Blue shifts in bacteriochlorophyll absorbance correlate with changed hydrogen bonding patterns in light-harvesting 2 mutants of *Rhodobacter sphaeroides* with alterations at α -Tyr-44 and α -Tyr-45

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A combination of Fourier-Transform (FT) resonance Raman spectroscopy and site-directed mutagenesis has been used to examine the function of two highly conserved aromatic residues, α -Tyr-44 and α -Tyr-45, in the light-harvesting 2 (LH2) complex of the photosynthetic bacterium *Rhodobacter sphaeroides*. In LH2 complexes, aromatic residues located at positions α -44 and α -45 are thought to be located near the putative binding site for bacteriochlorophyll, and alterations at these positions are known to produce blue shifts in bacteriochlorophyll absorbance. In the present work, mutant LH2 complexes carrying the alterations α -Tyr-44 $\rightarrow$ Phe, α -Tyr-45 $\rightarrow$ Phe and α -Tyr-44,-45 $\rightarrow$ Phe,Leu were examined. FT resonance Raman spectroscopy of the

resulting complexes shows the breakage of a hydrogen bond to the 2-acetyl carbonyl group of one of the B850 bacteriochlorophylls in the LH2 complex; in the double mutant, breakage of a second bond is probable. These results suggest that one of these hydrogen bonds is to α -Tyr-44, placing this residue in close proximity to ring I of one of the B850 bacteriochlorophyll *a* pigments. The breakage of one, then two, 2-acetyl carbonyl hydrogen bonds correlates well with the shift in the absorbance of the B850 pigments of 11 nm then 26 nm at 77 K. Thus a consistency between literature theoretical calculations and the observations from both absorption and FT resonance Raman spectroscopy is demonstrated.

INTRODUCTION

The photosynthetic apparatus of the purple bacterium *Rhodobacter sphaeroides* consists of a photochemical reaction centre (RC), surrounded and interconnected by light-harvesting (LH) complexes. The LH1 complex is the immediate donor of excitation energy to the reaction centre, and it receives energy in turn from the peripheral LH2 complex. All three complexes, the RC, LH1 and LH2, are intrinsic membrane proteins, and in the case of the RC, the structure has been determined to atomic resolution for *Rhodospseudomonas viridis* and *R. sphaeroides* [1–3]. Although the same level of information does not exist for the LH complexes, the primary sequences of many of the polypeptides are known; biochemical studies have allowed the topology of the polypeptides relative to the membrane to be determined, and information about the relative positions and orientation of the bacteriochlorophyll *a* (Bchl_a) pigments has been obtained by spectroscopic methods (see, for example, [4]). From these various studies, models for the α and β polypeptides have been derived in which each contains a single transmembrane α -helical domain flanked by surface-exposed regions [5–7]. The presence of bacteriochlorophyll and carotenoid pigments is of further help, since the orientations of these pigments and the distances between them can be determined with some accuracy. As an example, Kramer et al. [4] proposed an $\alpha\beta_2$ model for the LH2 complex of *R. sphaeroides* in which four B850 bacteriochlorophylls are arranged with their Q_y transitions parallel to the membrane plane approximately 21 Å (2.1 nm) away from the two B800 pigments.

Although the peripheral antenna complex from *R. sphaeroides* absorbs at 800 and 850 nm, antennae from other purple bacteria absorb at different wavelengths. For example, the cells of

