Stark Absorption Spectroscopy on the Carotenoids Bound to B800-820 and B800-850 type LH2 Complexes from a Purple Photosynthetic Bacterium, *Phaeospirillum molischianum* strain DSM120

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Abbreviations: *Phs.*, *Phaeospirillum*; RC, reaction center; NIR, near infrared; Bchls, bacteriochlorophylls; *Rps.*, *Rhodopseudomonas*; *Rba.*, *Rhodobacter*; HL, high light; LL, low light; PVA, poly vinyl alcohol;

Abstract

Stark absorption spectroscopy was applied to clarify the structural differences between carotenoids bound to the B800-820 and B800-850 LH2 complexes from a purple photosynthetic bacterium *Phaeospirillum (Phs.) molischianum* DSM120. The former complex is produced when the bacteria are grown under stressed conditions of low temperature and dim light. These two LH2 complexes bind carotenoids with similar composition, 20% lycopene and 80% rhodopin, each with the same number of conjugated C=C double bonds ($n = 11$). Quantitative classical and semi-quantum chemical analyses of Stark spectra recorded in the carotenoid absorption region reveal substantial differences in the $|\Delta\mu|$ values ($2 [D/f]$) for carotenoids bound to either B800-820 or B800-850 complexes. The origin of this striking difference in the $|\Delta\mu|$ values was analyzed using the X-ray crystal structure of the B800-850 LH2 complex from *Phs. molischianum* DSM119. Semi-empirical molecular orbital calculations predict structural deformations of the major carotenoid, rhodopin, bound within the B800-820 complex. We propose that simultaneous rotations around neighboring C=C and C-C bonds account for the differences in the $2 [D/f]$ of the $|\Delta\mu|$ value.

Introduction

The photosynthetic light-harvesting antenna absorbs light energy and transfers the excitation energy to the photosynthetic reaction center (RC).[1] In general, two types of antenna systems are found in purple photosynthetic bacteria. The peripheral LH2 antenna complex is characterized by two near-infrared (NIR) absorption bands at 800 and 850 nm arising from the Q_y transitions of two rings of bacteriochlorophylls (Bchls). Energy from LH2 complexes passes to the LH1 antenna complex that has a single ring of Bchls absorbing at 875 nm surrounding the RC. Thus, the LH2-LH1 antenna funnels excitation energy toward the RC where eventually the energy is trapped and transiently stored as a charge separation, which initiates a cascade of electron transfers. The efficiency of energy trapping in natural photosynthetic systems[2] presents some intriguing problems, particularly when artificial photosynthetic systems are envisaged that might power the production of solar fuels.[3] Elucidation of the relationship between the structure of light-harvesting pigment-protein complexes and their physiological functions is therefore important, and requires an understanding of their spectroscopic behavior.

The LH2 complexes of purple phototrophs provide convenient models for understanding the structural basis for the spectroscopic properties of antenna complexes. The X-ray crystal structure of the LH2 from *Rhodospseudomonas (Rps.) acidophila* 10050 was first reported at 2.5 Å resolution by McDermott *et al.*[4] and later at higher Å resolution by Papiz *et al.*[5] Koepke *et al.* reported the 2.4 Å X-ray structure of the LH2 from *Phaeospirillum (Phs.) molischianum* strain DSM119.[6] The overall structure of LH2 from *Rps. acidophila* is a nonameric ring, whereas the *Phs. molischianum* complex is an octamer of α - and β - polypeptide protomer pairs, each binding one carotenoid and three Bchl *a* molecules. In these complexes one Bchl *a* molecule is monomeric, and other two Bchl *a* molecules exist as a dimer. The 800 and 850 nm Q_y absorption maxima arise from the rings of Bchl *a* monomers and dimers, respectively, so this is often referred to as a B800-850 type LH2. Nevertheless, some species of purple photosynthetic bacteria produce another type of LH2 when grown low light and/or low temperature conditions, where the B850 absorption band shifts to blue, giving a B800-820 type LH2.[7] This blue-shifting response to stress varies between photosynthetic bacteria; sufficiently low light and low temperature (20°C) conditions completely shift LH2 production to the B800-820 type in the case of *Rps. acidophila* strain 7750 or *Phs. molischianum* strain DSM 120, whereas both B800-820 and B800-850 type LH2s are found in *Rps. acidophila* strain 7050 or *Phs. molischianum*

