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Identification of intramembrane hydrogen bonding between 13^1 keto group of bacteriochlorophyll and serine residue α27 in the LH2 light-harvesting complex

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Abstract

Intramembrane hydrogen bonding and its effect on the structural integrity of purple bacterial light-harvesting complex 2, LH2, have been assessed in the native membrane environment. A novel hydrogen bond has been identified by Raman resonance spectroscopy between a serine residue of the membrane-spanning region of LH2 α-subunit, and the C-13^1 keto carbonyl of bacteriochlorophyll (BChl) B850 bound to the β-subunit. Replacement of the serine by alanine disrupts this strong hydrogen bond, but this neither alters the strongly red-shifted absorption nor the structural arrangement of the BChls, as judged from circular dichroism. It also decreases only slightly the thermal stability of the mutated LH2 in the native membrane environment. The possibility is discussed that weak H-bonding between the C-13^1 keto carbonyl and a methyl hydrogen of the alanine replacing serine(−4) or the imidazole group of the nearby histidine maintains structural integrity in this very stable bacterial light-harvesting complex. A more widespread occurrence of H-bonding to C-13^1 not only in BChl, but also in chlorophyll proteins, is indicated by a theoretical analysis of chlorophyll/polypeptide contacts at <3.5 Å in the high-resolution structure of Photosystem I. Nearly half of the 96 chlorophylls have aa residues suitable as hydrogen bond donors to their keto groups.

Keywords: Intramembrane H-bonding; Raman resonance spectroscopy; Antenna complex; Photosystem I; Chlorophyll binding pocket; Heat denaturation

1. Introduction

α-Helical, integral membrane proteins display surprising simplicity in design, in spite of their wide and variable range of functions in the cell. Their central structural element is the transmembrane helix (TMH). Two or more TMH associate, in many cases, with additional ligands or cofactors to form distinctive membrane-spanning helix-bundles. Advances have been made in understanding the factors and forces that drive TMH devoid of prosthetic groups or ligands, to assemble to the helix-bundles in the membrane. At intramembrane helix–helix interfaces, interaction motifs have been proposed with H-bonding as a key factor in the helix–helix association [1]. In many cases, cofactors or ligands contribute to helix–helix interactions. For example, in photosynthetic proteins, (bacterio)chlorophylls ((B)Chl) are frequently inserted into TMH bundles and contribute extensively to the structure of (B)Chl-proteins (for recent reviews see Refs. [2,3]). Often, they are strictly required for correct folding and assembly of pigment protein complexes (see e.g. [4–9]).

The best understood interaction of (B)Chl molecules in their binding sites is the ligation of the central Mg ion by a suitable protein ligand, frequently a histidine residue [10]. The complex structures of the natural (B)Chl with up to four peripheral C=O groups, several asymmetric centers and the long-chain esterifying alcohols, point to multiple types of interactions between the Chl and the surrounding polypep-

Abbreviations: (B)Chl, (bacterio)chlorophyll; LH, light-harvesting complex; *Rb.*, *Rhodobacter*; *Rps.*, *Rhodopseudomonas*; TMH, transmembrane helix; amino acids in TMH regions are counted relative to the (B)Chl-binding histidine

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tide(s). Critical structural features have been identified for several binding sites by pigment exchange experiments [11,12]. Details of the interactions with carbonyl groups, which are of particular interest due to their H-bonding potential, have hitherto been particularly explored for the acetyl group of BChl. H-bonding between the acetyl group and neighbouring residues may modulate the functional properties, such as the excitation energies of BChl *a* in light-harvesting (LH) complexes from purple bacteria [13,14], or (though still under some dispute [15]), the redox energies of P870 in bacterial reaction centers [16]. H-bonding interaction with the other conjugated carbonyl group, the keto carbonyl at the isocyclic ring of BChl, has hitherto been much less understood. In particular, there has been, to the best of our knowledge, no site-selected mutant specifically designed to modulate the H-bonding state of these chemical groups within photosynthetic proteins.

