Characterization of the Light-Harvesting Antennas of Photosynthetic Purple Bacteria by Stark Spectroscopy. 2. LH2 Complexes: Influence of the Protein Environment

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We have performed low-temperature Stark spectroscopy on a variety of different LH2 complexes from four photosynthetic bacteria, with the aim of characterizing the electric field response of the B800 and B850 absorption properties as a function of the protein environment. The following LH2 complexes were investigated: B800-850 and B800-820 of *Rhodopseudomonas (Rps) acidophila*; B800-850, B800-840 (αTyr_{+13}→Phe), and B800-826 (αTyr_{+13}→Phe, αTyr_{+14}→Leu) of *Rhodobacter (Rb.) sphaeroides*; B800-850 and B800-830 (obtained at high LDAO) of *Ectothiorhodospira sp.*; and B800-850 of *Rhodospirillum (Rsp.) molischianum*. For all these cases the spectral blue shift of B850 has been assigned to the loss hydrogen-bonding interaction with the acetyl carbonyl of bacteriochlorophyll *a*. $|\Delta\boldsymbol{\mu}|$ values for the 850 nm bands as well as for the blue-shifted bands are all on the order of 3−4.5 D/*f*. The loss of hydrogen-bonding interactions has only small effects on $|\Delta\boldsymbol{\mu}|$ in these complexes. The values of the difference polarizability, Tr($\Delta\boldsymbol{\alpha}$), are large (600−1400 Å^3/*f*^{2}). The results are discussed in terms of crystal-structure-based models for LH2, in which pigment−pigment and pigment−protein interactions are considered; strong pigment−pigment interactions were found to be especially important. The values of $|\Delta\boldsymbol{\mu}|$ for the 800 nm band are small, 1.0−1.5 D/*f* for LH2 complexes from *Rb. sphaeroides* and *Rps. acidophila*. However, in *Rsp. molischianum* and *Ectothiorhodospira sp.* $|\Delta\boldsymbol{\mu}|$ values are much larger, of the order of 3 D/*f*. The difference in the B800 band is assigned to the difference in orientation of the B800 pigments in *Rsp. molischianum* and *Ectothiorhodospira sp.*, as compared to the *Rps. acidophila* and *Rb. sphaeroides*. Due to the difference in orientation, the interactions of the Bchl *a* with the surrounding protein and neighboring carotenoid pigments are also not identical.

Introduction

The photosynthetic light-harvesting antenna absorbs light energy and transfers the excitation energy to the photosynthetic reaction center (RC). In general, two types of antenna systems are found in photosynthetic purple bacteria: a core antenna complex, LH1, with a near-infrared (NIR) absorption band which at room temperature (RT) absorbs at 875 nm, B875, and a peripheral antenna complex, LH2, which is characterized by two NIR absorption bands, typically at 800 and 850 nm at RT, B800-850.[1,2] The antenna species most intimately connected to the RC is lowest in energy, and therefore the antenna funnels the excitations toward the RC where eventually the energy is trapped and the electron transfer reaction is triggered .[3]

It was found that the basic unit of LH1 and LH2 consists of a pair of small apo-proteins, α and β, each with a single membrane spanning α-helix.[1,2] In LH2 each $\alpha\beta$-pair (or $\alpha\beta$-subunit) binds three bacteriochlorophyll *a* (Bchl *a*) and 1−2 carotenoids. Recently, the high-resolution structures were published of LH2 complex from *Rhodopseudomonas (Rps.) acidophila*[4,5] and *Rhodospirillum (Rsp.) molischianum*.[6] In both cases LH2 is composed of a closely packed ring of a number of $\alpha\beta$-subunits, nine for *Rps. acidophila*, $(\alpha\beta)_9$, and eight for *Rsp. molischianum*, $(\alpha\beta)_8$. In the structures, the positions of the three Bchl *a* molecules and one carotenoid were revealed per $\alpha\beta$-subunit, while in the *Rps. acidophila* structure there is evidence for a second carotenoid.[5]

In *Rps. acidophila* the Bchl *a* molecules are arranged in two rings, one of 18 Bchl *a* molecules, with their porphyrin macrocycles approximately perpendicular to the membrane plane and with the Q_y transition making only a small angle with the plane of the membrane.[5] The second ring consists of nine Bchl *a* molecules which are oriented in the plane of the membrane. Combining structure and polarized light spectroscopy,[7,8] the 850 nm absorption band is assigned to the ring of 18 Bchl's and the 800 nm band to the ring of nine Bchl's. In the following we will refer to the ring of B850 molecules as "B850" and to the ring of B800 molecules as "B800". The 18 Bchl's are close together, with the Mg−Mg distance between two Bchl's within an $\alpha\beta$-protomer, about similar to that between neighboring Bchl's on adjacent subunits, about 9 Å.[4,5] The π-electron systems of the Bchl *a* molecules are close together and are possibly overlapping both at rings 1 and 3 of the Bchl *a* molecules.[5] Furthermore, for the Bchl *a* molecules within an $\alpha\beta$-subunit the electron density in the crystal structure was found to be continuous, and this is not the case for the electron densities of neighboring Bchl's on adjacent subunits.[4]