strain DSM119.[8] The basis for these absorption shifts lies in differences in the structures of the α - and/or β - polypeptides, which induce effects on the 820/850 nm binding sites; comparison of the X-ray structures of the B800-820 type LH2 from *Rps. acidophila* strain 7050 (3.0 Å resolution) and the B800-850 type LH2 from *Rps. acidophila* strain 10050 show differences in the hydrogen-bonding pattern between C3-acetyl group of BChl *a* dimer and $\alpha\beta$ -polypeptides.[9-11] Independently, the Tyr-Tyr motif adjacent to the B850 binding site in the *Rhodobacter sphaeroides* B800-850 LH2 complex was altered to Phe-Tyr and Phe-Leu using site-directed mutagenesis. B800-839 and B800-826 complexes, respectively, were produced, and Fourier-Transform resonance Raman spectroscopy showed that these shifts could be explained by the breakage of one, then two hydrogen bonds from the Tyr residues to the C2-acetyl carbonyls of the B850 pair of Bchls.[9,10]

Carotenoids are biological polyenes, and are found in all photosynthetic antenna complexes.[12] They enhance light harvesting efficiency by absorbing in the 400-550 nm spectral region, where Chls and Bchls absorb only weakly. Carotenoids also protection against excess light exposure.[13-15] Rhodopin glucoside, rhodopinal glucoside, and lycopene are the major carotenoids bound to the LH2 complexes from *acidophila* strain 10050, *Rps. acidophila* strain 7050, and *Phs. molischianum* strain DSM119, respectively.[6,11,16] Some reports show the interaction between carotenoids and aromatic residues is important for the binding of carotenoids to LH2 proteins.[17,18] Polar residues are also important; site-directed mutagenesis showed that the conserved residue β Arg-10 (numbered by counting back from the conserved B850 ligand His0) is closely associated with LH2 carotenoids. The β Arg-10→Glu mutation blue-shifts carotenoid absorbance maxima by approximately 6 nm, as well as attenuating the electrochromic response of LH2 carotenoids to an external membrane potential.[19] Another consequence of altering β Arg-10 is a blue-shifted and broadened B800 absorption band;[20,21] a subsequent FT-Raman study showed that Arg residue forms an H-bond with the C2 acetyl carbonyl of the monomeric BChl in LH2.[22] The β His-18→Ser mutation nearly abolishes B800 absorption and also carotenoid absorbance and electrochromic behaviour.[19] Each of these mutants has a normal carotenoid content, and energy transfer to B850 pigments is unaffected.[19] Although mutagenesis studies can identify important carotenoid interactions, they were applied to the *Rhodobacter (Rba.) sphaeroides* LH2 complex, for which there is only a Å projection structure;[23] higher resolutions, of at least 2 Å, are required for unequivocal assignment of all such interactions. To our knowledge, the only LH2 structure that precisely determines the electron density map of bound carotenoids is the

B800-850 type LH2 from *Rps. acidophila* strain 10050,[5] but the detailed structure and environment of carotenoids bound to B800-820 type LH2 complexes are still unknown and we therefore apply Stark absorption spectroscopy in the present work in order to obtain more information about carotenoids bound to this particular LH2 complex.

Stark absorption spectroscopy determines the changes of polarizability ($\Delta\alpha$) and static dipole moment ($\Delta\mu$) upon photo-excitation of pigments.[24,25] These parameters reflect both the structure of pigments and their electrostatic environment surrounding pigments. This spectroscopic method has been used successfully to investigate the molecular structure of carotenoids bound to the LH1 complex from *Rhodospirillum rubrum* strain S1,[26] and is used here to investigate the molecular structure of carotenoids bound within the B800-820 type LH2 complexes from *Phs. molischianum* strain DSM 120. This strain produces both B800-820 type and B800-850 type LH2 complexes, depending on the growth condition (*vide supra*), and each with similar carotenoid contents. The protein environments of the carotenoids will differ slightly, and therefore a direct spectral comparison of these two complexes should yield meaningful information on the similarities and differences of carotenoids bound to these LH2 complexes. Here, we show that the $\Delta\mu$ values of carotenoids reveal slight but substantial differences in torsion of carotenoids bound to B800-820 type and B800-850 type LH2 complexes.