Rhodospseudomonas acidophila strain 7750 can promote the synthesis of light-harvesting complexes that absorb either at 800/850 or at 800/820, depending on the growth conditions; similar effects can be seen, for example, with the purple bacterium *Rp. palustris* [8]. The physical mechanisms governing the absorbance of these complexes and the role of the protein are far from clear; different complexes appear to absorb at different wavelengths even though at first sight the protein sequences are not very different from each other [7]. This is an important factor in the light-harvesting process, however, since it is important for the protein to modulate the absorbance properties of the pigment in such a way that energy migrates from the peripheral LH2 complex to the LH1/RC core. In the present work we explore the factors that underlie the absorbance shifts observed when two residues, α -Tyr-44 and α -Tyr-45, are altered by site-directed mutagenesis to Phe and Leu respectively. It is already known that the changes α -Tyr-44 $\rightarrow$ Phe and α -Tyr-44,-45 $\rightarrow$ Phe,Leu produce alterations of the normal 800/850 nm absorbance spectrum of the LH2 complex to 800/838 and 800/826 nm respectively. Fluorescence excitation and emission spectroscopy, together with linear dichroism and circular dichroism measurements, indicate that this effect is specific to the B850 bacteriochlorophylls and that there are no observable rearrangements in the complex [9]; the α -Tyr-44,-45 $\rightarrow$ Phe,Leu B800/826 *R. sphaeroides* LH2 mutant has the same sequence at analogous positions in the α -subunit as the B800/820 light-harvesting complex of *Rp. acidophila* strain 7050 [8]. In the present work the specificity of these mutagenic changes in the *R. sphaeroides* LH2 has been examined by Fourier transform (FT) resonance Raman spectroscopy, a type of vibrational spectroscopy which is currently one of the only experimental methods which can yield submolecular information about the pigment binding sites in

bacteriochlorophyll pigment/protein complexes. In particular, this technique is sensitive to the hydrogen bonding states of the 2-acetyl and 9-keto carbonyl oxygen molecules which are linked to the conjugated bond system of the Bchl_a dihydrophorbin macrocycles [8,10,11]. This type of measurement is accurate even at room temperature, especially when applied to Bchl_a-containing proteins [12]. Using this method we show that the blue shifts in absorbance correlate with breakage of hydrogen bonds to the 2-acetyl carbonyl groups of the B850 pigments. The implications of these results for existing structural models of the LH2 complex are discussed.

MATERIALS AND METHODS

Media, antibiotics and growth conditions

Escherichia coli strains were grown in Luria broth. *R. sphaeroides* strains were grown under semi-aerobic/dark conditions in M22 + medium [13] supplemented with 0.1 % casamino acids for growth in liquid culture. For *E. coli*, tetracycline was used at a concentration of 10 µg/ml. For *R. sphaeroides*, antibiotic concentrations were: tetracycline, 1 µg/ml; neomycin, 20 µg/ml; streptomycin, 5 µg/ml.

Bacterial strains and plasmids

The bacterial strains used in this work include *E. coli* strain S17-1 {*thi pro hsdR- hsdM + recA RP4-2 (Tc::mu km::Tn7)* [14]} and *R. sphaeroides* strains DD13 and DD13/G1 (genomic deletion of *pucBA* and *pufBALMX*; insertion of Sm^R and Km^R genes [15]). The mobilizable plasmid used was pRK(CBC2) (Tc^R; derivative of pRK415; 4.4 kb fragment encompassing *pucBA* [15]).

DNA preparation, mutagenesis and sequencing

Plasmids were prepared using the alkaline lysis method. The codon for the required mutation was introduced into *R. sphaeroides* DNA using an appropriate mutagenic oligonucleotide according to the method of Kunkel [16], with a template based upon bacteriophage M13mp18 containing the *pucBA* genes. DNA sequencing was carried out using M13mp18 single-stranded sequencing methods by the dideoxy chain termination method of Sanger *et al.* [17].

Conjugative crosses

The mobilizable plasmids to be introduced into *R. sphaeroides* were first transformed into *E. coli* strain S17-1. Matings were then performed as described in [13]. Transconjugants were grown aerobically in the dark on plates of M22 + medium supplemented with appropriate antibiotics. In the case of matings performed to complement deletion/insertion mutants *in trans* using plasmids based on pRK(CBC2), tetracycline was included in the growth medium.

