Chlorophyll is attached to apoprotein in diastereotopically distinct ways, by β- and α-ligation. Both the β- and α-ligated chlorophylls of photosystem I are shown to have ample contacts to apoprotein within their proteinaceous binding sites, in particular, at C-13 of the isocyclic ring. The H-bonding patterns for the C-13β oxy groups, however, are clearly distinct for the β-ligated and α-ligated chlorophylls. The β-ligated chlorophylls frequently employ their C-13β oxy in H-bonds to neighboring helices and subunits. In contrast, the C-13α oxy of α-ligated chlorophylls are significantly less involved in H-bonding interactions, particularly to neighboring helices. Remarkably, in the peripheral antenna, light harvesting complex (LH2) from Rhodobacter sphaeroides, a single mutation in the α-subunit, introduced to eliminate H-bonding to the β-bacteriochlorophyll-B850, which is ligated in the “β-position,” results in significant thermal destabilization of the LH2 in the membrane. In addition, in comparison with wild type LH2, the expression level of wild type LH2, the presence of different substituents at the periphery of the tetrapyrrole skeleton, as well as its hydrogenation state, further distinguish these two groups of molecules. The structures of Chl and BChl are very similar. The bacteriochlorophyll-Bchls has been recognized to modulate the spectral and, although still disputed, redox properties of these molecules (10). H-bonding to the C-13δ keto carbonyl at the isocyclic ring, which is common to all bacteriochlorophylls and chlorophylls, seems to be widespread (see e.g. Refs. 8–11) but appears to have less or no effects on their electronic properties (10–12). In photosystem I (PS) from cyanobacteria (13), which comprises more than 10 TM subunits and contains nearly 100 Chl-binding pockets and thus constitutes a considerable data base for (bacterio)chlorophyll binding studies, the majority of the polar groups of Chls, in particular the C-13δ keto group, are likely to be H-bonded by the polypeptide residues in close vicinity (11, 14–16). The ligation to the central magnesium has long been recognized to be critical for the binding of BChl and thus the assembly of BChl proteins (17, 18). However, only recently, the stereochemical aspect in Chl ligation has been discussed for Chl protein assemblies (19, 20). Because of the dissymmetric arrangement of the substituents, the majority of the polypeptides, in particular the C-13δ keto group, are likely to be H-bonded by the polypeptide residues in close vicinity (11, 14–16).

The ligation to the central magnesium has long been recognized to be critical for the binding of BChl and thus the assembly of BChl proteins (17, 18). However, only recently, the stereochemical aspect in Chl ligation has been discussed for Chl protein assemblies (19, 20). Because of the dissymmetric arrangement of the substituents, the ligation to the central magnesium atom may occur from either the top (β-type) or the bottom (α-type) of the macrocycle. The two ligation states should differ in their energies, with the ligation from below being favored by ∼4 kJ/mol over ligation from above (21). In (B)Chl proteins, the two types of ligation are unevenly distributed. In PSI only 14 of the 96 Chl molecules are ligated in the β-position (19). It has been hypothesized that the stereochemistry of (B)Chl ligation by the protein matrix may considerably influence the photophysical properties of these pigments. In the case of (B)Chl ligated in the β-position, it has been proposed that ligation could result in a more pronounced red-shifted absorption, thus optimizing energy transfer (19). A very recent work, it has been proposed that the β-ligated Chl in light-harvesting complex II occupies key positions for energy transfer (22). This, however, has not been demonstrated by experimental studies. The role of the diastereotropic distinct ligation of (B)Chls thus remains to be substantiated.

Here, we have addressed the role of the diastereotropic Chl ligation by a combination of statistical analysis of PS I and site-specific mutagenesis of the (B)Chl-binding pockets of the peripheral LH2 antenna from
Rhodobacter sphaeroides. We show that there is a strong correlation between the ligation stereochemistry and the H-bonding patterns of the C=O groups of ring E with neighboring polypeptide in PS I. The C-13 keto carbonyl groups of the unfavorable β-ligated Chls primarily have H-bonding contacts with residues from neighboring helices and/or loop structures. In contrast, the C-13 keto carbonyl groups of α-ligated Chls have significantly less H-bonding contacts, and it is primarily to loop structures. As is obvious from the LH2 crystal structure (23, 24), both BChls that make up BChl-B850 are ligated in the β-position. Mutation of the H-bond donor to the C-13 keto of one of these BChl molecules results in the significant impairment of LH2 assembly. This supports the idea that ligation of (bacterial)chlorophyll in the β-position has a pronounced structural impact on (B)Chl proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Gene Transfer, and Growth Conditions—The bacterial strains used in this work include Escherichia coli strain S17-1 (thi pro hsdR7 hsdM7 recA4 RP4-2 (Tc::mu Kan::Tn7)) and Rh. sphaeroides strain DD13 (genomic deletion of both pucBA and pufBALMX; insertion of Smr and Kanr genes, respectively) (25). The mobilizable plasmids used were based on pRK75BC1 (Te8, derivative of pRK415; insertion of a 4.4-kb fragment encompassing pucBA); briefly, this expression vector contains the pucBA genes as a 420-base pair KpnI-BamHI insert (25). Growth conditions for E. coli and Rh. sphaeroides were as described in Ref. 26. For E. coli, tetracycline was used at concentrations of 10 μg/ml. For Rh. sphaeroides the antibiotics were tetracycline (1 μg/ml) and neomycin (10 or 20 μg/ml). Conjugative transfer of plasmid from E. coli S17-1 to Rh. sphaeroides was performed as described (26).

