, in intense contributions in the C−H stretching region at ca. 3000 cm^{-1} (data not shown). This is due to the fact that the amount of LH1 complexes in these membranes is not high enough for the Bchl contributions to be observed selectively. In these conditions, as reported previously (Beekman *et al.*, 1995), the carbonyl stretching frequency region of Bchl *a* is distorted by additional, broad, contributions at ca. 1650 cm^{-1}. It is possible to extract the Bchl signal from such FT-Raman spectra, by subtracting a FT-Raman spectrum of DD13 membranes, purified from bacteria in which neither of the operons encoding LH2 or LH1−RC complexes are present, using the C−H stretching region for normalisation (Beekman *et al.*, 1995). Such a difference spectrum is shown in Figure 2a. As a further control, Raman spectra with a 950 nm

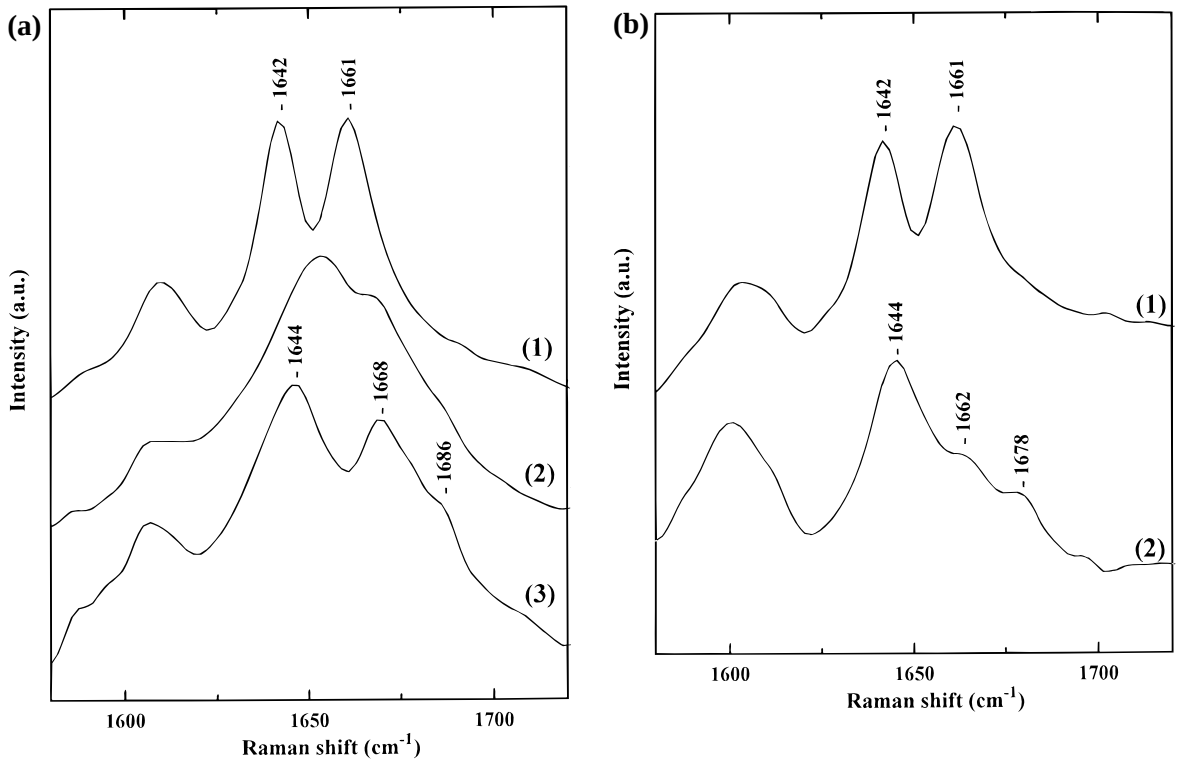

FIGURE 2: (a) FT Raman spectra (1580−1720 cm^{-1} region) of membranes from *Rb sphaeroides*: (1) WT in LH1-only strain; (2) β Asn_0 in LH1-only strains; and (3) computed spectra of the β Asn_0 mutant, after subtraction of membrane contributions (see text). (b) FT Raman spectra (1550−1750 cm^{-1} region) of membranes from *Rb sphaeroides*: (1) WT in LH1 + RC strain and (2) β Asn_0 in LH1 + RC strain.