Materials and Methods

Cell growth and protein purification

Preparations of the B800-850 and B800-820 type LH2 complexes have already been reported.[27] Briefly, the cells of *Phs. molischianum* DSM120 (DSMZ, Germany) were grown anaerobically in DSM 27 medium at 25°C. The irradiation conditions were carefully adjusted for selective expression of B800-850 type LH2 or B800-820 type LH2 antenna, using 400 mL flat-sided glass bottles with continuous stirring, with high light (HL) ($>25 \text{ Wm}^{-2}$) or low light (LL) ($<1.5 \text{ Wm}^{-2}$) irradiation to favor B800-850 type LH2 or B800-820 type LH2, respectively. Photosynthetic membranes were purified from HL or LL cells by 20/40% stepwise sucrose density gradient centrifugation (158,000 g, 4 hrs, 4°C) after passing through French press (18k psi). Solubilization was performed for the membranes (maximum absorbance of 60 cm^{-1}) by 1% LDAO in buffer A (50 mM Tris-HCl, 1 mM EDTA, pH 7.8). Following

centrifugation (158,000 g, 1 hr, 4°C) the complexes in the supernatant were purified using a 15/20/25/30/35/40% stepwise sucrose density gradient in buffer A containing 0.1% LDAO, centrifuged for 100,000 g for 40 hrs at 4°C. Bands containing LH complexes were collected and applied to an ion-exchange column (DEAE Sepharose, Sigma) with a linear gradient elution from 250 mM to 500 mM NaCl solution that contains 1 mM EDTA, 20 mM HEPES, 0.1% LDAO, pH 7.8 (buffer C). For B800-820 type LH2, a second ion exchange column with lower pH was performed. Both B800-850 type LH2 and B800-820 type LH2 were further purified using a gel filtration column (Superdex 200, GE Healthcare Bio-Sciences). The sample solution was diluted with buffer D (20 mM Tris-HCl pH 8.0 containing 0.03% DDM (*n*-dodecyl- β -maltoside)) followed by ultrafiltration (Amicon Ultra-4, 50 kD, Millipore, MA, USA). The procedure was repeated several times to exclude HEPES thoroughly. This is because that the contamination of HEPES harms the Stark absorption measurement in polyvinyl alcohol (PVA) films.

Spectroscopic measurements

50 mg of poly vinyl alcohol (PVA) was dissolved in 1 ml of the sample solution (absorbance at 800 nm was adjusted to be 20) in buffer D. Then the sample/PVA solution was cast to form a film on top of the gold electrodes of a sample cell for spectroscopic measurements. Steady-state absorption and Stark absorption spectra were recorded using a home-built setup as previously described.[28] For the measurements of steady-state absorption spectra, a xenon lamp (150W, L2274, Hamamatsu) was replaced by a tungsten-halogen lamp (55W, H3-12V55W, Phoenix) for the white continuum. Output from the monochromator (SpectraPro 150, Acton Research) was passing through a quartz polarizer (Gran-Thomson prism), to achieve linear polarization. The angle χ between the externally applied electric field and the linear polarization light was fixed to the magic angle (54.7°). The sample cell was mounted in a cryostat (Optistat-DN, Oxford Instruments, UK) and the temperature was set to 77 K. A sinusoidal electric field of approximately 40 kV/cm with a frequency $f = 500$ Hz was applied to the electrodes of the sample cell. The intensity of the light transmitted through the sample cell was detected by a silicon photodiode (Hamamatsu, S1336-8BQ). The AC component of the signal selectively amplified the second harmonics of f using a dual phase lock-in amplifier (NF, 5610B). The DC component was detected with a digital multimeter (Fluke, 45). Both the DC and AC components

were recorded simultaneously and converted to the electric field-induced difference absorbance, ΔA and to the absorbance A . Reference signals to calculate A were recorded with a Stark cell without samples.

Analysis of Stark absorption spectra

A Stark absorption spectrum, $\Delta A(\nu)$ of the samples immobilized isotropically in PVA films can be described as a linear combination of zero-th, first and second order derivatives of the original absorption spectrum, $A(\nu)$: [29]

$$\Delta A(\nu) = \left[A_\chi \cdot A(\nu) + B_\chi \cdot \frac{\nu}{15hc} \frac{d\left(A(\nu)/\nu\right)}{d\nu} + C_\chi \cdot \frac{\nu}{30h^2c^2} \cdot \frac{d^2\left(A(\nu)/\nu\right)}{d\nu^2} \right] |f \cdot \mathbf{E}_{ext}|^2 \quad (1)$$

Here ν is the frequency of incident light, f is the correcting factor against the local electric field, and $\mathbf{E}_{ext}$ is the magnitude of the externally applied electric field. When χ is 54.7° , the coefficients B_χ and C_χ can be simply written by $\Delta\alpha$ (the difference in polarizability between the ground and excited states upon photoexcitation) and $\Delta\mu$ (the difference in the static dipole moment between the ground and excited states upon photoexcitation), respectively, as follows:

$$B_\chi \approx \frac{2}{5} \text{Tr}(\Delta\alpha) \quad (2)$$

$$C_\chi = 5|\Delta\mu|^2 \quad (3)$$

Therefore, $\text{Tr}(\Delta\alpha)$ and $|\Delta\mu|$ can be determined from the coefficients B_χ and C_χ after the least-squares fitting of Eq. (1) to the experimentally observed Stark absorption spectrum.