Construction of Mutant LH2—LH2 with glycine instead of serine, valine, or leucine at positions −4, +3, or +4 of the α-subunit, resulting in α-S(−4)G, α-V(+3)G, or α-L(+4)G were constructed by site-directed mutagenesis (QuikChange II, Stratagene) by directly mutating pucA in pRK75BC1.

Instrumentation—In situ absorbance spectroscopy of whole cells in bacterial colonies was performed on a dual fiber model SD2000 spectrophotometer equipped with a reflexion probe (Avantes, Ostfildern, Germany) as described previously (11, 27). FT-Raman spectra and CD spectra of purified membranes and isolated LH2s were recorded using a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module equipped with a continuous Nd:YAG laser and on a Dichrograph (Jasco I-715) as described previously (28).

Protein Determination—Total protein content in membranes was determined by using the BCA™ kit (Pierce). Sample concentration ranges, used for protein determination, were such that contributions to the absorption at 562 nm from sources other than the peptide intercalating agent were negligible (sample absorption, A562 < 0.01). For the estimation of LH2 protein content, the extinction coefficient of B850 BChl was taken as 120 mm−1 cm−1 (29).

Preparation of Intracytoplasmic Membranes—The membranes were prepared from cells grown semi-aerobically in the dark by disruption in a French pressure cell and subsequent centrifugation on a sucrose step gradient (27).

Purification of LH2 Complexes—To purify LH2s, the membranes were solubilized as described (30). Following solubilization, the mixture was centrifuged, and LH2s were purified by DEAE-Sepharose and size exclusion chromatography (30).

The numbering specifies the amino acid position relative to the histidine, designated His (0), which binds the central magnesium of the α-BChl-850. The number following BChl indicates the maximum absorption of the red-most absorption band of BChl.

![FIGURE 1. Diastereotopic ligation of chlorophyll. In the α-ligated (B)Chl, the histidine ligand (l) to the central magnesium atom and the 17-propionic acid phytol are pointing to opposite sides of the chlorin macrocycle, while in the β-ligated (B)Chl they are positioned on the same side. Note that for the C-13 keto carbonyl and methoxy groups the positioning relative to the protein ligand is reversed. Chlorin structure and numbering system are according to IUPAC rules (66). R1 is either acetyl (BChl) or vinyl (Chl). Note that R3, (ethyl), R5, (carboxymethoxy) and R4 (hydrogen) are identical in BChl and Chl.](image-url)
for the two diastereotopic ligation forms. Naturally, histidine is frequently close to the nitrogen atoms, which bind the central magnesium atoms. Notably, the aromatic residue, phenylalanine, is often found to be close to Chl, in particular around atoms that have little contact to histidine residues. Contacts to phenylalanine residues are entirely absent at C-14, C-15, C-16, and N-24 of ring D where histidines are frequently observed. Next to these two residues, the aliphatic residues leucine, isoleucine, and valine as well as alanine and the aromatic residues tyrosine and tryptophan make up most of the residues in the Chl-binding pockets. Polar residues are present to a lesser degree but are equally distributed, whereas charged residues are rarely found in close vicinity of the Chls (Fig. 2, A and C).