excitation from a Ti-sapphire laser were recorded, which ensures better resonance conditions with the Q_y transition of the Bchl molecules, and these spectra were very similar to those obtained by the difference method with a 1064 nm excitation (data not shown). Despite the change in the liganding residue there is little change in the 1612 cm^{-1} band which indicates that the affected Bchl is still pentacoordinated. The band arising from the acetyl carbonyl stretching modes is at a similar position as in the WT spectra, although broader than that in the WT. This suggests for this mutant, that some structural disorder exists in the binding site of at least one Bchl. The band arising from the keto carbonyl stretching frequencies is highly perturbed, being less intense and broader, and new components are observed between 1675 and 1686 cm^{-1}.

In the membranes from strains synthesizing RCs there is now sufficient βAsn_0 LH1 to dominate the FT-Raman signal, as indicated by the absence of sizeable contribution of the membrane components such as phospholipids, which may be verified by the absence of strong signal in the 3000 cm^{-1} region where the C−H stretching modes of the aliphatic chains of these molecules contribute (data not shown). In the experimental conditions used, in which the primary donor is in its oxidised state, the main contribution of the RC is a strong band at ca. 1600 cm^{-1}, which arises from the methine bridge stretching mode of oxidized P [see e.g. Olsen *et al.* (1994) and spectra herein]. However, contributions in the carbonyl stretching frequency region of P are relatively small (Mattioli *et al.*, 1993) and do not interfere with the LH1 signal. Thus, it may be safely concluded that the contributions in the 1620−1700 cm^{-1} region arise from the carbonyl stretching frequencies of the carbonyl groups of the β Asn_0 mutant. The band at ca. 1644 cm^{-1} (Figure 2) is very similar to that observed in the WT spectra, although broader, and therefore originates from both the acetyl carbonyl stretching frequencies from both the Bchl molecules. In contrast, there is a substantial attenuation of the band arising from the C_9 keto stretching frequencies, accompanied by a small upshift to 1662 cm^{-1} and the appearence of an additional peak at 1678 cm^{-1}. This new feature can only be attributed to the stretching frequency of a less strongly interacting C_9 keto carbonyl group, and must therefore have arisen from the weakening of an H-bond to the C_9 keto carbonyl of one of the B880 Bchls.

DISCUSSION

The results of the mutagenesis of LH1 α His_0 presented in this paper, showing that no complex is formed when this residue is changed, are in accord with those of Bylina *et al.* (1988) for the equivalent residue in *Rb capsulatus*. One conclusion which may be drawn from our work is that the LH1 Bchl binding site is much more demanding, in terms of the Bchl ligands, than the binding site for the primary donor of electron (P) in bacterial RCs. Indeed, in the latter, it is possible to replace each of the histidine residues that form the ligands to P by a series of residues, such as glycine, glutamine, asparagine, leucine, in the latter case the primary donor becomes a Bchl/Bpheo heterodimer (Bylina & Youvan, 1988). As would be expected, when the substitute residue is able to ligand a Mg ion the physicochemical properties of P are well-conserved (Bylina & Youvan, 1988); however, when a glycine residue is employed, P is still unchanged, and this has been interpreted as evidence that a water molecule is interpolated between the glycine and the Mg ion (Goldsmith *et al.*, 1996). In the case of LH1, none of the changes we have introduced on the α polypeptide resulted in the presence of functional proteins in the membrane, and most of the changes affecting the β His_0