Resonance Raman (RR) spectroscopy has been very useful in dissecting the physicochemical mechanisms underlying the interactions among (B)Chl and the surrounding polypeptide [13,17]. In particular, RR is sensitive to intermolecular interactions of the C-13[1]-keto and C-3-acetyl groups conjugated to the macrocyclic π-system as well as to the properties of their immediate polypeptide environments [18]. Low-energy shifts of up to 40 cm^{-1} of the C=O stretching modes are diagnostic of the strength of H-bonding, while the dielectric constant (polarity) of the binding pocket may tune their frequency by 10–20 cm^{-1} [19]. H-bonding interactions have thus been identified in photosynthetic proteins, in particular, H-bonding to the 13^1 keto carbonyl of BChl and possible partners were suggested [20,21].

In a systematic approach to investigate structural and functional aspects of BChl-proteins, in particular intramembrane interactions in the highly stable LH2 from *Rhodobacter (Rb.) sphaeroides* in a membrane system, we have started to express mutants modified in the membrane-spanning region in a background strain of this organism lacking all the BChl complexes [22]. Here, we focus on H-bonding to C-13[1] by replacing serine at position -4^1 of the α-subunit (α-S(−4)) of LH2 in *Rb. sphaeroides*, which potentially participates in H-bonding to BChl-B850, with alanine, the residue which occupies position −4 in LH2-α in most other purple bacteria [22,23]. The results suggest that the 13^1-keto group of β-BChl-B850[1] is strongly H-bonded to the hydroxyl group of serine(−4), yet disruption of this bond neither significantly affects the stability nor the structure of the complex. Extension of the theoretical analysis beyond BChl proteins, to the nearly 100 binding pockets of Chl *a* in Photosystem I (PSI) from *Thermosynechococcus elongatus* (previously *Synechococcus elongatus*) [10], indicates that intramembrane H-bonding is not uncommon between TMH residues and (B)Chl, in particular C-13[1] keto carbonyl groups.

[1] The numbering specifies the aa position relative to the histidine, designated His (0), which binds the central Mg of the α-BChl-850. The number following BChl indicates the maximum absorption of the BChl's red most absorption band.

2. Materials and methods

2.1. Bacterial strains, plasmids, gene transfer and growth conditions

The bacterial strains used in this work include *E. coli* strain S17-1 ((*thi pro hsdR*$^-$ *hsdM*$^+$ *recA* RP4-2 (Tc::mu Kan::Tn7), *Rb. sphaeroides* strain DD13 (genomic deletion of both *pucBA* and *pufBALMX*; insertion of Sm^R and Kan^R genes, respectively) [24]. The mobilizable plasmids used were based on pRKCBC1 (Tc^R, derivative of pRK415; insertion of a 4.4 kb fragment encompassing *pucBAC*); briefly, this expression vector contains the *pucBA* genes as a 420 base pair *Kpn*I–*Bam*HI insert [24]. Growth conditions for *E. coli* and *Rb. sphaeroides* were as described in Ref. [25]. For *E. coli*, tetracycline was used at concentrations of 10 μg/ml. For *Rb. sphaeroides* antibiotics were tetracycline (1 μg/ml) and neomycin (10 or 20 μg/ml). Conjugative transfer of plasmid from *E. coli* S17-1 to *Rb. sphaeroides* was performed as described [25].

2.2. Construction of mutant LH2

For the construction of LH2 complex with alanine at position −4, α-S(−4)A, by site-directed mutagenesis (Quick change, Stratagene), *pucA* was subcloned into Topo-TA using an engineered *Hin*dIII site between *pucB* and *pucA*, just downstream of the stop codon of *pucB*, and a *Bam*HI site downstream of the *pucA* coding region.

2.3. Instrumentation

UV–Vis-absorbance spectra were recorded on a Lamda 25 spectrophotometer (Perkin Elmer), circular dichroism (CD) spectra on a Dichrograph CD6 (Jobin Yvon). FT-Raman spectra were recorded using a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module equipped with a continuous Nd:YAG laser, as described previously [13].

2.4. Preparation of intracytoplasmic membranes

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 [22].

