Chapter 27

Assembly of Model Bacteriochlorophyll Proteins in the Native Lipid Environment

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Summary 387
I. Introduction 388
II. Bacteriochlorophyll Proteins with Model Transmembrane Helices 388
A. Design and Expression 388
B. Spectral Properties 390
III. Assembly Motifs of (Bacterio)chlorophyll Proteins 391
A. Statistical Analyses of (Bacterio)chlorophyll Binding Proteins 391
B. Identification of Assembly Motifs by 'Rescue Mutagenesis' 392
C. H-Bonding at the Bacteriochlorophyll/Protein Interface 393
Acknowledgments 394
References 394

Summary

Protein design is used as an approach to further the understanding of membrane protein assembly, in particular, the assembly of transmembrane (bacterio)chlorophyll-binding pockets. Pigment-protein interaction motifs have been explored by (i) use of model proteins in which the native amino acid sequence in the pigment binding pockets are substantially altered and (ii) theoretical analyses of binding pockets of natural photosynthetic proteins. The bacteriochlorophyll binding sites of light harvesting complex 2, LH2, are replaced by model sites and expressed in vivo by the use of a modified *Rhodobacter sphaeroides* strain. The artificial helices are shown to bind bacteriochlorophyll and support the assembly of light-harvesting active complexes in the native membrane. A H-bond, which has been introduced at the membrane embedded bacteriochlorophyll/helix model site, is shown to drive the assembly of the model LH2 complex. Statistical analyses of natural (bacterio)chlorophyll binding pockets reveal the presence of distinct interaction motifs at the pigment/helix interface. One example is intra-membrane H-bonding between the pigments and the surrounding polypeptides, particularly between the chlorophylls' $C13^1$ keto carbonyl groups and the residues of the binding helices. With this system at hand, specific interaction motifs, such as the H-bonding motif, and their contribution to the folding and assembly can now be directly addressed within a highly simplified sequence context and in the polypeptides' native membrane environement.

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I. Introduction

Photosynthetic pigment protein complexes chiefly belong to the family of integral membrane proteins. The transmembrane α-helix (TMH), the central structural element of most membrane proteins, traverses the lipid bilayer and usually binds, together with adjoining TMHs, the photosynthetic cofactors, primarily (bacterio)chlorophyll ((B)Chl) and carotenoids. In nature, such membrane-embedded pigment-helix-structures are formed either by oligomerization of monotopic polypeptides (as in light-harvesting (LH) systems of photosynthetic bacteria) or by association of helices from polytopic helix-bundle polypeptides (as in most other LH proteins and reaction center complexes). Recently, the understanding of membrane protein folding, in particular interactions at helix-helix interfaces, has been greatly advanced by the increasing numbers of high resolution structures, by the availability of genomic sequencing data (Arkin et al., 1997; Wallin and von Heijne, 1998; Senes et al., 2000) and by a number of model studies (Choma et al., 2000; Zhou et al., 2000, 2001; Gratkowski et al., 2001; Lear et al., 2001). In the case of BChl- and Chl-proteins, however, additional factors have to be accounted for as these molecules may contribute significantly to the pigment protein assembly in the bilayer (Kim et al., 1994; Davis et al., 1995; Plumley and Schmidt, 1995; Remelli et al., 1999; Horn and Paulsen, 2002; Schmid et al., 2002).

The factors which drive the assembly of (B)Chl and polypeptide into unique arrangements have been addressed in numerous studies. Four principal experimental approaches have previously been used to investigate the interplay between the proteins and pigments in Chl- and BChl-proteins. These are: firstly, exchange of pigment with chemically modified pigment in natural pigment-protein complexes in vitro (Scheer and Hartwig, 1995; Lapouge et al., 2000; Chapter 26, Paulsen); secondly, chemical synthesis of *de novo* proteins or of truncated versions of natural proteins followed by reconstitution of the complex in vitro (Davis et al., 1997; Meadows et al., 1998; Kehoe et al., 1998; Todd et al., 1998, 1999; Kashiwada et al., 1999; Rau et al., 2001; Chapter 24, Noy et al.; Chapter 25, Nango); thirdly, mutagenesis and over-expression of the gene, followed by reconstitution of the pigments in vitro (Bassi et al., 1999; Heinemann and Paulsen, 1999; Remelli et al., 1999; Lapouge et al., 2000; Chapter 26, Paulsen); and, fourthly, site-directed or combinatorial mutagenesis combined with assembly of the complex in vivo (Fowler et al., 1992; Olsen et al., 1994; Hu et al., 1998).