Chl protein Interactions Vary Depending on the Chl Ligation State—Fig. 2 (B and D) show the contact maps for β- and α-ligated Chl with their respective binding helices, i.e. the helices that carry the ligation residue to the central Mg of the Chls. These contact maps are clearly distinct for the two diastereotopic forms. The main differences are observed at the rings E and their substituents. In case of the β-ligated Chl (Fig. 2B), there exist very few close contacts between the residues of the helices and the atoms of ring E. Remarkably, the keto carboxyl and methoxy groups of C-131 and C-133 of the β-ligated Chls are not found in the vicinity of the residues of the binding helices in PS I (Fig. 2B). In contrast, the atoms of ring E of the α-ligated Chls are very frequently found in close vicinity of these residues (Fig. 2D). Both the C-131 and C-133 oxo as well as C-133 of the α-ligated Chl are frequently in close contact with the residues of the binding helices. Additional differences in the Chl-binding sites are observed for the atoms of ring D and the 17-propionic phytol (Fig. 2D). In the case of the α-ligated Chls, contacts are conspicuously low. These findings show that significant differences exist between the binding pockets of Chls, depending on the ligation state. The C-13 substituents of Chls ligated in the α-position are facing the binding helix, and thus, they are often in close contact with residues of these helices. The C-13 substituents of Chls ligated in the β-position, however, face to the opposite side and are not in contact with the binding helix. Nevertheless there is a substantial number of contacts between the C-13 substituents of the Chls ligated in the β-position and the protein environment as shown in Fig. 2A.

Chls Ligated in the β-Position Associate with Residues of Adjacent Helices—The interactions of the C-131 oxo groups of the β-ligated and α-ligated Chls in photosystem I and photosystem II are listed in Table 1. These data show that the oxo atoms of β-ligated Chls in both PS I and PS II are exclusively found in close contact with residues of adjacent helices or loop structures but not with residues of the helices which carry the magnesium ligand (Table 1). Remarkably, these close contacts include the helices of other subunits, as well as those of the same apoprotein. Most of the contacts of the C-131 oxo involve residues of neighboring helices (44% in PS I and 57% in PS II) either from the apoproteins, or from neighboring subunits. The remaining C-131 oxo atoms of the Chls ligated in the β-position are in close contact with other Chls and residues located in loop structures. Interestingly, a very different picture emerges for the C-133 oxo atoms of α-ligated Chl. Here, the majority of contacts (47% in PS I and 39% in PS II) are to residues of adjacent loop structures, whereas contacts to neighboring helices (10% in PS I and 25% in PS II) are less frequent, in particular to different subunits (3% in PS I and 7% in PS II).

Chls Ligated in the β-Position Are H-bonded to the Residues of Adjacent Helices—Almost all of the observed contacts between the C-133 oxo of the Chl molecules ligated in the β-position and the surrounding residues are identified as H-bonding interactions (Table 1) as judged by the distance and angle between the interacting atoms (not shown). All of the contacts to the helices of neighboring subunits and most of the contacts to adjacent helices within the apoproteins are identified as H-bonds. Essentially, these findings are very similar for the two photosystems with the exception that the majority of contacts to loop structures are not identified as H-bonds in PS II yet are identified as H-bonds in PS I. The reason for this discrepancy is presently unknown. It maybe related to the lower resolution in the case of the PS II structure introducing a considerable error. Remarkably, in the case of the Chls ligated in the α-position, very few of the contacts to adjacent helices are identified as H-bonding interactions. Of the contacts to loops structures (in PS I) and binding helices approximately half meet the criteria for H-bonding interactions (Table 1). In PS II, very few H-bonds are identified in the case of the contacts between the α-ligated Chls and loop structures as has been already observed for β-ligated Chls (see above).
In conclusion, the statistical analyses of PS I and PS II suggests that the binding pockets of the two Chl ligation forms are clearly distinct. In particular, the C-13 substituents are found in different protein environments. In the majority of Chls, which are the Chls ligated in the α-position, the oxo atoms of the C-13\(^1\) are in the close vicinity of loop structures and the helices carrying the magnesium ligand as well as adjacent helices. A considerable percentage of H-bonding interactions are merely found for the contacts to loop structures (in PS I). In contrast, the C-13\(^1\) oxo of the Chls ligated in the β-position are chiefly engaged in H-bonds, in particular with helices other than the one ligating the central Mg. It appears thus that the C=O groups of β-ligated Chls mediate interactions between adjacent helices, likely critically contributing to their packing and assembly. H-bonding, in particular involving the β-ligated Chls, may thus be a critical, hitherto unrecognized, assembly motif in (B)Chl proteins.

**Mutagenesis of the H-bond Donor to β-Ligated BChl-B850 Significantly Hampers LH2 Assembly**—To experimentally explore the role of H-bonding to the C-13\(^1\) keto carbonyl group of (B)Chl that is ligated in the β-position, we chose the peripheral antenna complexes, LH2, from the purple bacterium *R. sphaeroides*. This complex contains three bacteriochlorophyll molecules, two are bound in the β-position (BChl-B850) and one is bound in the α-position (BChl-B800). Although the pigments used for the statistical analysis of Chl protein interactions (Fig. 2 and Table 1) are Chl\(\alpha\), the LH2s contain Chl\(\alpha\). The overall structures of BChl and Chl, however, are very similar (Fig. 1). In particular, ring C and E including the C-13 substituents are identical in the two molecules. It has previously been shown that Chl molecules may replace BChl in certain photosynthetic complexes (33), demonstrating their similar structural basis for binding. Moreover, in these reconstitution studies, the C-13 substituents have been identified as a critical structural feature for stable binding (34). Thus, exploring the role of the diastereoptic ligation of chlorophylls by use of BChl-binding proteins should be a valid approach for both BChl and Chl molecules.