2.5. Thermal denaturation of LH2

Purified membranes, wt LH2 and LH2 α-S(−4)A were adjusted to an $A_{850\ nm} = 8\ cm^{-1}$ in Tris (10 mM, pH 8)

EDTA (1 mM) and placed into 1 mm thermostated cylindrical quartz cuvette. Samples were heated from 15 to 95 °C at a constant rate of 1 °C min^{-1} and the CD signal at 845 nm was recorded every 0.05 °C (integration time 0.2 s). Thermal denaturation was irreversible and accompanied by an increase in sample turbidity at higher *T*. The increase in sample turbidity (as reproduced by the addition of milk to LH2 membrane samples) does not significantly affect the NIR-CD spectrum.

2.6. Modeling and statistical analysis

Modeling of the interaction between the α-TMH and the BChl-B850 was done by use of the high resolution data of *Rhodopseudomonas (Rps.) acidophila* [26]. Replacements of α-TMH residues (*Rps. acidophila>Rb. sphaeroides*) and subsequent energy minimizations were done using WebLab Viewer 3.7. For the statistical analysis of Chl/polypeptide contacts, the high-resolution structure of PSI from *Thermosynechococcus* [2] was investigated using the same software. All protein atoms have been analysed within radii ≤ 3.5 Å around the keto carbonyl's oxygen atoms at C-13^1, C-13^3 and C-17^3 and the C-3 vinyl group.

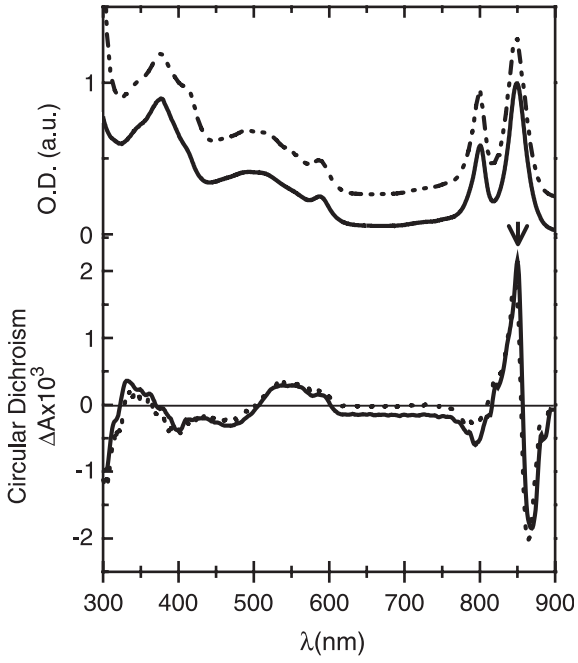

Fig. 1. Optical absorption (top) and CD spectra (bottom) of wt LH2 (—) and LH2 α-S(−4)A (- - -). Absorption spectra are vertically displaced for clarity.

3. Results and discussion

In order to study possible H-bonding partners to the keto carbonyls of BChl-B850 in LH2, α-serine(−4) was replaced by alanine. α-serine(−4) is close to the isocyclic rings of BChl-B850 and has been proposed to partake in H-bonding to BChl-B850 [21,23]. The absorption and CD spectra of purified membranes of wt LH2 complex and LH2 complex with α-S(−4)A are nearly identical, with Q_y absorption at 849 nm, and a non-conservative, S-shaped CD signal centered at 855 nm that is characteristic for the CD signal of LH2 from *Rb. sphaeroides* (Fig. 1) [27]. Thus, the exchange of serine by alanine at position −4 in α-LH2 apparently neither results in significant alterations of the BChl excitation energies, nor in the pigment arrangement.