In the model synthesis approach, synthetic α-helical peptides have been made which are comprised of bundles of amphipathic helices; although they are water-soluble, they bind cofactors in the hydrophobic interior and mimic important features of membrane-inserted redox-proteins. The first examples of this approach were the heme-binding maquettes from the groups of de Grado and Dutton (Robertson et al., 1994; Gibney et al., 1997). Lately, the *de novo* design of a BChl-binding site has been accomplished by synthesis of an orthogonal, four-helix protein. It mimics the coordination of (B)Chl-derivatives through one or two His residues located in the hydrophobic interior of the helix bundle, which is similar to the situation in natural (B)Chl-proteins. In a first attempt, the Chls were bound covalently to the helices to enable efficient Chl incorporation (Rau et al., 2001), but more recently, non-covalent binding has been achieved (Kashiwada et al., 1999; Snigula et al., 2001).

These first two approaches each depend on reconstitution with the pigments in a non-native system and they require the use of aqueous systems either by use of detergents or by rendering the helices water-soluble. A new approach has recently been introduced, namely, the in vivo use of artificial model TMHs to explore both BChl-binding and the assembly of light-harvesting systems in their native lipid-environment.

II. Bacteriochlorophyll Proteins with Model Transmembrane Helices

A. Design and Expression

The design of the model BChl-proteins is based on the peripheral light-harvesting complex 2 (LH2) from purple bacterium, *Rhodobacter (Rba.) sphaeroides*. LH2 is made of two short α-helical membrane proteins: the α- (LH2-α) and β- (LH2-β) subunits together with three BChl and one or two caroteinoid molecules. In essence, the membrane-embedded BChl-binding pocket of LH2 subunits has been re-designed by replacing the large portions of the

Abbreviations: (B)Chl – (bacterio)chlorophyll; CD – circular dichroism; ET – energy transfer; LH – light harvesting; *Rba.* – *Rhodobacter*; *Rps.* – *Rhodopseudomonas*; TMH – transmembrane helix; wt – wild type

helix regions, which make up the scaffold for the BChls, with simplified alternating alanine-leucine stretches (AL) (Fig. 1). In vivo expression of these model LH proteins has been accomplished by use of an engineered strain of the purple bacterium, *Rba. sphaeroides* (Jones et al., 1992); it lacks the operons encoding the apoproteins of the photosynthetic apparatus, but provides the assembly factors, pigments and membranes which are required to test the fitness of the model proteins as light harvesting units within a near-native system. Difficulties, likely to arise during expression of heterologous or artificial proteins in situ (Steiner and Scheer, 1985; McDermott et al., 1995; Drews, 1996; McGlynn et al., 1996; Braun and von Heijne, unpublished), have been initially avoided by retaining polypeptide domains which do not directly participate in pigment binding, namely, the domains that protrude out of the membrane. These, however, appear to be critical for the targetting and insertion of the helices into the membrane and for the overall assembly of the complex.