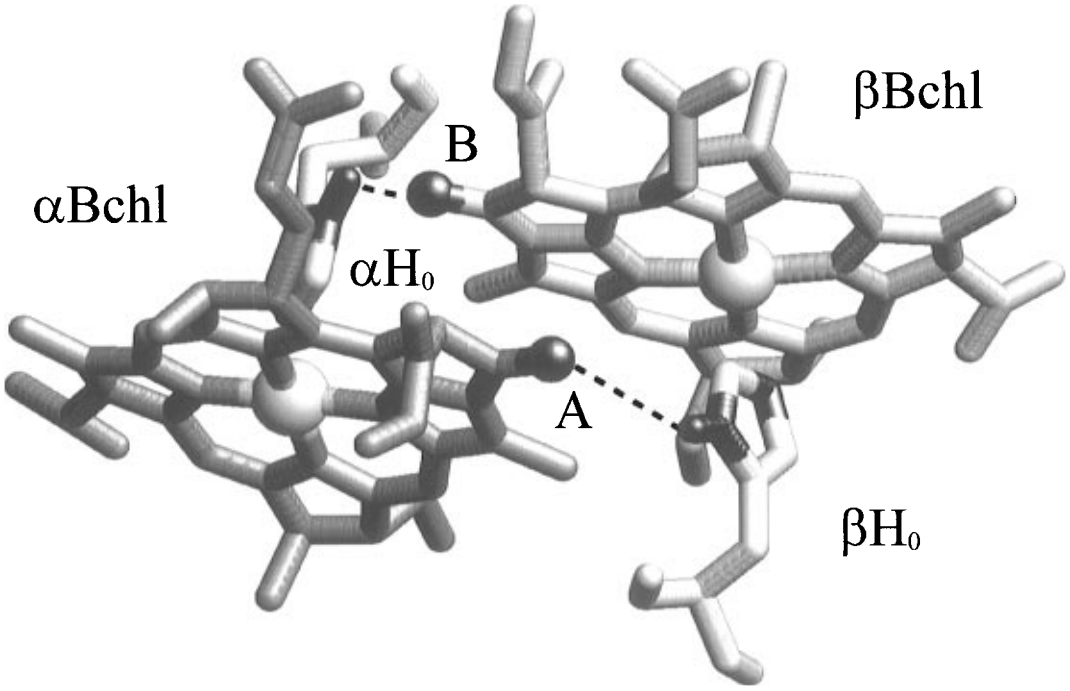

FIGURE 3: Schematic model of the B880 binding site viewed approximately perpendicular to the membrane plane. The His residues and the Bchl orientations are based upon the *Rps molischianum* structure. The proposed H-bonds are represented by the black dashed lines. The His residues belongint to the α and β subunits are indicated on the diagram as αH_0 and βH_0, respectively. The respective C_9 keto carbonyl for each Bchl is labeled A or B.

appear to be incompatible with the presence of functional LH1 in the photosynthetic membrane, even when those potentially allow the liganding of the Mg atom of the bound Bchl molecule. Moreover, in the RC, the mutations affecting the ligands of P do not significantly perturb the interactions between the carbonyl groups of P and the surrounding protein (Goldsmith *et al.*, 1996; Allen *et al.*, 1996). In LH1, by contrast, the only mutation resulting in the presence of complexes in the photosynthetic membrane induces a clear perturbation of the binding site of one of the Bchl, as evidenced by FT-Raman spectroscopy. This difference in tolerance between the RC and antenna binding sites is possibly due to the fact that RC is a more "globular" membrane protein, in which P is enclosed far more completely by the protein structure. In this case the loss of the Mg atom, together with the attendant stabilizing forces, is tolerated, because the Mg ligands represent only a small fraction of the energy which maintains the protein structure. However, it is also possible that energy terms arising from structural constraints are higher in LH1 than in RC, and that mutations targetting the His β_0 residue destabilise these complexes through, for example, steric hindrances. The same argument might also apply to the LH2 α Asn_0 and β Asn_0 mutants, as it appears that these binding sites are even more demanding than those of LH1.

These steric constraints in the B880 binding pocket of LH1 may be due to the interactions between the Bchls, and also between the Bchls and the polypeptides. A comparison of the protein sequences of the *Rb sphaeroides* α and β polypeptides with that of a naturally occurring complex that has Asn in place of His_0 in the β polypeptide, the *Ectothiorhodospira halochloris* β polypeptide, is shown in Chart 1 together with the corresponding sequences of the LH1 α and β polypeptides of *Rb sphaeroides* (Wagner-Huber *et al.*, 1988). The volumes of the relevant side chains are Gly, 66 Å^3; Ala, 92 Å^3; Asn, 135 Å^3; Val, 142 Å^3; His, 167 Å^3; Leu, 168 Å^3.