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)A mutant by RR spectroscopy of the BChl keto carbonyl stretching modes. In apolar solvents and in the absence of H-bonding interactions, the 13^1 keto stretching mode of BChl *a* is located at 1685 cm^{-1}, that of the 3-acetyl carbonyl group 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, e.g. a mutation. In the RR spectrum of wt LH2, five bands in the carbonyl stretching modes region (1620–1710 cm^{-1}) are resolved (Fig. 2), similar to the findings in Ref. [23]. The bands at 1627 and 1635 cm^{-1} have been attributed to the C-3 acetyl groups of the BChl-B850 [13]. 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 [28]. The remaining bands have been attributed to the 13^1 keto carbonyl of the BChl-B850 (1651 and 1677 cm^{-1}) and of the BChl-B800 (1701) cm^{-1} [23,29]. The considerable downshift of one of the BChl-B850 13^1 C=O bands to 1651 cm^{-1} has been proposed to reflect strong H-bonding [23], while the band at 1677 cm^{-1} is downshifted only by about 8 cm^{-1} relative to the band in organic solvent [19]. In the α-S(−4)A mutant, only three carbonyl bands are resolved (Fig. 2) in the carbonyl region. 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} gained intensity and is slightly downshifted to 1674 cm^{-1}. The FT-Raman spectrum of the *Rb. sphaeroides* α-S(−4)A mutant is very similar to the spectra of LH2 from *Rb. capsulatus* and *Rps. acidophila* [23]. 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 [30]. It has been attributed to a minor reorganization of the carboxy terminal end of the α-polypeptide, due to the change in the chemical structure of the carotenoid. The similarity in the RR spectra indicate that similar minor reorganization may occur upon the exchange of serine(−4) with alanine in α-S(−4)A mutant. The main change in the FT-Raman spectra of the α-S(−4)A mutant

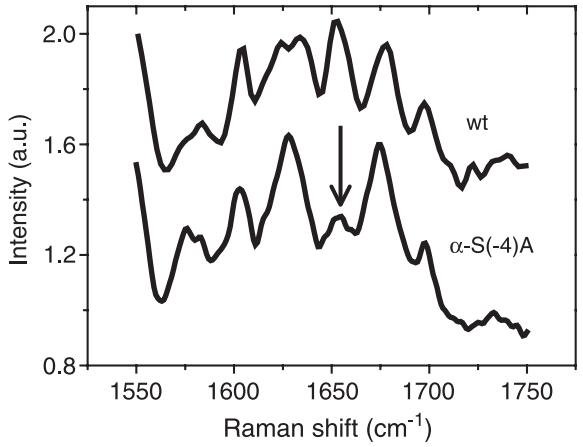

Fig. 2. Resonance Raman spectra of wt LH2 (top) and LH2 α-S(−4)A. The shift of the 1651 cm^{-1} band to 1677 cm^{-1} is indicated by the arrow.

LH2 is, however, the considerable shift of the 1651 cm^{-1} band to 1674 cm^{-1}. This shift indicates that the H-bond to the 13^1 keto carbonyl has been disrupted or significantly weakened. This study thus constitutes the first direct experimental evidence of such a strong H-bond between the protein and the keto carbonyl group of one of the BChl-B850 molecules.

To model putative H-bond(s) between the BChl-B850 and serine(−4), the high-resolution structure of *Rps. acidophila* was used to substitute residues 18–37 of the α-subunit with the respective residues of the *Rb. sphaeroides* (Fig. 3). In the vicinity (<3 Å) of the hydroxyl group of serine(−4), two C=O groups are found, i.e. the C-13^1-keto carbonyl of β-BChl-B850 and the C-17^3 ester-carbonyl of the α-BChl-B850. The distance between the hydrogen of the serine OH-group to the C-13^1 oxygen is 2.4 Å and the angle O–H–O is 112°, which compares favorably with the ideal values of ≤2.7 Å and 120°, respectively. The distance between the hydroxyl hydrogen and the C-17^3 carbonyl oxygen is only slightly larger (2.9 Å), but the angle between the hydroxyl bond and the carbonyl oxygen of α-BChl-B850 (78°) deviates significantly from the ideal angle. This would support an H-bond to the 13^1 C=O group of β-BChl-B850 (and the assignment of the 1651 cm^{-1}-Raman band to this pigment) in wt LH2 from *Rb. sphaeroides*. The next nearest C=O group (C-13^3 of β-BChl-B850) is located approximately 5 Å from the hydroxyl group, which is too far for an H-bond. It should be mentioned, however, that there is currently no force field available for BChl, and energy minimization therefore does not include the BChl macrocycles. While this limits the model, significant rearrangements of the pigments are unlikely from the conservation of the CD in the α-S(−4)A mutant. We may thus conclude, (taking into account the *Rps. acidophila* structure), that the H-bond is formed between the keto carbonyl group of the BChl-B850, which is bound to the β-polypeptide, and the serine(−4) of the α-polypeptide. In wt LH2 from *Rb. sphaeroides*, the 1651 and 1677 cm^{-1} bands thus arise from the keto carbonyl groups of the β-bound and α-bound BChl-B850 molecules, respectively. This attribution is consistent with the observation that FT-Raman spectra of LH2 proteins which exhibit in their primary sequence an alanine at position −4 (such as *Rps. palustris*, *Rb. capsulatus*, *Rps. acidophila*), closely resemble the spectrum of mutant LH2 α-S(−4)A [23], while that of LH2 from *Rb. sulphidophilus* (now *Rhodovulum sulphidophilum*), the only other species with serine at position −4, closely resembles the FT-Raman spectra of wt LH2 from *Rb. sphaeroides*.