A contiguous stretch of 16 residues is replaced from α–Val–7[1] to α-Thr+6 in the LH2-α TMH, and another of 12 residues is replaced from β-Gly–7 to β-Ala+4 in the LH2-β TMH (Fig. 1). In wild type (wt) LH2, these stretches include all the residues that are proposed to interact with the BChl-B850 of both of the α- and β-subunits at distances of <4.5Å, except for residues α-Trp+9, α-Tyr+13 and α-Tyr+14 as well as β-Thr+7 and β-Trp+9, which lie outside of the TMH (McDermott et al., 1995; Prince et al., 1997). To retain the ligation with the central metal of the BChl, His0[1] was not replaced, nor was the neighboring α-Ile-1 residue replaced because of its proximity to the Mg-coordination site on the BChl-helix interface (McDermott et al., 1995). Alanine residues already occur in the wt sequence at positions –4, +1, +2 (for LH-α) and at positions –4, –1, +3, +4 (for LH2-β), while leucine residues already occur at position +4 (for LH2-α) and –3, +2 (for LH2-β). This results in a total of eight 'new' residues in the 16-residue-long stretch and five 'new' residues in the 12-residue-long stretch, enclosing all TMH residues of the BChl-B850 site. Obviously, when screening LH2 sequences for

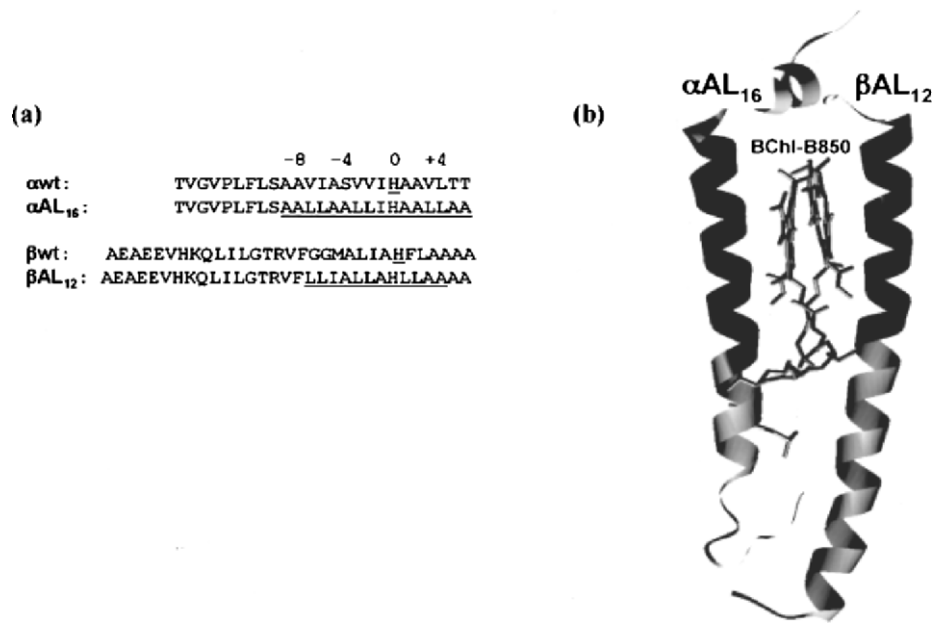

Fig. 1. LH2 complex with model helices as BChl binding sites. (a) Amino acid sequences of the model helices α-AL_{16} and β-AL_{12}. The model sequences which replace the native sequences in α-AL_{16} and β-AL_{12} as well as His0[1], which binds the central Mg are underlined. Wt sequences are shown above the model sequences. (b) Schematic view of model LH2 complexes with α-AL_{16} and β-AL_{12}. The helix stretches which are replaced by the model sequences are shown in dark grey, the native sequences and the BChl-B850 dimer are shown in light grey. For clarity, only the polypeptide's backbone and the BChl-B850 are shown.

[1] The numbering specifies the aa position relative to the histidine, designated His (0), which binds the central Mg of the α- or β-BChl-850. Positive numbers indicate the C-terminal, negative the N-terminal direction.

lower overall Ala-Leu content from other purple non-sulfur bacteria, we found that the Ala and Leu content is usually relatively high in this stretch of the TMH. The novel LH2 complexes consist of assemblies either of chimeric α-subunits and wt β-subunits and vice versa, or of both chimeric α-subunits and chimeric β-subunits (Fig. 1b, see also 3.1).