Structure Modeling.

The amino acid sequences of the LH2 protein molecules in *Phs. molischianum* DSM120 were predicted by the analysis of the genomic DNA sequence of this bacterium[27] as shown in Table 1. The three-dimensional structures of the LH2 polypeptides were predicted using I-TASSER (threading/assembly/ refinement),[30] which is available at <http://zhang.bioinformatics.ku.edu/I-TASSER>. The predicted structures were further optimized using the UCSF Chimera program and the MatchMaker tool[31] on the basis of the X-ray structure of the LH2 from *Phs. molischianum* strain DSM119 (PDB/1LGH). The predicted protein structures were superimposed on the crystallographically determined LH2 structure by first creating pairwise sequence alignments, then fitting the aligned residue pairs.

Pigment analysis

Carotenoids and Bchls were extracted from the B800-820 type and B800-850 type LH2 complexes by a method similar to that described in the literature.[32] The sample solution was injected onto a syringe filter (PTFE 13 nm diameter, 0.2 mm pore size, Whatman), for adsorption of the pigment-protein complexes. The filter was dried under N₂ gas flow and bacteriochlorophyll component bound to the LH2 complexes were extracted with methanol. Carotenoids were then extracted from the resultant filter with acetone and injected into an HPLC system (LC 10Av and SPD M20A, Shimadzu, Japan) equipped with a silica-gel column (5SL-II, Nacalai tesque Inc., Japan) by an isocratic elution of 12.0% tetrahydrofuran (THF) in *n*-hexane. Peaks were assigned by comparing the elution time and the absorption spectra at each peak position with references.[33,34]

Results

Analysis of pigment composition

Figure 1(a) and 1(b) show the HPLC elution profiles of the carotenoids from B800-820 and B800-850 type LH2 complexes from *Phs. molischianum* strain DSM120, respectively. Peak 1 (1') corresponds to all-*trans* lycopene, while Peak 2 (2') to all-*trans* rhodopin. Minor peaks are due to *cis* isomers of lycopene and rhodopin. Figure 1(c) shows the absorption spectra of all-*trans* lycopene and all-*trans* rhodopin detected with a PDA detector of the HPLC system. Since both lycopene and rhodopin have the same number of conjugated C=C bonds ($n=11$), these two carotenoids have very similar spectral features. The compositions of the carotenoids bound to the LH2 complexes were determined using the area under the peaks of HPLC elution profiles. The B800-820 type LH2 contains 77% rhodopin and 9% lycopene, while B800-850 type LH2 contains 81% rhodopin and 12% lycopene.

Spectroscopic measurements

The room temperature absorption spectra of the B800-820 (B800-850) type LH2 are displayed in Figure 2. The 370 nm and 590 nm bands correspond to the Soret and Q_x transitions of BChl *a*, respectively. The absorption maxima at 799 nm and 821 nm (799 nm and 847 nm) for B800-820 (B800-850) type LH2 are associated with the BChl *a* Q_y transition. The absorption bands in the 400-540 nm regions arise from the S_0 - S_2 transition of the carotenoids (~80% rhodopin and ~10% lycopene). The 77 K S_0 - S_2 absorption spectra of the carotenoids bound to B800-820 (B800-850) type LH2 dispersed in PVA (Figure 3) show the typical vibrational structure of carotenoids, assigned to the 0-0, 0-1 and 0-2 transition bands. This vibrational structure arises from the C-C and C=C stretching modes in the S_2 state. The frequencies of C-C and C=C stretching modes in the ground (S_0) state are 1155 cm^{-1} and 1513 cm^{-1} for lycopene in *n*-hexane;[35] essentially similar frequencies of the C-C and C=C modes can be expected for rhodopin with the same number of C=C bonds. If we assume the harmonic approximation for the potential energy surface of both the S_0 and S_2 states, similar vibrational progressions can be justified for both S_0 and S_2 states.[36] The plateau shape of the 0-1 absorption band around 20000 cm^{-1} shown in the top panel of Figure 3(a), 3(b) hence indicates the degeneracy of the C-C and C=C vibrational modes, which can be identified separately at low temperature.