Curiously, the stretching frequency of the keto carbonyls seem shifted even in the absence of serine to somewhat lower energies (1670 cm^{-1} in *Rps. acidophila*) compared to that in aprotic solvent, indicative of either a polar environment around the keto carbonyl [19] or a weak H-bond even in the absence of serine(−4). Recently, high-resolution structural data have suggested that CH groups, such as methyl hydrogens, could act as H-bond donors [31,32] (for a recent review, see Ref. [33]). It therefore may be possible that the alanine residue at position −4 is involved in weak H-bond interactions with the C-13^1 keto carbonyl, and that this bond may be accountable for the shift of the stretching mode to ~1674 cm^{-1} compared to ~1680 cm^{-1} in organic solvent. Based on the structural data, statistical potentials have been developed to quantitatively

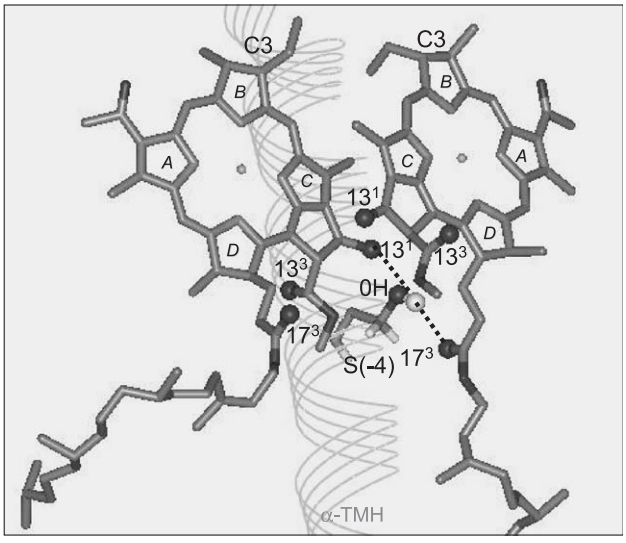

Fig. 3. Putative H-bonds at the BChl-B850/α-TMH interface in LH2 complexes from *Rb. sphaeroides*. In the high-resolution structure (PDB file:1KZU) of *Rps. acidophila* [26], residues 18–37 of the α-subunit have been replaced with residues 18–37 of the α-subunit of *Rb. sphaeroides* (sequence P02946). Energy minimization has been carried out on the replaced TMH stretch and the BChl-B850 carbomethoxy groups using the molecular modeling and visualization software WebLab ViewerPro™ 3.7 (Molecular Simulations Inc.). For clarity, only the α-TMH, BChl-B850 and α-serine(−4) are depicted in detail. The hydroxyl hydrogen of serine and the oxygen 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 α-BChl-B850 and the C-17^3 carbonyl group of the β-BChl-B850, are indicated by dotted lines.

estimate the energy of such H-bonds. Accordingly, H-bonding to methyl hydrogens have been estimated to amount to about 2 kJ/mol [34]. The 15 cm^{-1} downshift of the keto carbonyl band as observed in the presence of serine(−4), however, has been correlated with stronger H-bonding (~ 10 kJ/mol) [35].