B. Spectral Properties

The novel LH2 complexes with α-AL_{16} or β-AL_{12} support not only binding of BChl but also its association into structures with a geometry producing the red-shift characteristic of B850 pigments and of a natural B800 binding site. The absorption bands of the novel LH2 with model TMH have the 'red shifted' transition maxima, namely, at ~852 nm for α-AL_{16} (see Fig. 2a) and at ~848 nm for β-$AL_{12,}$ which is typical for the B850 pigments of LH2 in membranes. The absorption at 800 nm of the monomeric, weakly coupled BChl, proposed to be bound to the terminal N-formylated α-Met (McDermott et al., 1995), is well preserved in the model LH2, and is indicative of an intact B800 binding site in this complex (Fig. 2a). There are minor, but noticeable, red-shifts from 849 to 853 nm in the Q_y-transition of α-AL_{16} which may indicate certain minor alterations in the BChl-850 geometry. The structural information responsible for the shift in the absorption of the Q_y band from 770 nm ('free' BChl) to 850 nm is, however, clearly retained if the stretches α-Val–7 to Thr+6 or β-Gly–7 to Ala+4 (Fig. 1a) are replaced by the much simplified Ala-Leu sequence. It should be noted, that the spectral properties of the BChl-B850 may be further tuned by altering the surrounding sequence in the TMH. For example, a mutagenesis study in *Rba. capsulatus* showed that combinatorial changes of 3, 4, 5 and 6 residues in the TMH of the β-subunit caused spectral shifts of the B800-850 absorption (Hu et al., 1998). In the light of these findings, it is remarkable that the simplified Ala-Leu sequence in the vicinity of BChl-B850 did not produce significantly altered spectral properties.

The BChl arrangement in LH2 with α- or β-AL seems to be largely preserved judged by the circular dichroism (CD) spectra, which serve as fingerprints for the BChl geometries (Cogdell and Scheer, 1985; van Grondelle, 1985; Braun et al., 1990; Braun and Scherz, 1991; Koolhaus et al., 1998). Typically, there is a conservative, S-shaped CD signal in the 'near IR' with a positive peak near 848 nm and a negative trough near 872 nm (see Fig. 2b), very similar to those observed in wt LH2 (Bandilla et al., 1998; Georgakopoulou et al., 2002). In addition, the optical activity of BChl-B800, seen as a negative trough near 800nm is retained in the α-AL_{16} complex (Fig. 2b).

The fitness of the model TMH to support proper light harvesting function of the complex is demon-

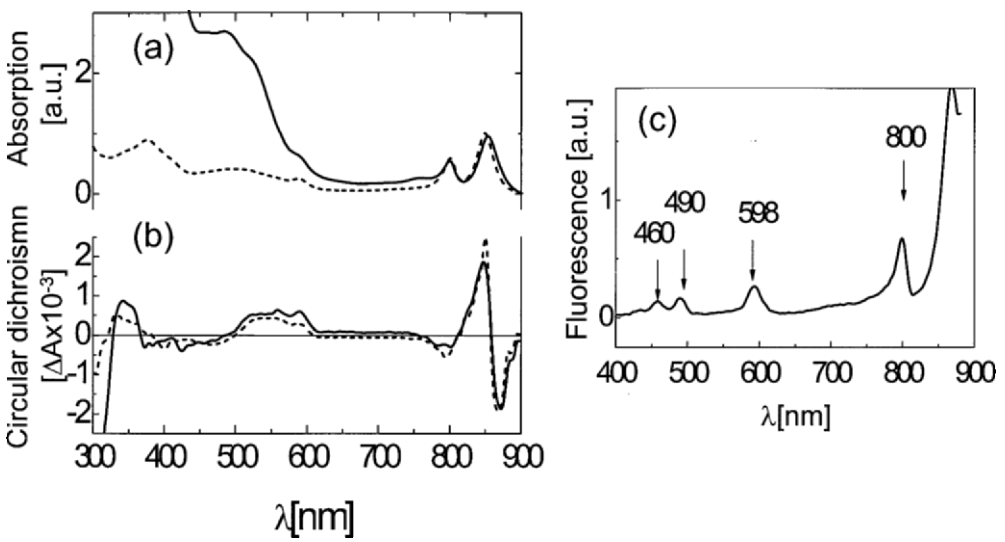

Fig. 2. Absorption (a), circular dichroism (b) and fluorescence excitation (c) spectra of model LH2 complexes with α-AL_{16} model LH2 (———) and wt LH2(- - - - -).