Preparation of intracytoplasmic membranes

Membranes were prepared from cells grown semi-aerobically in the dark by disruption in a French press cell, and were purified by harvesting from the interface of sucrose step gradients (15 %/40 %, w/w) after centrifugation.

Spectroscopy

Following conjugative transfer, antibiotic-resistant colonies were screened for the presence or absence of LH2 complexes using a Guided Wave model 260 fibre optic spectrophotometer (Guided Wave Inc., 1590 Golden Foothill Parkway, El Dorado Hills, CA 95630, U.S.A.). FT resonance Raman spectra were recorded using a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module. Excitation at 1064 nm was provided by a continuous Nd:Yag laser. The set-up, laser powers and sample behaviour have been extensively described [18]. The typical resolution of FT resonance Raman spectra was 4 cm⁻¹. All spectra were recorded at room temperature with 180° back-scattering geometry from pellets or concentrated solutions held in standard aluminium cups. Depending on the sample, spectra are the result of 5000–20000 co-added interferograms.

RESULTS

WT (wild-type) *R. sphaeroides* LH2

Figure 1 shows the high-frequency region (1550–1750 cm⁻¹) of the Raman spectra obtained from *R. sphaeroides* membranes in which LH2 genes have been expressed in a double-deletion DD13 strain (RC⁻, LH1⁻, LH2⁻) [15]; this includes both the WT genes and the following mutant LH2 genes: (a) α-Tyr-44 → Phe, (b) α-Tyr-44,Tyr-45 → Phe,Leu and (c) α-Tyr-45 → Phe; all of which have been described previously [9,19].

The Raman signals for the WT LH2 complex of *R. sphaeroides* have been described and assigned previously [20]. Briefly, the signals between 1620 and 1700 cm⁻¹ arise from the stretching modes of either the 2-acetyl or 9-keto carbonyl substituents found in the dihydrophorbin macrocycles of the bacterio-

Figure 1 FT Raman spectra of plasmid-borne WT and mutant intracytoplasmic LH2 membranes from *R. sphaeroides* strain DD13 in the 1550–1750 cm⁻¹ carbonyl stretching region.

a, α-Tyr-44,α-Tyr-45 → Phe,Leu LH2 double mutant; b, α-Tyr-45 → Phe mutant LH2; c, α-Tyr-44 → Phe mutant LH2; d, WT LH2.

chlorophyll pigments in the LH2 pigment–protein complexes; the frequencies of these signals depend on the state of interaction of the carbonyls. In the absence of any intermolecular interaction, the band arising from the stretching mode of the 2-acetyl carbonyl groups is seen at 1660 cm^{-1} , and upon the formation of a hydrogen bond between this group and a neighbouring molecule, the frequency may shift down to approx. 1620 cm^{-1} , the amplitude of the shift partly depending on the strength of the H-bond formed. Similarly, the 9-keto groups give rise to a Raman peak at approx. 1695 cm^{-1} which on H-bonding shifts to approx 1660 cm^{-1} [20].

The stoichiometry of the LH2 complex is such that each minimal unit of the complex is thought to contain three Bchl_a molecules [21]; therefore, since there are two carbonyl groups (2-acetyl and 9-keto), that could give rise to a signal in this region of the Raman spectrum for each Bchl_a molecule, one would expect there to be six peaks, one for each of the carbonyl groups, which might all be expected to be in different H-bonding environments. In reality, however, five frequencies are seen in the carbonyl stretching region ($1620\text{--}1700\text{ cm}^{-1}$) of the FT Raman spectrum for membranes of the *R. sphaeroides* strain containing the LH2 as the sole complex, at approx. 1628, 1635, 1652, 1679 and 1699 cm^{-1} (Figure 1); these five frequencies match those already published for detergent-solubilized isolated LH2 complexes prepared from *R. sphaeroides* [12,20]. The bands at 1628 and 1635 cm^{-1} most probably arise from H-bonded 2-acetyl Bchl_a carbonyl groups, whereas the bands at 1679 and 1699 cm^{-1} can be readily attributed to a weakly H-bonded and a free-from-interaction 9-keto Bchl_a carbonyl group respectively. The attribution of the remaining fifth band at 1652 cm^{-1} is slightly more ambiguous, as this band could arise from either a weakly interacting 2-acetyl Bchl_a carbonyl group or a strongly H-bonded 9-keto group. As only five frequencies can be observed in the Raman spectrum, one of the Raman peaks must be degenerate, being made up of the contributions from two different carbonyl groups.