Alternatively, the polar NH groups of the liganding histidine residues, which do not participate in complexation of the central Mg atom, are also close (less than 4 Å) to the BChl-B850 keto groups [26]. The presence of this highly dipolar imidazole side chain in the environment of these carbonyl groups may explain their low frequency. In LH1, the downshifts of the stretching frequency of the keto carbonyl groups to 1660 cm^{-1} as compared to free C=O groups at around 1685 cm^{-1} have been shown to be related to the presence of the liganding histidines, possibly by formation of H-bonds between the histidine's NH group and the keto carbonyl of the red-most absorbing BChl molecules [20].

H-bonding to (B)Chl 13^1 keto carbonyl group has been clearly recognized in antenna–RC complex [36,37], in PSI [38] and in bacterial reaction centers [16,39,40]. However, there is little information whether such H-bonding interactions affect the excitation energies of the bonded (B)Chl, and whether it contributes to the complex's stability. H-bonding to the acetyl carbonyl groups of BChl seems to be correlated with red-shifting of the Q_y band for both monomeric and coupled BChl molecules [13,23,28,41], although the underlying mechanism for the red shift is still not completely understood. In contrast, the electronic absorption spectra of the *Rb. sphaeroides* α-S(−4) mutant LH2 is very similar to that of the wt LH2 (Fig. 1). Therefore, it appears that, in these complexes at least, protein-BChl H-bonds involving the C-13^1 keto carbonyl do not significantly influence the electronic properties of the BChl molecules, contrary to what had been proposed from molecular calculations [42].

To explore the effect of the identified H-bond on the thermal stability of LH2, we monitored the CD signal of wt and mutant complexes in their native lipid environment during heat denaturation (Fig. 4). LH2 complexes exhibit irreversible, cooperative thermal unfolding transitions and can be described as an irreversible two state process [43]. In the case of the irreversible two-state process, the denaturation is kinetically controlled and thus depends on the heating rate. At a heating rate of 1 °C min^{-1}, the melting temperature of wt LH2 in purified membranes (T_m = 68 °C) is very similar to the T_m value of LH2 in octylglucoside and is distinct from the T_m in LDAO [44]. Interestingly, the denaturation process within the native membrane covers a much wider temperature range compared to the process in detergent. In addition, denaturation is accompanied by a strong increase in the sample turbidity (not shown), indicative of major reorganization of the lipid bilayer during LH2 disassembly [45]. The denaturation curve of the LH2 mutant α-S(−4)A is very similar to that of the wt LH2. It is

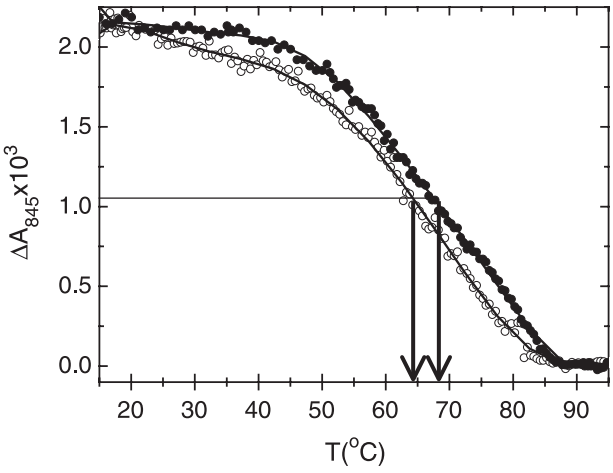

Fig. 4. Thermal denaturation of wt and mutant LH2. Changes of the CD signal at 845 nm during heating of suspended membranes containing wt LH2 (filled circles) and of LH2 α-S(−4)A (open circles). The transition temperatures T_m of wt LH2 and LH2 α-S(−4)A are indicated by the arrows. Heating speed: 1 °C min^{-1}.

similarly broad and its T_m is only slightly reduced to 65 °C, indicating only a minor decrease, if any, in the thermal stability of LH2 α-S(−4)A. This is further supported by very similar expression levels for wt LH2 and LH2 α-S(−4)A (nor shown). It should be emphasized again that this study has been performed using purified membranes. Under these conditions, which may be considered near native, the strong H-bond between serine(−4) and the C-13^1 keto carbonyl of the BChl-B850 apparently does not contribute significantly to the stability of the fully assembled LH2 complex.