Figure 3 also shows the results of Stark absorption measurements. The Stark absorption spectrum can usually be reproduced by the spectral fitting using zero-th, first, and second order derivative waveforms of the absorption spectrum. Figure 3 shows the results of performing such a spectral fitting, which allow determination of the nonlinear optical parameters ($\text{Tr}(\Delta\alpha)$ and $|\Delta\mu|$) of carotenoids bound to B800-820 and B800-850 complexes. The results of spectral fitting, summarized in Table 2, show that there is a deviation in the 0-1 absorption band region, which is particularly pronounced in the case of the B800-820 complex. This discrepancy may be due to different $\text{Tr}(\Delta\alpha)$ and $|\Delta\mu|$ values for each vibrational structure of the absorption band. In order to eliminate this ambiguity and to improve the quality of our spectral fits, we convoluted the absorption spectra of the carotenoid region with several Gaussian profiles that correspond to the vibrational progression of the C-C and C=C stretching modes.[37] The 0-0 bands were fitted with one Gaussian sub-band (g1), whereas two Gaussian sub-bands that correspond to C-C and C=C stretchings (g2 and g3, respectively) were used to fit the 0-1 bands, and overtones and combination tones of these vibrational modes were used to fit the 0-2 and 0-3 bands. The Stark absorption spectra were satisfactorily reproduced by the fitting using the zero-th, first and second order derivatives of these Gaussian sub-bands, and the results are shown in Figure 4. The fitting parameters of Eq. (1) were determined for each Gaussian sub-band independently. Table 2 shows these parameters for only the Gaussian sub-band (g1), since this particular band is unaffected by vibronic interactions and its purely electronic character can be discussed.

Discussion

Analysis of the Stark absorption spectra using whole absorption spectra

The Stark absorption spectra of carotenoids bound to the B800-820 and B800-850 type LH2 complexes isolated from *Phs. molischianum* strain DSM120 were fitted with the combination of the zero-th, first, and second order derivative waveforms of the absorption spectra (see Figure 3). The results are satisfactory, although the perfect fits can be achieved by further analysis by spectral deconvolution of the absorption spectra, which is generally accepted to provide a qualitative interpretation of Stark absorption spectra.[38] In this section we discuss the meaning of the nonlinear optical parameters determined by this rather classical treatment (see Table 2).

Both the B800-820 and B800-850 complexes show similar $\text{Tr}(\Delta\alpha)$ values around 600 [$\text{\AA}^3/f^2$], similar to that of the LH2 complex from *Rba. sphaeroides*. [39] On the other hand, the $|\Delta\mu|$ values differ: the B800-820 type LH2 shows 5.5 ± 1.2 [D/f], while the B800-850 type LH2 shows 3.4 ± 1.9 [D/f]. Below we discuss the origin of the larger $|\Delta\mu|$ value for the B800-820 complex.

The $\Delta\mu$ values of carotenoids are mainly caused by the contribution of the induced dipole moment ($\Delta\mu_{\text{ind}}$) because of the symmetrical structure of the π -conjugated system. [38,40] However, it has been shown that there is an additional small contribution of the intrinsic dipole moment ($\Delta\mu_i$) caused by structural deformation as well as accidental degeneracy of the electronic states. [28] In fact we have already demonstrated that the $\Delta\mu$ values of carotenoids bound to pigment-protein complexes can be explained by the combination of $\Delta\mu_{\text{ind}}$ and $\Delta\mu_i$ as shown in Eq. (4). [26]

$$\Delta\mu = \Delta\mu_{\text{ind}} + \Delta\mu_i = \Delta\alpha \cdot E_p + \Delta\mu_i \quad (4)$$

Here, E_p represents the local electric field surrounding the carotenoid molecule. Therefore, the $\Delta\mu_{\text{ind}}$ values reflect the effect caused by the electrostatic environment surrounding carotenoids, and the $\Delta\mu_i$ values reflect the structural deformation of carotenoids. [26]

Since both the B800-820 and B800-850 complexes show the similar $\text{Tr}(\Delta\alpha)$ values, then according to the above theoretical considerations it can be concluded that the 2 [D/f] difference in $\Delta\mu$ is solely due to the difference of the $\Delta\mu_i$ values, indicating that interactions between carotenoids and the surrounding protein environment causes severe structural deformation of carotenoids. However, before proceeding with more discussion, we attempt a more sophisticated analysis of our Stark spectra, since perfect fitting of the Stark spectra have not been achieved by classical treatment.