We have previously shown that the C-13\(^1\) keto carbonyl group of BChl-B850 of the β-subunit is H-bonded to the hydroxyl group of serine(−4) of the α-subunit (11) (Fig. 3, A and B). To study the impact of this H-bonding motif on the assembly of LH2, we have now replaced serine(−4) by a glycine residue. This mutation removes the side chain in the close vicinity of C-13\(^1\) oxo of the β-BChl-B850 and should thus effectively disrupt the H-bond to α-serine(−4). The assembly of the complex is monitored by absorption spectroscopy, because significant spectral alterations accompany the BChl-BChl and BChl-polyester association to the native LH2. Most pronounced are the red shifts of the \(Q_s\) transitions of the BChl-B850 and BChl-B800 from \(\sim 770\) nm in organic solvent to \(\sim 848\) and \(800\) nm. The near infrared absorption spectra of LH2 α-S(−4)G or LH2 WT are shown in Fig. 4. The replacement of the α-serine(−4) with glycine results in significant reduction of the 800- and \(\sim 850\)-nm absorption bands relative to the absorption band at \(\sim 760\) nm (Fig. 4). The spectrum of LH2 α-S(−4)G has a major band

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**TABLE 1**

H-bonding interactions of the Chl ligated in the β- and α-positions in PS I and II

<table>
<thead>
<tr>
<th></th>
<th>Chl β</th>
<th>Chl α</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>Neighboring pigments</td>
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<td>29</td>
</tr>
</tbody>
</table>

* H-bonds between Chl molecules that are ligated by their central magnesium to one subunit and residues of another subunit (e.g. Chl is ligated to subunit A, the C-13\(^1\) group makes a H-bond to a residue of subunit B).

* H-bonds between Chls that are ligated to one TM helix and the residues of neighboring TM helices (other than the binding helices within one subunit).

* H-bonds between Chl and residues of the binding helices.

* H-bonds between Chl and residues from loop structures (including helices parallel to the membrane).

* H-bonds between Chl and neighboring Chl or carotenoids (contacts to Chl are only included if no contacts to polypeptide are present).

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**FIGURE 3.** H-bonding between the β-ligated BChl-B850 and their apoproteins in LH2. **A**, view of the β-ligated BChl-B850 and their binding helices in the LH2 high resolution structure of *R. acidophila* (22). The ligation to the central magnesium and the H-bonds are indicated by lines. **B**, close-up view of the H-bonds between the C-13\(^1\) oxo of the β-ligated BChl-B850 and the residues at position −4 of α-subunit (blue) as well as β-subunit (red). The H-bonds are indicated by the dotted line. **C**, amino acid sequence of the TM helices (TMH) of the LH2 β- and α-subunits. The histidine ligand to the central Mg\(^{2+}\) atom and the residues that have been mutated are underlined.
at 755–765 nm, typical of “free” BChl, mixed with metabolic precursors or degradation products of BChl. This indicates that free BChl accumulates in cells expressing LH2 α-S(−4)G because of either the disturbed assembly of LH2 or the enhanced disintegration of already assembled LH2s. In addition, LH2 α-S(−4)G is expressed only transiently. In cells expressing WT LH2, the assembled complex is detected for nearly 10 days, whereas in cells expressing LH2 α-S(−4)G, assembled complexes are observed only at the onset of growth but are absent from the cells beyond day two (not shown). This is further supported by directly measuring the amount of assembled LH2 in the membrane. Quantification of total protein content versus LH2 content in the membrane indicates that the ratio of total protein to LH2 (as judged by the B850 content) is ~10:2 for WT LH2 and 10:0.5 for LH2 S(−4)G (Table 2). Thus, WT LH2 seems to be approximately four times more abundant in the membranes than LH2 S(−4)G. This directly demonstrates that the replacement of serine with glycine results in reduced amounts of LH2 in the mutant cells.