strated by energy transfer within the pigment assembly. The similarity of the excitation spectra (Fig. 2c) with the absorption spectra shows that efficient energy transfer takes place from BChl-B800 to BChl-B850. In particular, there is a pronounced 800 nm excitation band when detecting at 900 nm (Fig. 2c). Energy transfer (ET) occurs not only from B800 to B850 but also from carotenoids to BChl-B850, as indicated by the excitation bands in the spectra range of the caroteinoids of the complex (455–550nm). ET from carotenoid to BChl has only been observed in pigment-protein complexes or covalent constructs (Gust et al., 1993) and never in BChl-Car mixtures.

Thus, the model BChl-B850 site supports both pigment-protein assembly and the function of this complex as a light harvesting unit. Further, the residues in this region of the TMH, notably also at the BChl-protein interface, do not seem to be critical for specifying either the BChl-B850 array or the light-harvesting function in LH2. Possibly, the minimal requirements for this site in LH2 are fulfilled already by His, which binds the central Mg of BChl, together with a few key residues, such as the aromatic residues anchored in the bilayer interface which have been shown to form H-bonds with BChl-B850 (Fowler et al., 1992, 1994). Indeed, the sequence variability around the BChl-B850-binding region is high and most residues in the BChl-binding site with the exception α-His0 and β-Ala–4 are not strictly conserved (Braun et al., 2002). In view of that, it may not be surprising that additional extension of the AALL model sequence three residues towards the N-terminus of the TMH in LH2-α, does not impair BChl-B850 assembly. There is, however, significant loss of BChl-B800, indicating the importance of residues in this region for the binding of BChl-B800. Furthermore, the extension of the AALL sequence by only one residue towards the N-terminus of β-AL_{12} results in the total loss of LH2 complex assembly.

III. Assembly Motifs of (Bacterio)chlorophyll Proteins

Simultaneous replacement of the native TMH with the model AL (see Fig. 1) in both, the LH2 α- and β-subunits, abolishes assembled LH2 from the membrane. In order to rescue assembly of the novel LH2 complex, we aimed at designing stabilizing interaction motifs at the model protein/BChl interface. To that end, theoretical analyses of (B)Chl-proteins, particularly the BChl-binding pockets, have been undertaken.

A. Statistical Analyses of (Bacterio)chlorophyll Binding Proteins

Several strategies have been employed for the prediction of potential assembly motifs: Firstly, statistical analysis of existing high resolution structures, in particular, Photosystem I (PSI) with nearly 100 Chl binding pockets; secondly, alignment of aa sequences of LH2 subunits; and, thirdly, computational analysis of the aa distribution in putative (B)Chl-binding pockets of 'non-homologous' (B)Chl-proteins retrieved from protein databases.

In Photosystem I, remarkably consistent patterns of interactions were found (Fig. 3) at the Chl/TMH interface, i.e., between the binding helix and its attached Chl (Garcia-Martin et al., 2005). The macrocycle substituents chiefly interact with residues at the positions –4, +3, –1 and ±7 (relative to the liganding His0), most noticeably the $C13^2$ oxo groups interact almost exclusively with residues at –4 of the binding helix while the $C3^2$ of the vinyl group primarily interacts with residues at +3 and, to a lesser extent, with residues at –1, –4 and –7 (see Fig. 3). Sequence alignment and mutation 'hot spot' studies indicate two conserved residues besides the His0 in the TMH of the LH2 α-subunit: one of these two residues is located at position –4 (Braun et al., 2002). The sequence analysis showed that specific residues are found with significantly higher or lower probability than expected from random distribution at the positions which are critical for Chl binding (Table 1; Garcia-Martin et al., 2005). These findings point towards distinct amino acid motifs, which bind (B)Chl to its apoproteins.