In the region $1595\text{--}1610\text{ cm}^{-1}$ of the Raman spectrum, there is a signal at approx. 1607 cm^{-1} (also shown in Figure 1) which arises from stretching modes of the methine bridges of the Bchl_a molecules [22]; this band is sensitive to the co-ordination of the central Mg atom of the Bchl_a molecule, being located at approx. 1595 cm^{-1} when the Mg atom binds two axial ligands (six-coordination) and at approx. 1605 cm^{-1} when it bonds on one axial ligand (five-coordination) [22]. Figure 1 illustrates that the methine bridge stretching modes in all the samples examined are located at $1605\text{--}1607\text{ cm}^{-1}$, indicating that the Bchl_a central Mg is five-coordinate for both the mutant LH2 complexes as well as the WT [20]. Also in the spectra is a shoulder at approx. 1585 cm^{-1} ; the intensity of this band appears to be highest in the $\alpha\text{-Tyr-44} \rightarrow \text{Phe}$ LH2 (described further below). The intensity of this band has already been reported to vary according to the bacterial strain involved (see Figure 5 in [20]), but the variation cannot be ascribed to a particular structural property of the Bchl_a binding site [23]. It is worth noting, however, that the frequency of the band is too low for it to have arisen from a methine bridge stretching mode [22].

The $\alpha\text{-Tyr-44} \rightarrow \text{Phe}$ mutant

Examination of the carbonyl stretching region ($1620\text{--}1700\text{ cm}^{-1}$) of the FT Raman spectrum of membranes from one of the LH2 mutants, $\alpha\text{-Tyr-44} \rightarrow \text{Phe}$, reveals a clear difference from the WT spectrum (Figure 1). Whereas four of the five frequencies are the same as those observed in the WT spectrum (at approx. 1628,

1652 , 1675 and 1699 cm^{-1}), the contribution at approx. 1635 cm^{-1} has disappeared and a band has appeared at approx. 1659 cm^{-1} , a position where no peak is observed in the WT spectrum. This frequency is observed *in vitro* in situations where the 2-acetyl carbonyl groups are free from intermolecular interactions [11]. The signal at 1635 cm^{-1} , in contrast, unambiguously indicated the presence of an H-bonded 2-acetyl group, as described earlier. Therefore the inevitable conclusion is that the $\alpha\text{-Tyr-44} \rightarrow \text{Phe}$ mutation breaks an H-bond between the nearby LH2 polypeptide and one of the 2-acetyl carbonyl groups of one of the Bchl_a molecules. In these spectra, the contribution at 1679 cm^{-1} in the WT seems to be slightly down-shifted in the $\alpha\text{-Tyr-44} \rightarrow \text{Phe}$ mutant to 1675 cm^{-1} . Although small, this variation could suggest a slight strengthening of a hydrogen bond to one of the Bchl_a 9-oxo carbonyl groups. However, the presence of the contribution at approx. 1660 cm^{-1} with probable width of 13 cm^{-1} is expected to perturb the baseline of the 1679 cm^{-1} signal, and could also account for this small apparent down-shift. The fact that the other three Raman peaks seen in the mutant spectrum remain unchanged, relative to those from the WT LH2 complex, indicates that the effect of this mutation on the H-bonding pattern in the protein–pigment complex is highly localized, and the overall perturbation to the LH2 complex ongoing from WT to mutant is limited.