To test whether the introduction of a glycine residue per se at the BChl-B850/helix interface results in the loss of assembled LH2 from the membrane, we have replaced residues that are found at the BChl-B850/TM helix interface with glycine. Next to histidine(0)° and serine(−4), there are two additional residues in close contact to the BChl-B850. One of them is α-valine(+3), which is in close contact with both the α- and β-BChl-B850 (35), in particular, with the C8° of the ethyl group at ring B of α-BChl-B850 and C-8, C-9, and C-10 of the β-BChl-B850 (not shown). The absorption spectrum of LH2 with α-V(+3)G clearly has the typical BChl-B800 and B850 absorption bands with maxima at ~845 and 800 nm (Fig. 4). There occurs a minor blue shift (from 848 to 845 nm) in the absorption band indicative of some slight structural rearrangements in LH2 α-V(+3)G. There also appears a minor absorption band with maximum at ~760 nm, indicative that assembly in LH2 α-V(+3)G is somewhat impaired (Fig. 4).

In addition, leucine(+4), which is strictly conserved in the α-subunits of LH2s (33), has been replaced by glycine. The absorption spectrum of LH2 with α-L(+4)G clearly has the typical BChl-B800 and B850 absorption bands with maxima at ~848 and 800 nm, respectively, indicative of assembled LH2, whereas the absorption band of free BChl is entirely absent. The packing values are very different for glycine and valine as well as leucine residues (0.57 versus 0.48) at membrane embedded helix-helix interfaces, whereas they are rather similar for glycine and serine (0.57 versus 0.534) (36). These altered packing values may thus account for the minor destabilization of LH2 α-V(+3)G. In addition, glycine has been shown to be helix destabilizing when placed within the last 5 residues of a TM helix (37). Both valine(+3) and leucine(+4) are positioned close to the C-terminal end of LH2-α in contrast to serine(−4), which is close to the helix core. The glycine residues at positions +3 and +4 thus entail multiple destabilizing affects on the LH2 unlikely to arise at position −4. Taken together, these data indicate that the stable assembly of the antenna complexes is significantly impaired by the disruption of the H-bond to the BChl-B850, which is ligated in the β-position.

**H-bonding State to β-Ligated BChl-B850 Probed by Resonance Raman Spectroscopy—**The interaction state of BChl, especially its H-bonding interactions with the surrounding polypeptide, has been examined in native LH2 and the α-S(−4)G mutant by resonance Raman spectroscopy (Fig. 5). In apolar solvents and in the absence of H-bonding interactions, the 13° keto stretching mode of BChl α is located at ~1685 cm⁻¹, and that of the 3-acetyl carbonyl group is at ~1663 cm⁻¹. Close to the bands arising from the stretching modes of these groups, on the lower frequency side, there is a band arising from the methine bridge stretching modes of the (B)Chl molecules. This band, which is sensitive to the conformation of the (B)Chl macrocycle, may be used for comparing the intensity of the different bands from the carbonyl stretching modes, thus assessing what happens in this spectral region upon, for example, a mutation. In the RR spectrum of WT LH2, five bands in the carbonyl stretching mode region (1620–1710 cm⁻¹) are resolved (Fig. 5), similar to the findings of Ref. 38. The bands at 1627 and 1635 cm⁻¹ have been attributed to the C-3 acetyl groups of the BChl-B850 (27). The stretching mode of the acetyl carbonyl of the BChl-B800 also contributes in this frequency range, but it is very weak in FT-Raman spectra (39). The remaining bands have been attributed to the 13° keto carbonyl of the BChl-B850 (1651 and 1677 cm⁻¹) and of the BChl-B800 (1701 cm⁻¹) (38, 40). The considerable downshift of one of the BChl-B850 13° keto carbonyl bands to 1651 cm⁻¹ has been proposed to reflect strong H-bonding (38), whereas the band at 1677 cm⁻¹ is downshifted only by ~8 cm⁻¹ relative to the band in organic solvent (41). More recently, this H-bond has been assigned by site-directed mutagenesis to the 13° keto group of β-BChl-B850 interacting with the hydroxy group of α-serine(−4) (29). In the α-S(−4)G mutant only three carbonyl bands are resolved (Fig. 5). The two low frequency bands at 1627 and 1635 cm⁻¹ are replaced by an intense 1630-cm⁻¹ band. The band at 1651 cm⁻¹ is nearly absent, whereas that at 1677 cm⁻¹ has gained intensity and is slightly downshifted to 1674 cm⁻¹. The FT-Raman spectrum of the *Rb. sphaeroides* α-S(−4)G mutant is very similar to the spectra of LH2 from WT *Rhodobacter capsulatus* and *Rhodopseudomonas acidiphila* (38), where the keto carbonyl groups are expected to be free from close protein-interactions. The merging of the 1627–1635-cm⁻¹ bands has already been observed previously in the LH2 spectra from *Rb. sphaeroides* G1C, which produces as the major carotenoid neurosporene (42). It has been attributed to a minor reorganization of the C-terminal end of the α polypeptide, because of the change in the chemical structure of the carotenoid. The similarity in the RR spectra indicates that a similar minor reorganization may occur upon the exchange of serine(−4) with glycine in α-S(−4)G mutant. The main change in the
FT-Raman spectra of the α-S(−4)G mutant LH2 is, however, the considerable up-shift of the 1651 cm⁻¹ band to ~1674 cm⁻¹. This shift indicates that the H-bond to the 13¹ keto carbonyl has been disrupted or significantly weakened. This value (1674 cm⁻¹) is only slightly down-shifted as compared with the expected stretching frequency for a free keto carbonyl group (1680 cm⁻¹). Such a value may be due to a particularly polar environment of this keto carbonyl provided, for instance, by the proximity of the imidazole ring of the histidine 0 of the β polypeptide (11). It is of note that, upon the serine to glycine mutation, when the 1651-cm⁻¹ component shifts, a clear but minor component appears at 1655 cm⁻¹ as a shoulder to the large 1674 cm⁻¹ band. This component is also present in the spectra of mutant LH2 S(−4)A where the serine has been replaced by alanine, although less visible because of the larger shift of the 1651 cm⁻¹ (11). The intensity of this smaller component is ~10% of that of the main C=O stretching band. The origin of this minor component is unclear, and it could suggest some inhomogeneity in the sample. However, it is not uncommon that such a minor component is found in the resonance Raman spectrum of bacterial light harvesting complexes. In WT LH1, isolated or in the membrane, a small component at ~1680 cm⁻¹ has been described, although all of the stretching modes from the four keto groups of the two unequivalent BChls have been attributed in these complexes. These smaller components could be the molecular reflect of the intrinsic disorder in the LH structure, which, in the case of LH2, might result in the breathing of the H-bonds in these complexes (1655 is the expected frequency for free-from-interaction acetyl carbonyl groups).