Analysis of the Stark absorption spectra using Gaussian profiles

As shown in Figure 4, Stark absorption spectra were perfectly fitted with the derivative waveforms of Gaussian sub-bands that were obtained by spectral convolution of absorption spectra. This treatment basically follows quantum mechanical rules that govern the shape of the absorption spectra. By focusing on the g1 (0-0) sub-band, we can eliminate the vibronic effect and can discuss the purely electronic character of the transition. The nonlinear optical parameters ($\text{Tr}(\Delta\alpha)$ and $|\Delta\mu|$) for the g1 sub-band

were determined and are shown in Table 2. It is interesting to note that the B800-820 complex shows a smaller $\text{Tr}(\Delta\alpha)$ than B800-850 complex, but a larger $|\Delta\mu|$ value. According to the discussion in the previous section, a smaller $\text{Tr}(\Delta\alpha)$ corresponds to the induction of smaller $\Delta\mu_{\text{ind}}$ value, if both the B800-820 and B800-850 complexes produce similar electrostatic field around carotenoids. In the subsequent section, we discuss the origin of the ~ 2 [D/f] larger value of $|\Delta\mu|$ for the B800-820 complex.

Structure modeling of the LH2 complexes and the prediction of structural deformation of rhodopin

Table 1 compares the amino acid sequences of α - and β -polypeptides of the B800-820 and B800-850 type LH2 complexes. Major differences were found for the α -polypeptide: (Asn, Ala)₃, (Pro, Leu)₄, (Tyr, His)₈, (Leu, Ile)₁₄, (Val, Ala)₄₁, (Lys, Gln)₅₃, (Val, Asn)₅₆. For the β -polypeptides: (Phe, Val)₁₈, (His, Asn)₁₉. In order to predict how carotenoid molecules can interact with these amino acid residues, modeled structures were constructed for both B800-820 and B800-850 type LH2 complexes from *Phs. molischianum* DSM120 *in silico* with reference to the structure of the LH2 complex from *Phs. molischianum* DSM119 whose structure has already been determined by X-ray crystallography.[6] Lycopenes in the DMS119 structure were swapped, which places the hydroxyl group of rhodopin adjacent to the B850 bacteriochlorophyll *a* dimers, according to Nakagawa *et al.*[26] Figure 5 displays the rhodopin molecule modeled into both the B800-820 and B800-850 type LH2 complexes together with nearby amino acids. Close inspection of these models suggests a different environment for the cytoplasmic half of the rhodopin molecule, particularly around the 18th amino acid of the β -polypeptides. The β_2 -polypeptide of B800-820 complex possesses aromatic Phe₁₈, while both β_1 - and β_3 -polypeptides possess aliphatic Val₁₈. This small but distinct difference may induce the steric hindrance of Phe₁₈ around C21-C24 region of the bound carotenoid, rhodopin (see Figure 6 for the numbering of carbon atoms), and may cause the twisting and/or bending of the bound carotenoid around this region.

Molecular orbital calculations and the prediction of $|\Delta\mu|$ values of various conformers of rhodopin molecule

The intrinsic $|\Delta\mu|$ of carotenoids can be generated by their asymmetric deformation. According to the discussion based on the modeled structures shown above, the major structural deformation between rhodopin and the surrounding protein is suggested to localize around the C21-C24 region of the rhodopin rather than the periplasmic part of the molecule with the hydroxyl group. Since it has already been shown that the $|\Delta\mu|$ values of carotenoids can be predicted satisfactorily using semi-empirical molecular orbital calculations,[26] we have adopted a similar protocol to estimate the possible twisting of the rhodopin molecule to produce the 2 [D/f] difference of the $|\Delta\mu|$ values between B800-820 and B800-850 LH2 complexes. Since there are so many degrees of freedom of rotation around the C-C and/or C=C bonds of rhodopin, we have mainly focused our attention on the C17-C26 region. The simulations of $|\Delta\mu|$ are shown in Figure 6. The most stable (no-twisting) structure of the rhodopin was predicted to be at a $|\Delta\mu|$ value of 0.2 [D].