Interestingly, the structural analysis of the Chl binding pockets indicate that H-bonding at the Chl/TMH interface is wide-spread: nearly half of the 100 Chl in PS I are likely to be H-bonded to the surrounding polypeptide, frequently through the $C13^1$ carbonyl group oxygen, but the oxygen atoms of other Chl substituents may also participate in H-bonding (Liebl et al., 1996; Braun et al., 2003). The residues participating in these bonds vary: for the H-bonds to the $C13^1$ oxo group, it included backbone NH (10×), heteroatoms of aromatic residues (13× Trp and Tyr, 7× His), side chain amino (2× Lys), guanidinium (5× Arg), amide (5× Asn or Gln) and hydroxl groups (4× Ser or Thr).

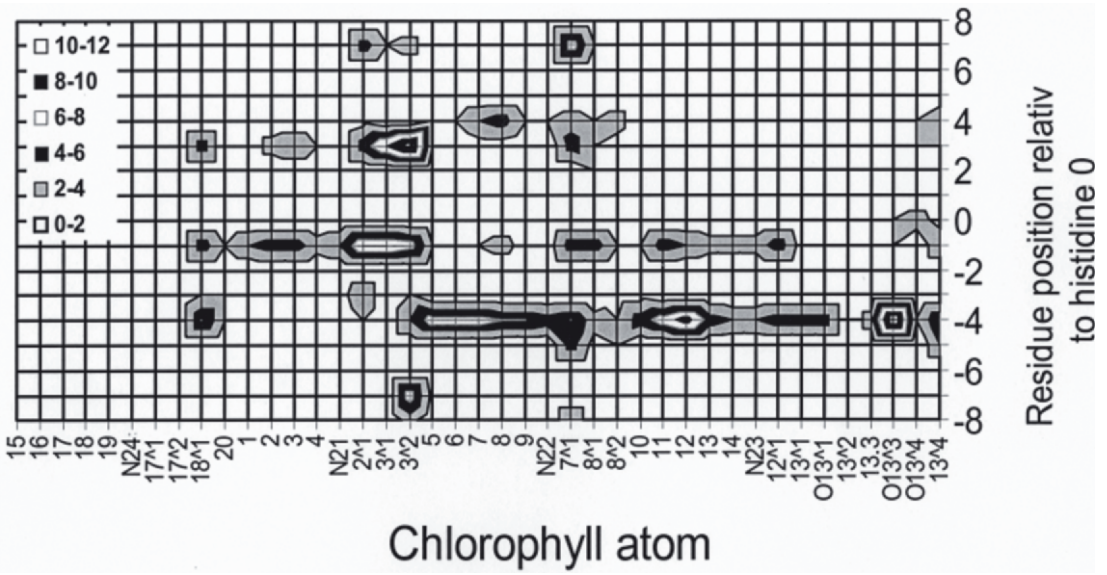

Fig. 3. Statistical analysis of Chl-binding pockets in Photosystem I from the structure of PS I (Fromme et al., 2001). Position dependent interactions between the atoms of the Chl macrocycle and the residues at positions ±8 relative to the His0 a distance ≤ 3.5Å. The shading code (top) corresponds to the interaction frequencies (see inset).

H-bonds have previously been identified in antenna and reaction center complexes (Fowler et al., 1994; Gall et al., 1997; Olsen et al., 1997; Sturgis and Robert, 1997). The currently identified H-bonds seem to participate primarily by modulating of the spectral properties of the BChls and, in reaction centers, in modulation of the redox potential (Chapter 19, Allen and Williams). It is not yet understood if polar interactions, in particular H-bonds, also contribute to the stability of the (B)Chl-polypeptide association in the membrane. The contribution of such intra-membrane H-bonding at BChl/TMHs interfaces has been studied in LH2 with the model helices (Kwa et al., 2004; Garcia-Martin et al., 2005).