**Decrease in the Thermal Stability of LH2 Complex α-S(−4)G**—To examine the contribution of the H-bond between α-serine(−4) and BChl-B850, the thermal stability of the LH2 α-S(−4)G complexes has been determined by monitoring the CD signal of BChl-B850 during heat denaturation (see Fig. 6). As BChl-BChl couplings mainly contribute to the typical CD signal (43–47), it is agreed that its exact shape serves as a fingerprint for the BChl and carotenoid arrangement in the LH2. The overall CD spectrum of LH2 WT and LH2 S(−4)G is very similar (see Fig. 6A). The typical CD signal of the BChl-B850 is conservative and S-shaped with extrema at 845 (+) and 862 nm (−) (Fig. 6A). Upon heating the dissociation/unfolding of the complex leads to disruption of the BChl-BChl coupling and consequently to the loss of the CD signal at 845 and 862 nm. As shown, both WT LH2 and LH2 α-S(−4)G exhibit cooperative thermal unfolding transitions in the native membrane (Fig. 6B) and solubilized in detergent β-octylglucoside (Fig. 6C). Interestingly, the denaturation process within the native membrane of WT LH2 covers a much wider temperature range compared with the process in detergent (48). In addition, denaturation is accompanied by a strong increase in the sample turbidity indicative of major reorganization of the lipid bilayer during LH2 denaturation (49). The thermal stability of LH2 α-S(−4)G, as compared with WT LH2, is clearly reduced. The difference in the thermal stability is even more evident in purified complexes, e.g. in detergent as compared with the native membrane (Fig. 6C), suggesting that the decrease in structural stability is not related to changes in the membrane lipid composition or the protein/lipid ratio. The midpoint of transition is shifted to lower temperatures, and most pronouncedly, the cooperativity of denaturation is altered. In the membrane, a much wider temperature range is covered during denaturation for WT LH2 in comparison with LH2 α-S(−4)G, indicative of changes either in the unfolding pathways and/or denaturation rates in the mutant (50). Possibly, in LH2 α-S(−4)G, the rate of dissociation of the BChl from the apoproteins is accelerated in LH2 α-S(−4)G, resulting in the altered cooperativity. Similar alterations have been observed in the cooperativity of the thermal denaturation of BChl model proteins with simplified BChl-B850-binding sites (27). In any case, the acceleration of the decay of the CD signal and the shift to lower temperatures in LH2 α-S(−4)G in comparison with WT LH2, shows that the removal of the H-bond results in an inherently less stable complex. If, *a priori*, that also less complex is assembled cannot be excluded at this point.
The formation of H-bonds is energetically far more favorable in the hydrophobic interior of the lipid bilayer than in a hydrophilic milieu, because of the low effective dielectric environment of the lipids. This has also been demonstrated experimentally by comparison of the formation of intramolecular H-bonding of model compounds in Me2SO and water (51). Nevertheless, additional effects such as packing defects could contribute to the significantly impaired assembly of LH2 in the membrane.