Chart 1

		-4							0		+3						
R. sphaeroides	LH1α	L	F	L	L	A	V	M	I	H	L	I	L	L	S	T	
R. sphaeroides	LH1β	F	S	A	V	A	I	V	A	H	L	A	V	Y	I	W	
E. halochloris	β polypeptide	Y	W	S	I	G	L	I	A	N	A	L	A	Y	A	W	

The residues that are probably closest to the Bchl are underlined. As can be seen, at positions −1 and +3 the α polypeptide has much larger residues than either of the β polypeptides, while at positions −4 and +3, which are likely to be adjacent to the Bchls, the smallest residues are found in the *E. halochloris* β polypeptide. Therefore the LH1$^-$ phenotype of the α His_0 mutants and the low levels of the β N_0 mutant LH1 in *Rb sphaeroides* may be linked to the disposition of the larger residues in this region of the helix, compared to those of the *E. halochloris* β polypeptide. This could be particularly significant for Ala_{-4}, which is conserved among most LH1 complexes (Brunisholz & Zuber, 1992); also, it has been shown by site-selected mutagenesis that no residue larger than Val is tolerated at that position (Bylina *et al.*, 1988). The failure of Asn and Gln to replace α His_0 as the fifth ligand of the central Mg ion suggests that a very specific relative geometry of the α polypeptides, forming the inner ring of the complex, and the corresponding Bchl macrocycles is required in order for the complex to assemble. In contrast, the β polypeptides on the outside of the complex may be in a less constrained environment, permitting greater flexibility in the length and orientation of the Bchl ligand. Further mutagenesis of the residues that face the Bchls on the α and β polypeptides should reveal information on these putative protein steric constraints and possibly increase the stability of the mutated complex.

The co-expression of RCs is clearly advantageous for the expression of mutated LH1 complex, since their levels in membrane are increased fourfold relative to those in the LH1-only strain. This may be the consequence of an interaction between these complexes, either because RCs act as stabilizing scaffolds for the antenna complex or because their presence enhances insertion of LH polypeptides into the membrane. The small difference between the Q_y absorption maxima of WT LH1 in the absence and presence of RCs (Table 2) also indicates that the properties of LH1 are influenced by the presence of RCs and PufX.

The more pronounced blue shift of β Asn_0 LH1 relative to that of the WT complex may be a consequence of the relatively low antenna levels in these membranes, the latter influencing the aggregation state of the complexes. Comparable spectral shifts have been observed with *Rb sphaeroides* LH1 mutants where C-terminal truncations of the α-polypeptide led to reduced antenna levels (McGlynn *et al.*, 1996), and these were attributed to a smaller aggregation state of the complex. Alternatively, these shifts may be related to an alteration of the H-bonding to the C_9 keto carbonyls of the Bchls. However, blue shifts of the absorption maxima in α Y_{+11} and F_{+11} mutants, accompanying the perturbation of the H-bond to one of the C_2 acetyl carbonyls, generally were considerably larger. Alterations in the H-bonding of the C_9 keto carbonyls resulting from the replacement of His by Asn may therefore have relatively little or no direct effect upon the absorption red shift of the complex, a conclusion that mirrors the situation in LH2 complexes (Sturgis *et al.*, 1995).

Comparison of the Raman spectra leads to the conclusion that half of the 1661 cm^{-1} band appears to upshift in the β Asn_0 mutants. This demonstrates that this band is degenerate, as predicted in earlier studies (Olsen *et al.*, 1994), and that it must account for each of the keto carbonyl groups of the Bchl bound to the LH1 protein, both being strongly H-bonded in the WT complex. In the β Asn_0 mutant, one of these H-bonds is either broken or considerably weakened. As previously concluded for LH2 proteins (Sturgis *et al.*, 1995), modifying the interaction state of a keto carbonyl group seems to have only a very limited influence on the absorption and fluorescence properties of the complex. Thus, the breakage of an H-bond between a C_2 acetyl carbonyl and the protein generally results in a blue shift in the absorption of the protein of ca. 10−15 nm, in both LH2 and LH1 (Fowler *et al.*, 1994; Olsen *et al.*, 1994; Sturgis *et al.*, 1995, 1996), whereas the absorption changes directly induced by the β Asn_0 mutation are at most 3 nm.