The $\alpha\text{-Tyr-45} \rightarrow \text{Phe}$ mutant

In contrast, examination of the carbonyl stretching region of the FT Raman spectrum from the second mutant, in which $\alpha\text{-Tyr-45}$ is changed to a Phe, shows that the spectrum of this mutant differs from that of the WT complex in a number of ways (Figure 1). Firstly, the signal at 1628 cm^{-1} , which arises from an H-bonded 2-acetyl Bchl_a carbonyl group in the WT, is absent. Secondly, the band at 1652 cm^{-1} , which in the WT is thought to arise from either a weakly interacting 2-acetyl Bchl_a carbonyl group or a strongly H-bonded 9-keto carbonyl group, is shifted slightly to lower frequencies and is relatively more intense when normalized to other bands (such as that at 735 cm^{-1} , which arises from vibrations of the Bchl_a dihydrophorbins ring and is unlikely to be affected by the $\alpha\text{-Tyr-45} \rightarrow \text{Phe}$ mutation). Finally, the band at 1679 cm^{-1} , attributed in the WT to a weakly H-bonded 9-keto Bchl_a carbonyl group, is down-shifted to approx. 1672 cm^{-1} and has a lower intensity relative to the comparable peak in the WT spectrum.

It is difficult to establish, at first sight, whether the increase in intensity of the Raman signal at 1652 cm^{-1} is linked to the reduction of intensity of the signal at 1628 cm^{-1} (attributed in the WT to an H-bonded 2-acetyl group) or to the reduction of intensity of the signal at 1679 cm^{-1} (attributed to a free-from-interaction 9-keto group). The increase in intensity of the band at 1652 cm^{-1} corresponds to the appearance of a new component at approx. 1649 cm^{-1} . This component might arise from a down-shift of the signal at 1679 cm^{-1} , which is observed in the WT and which can be attributed to a weakly bonded 9-keto carbonyl. However, as a 1672 cm^{-1} band is still present in the mutant, this would imply that in the WT the 1679 cm^{-1} band is degenerate, being composed of a 1679 cm^{-1} component (which is sensitive to the $\alpha\text{-Tyr-45} \rightarrow \text{Phe}$ mutation), plus a weaker component at 1672 cm^{-1} (which is not sensitive to the change at $\alpha\text{-Tyr-45}$). This would lead to an hypothesis in which the Raman signals from the carbonyl groups of the three Bchl_a pigments present in the WT LH2 were located at 1628, 1635, 1659, 1672, 1679 and 1699 cm^{-1} . However, this hypothesis is incorrect for the following reasons. As the 1628 and 1635 cm^{-1} signals are affected by the $\alpha\text{-Tyr-45} \rightarrow \text{Phe}$ and the $\alpha\text{-Tyr-44} \rightarrow \text{Phe}$ mutations respectively, one might

expect that there would be no band present for the double α -Tyr-44,-45 mutant in the 1620–1640 cm^{-1} region; however, this is not the case, as in the double mutant a signal at 1628 cm^{-1} is still present. Thus we conclude that there are three bands present in the 1620–1640 cm^{-1} region of the Raman spectrum of the WT LH2, one at 1628 cm^{-1} (sensitive to the α -Tyr-45 $\rightarrow$ Phe mutation), one at 1635 cm^{-1} (sensitive to the α -Tyr-44 $\rightarrow$ Phe mutation) and finally a weak signal at approx. 1628 cm^{-1} which is not sensitive to either of the α -Tyr mutations, and which arises from the intermolecular interactions of the 2-acetyl carbonyl group of the B800 molecule. Therefore the six carbonyl signals in the WT LH2 are located at 1628, 1628, 1635, 1659, 1679 and 1699 cm^{-1} , with no WT contribution at 1672 cm^{-1} . In the Raman spectrum for the α -Tyr-45 $\rightarrow$ Phe mutant LH2, the 1672 cm^{-1} band most probably arises from the WT 1679 cm^{-1} signal, slightly down-shifted and lowered in relative intensity. The additional contribution at 1649 cm^{-1} to the α -Tyr-45 $\rightarrow$ Phe spectrum is due to the shifting of the WT band at 1628 cm^{-1} ; a shift that is associated with a weakening or even the complete breakage of an H-bond to the 2-acetyl carbonyl of one of the B850 Bchl a molecules.