In order to fulfill the requirement that rhodopin is bound to the LH2 complexes with *trans*-conformation and at the same time induces the 2 [D/f] difference of the $|\Delta\mu|$ value by twisting, neighboring C-C and C=C bonds were simultaneously rotated. Figure 6 shows all the results of this simultaneous rotation; deviations with a value of 2 [D] are in yellow-green. In contrast to the single C=C bond rotation, a deviation of 2[D] can be induced by simultaneously rotating neighboring C-C and C=C bonds. This modeling predicts structural deformation localizes to the C21-C24 region of rhodopsin. One of the possible twisted structures that can produce a $|\Delta\mu|$ value of 2 [D], which is predicted to be (C20-C21=C22-C23, C21=C22-C23=C24) = (70°, 110°). In this case the *cis*-conformer like structure induced by the rotation around the C21=C22 bond is relaxed by the simultaneous rotation around the neighboring C22-C23 bond, and hence can be accommodated into the binding site of the LH2 complex.

Conclusion

Stark absorption spectroscopy was applied to the carotenoid, rhodopin, bound to the B800-820 and B800-850 complexes from *Phs. molischianum* DSM119. Both classical and semi-quantum mechanical analyses of the observed Stark absorption spectra showed that $|\Delta\mu|$ was larger by a value of (2 [D/f]) for rhodopin bound to the B800-820 LH2 complex, compared with the B800-850 LH2. Although it is still rather speculative, the origin of this severe difference was discussed based on the modeled structure of the LH2 complexes as well as the results of semi-empirical molecular

calculations. It was suggested that simultaneous rotation around neighboring C=C and C-C bonds might be required to explain the induction of this large deviation of the $|\Delta\mu|$ value when rhodopin is embedded in protein environment. The plausible position of the rotation is postulated to be located around C21-C24 bonds of rhodopin. Future experimental work will be needed to test the validity of this hypothesis. However, Stark absorption spectroscopy presented here for sure highlight the possibility to be able to discuss the subtle change of molecular structures of carotenoids bound to the photosynthetic light-harvesting complexes.

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Table 1. Amino-acid sequences of α - and β -polypeptides of the LH2 protein from *Phs. molischianum* DSM120 that were deduced from the genomic analysis. Shadowed amino-acids correspond to those apparent difference was found between B800-820 and B800-850 LH2 proteins. Underlined amino-acids are those exist near-by a carotenoid molecule. There are two different genomic codes that produce β -polypeptides of B800-850 LH2 proteins (β_1 and β_3).

	1	11	21	31	41	51					
B800-850 (α_1)	MSAL	KDDHKI	WLVI	NPSTWLP	VIWVVAVVVA	ISVHA	AVLA	APGF	NWIALGAA	QSANK	
B800-820 (α_2)	MSN	PKDDYK	IWLVL	NPSTWLP	VIWVVAVVVA	ISVHA	AVLA	VPGF	NWIALGAAK	SAVK	
	1	11	21	31	41						
B800-850 (β_1)	.MAE	RSLSGL	TVEE	A	AVEVNE	QFKTTFS	AFLL	IAA	VAHVLV	VWVKPWF	
(β_3)	.MAE	RSLSGL	T	EEEE	A	VEVND	QFKTTFS	AFLL	IAA	VAHVLV	VWVKPWF
B800-820 (β_2)	MAE	KRSLSGL	T	EEEE	A	VEFHD	QFKTTFS	AFLL	IAA	VAHVLV	VWVKPWF

Table2. Nonlinear optical parameters of carotenoids bound to the B800-820 and B800-850 LH2 complexes from *Phs. molischianum* DSM120.

	B800-820		B800-850	
	Tr($\Delta\alpha$) [$\text{\AA}^3/\text{f}^2$]	$ \Delta\mu $ [D/f]	Tr($\Delta\alpha$) [$\text{\AA}^3/\text{f}^2$]	$ \Delta\mu $ [D/f]
Whole carotenoid region	600 $\pm$ 30	5.5 $\pm$ 1.2	590 $\pm$ 20	3.4 $\pm$ 0.9
g1	350 $\pm$ 20	5.9 $\pm$ 1.2	470 $\pm$ 40	3.8 $\pm$ 1.5

Figure Captions

Figure 1. HPLC elution profiles of pigments extracted from (a) B800-820 and (b) B800-850 type LH2 complexes from *Phs. molischianum* DSM120. Peak 1 (1') and Peak 2 (2') are assigned to lycopene and rhodopin, respectively. Absorption Spectra of lycopene and rhodopin recorded by a PDA detector of the HPLC system are shown in (c). Chemical structures of lycopene and rhodopin are displayed on top panel.

Figure 2. Absorption spectra of (a) B800-820 and (b) B800-850 complexes isolated from *Phs. molischianum* DSM120 in Tris-HCl (pH=8.0) buffer solution at room temperature.