Given that wt LH2 is comprised of nine αβ-protomers, and that each H-bond should ideally contribute 20–24 kJ/mol [46], it is somewhat surprising that there is hardly a difference in melting temperatures between wt and mutant complex. However, the contributions of intramembrane H-bonds to the overall conformational stability seem to vary and are not yet understood. For instance, single strongly polar amino acids (e.g. Gln, Arg) were sufficient to drive helix–helix interactions in model TMH systems [47,48], while single amino acids of the less polar residues (Ser, Thr) were not able to promote significant helix–helix association [48,49]. Specific combinations of serine and threonine residues have been shown to participate in cooperative and stabilizing helix–helix interactions [50]. Intrahelical H-bonds involving single serine residues, however, have even been shown to destabilize TMHs by promoting bends in the backbone possibly facilitating conformational changes in membrane proteins [51].

The H-bond which has been identified between the C-13^1 keto carbonyl of the β-BChl-B850 and α-serine(−4) in this study does not seem to be a major factor for the stability of LH2. One possible explanation could be that the serine's hydroxyl hydrogen is substituted by another, weaker H-bond donor in the mutant α-S(−4), involving either α-

alanine(−4), or the nearby α-histidine(0). Formation of a hydrogen bond between these residues and BChl may partly compensate for the loss of the H-bond to the OH of serine(−4) and at the same time may be accountable for the relatively low stretching frequency (1674 cm^{-1} compared to 1685 cm^{-1} in organic solvent) of the keto group even in the absence of serine(−4) (Fig. 2). However, if such a bond is formed, it should be weaker than the H-bond with the hydroxyl group of serine (−4), as noticeable from the pronounced upshift of the Raman stretching of the keto carbonyl (Fig. 2). Therefore, to account for the comparable thermal stability of wt LH2 and LH2 α-S(−4)A, additional alteration, such as favorable packing effects, may take effect which could compensate for the loss of the H-bonding energy.

As discussed before, H-bonds to C-13^1 are not uncommon in BChl proteins. We therefore undertook a search if such bonds are also found in chlorophyll proteins, containing Chl *a*, a pigment lacking the 3-acetyl group and preserving only the one at C-13^1. The immediate environment was searched around the C-13^1, C-13^3 and C-17^3 carbonyl groups in the Chl binding pockets of PSI from *Thermosynechococcus* [10]. The 96-well resolved Chl molecules currently provide the largest database suitable for a statistical analysis. Chl-polypeptide contacts are shown in Fig. 5 for the C-13^1, C-13^3, and C-17^3 carbonyl groups, at radii ≤3.5 Å around the oxygens. They are categorized into 'non-polar' and 'polar', the latter including charged atoms of H-bonding potential. Similar contacts with the non-polar C-3 vinyl group of Chl *a* are also shown for comparison. Of the three C=O groups, the one at C-13^1 has significantly higher percentages of polar contacts than the other two (and the C-3 vinyl groups). 'Polar' contacts