B. Identification of Assembly Motifs by 'Rescue Mutagenesis'

With the intention to construct a H-bonding motif at the BChl/helix interface in the model proteins, residues with potential H-bonding properties have, therefore, been placed at position –4 in close vicinity of the C13 oxo groups of BChl-B850. The restoration of the model LH2 with α-AL_{16} and β-AL_{12} is achieved by the insertion of a single serine residue at –4 in α-AL_{16} (Fig. 4). This approach enables us to identify and analyze in depth local BChl-protein interactions

Table 1. Occurrences of aa residues in (B)Chl-binding sites. A set of BChl-binding proteins (2104 TM residues) has been investigated by computer based sequence analysis. The position of the residue in the helix relative to the histidine ligand are listed according their interaction frequencies with Chl (see Fig. 3). Bold type letters indicate deviation from random with a confidence interval of 95%, the regular type letters of 90% and the small letters of 85%.

Position	More frequent	Less frequent
-4	**D** A F	H L
-1	**H**	**L**
3	**L W K** H	M
7	**W C** P	A M
4	**A** G	H W
-8	**L N Q R** P Y K	A M V
-3	G	
-5	**S D E L**	
-7	**C** G Q	A F I
2	**D** W G	
5	**G** I	L W

in the model sequence context. The implementation of a strong H-bond at the BChl/TMH interface, most likely between the re-introduced serine at position –4 in α-AL_{16} (α-$AL_{16/S-4}$) and the $C13^1$ oxo group of β-BChl-B850, has been confirmed by resonance