Although the changes in the Raman spectrum on going from the WT LH2 to the α -Tyr-45 $\rightarrow$ Phe mutant cannot be unequivocally interpreted, it may be concluded that the H-bonding pattern for one of the 2-acetyl carbonyl groups is disrupted by the change, and also that the mutation induces a reorganization of the B850 Bchl a binding site, affecting at least two of the interactions associated with the B850 pair. This is a weakening of the other 2-acetyl carbonyl interaction and an enhancement of a 9-keto carbonyl interaction.

The α -Tyr-44,-45 $\rightarrow$ Phe,Leu double mutant

The FT Raman spectrum of the α -Tyr-44,-45 $\rightarrow$ Phe,Leu double mutant LH2 (Figure 1) is seen to be significantly perturbed in relation to the WT spectrum, and an interpretation is only possible by reference to the spectra obtained from the single α -Tyr-44 $\rightarrow$ Phe and α -Tyr-45 $\rightarrow$ Phe mutants described earlier. The spectrum shows a peak of reduced intensity at 1628 cm^{-1} (relative to the 1609 cm^{-1} peak as compared with the WT spectrum), a complex cluster of peaks in the region 1652–1672 cm^{-1} (including a peak at 1672 cm^{-1} of reduced intensity) and a peak at 1699 cm^{-1} .

Such a spectrum could arise as a result of the breaking of two 2-acetyl carbonyl group H-bonds and the strengthening of the H-bond to one of the 9-keto carbonyl groups. The breaking of the first 2-acetyl carbonyl group H-bond, signalled by a shifting of the Raman peak at 1635 cm^{-1} in the WT LH2 to 1659 cm^{-1} in the double α -Tyr-44,-45 $\rightarrow$ Phe,Leu mutant, is the same as is seen in the single α -Tyr-44 $\rightarrow$ Phe mutant LH2. The breaking of the second 2-acetyl carbonyl group H-bond is suggested by an additional contribution to the double α -Tyr-44,-45 $\rightarrow$ Phe,Leu Raman spectrum at a frequency higher than 1650 cm^{-1} (i.e. within the cluster of carbonyl signals in the region 1652–1672 cm^{-1}); the same effect is seen, to a lesser extent, in the single α -Tyr-45 $\rightarrow$ Phe mutant, in which there is a greater relative signal at 1652 cm^{-1} . The strengthening of the H-bond to one of the 9-oxo carbonyl groups is suggested by a shift of the peak at 1679 cm^{-1} in the WT LH2 to a peak at 1672 cm^{-1} in the double α -Tyr-44,-45 $\rightarrow$ Phe,Leu Raman spectrum (i.e. within the cluster of carbonyl signals in the region 1652–1672 cm^{-1}). This effect is also seen in the Raman spectrum of the α -Tyr-45 $\rightarrow$ Phe mutant LH2 described earlier.