Curiously, in the WT sequence context, mutating serine to alanine resulted in the loss of the strong H-bond without significantly destabilizing the LH2 (11). Recently, high resolution structural data have suggested that CH groups, such as methyl hydrogens, could also act as H-bond donors (52, 53). It therefore may be possible that the alanine residue at position −4 is also involved in, albeit weaker, H-bond interactions with the C-13\textsuperscript{1} keto carbonyl and partly compensates for the disruption of the strong bond to serine(−4). Alternatively, alanine, which has a packing value closely similar to serine, entirely compensates for serine in its role to sequestrate the polar keto carbonyl group from the hydrophobic lipid core, surrounding TM helix, and pigments and thereby to prevent incorrect folds. In any case, the removal of the side chain in close vicinity of the C-13\textsuperscript{1} keto carbonyl group significantly impairs the assembly of the LH2. Apparently, the H-bonding interactions between the serine(−4) of the α-subunit and the C-13\textsuperscript{1} o xo of the β-ligated BChl-B850 represent an essential interaction motif for the assembly of this light harvesting complex.

**H-bonding Motif Occurs in the Inner Antenna Proteins of Photosystems I and II**—A conspicuous H-bond network between Chls that are ligated in the β-position and TM helices are observed in the major subunits A and B of PS I (Fig. 7). The six N-terminal helices of subunit A and B, which are organized into trimers of helix pairs (AB, CD, and EF), make up the inner core antenna of PS I. The five remaining C-terminal helices make up the reaction center (13). As is obvious from the high resolution data, each helix pair has few protein-protein contacts with the other pairs in the TM domains. Just the TM helices of the EF pairs have hydrophobic contacts to the C-terminal helices making up the reaction centers. The three helix pairs each bind at least one Chl that is ligated in the β-position. (Fig. 7). Remarkably, all of these Chls are ligated by their central magnesium to one of the helices that make up the pairs and at the same time are H-bonded to one of the helices that make up an adjacent pair. We note that this assembly motif found in the A subunit is strictly conserved in the B subunit. Therefore, it may be possible that, as in the case of the LH2, the Chls ligated in the β-position critically contribute to the association of the helix pairs into trimers and thus are critical for assembling the inner core antenna of PS I. In line with the high homology between photosystem I and II (54), we found such a motif also in the light harvesting protein CP47 (not shown). This suggests that the H-bonding network between Chls ligated in the β-position may equally critical for the assembly of the inner antenna proteins of PS II. Contrary to the α- and β-subunits in LH2, the helices of the antenna of the plant photosystems are covalently connected. This, however, has little impact on the folding and assembly, because individual TM helices are considered structurally independent folding domains, mostly like distinct subunits (55).

We have previously shown that H-bonding at the helix/BChl interface may drive the assembly of model BChl proteins (27). Furthermore, in recent works with synthetic peptides (56, 57), BChl-H-bonding interactions with surrounding polypeptide have been shown to be critical for LH1 subunit type complex assembly. In summary, we show here that H-bonding at the (B)Chl/helix interface may significantly contribute to the assembly of natural proteins within their native lipid environment. The H-bonding motif involves Chls that are ligated in the β-position by a ligand attached to one TM helix and are H-bonded to residues from adjacent helices. In the B-ligation state, the polar C-13 keto carbonyl and the methoxy groups are averted from the binding helices and thus are available for tertiary interactions with structural elements other than their immediate binding helices.

**DISCUSSION**

Ligation to the central magnesium has long been recognized as commonly critical for the binding of (B)Chl and thus the assembly of (B)Chl proteins (17, 18). The ligation of the central magnesium atom may occur from either the top (β-type) or the bottom side (α-type) of the macrocycle. In (B)Chl proteins, the two types of ligation are unevenly distributed. In PS I only 14 of the 96 Chl molecules (19, 20) and in PS II 7 of 36 Chl\textsuperscript{5} are ligated in the β-position. The ligands and the exact position of the diastereomers are all very conserved (22), suggesting a critical role for these Chls. In the present study we have examined the proteic environment and H-bonding state of the Chl ligated in the β-position in comparison with the Chl ligated in the α-position in two photosystems. Our data show (i) that Chl ligated in the β-position (in contrast to the Chl ligated in the α-position) are frequently involved in H-bonding interactions with adjacent helices and (ii) that disruption of such a H-bonding motif leads to significant structural destabilization of the BChl-binding peripheral antenna of Rb. sphaeroides. Based on this work a structural role of the (B)Chl ligated in the β-position is suggested.