Figure 3. Absorption spectra and their first and second derivative waveforms as well as Stark absorption spectra (open circles) of (a) B800-820 and (b) B800-850 LH2 complexes from *Phs. molischianum* DSM120 in PVA polymer film at room temperature. Red solid lines show the results of the spectral fitting based on the classical method using whole range of absorption spectra and their derivative waveforms.

Figure 4. The results of the spectral deconvolution of the absorption spectra (top panels) using Gaussian profiles based on a quantum mechanical model of (a) B800-820 and (b) B800-850 LH2 complexes from *Phs. molischianum* DSM120 in PVA polymer film at room temperature. The first and second derivative waveforms of each of the Gaussian profiles are shown in middle panels. The bottom panels show the results of the spectral fitting (red solid lines) for the Stark absorption spectra (open circles) using the combination of the zero-th, first, and second order derivative waveforms of the Gaussian profiles.

Figure 5. Modeled structures of rhodopin bound to the B800-820 and B800-850 LH2 complexes from *Phs. molischianum* DSM120. The structures of surrounding amino-acids are also shown in the figure.

Figure 6. Theoretically predicted $|\Delta\mu|$ values of rhodopin when rotating around the neighboring C=C and C-C bonds simultaneously.

Figure 1. (a) A schematic illustration of the set-up for Stark absorption measurements and (b) the structure of a sample cell. Direction of externally applied electric field and the polarization of light are shown with arrows.

Figure 2. HPLC elution profiles of pigments extracted from (a) B800-820 and (b) B800-850 type LH2 complexes from *Phs. molischianum* DSM120. Peak 1 (1') and Peak 2 (2') are assigned to lycopene and rhodopin, respectively. Absorption Spectra of lycopene and rhodopin recorded by a PDA detector of the HPLC system are shown in (c). Chemical structures of lycopene and rhodopin are displayed on top panel.

Figure 3. Absorption spectra of (a) B800-820 and (b) B800-850 type LH2 complexes isolated from *Phs. molischianum* DSM120 in Tris-HCl (pH=8.0) buffer solution at room temperature (top), in poly vinyl alcohol (PVA) at room temperature (middle), and in PVA at 77 K (bottom).

Figure 4. Normalized absorption spectra (top panels) and Stark absorption spectra (bottom panels, open circles) of (a) B800-820 and (b) B800-850 LH2 complexes from *Phs. molischianum* DSM120 in PVA polymer film at 77K. The magnitude of Stark spectra was normalized against the concentration of the samples as well as the intensity of applied electric field (48 kV/cm for B800-820 and 30 kV/cm for B800-850 complexes). The thickness of the film of B800-820 sample was estimated to be 6.4 μm and that of B800-850 sample to be 9.6 μm . Red solid lines in the bottom panels show the results of the spectral fitting based on the classical method using whole range of absorption spectra and their derivative waveforms. The second and third panels show how the first and second derivative components of the absorption spectra contribute to reproduce the Stark absorption spectra. Here, B is $(B_{\chi} \nu d(A(\nu)/\nu))/(15hc \cdot d\nu)$ and C is $(C_{\chi} \nu d^2(A(\nu)/\nu))/(30h^2c^2 \cdot d\nu^2)$.

Figure 5. The results of the spectral deconvolution of the absorption spectra (top panels) using Gaussian profiles based on a quantum mechanical model of (a) B800-820 and (b) B800-850 LH2 complexes from *Phs. molischianum* DSM120 in PVA polymer film at 77 K. The bottom panels show the results of the spectral fitting (red solid lines) for the Stark absorption spectra (open circles) using the combination of the zero-th, first, and second order derivative waveforms of the Gaussian profiles. The second and third panels show how the first and second derivative waveforms of the g1, g2 and g3 Gaussian profiles contribute to reproduce the Stark absorption spectra. Here, B is $(B_{\chi} \cdot \nu d(A(\nu)/\nu))/(15hc \cdot d\nu)$ and C is $(C_{\chi} \cdot \nu \cdot d^2(A(\nu)/\nu))/(30h^2c^2 \cdot d\nu^2)$.

Figure 6. Modeled structures of rhodopin bound to the B800-820 and B800-850 LH2 complexes from *Phs. molischianum* DSM120. The structures of surrounding amino-acids are also shown in the figure.

Figure 7. Theoretically predicted $|\Delta\mu|$ values of rhodopsin when rotating around the neighboring C=C and C-C bonds simultaneously.

Graphical Abstract