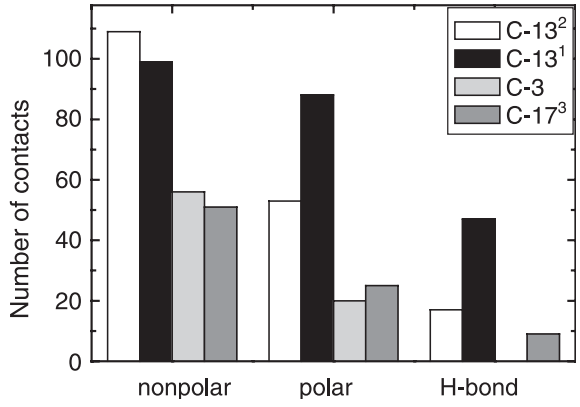

Fig. 5. Chl *a*–apoprotein interactions in PSI. Contact atoms at ≤3.5 Å distance from the 13^1, 13^3 and 17^3 carbonyl oxygens, as well as the 3^2 carbon of the 3-vinyl group, have been categorized into 'non-polar' and 'polar' groups. Potential H-bonds to 'polar' atoms have been identified by use of the WebLab ViewerPro™ 3.7. Numbers of contacts and H-bonds are shown for the keto carbonyl oxygens at C-13^3 (white columns), at C-13^1 (black columns) and at C-17^3 (dark grey columns), and for the vinyl groups at C-3 (light grey columns). All contacts ($r \leq 3.5$ Å) are listed between Chl *a* and the apoproteins. 'Polar' groups also include H-bond contacts.

around the C-13^1 oxygen are almost as frequent as 'non-polar' ones, and of the former nearly 60% potentially participate in H-bonds judged on the intermolecular distances and angles. While H-bonds cannot be positively identified in the crystal structure, the RR findings that a considerable fraction of the keto carbonyls of antenna Chl in PSI is being involved in H-bonding strongly supports this analysis [36,37]. The protein groups participating in these bonds include 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 hydroxyl groups (4× Ser or Thr). The RR spectra suggest that the H-bonds to the C-13^1 keto carbonyl diverge in strength [36], which agrees well with our findings of a high variation in the nature of H-bond donors. In contrast, about 80% of the contacts with the C-3 vinyl groups are 'non-polar' and, obviously, no potential H-bonds are found at these positions. This shows that, in spite of the predominantly intramembrane location of Chl, the Chl's keto carbonyls frequently interact with 'polar' protein groups and apparently are H-bonded to the TM domains. It should be pointed out that in the structure of PSI, eight alanine residues are found in close vicinity (<3.5 Å) of the C-13^1 keto, and in seven of these methyl-hydrogens are found at distances and angles close to the ones ideal for H-bonding (not shown).

Besides the C-13^1 carbonyl groups, the C-13^3 and C-17^3 carbonyl groups are also involved in potential H-bonding, even if to a notably lower extent. Yet, while the total number of C-13^3 C=O contacts is approximately equal to that of the C-13^1 C=O, only ~25% of them are 'polar' and comparatively fewer potential H-bonds are detected (Fig. 4). Among them, hydroxyl groups are relatively frequent, four H-bonds out of twelve involve the OH of serine residues. The C-17^3 groups, which are linked to the long, hydrocarbon phytol tail, are clearly less frequently involved in both polar and non-polar interactions: there are only about 40% of total contacts compared to C-13^1 or -13^3, with again half of them polar (6× His 1× Arg, 1× Asn, 1× Tyr). Among the latter, H-bonds are frequent with the histidines that ligand the central Mg of the Chl. Thus, it appears that H-bonding between the apoproteins and keto carbonyls, in particular, C-13^1, is a consistent feature in PSI, too. Future work will be required to take apart the significance of H-bonding, in particular of hitherto unrecognized H-bonds, for assembly and stability of membrane-embedded (B)Chl protein complexes.

4. Conclusions

In conclusion, the C-13^1 keto carbonyl of one of the BChl, most likely β-BChl-B850, in the photosynthetic antenna, LH2 of *Rb. sphaeroides*, is strongly H-bonded. Yet, disruption of this H-bond, which seems frequent in bacterial photosynthetic proteins, apparently neither alters

the strongly red-shifted absorption nor the structural arrangement of the BChl-B850, and only slightly decreases the thermal stability of LH2. With alanine consistently replacing serine in most LH2 from other purple bacteria, weak H-bonding may be involved in this compensation, either directly with this alanine or with the nearby histidine. The occurrence of H-bonding to the 13^1-C=O group of Chl *a* seems to be frequent as well. Statistical analysis of the 96 binding sites of PSI confirm that the C-13^1 keto carbonyl groups are repeatedly involved in H-bond interactions. Together with vibrational spectroscopy of these and other proteins, this shows that intramembrane H-bonding between TMH's residues and (B)Chl, in particular C-13^1 keto carbonyl group, which is present in all known chlorophylls, is indeed widespread in photosynthetic proteins.

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