Using the photosystems as a data base, we found that approximately half of the Chl ligated in the β-position are involved in H-bonding interactions with adjacent TM helices. In contrast, the Chls ligated in the α-position are hardly involved in H-bonding interactions with adjacent TM helices (4–8% of all contacts to residues within 4Å). Both diastereomers, however, are involved in H-bonding interactions (21–25%) to residues of loop structures in PS I. Much less frequent are such H-bonds observed in PS II. This possibly indicates that H-bonding interactions are less critical for the structural organization of loop structures in PS II. Alternatively, H-bonding may alter the redox potential of (B)Chl molecules, and thus the diverse H-bonding states in the two photosystems potentially reflect functional modulation (10). The statistical analysis of PS I and PS II indicates that H-bonding between the C-13\textsuperscript{1} keto carbonyl group of Chl ligated in the β-position and the residues of adjacent helices maybe a critical factor in the stable assembly of the Chl proteins, in T. S. Balaban and P. Braun, unpublished observation.
particular the packing of Chl/helix associates in the inner core of the proteins. Other Chl-binding proteins such as the light-harvesting complex II from green plants also contain Chl ligated both in the β- and α-positions: three Chl of 14 Chl/monomer are ligated in the β-position (58). Interestingly, the C-131 and C-132 keto carbonyl groups of one of these β-positioned Chl are involved in H-bonding interactions via an H2O molecule to the residue of an adjacent monomer (58). Chl ligated in the peripheral antenna complex from purple bacteria, the organization of the TM helices has been explored experimentally. In the reaction center of Rh. sphaeroides (59, 60), there are four BChl that are all ligated in the α-position. Neither of the C-13 keto carbonyl groups of these four BChl are involved in H-bonding interactions with neighboring protein residues. It should be pointed out that each of these four BChl are part of the electron transport chain and thus have a functional role in the primary charge separation, contrary to the vast majority of Chl in PS I and PS II, which are not involved in electron transfer.

The Fenna-Matthews-Olson protein is a soluble, β-sheet protein, in which the BChl are packed in the core of the protein surrounded by the β-sheets (61). Nevertheless, two of the seven BChls in this protein are ligated in the β-position; however, neither the α-ligated nor the β-ligated Chl are involved in H-bonding interactions (except for one α-ligated BChl). In this β-sheet protein, however, the principles for Chl/protein assembly are likely to be quite different because of the fundamental different folds.

The notion that Chl ligated in the β-position critically contribute to the organization of the TM helices has been explored experimentally in the peripheral antenna complex from purple bacteria, Rh. sphaeroides. The LH2 used as a model protein, contains BChl instead of Chl, which has been used for the structural analysis. The overall structures of BChl and Chl are very similar, in particular, at ring C and E including the C-13 substituents, which have been identified previously to be critical for binding (34) and are directly addressed in this study.

The significantly reduced thermal stability of the LH2 in which the H-bond to the β-ligated BChl-B850 is removed shows that this H-bonding motif has a significant impact on the structural stability of the complex. The decrease in thermal stability of the mutant is much more pronounced in the detergent as compared with the native membrane, indicating that the lipid environment stabilizes the mutant complex. It has been previously shown that native lipids stabilize the oligomeric state of tetrameric potassium channel (62). This is further supported by the 4-fold reduction of the mutant LH2 in the membrane relative to total protein. Obviously, it cannot be excluded at this point that the assembly of model BChl proteins may also be driven by intramembrane H-bonding between the BChl and its binding polypeptide (27). Here, we show that H-bonding to the C-13 keto carbonyl group may significantly affect the structural stability of native BChl proteins in their natural environment. The H-bonding motif involves Chls that are ligated in the β-position by a ligand attached to one TM helix and are H-bonded to residues from adjacent helices. In the β-ligation state, the polar keto carbonyl and methoxy groups at ring E point away from the binding helices and thus are available for tertiary interactions with structural elements other than their immediate binding helices. Therefore, BChl molecules that are ligated in the β-position are likely to have a key structural impact, particularly on the packing of BChl/helix associates with each other. Situated at a critical position, the interface between two subunits as in the oligomeric LH2 makes this motif a key factor in the stable assembly of this BChl protein. Additional examples may be found in the inner antenna of the green plant photosystems. In the case of these proteins, the impact of the β-ligated Chl may even be more dramatic as gauged from their central location for the arrangement of the helices (Fig. 7). Further experiments will be needed to test whether the H-bonds to the β-ligated Chl are key factors in the stable assembly of these Chl-binding proteins.

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Structural Role of β-Ligated (Bacterio)chlorophyll