The residual peak at approx. 1628 cm^{-1} in the α -Tyr-44,-45 $\rightarrow$ Phe,Leu Raman spectrum is probably a signal from one of the

carbonyl groups on the B800 Bchl a pigment. This is in accordance with previous observations [20] in which WT LH2 membranes from *R. sphaeroides* were compared with LH2 membranes that had been B800-depleted by addition of detergents in order to demonstrate that the Raman signals due to the B800 Bchl a occur at approx. 1628 cm^{-1} (H-bonded 2-acetyl carbonyl) and at approx. 1700 cm^{-1} (interaction-free 9-oxo carbonyl). Similarly, in work on the membranes from a mutant LH2 complex in which a residue possibly involved in the B800 binding site, β -His-21, has been changed to a Ser residue, leading to an attenuation of the B800 absorbance peak, the Raman peak at approx. 1628 cm^{-1} is also attenuated (G. J. S. Fowler, B. Robert and C. N. Hunter, unpublished work).

DISCUSSION

This study builds upon the earlier work of Fowler et al. [9], which demonstrated that the aromatic residues α -Tyr-44 and α -Tyr-45 in the LH2 complex of *R. sphaeroides* exert an effect on bacteriochlorophyll B850 absorbance *in vivo*. The precise physico-chemical mechanisms through which these residues were controlling the absorption maximum of these pigments was unknown; it had been suggested that the basis for this effect was a stacking interaction between the ring systems of the tyrosine and the tetrapyrrole macrocycle [26]. There is a precedent for this kind of association; for example, in the interaction of TrpM252 and the quinone Q_A in the photosynthetic reaction centre [1].

However, the results of resonance Raman spectroscopy on these mutants provides the first clear experimental evidence that the blue shifts in LH2 absorbance are manifested as a consequence of breaking of hydrogen bonds to the 2-acetyl group of each bacteriochlorophyll B850 molecule, probably within the $\alpha\beta$ minimal unit. This is the most straightforward interpretation of the data, although it should be noted that α -Tyr-44, α -Tyr-45 may govern the formation of these hydrogen bonds, rather than being the direct participants. The case for a single specific effect of bond breakage is easier to make for α -Tyr-44 than for α -Tyr-45; in the latter example, we also see a strengthened hydrogen bond to a 9-keto group, and there may well be larger scale rearrangements for this alteration. This is also the case for the double mutation α -Tyr-44,-45 $\rightarrow$ Phe,Leu. One consequence of modelling the interaction of α -Tyr-44 with one of the bacteriochlorophyll B850 molecules is that this places constraints on the distance between the participants; in order for the model to show a realistic separation between the molecules, one needs to include in the putative structure a turn in the α polypeptide near the membrane interface, as modelled in Figure 2. For the *R. sphaeroides* LH2 complexes the presence of a proline residue near the membrane interface is consistent with this suggestion. However, it might also be the case that α -Tyr-45 forms a hydrogen bond with the 2-acetyl group of a bacteriochlorophyll B850 molecule from another $\alpha\beta$ dimer pair; the consequence of the α -Tyr-45 $\rightarrow$ Phe alteration could then be to promote more structural rearrangement than obtained with the α -Tyr-44 $\rightarrow$ Phe change.

It is interesting to note in this context than an analogous residue in the LH1 of *R. sphaeroides*, α -Trp-43, also appears to form a hydrogen bond with a 2-acetyl of one of the bacteriochlorophyll B875 pigments [25]. Similarly, work on a rather anomalous light-harvesting complex from *Rs. molischianum* shows that this complex has an amino-acid sequence and a Raman spectrum similar to those of *R. sphaeroides* LH1, while having an absorption spectrum similar to that of the *R. sphaeroides* LH2 complex, i.e. absorbing at 800 and 850 nm; the 2-acetyl carbonyl groups in the *Rs. molischianum* light-harvesting complex both appear to be strongly hydrogen-bonded [10].

Figure 2 Representation of the $\alpha\beta$ terminal unit of the *R. sphaeroides* LH2 pigment-protein complex

The figure shows a model of a possible H-bonding interaction between the 2-acetyl carbonyl of a B850 bacteriochlorophyll pigment and the aromatic α -Tyr-44 residue. The polypeptide backbones of the α - and β -subunits are shown as ribbons; (a) and (b) are views at 90° to each other, both looking along the plane of the membrane. The bacteriochlorophyll and tyrosine molecules that interact through a hydrogen bond are shown in black; for ease of representation they are shown on adjacent α - and β -subunits, but this should not rule out the possibility that the hydrogen bonding occurs between adjacent $\alpha\beta$ dimers.