Site-directed modification of the β His_0 residue is highly specific, with regard to the effects on the Raman spectra. This is a counterpart to previous work, in which the C_9 keto carbonyl H-bonds were largely undisturbed in mutants of the LH1 of *Rb sphaeroides* (Olsen, 1994; Olsen *et al.*, 1994; Sturgis *et al.*, 1997) in which the C_2 acetyl carbonyl H-bond status of the B880 Bchls were perturbed and broken. Therefore it is proposed that the coordinating His residue on the β-polypeptide is the H-bond partner of the C_9 keto carbonyl of the α bound Bchl, and that it is this H-bond that has been perturbed by the β Asn_0 mutation. It should be noted that mutations of other highly conserved residues close to the Bchls have been constructed, such as αS_{+5} and αT_{+6}; these were possible candidates as donors of an H-bond to the C_9 keto carbonyls but the alteration to Ala, which is not capable of H-bonding, did not significantly alter the Raman spectrum (Sturgis and Olsen, unpublished data). These results are in good agreement with the structure of the binding site of the LH2 of *Rsp molischianum* (Koepke *et al.*, 1996) which has been shown to be closely related to that of LH1 (Germeroth *et al.*, 1993). Indeed, in these complexes, no amino acid side chain is close enough to the keto group of each Bchl to be a likely candidate for interacting with them, other than the imidazole rings of the α_0 and β_0 histidines. We therefore propose a structural model of the LH1 Bchl binding site which accounts for FT-Raman data on the β Asn_0 mutation. In this model, the Bchls in a minimal unit are arranged in a head-to-head configuration, similar to that seen in the LH2 structures, but with an imidazole conformation such that it results in the C_9 keto carbonyl of the Bchl liganded to the α polypeptide approaching the protonated nitrogen of the His residue on the β polypeptide, and *vice versa*. The symmetry of this model is a first approximation, accounting for the degeneracy of the C_9 keto peak in at room temperature, although this degeneracy could be resolved at low temperature (Robert & Lutz, 1985; Sturgis & Robert, 1997). Such a model is summarized in Figure 3, which was built from the BChl binding site of the *Rsp molischianum* LH2 (Koepke *et al.*, 1996). It must be noted that, because of the similarity between the Raman frequencies observed for *Rsp molischianum* LH2 and LH1 proteins, the length and geometry of the H-bonds involved in the BChl binding sites should be very well conserved among these complexes. Because the Asn coordinating residue is smaller than the His and also has a different disposition of atoms capable of H-bonding, it is likely that the exchange of Asn for His partially disrupts the H-bond to the α bound Bchl C_9 keto carbonyl.

REFERENCES

Allen, J. P., Artz, K., Lin, X., Williams, J. C., Ivancich, A., Albouy, D., Mattioli, T. A., Fetsch, A., Kuhn, M., & Lubitz, W. (1996) *Biochemistry 35*, 6612−6619.

Beekman, L. M. P., Visschers, R. W., Monshouwer, R., van Mourik, F., van Stokkum, I.H. M., Dawson, M. H., Mattioli, T. A., McGlynn, P., Hunter, C. N., Robert, B., van Grondelle, R., & Jones, M. R. (1995) *Biochemistry 34*, 14712−14721.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Brunisholz, R. A., & Zuber, H. (1992) *Photochem. Photobiol B 15*, 113−140.

Bylina, E. J., & Youvan, D. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7226−7230.

Bylina, E. J., Robles, S. J., & Youvan, D. C. (1988) *Isr. J. Chem. 28*, 73−78.