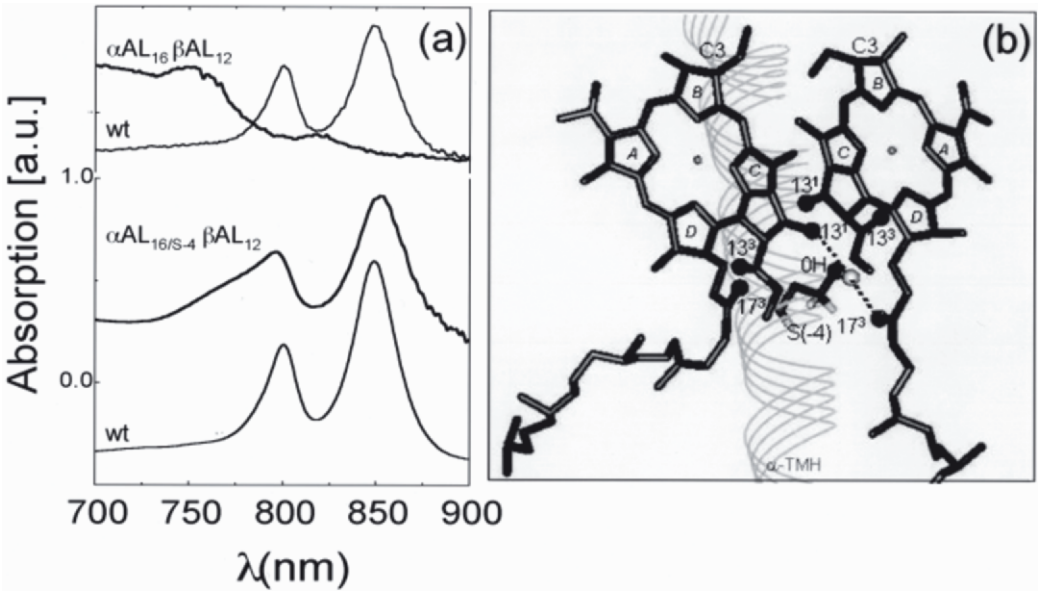

Fig. 4. H-bonding drives assembly of LH2 complex with model BChl binding site. (a) 'In situ' 'near IR' absorption spectra of LH2 wt and of model LH2 with $\alpha AL_{16}/\beta AL_{12}$ and with $\alpha AL_{16/S-4}/\beta AL_{12}$. (b) Model of H-bonds at the BChl-B850/TMH interface in LH2 from *Rba. sphaeroides*. In the high resolution structure of LH2 from *Rps. acidophila* (McDermott et al., 1995), residues 18–37 of the α-subunit have been replaced with residues 18–37 of the α-subunit of *Rba. sphaeroides*. Energy minimisation has been carried out on the replaced stretch and the BChl-B850 carbomethoxy groups (software WebLab ViewerPro™ 3.7). BChl-B850 and α-serine –4 are depicted in detail. The OH of serine and the O atoms of the C-13^1, C13^3 and C17^3 carbonyl groups are shown as spheres. Putative H-bonds to the C-13^1 carbonyl group of β-BChl-B850 and to the C-17^3 carbonyl group of the α-BChl-B850, are indicated by dotted lines.

Raman spectroscopy (Kwa et al., 2004) and by protein modeling (Braun et al., 2003; Fig. 4.) based on the high resolution structure of *Rhodopseudomonas* (*Rps.*) *acidophila* (McDermott et al., 1995). The BChl arrangment in α-$AL_{16/S-4}$ is very similar to wt LH2 as confirmed by absorption, CD, fluorescence excitation and resonance Raman spectroscopy. By use of this 'rescue-mutagenesis' approach we have thus identified H-bonding as a key assembly motif in the model BChl-proteins.

C. H-Bonding at the Bacteriochlorophyll/Protein Interface

In the model LH2 with α-AL_{16}, quantification of the complex content in membranes as well as thermal denturation experiments indicate that replacement of the native TMH in the BChl-B850 region by the Ala-Leu repeat sequence leads to (i) reduction of the model antenna complexes in the membrane and (ii) destabilized assembly of the complex. Remarkably, with serine at position –4, both expression levels and the thermal stability of the complex significantly improved and came close to the thermal stability of wt LH2 (Kwa et al., 2004; Garcia-Martin et al., 2005).

However, exchange by most other residues tested, for example, tryptophane, tyrosine, phenylalanine, cysteine, lysine, aspartate, asparagine, glutamate and glutamine, resulted in total loss of LH2; and, exchange of serine –4 by a threonine or alanine, resulted in more or less pronounced reduction of assembled complexes relative to α-AL_{16}. Thus, the residue at position –4 has a key function in the assembly of the model BChl-protein, although disturbances may be amplified by the inherent instability of LH2 with α-AL_{16}: this could be compensated in the native complex by other effects.

Curiously, removal of the H-bond between serine –4 and the C13^1 oxo group only slightly affected the thermal stability in wt LH2 (Braun et al., 2003). Furthermore, most LH2-α in related purple bacteria species possess an alanine residue at –4. Thus, contrary to the situation in the model proteins, the strong H-bond at the BChl/TMH_{wt} interface of *Rba. sphaeroides* LH2 apparently does not contribute critically to either the spectral properties or the stability of the native wt complex. One possible explanation for this apparent discrepancy maybe that alanine –4 favorably interacts

with the 13^1 group either by H-bonding interactions with Cβ-H of the methyl side chain (Jiang and Lai, 2002) or by close packing interactions within the native sequence context. These interactions may be prevented in the model sequence context due to the slight reorganization of the pigments as reflected in minor red shifts of the red-most transition (Fig. 2). Nevertheless, in the context of the model sequences, a single intra-membrane H-bond at the BChl/TMH interface converts the highly unstable model BChl protein complex into a complex with almost native-like thermal stability and expression levels in the membrane. To what extent the H-bond increases the inherent stability of the complex or its assembly in the membrane has not yet been determined. Using this system, however, the contribution of distinct interaction motifs such as H-bonding between a (B)Chl and its binding polypeptide can now be directly assessed within the native membrane environement.

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