Although the 800/850 (LH2) complex of *Rs. molischianum* does not possess tyrosine residues equivalent to the α -Tyr-44 and α -Tyr-45 residues in the LH2 of *R. sphaeroides*, and therefore cannot form the same Bchl_a-protein hydrogen bonding patterns, it does possess an aromatic Trp residue equivalent to the α -Trp-43 in the *R. sphaeroides* LH1 complex. The type of Trp-Bchl_a hydrogen bonding pattern that gives rise to a Bchl_a molecule absorbing at 875 nm in the *R. sphaeroides* LH1 complex [25] can presumably be invoked to explain the absorbance of the 850 nm absorbing Bchl_a in the *Rs. molischianum* LH2 complex. Moreover, recent resonance Raman experiments performed on light-harvesting complexes that naturally absorb at 820 nm seem to indicate that the H-bonding pattern in these complexes is modified relative to that seen in complexes from the same bacterial species that absorb at 850 nm (J. N. Sturgis, R. J. Cogdell and B. Robert, unpublished work).

For the *R. sphaeroides* LH2 mutants described in the present work, the breakage of one, then two, hydrogen bonds correlates well with the shift in the absorbance peaks of the B850 pigments of 11 nm and then 26 nm at 77 K [9]. This correlation agrees well with theoretical calculations on the effect of hydrogen bonding to the chlorophyll macrocycle [26], which demonstrate that the formation of an H-bond to the 2-acetyl carbonyl group of one of the Bchl_a pigments in a Bchl_a dimer shifts the absorbance of the dimer 10 nm toward the red; this shows a consistency between theoretical calculations and the observations from both absorption and FT resonance Raman spectroscopy. Furthermore, similar mutagenesis studies on the *R. sphaeroides* reaction centre, in which Phe-195 on the M-subunit was changed to a tyrosine,

resulted in a 10 nm red-shift in the absorbance of the special-pair bacteriochlorophylls [12]. This is the converse of the changes described in the present work, in which blue shifts were examined. It is significant that the M-Phe-195 $\rightarrow$ Tyr change creates a hydrogen bond to the 2-acetyl carbonyl group of the Bchl_a of the special pair [27]. Taken together with the LH complex work, it is reasonable to conclude that the effect of hydrogen bonding from tyrosine residues to bacteriochlorophylls *in vivo* is to fine-tune their absorbance maxima over a 20 nm range.

There is evidence, however, that it is possible to have absorbance shifts of 10 nm or more through mechanisms that are probably more to do with macromolecular effects (such as subunit aggregation and rearrangement) than with pigment-protein H-bonding patterns; this can be seen from detergent work on isolated LH1 complexes [28] and from work on other site-directed mutants of LH2 [29]. Although the changes in Raman signal seen with the α -Tyr-44 mutation seem to clearly indicate that this residue is directly involved in H-bonding to the 2-acetyl carbonyl of a 850 nm-absorbing Bchl_a, alterations in the Raman spectra due to the α -Tyr-45 mutation could be more indirect. Indeed, the α -Tyr-45 mutation could induce a perturbation of the H-bond pattern of the light-harvesting complex, which in turn could modify the H-bonding ability of other residues which would normally interact directly with Bchl_a molecules in their binding sites. Thus, instead of being the cause of the absorbance changes in the B850 pigments, the alteration of H-bonding patterns seen in the present paper may, in fact, be a symptom (in parallel with the B850 absorbance shifts) of larger changes in the mutant LH2 pigment-protein complexes. Further

site-directed engineering of carefully chosen mutations into the LH2 complex should clarify this point more fully.

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