Callahan, P. M., & Cotton, T. M. (1987) *J. Am. Chem. Soc. 109*, 7001−7007.

Chothia, C. (1984) *Annu. Rev. Biochem. 53*, 537−572.

Cotton, T. M., & Van Duyne, R. P. (1981) *J. Am. Chem. Soc. 103*, 6020−6026.

Crielaard, W., Visschers, R. W., Fowler, G. J. S., van Grondelle, R., Hellingwerf, K. J., & Hunter, C. N. (1994) *Biochim. Biophys. Acta 1183*, 473−483.

Fowler, G. J. S., Sockalingum, G. D., Robert, B., & Hunter, C. N. (1994) *Biochem. J. 299*, 695−700.

Germeroth, L., Lottspeich, F., Robert, B., & Michel, H, (1993) *Biochemistry 32*, 5615−5621.

Goldsmith, J. O., King, B., & Boxer, S. G. (1996) *Biochemistry 35*, 2421−2428.

Karrasch, S., Bullough, P. A., & Ghosh, R. (1995) *EMBO J. 14*, 631−638.

Koepke, J., Hu, X., Muenke, C., Schulten, K., & Michel, H. (1996) *Structure 4*, 581−597.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
Jones, M. R., Fowler, G. J. S., Gibson, L. C. D., Grief, G. G., Olsen, J. D., Crielaard, W., & Hunter, C. N. (1992a) *Mol. Microbiol. 6*, 1173−1184.
Jones, M. R., Visschers, R. W., van Grondelle, R., & Hunter, C. N. (1992b) *Biochemistry 31*, 4458−4465.
Lutz, M., & Robert, B. (1988) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Vol. 3, p 347, Wiley-Interscience, New York.
MacDermott, G., Prince, S. M, Freer, A. A., Hawthornthwaite-Lawless, A. M., Papiz, M. Z., Cogdell, R. J., & Isaacs, N. W. (1995) *Nature 374*, 517−521.
Mattioli, T. A., Hoffman, A., Sockalingum, D. G., Schrader, B., Robert, B., & Lutz, M. (1991) *Biochemistry 30*, 4648−4654.
Mattioli, T. A., Hoffmann, A., Sockalingum, D. G., Schrader, B., Robert, B., & Lutz, M. (1993) *Spectrochimica Acta 49A*, 785−799.
McGlynn, P., Westerhuis, W. H. J., Jones, M. R., & Hunter, C. N. (1996) *J. Biol. Chem. 271*, 3285−3292.
Näveke, A., Lapouge, K., Sturgis, J. N., Hartwitch, G., Simonin, I., Scheer, H., & Robert, B. (1997) *J. Raman Spectrosc.* (in press).
Olsen, J. D., Sockalingum, G. D., Robert, B., & Hunter, C. N. (1994) *Proc Natl Acad Sci U.S.A. 91*, 7124−7128.
Olsen, J. D. (1994) Ph.D. Thesis, University of Sheffield, U.K.
Olsen, J. D., & Hunter, C. N. (1994) *Photochem. Photobiol. 60*, 521−535.
Robert, B., & Lutz, M. (1985) *Biochim. Biophys. Acta 807*, 10−23.
Sanger, F., Nickler, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Simon, R., Preifer, U., & Puhler, A. (1983) *BioTechnology 1*, 784−791.
Sturgis, J. N., & Robert, B. (1994) *J. Mol. Biol. 238*, 445−454.
Sturgis, J., Olsen, J. D., Robert, B., & Hunter, C. N. (1997) *Biochemistry 36*, 2772−2778.
Tabor, S., & Richardson, C. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4767−4771.
Tabor, S., & Richardson, C. C. (1989a) *J. Biol. Chem. 264*, 6447−6458.
Tabor, S., & Richardson, C. C. (1989b) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4076−4080.
Wagner-Huber, R., Brunisholz, R. A., Bissig, I., Frank, G., & Zuber, H. (1988) *FEBS Lett. 233*, 7−11.
Westerhuis, W. H. J. (1994) Ph.D. Thesis, Rutgers University, New Brunswick, NJ.

BI9710481