Since distances are small, interactions between pigments are large. This was earlier concluded from circular dichroism (CD) measurements, where a large doublet signal is observed around 850 nm, indicating strong excitonic interactions.[9−11] The CD

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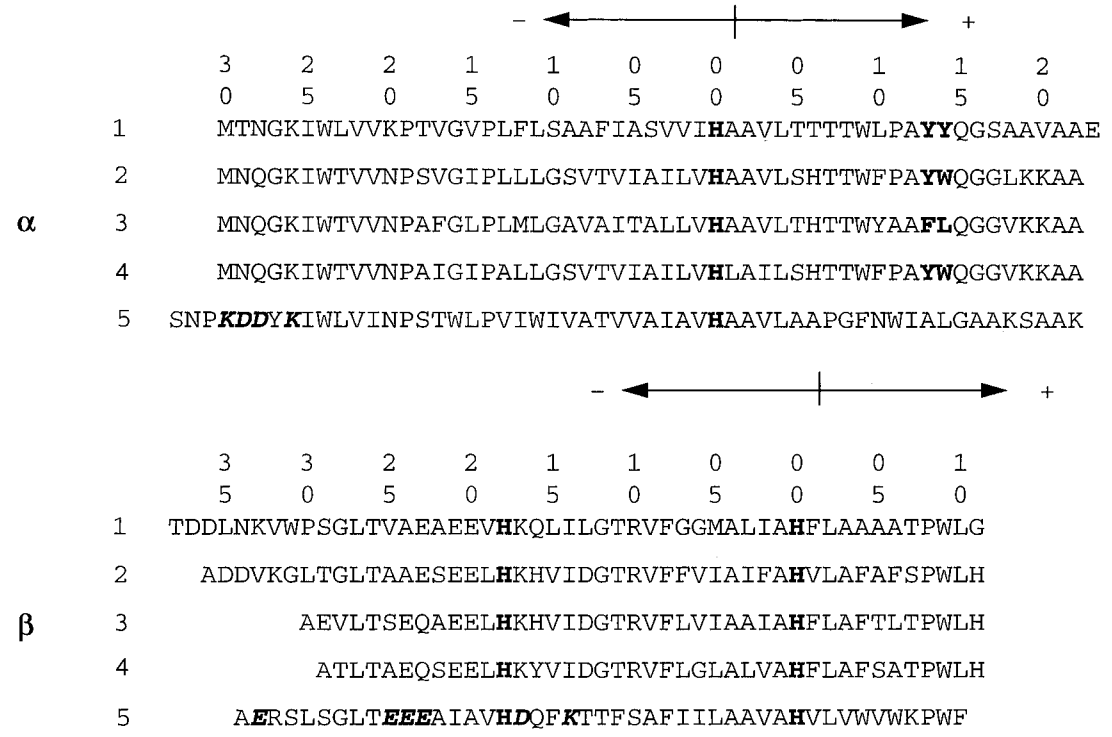

Figure 1. Amino acid sequences of the LH2 complexes.[2] 1α and *β Rb. sphaeroides* strain 2.4.1 B800-850 complex. 2α and *β Rps. acidophila* strain 7050, B800-850. 3α and *β Rps. acidophila* strain 7050, B800-820. 4α and *β Rps. acidophila* strain 10050: B800-850. 5α and *β Rsp. molischianum*.[6] The sequences are aligned to their conserved histidine (H) given in bold characters, residues which ligate the 850 Bchl *a* molecules. Note the YY (Tyr, Tyr) sequence in the α-subunit of the *Rb. sphaeroides* (1α+13 and +14), this is YW (Tyr, Trp) in both *Rps. acidophila* B800-850 strains. In *Rps. acidophila* B800-820 complex the sequence is changed to FL. Although the sequences of *Rsp. molischianum* is aligned to the ligating H residues, the numbering of the amino acid residues is adjusted to numbering as used in ref 6 to avoid confusion. In the *Rsp. molischianum* sequences the charged residues are given in bold/italic.

signal at 800 nm in *Rps. acidophila* and *Rhodobacter (Rb.) sphaeroides* is much less intense, which can be equally well explained from the structure, since these pigments are much further (∼20 Å) apart and furthermore, the CD is expected to be small due to the in plane orientation.[12,13] The similarity between LH2 of *Rb. sphaeroides* and *Rps. acidophila* both spectrally[1,7,11,14] and chemically[1,2] is most striking and suggests that *Rb. sphaeroides* LH2 has a comparable structure as reported in refs 4 and 5. However, electron microscopy on *Rb. sphaeroides* LH2 complexes suggests a hexameric structure indicating a $(\alpha\beta)_6$ organization.[15]

Rsp. molischianum LH2 is built up of eight $\alpha\beta$-subunits and has a similar overall arrangement of the pigments as in *Rps. acidophila*.[6] The only apparent difference is found in the orientation of the B800 pigments. Compared to the arrangement in *Rps. acidophila*, the B800 pigments are rotated over about 90° in the plane of the porphyrin ring, which is tilted to make a 38° angle with the membrane plane.[6] This results in a ∼10° out-of-plane orientation of the Q_y transition dipoles and a ∼30° of plane orientation of the Q_x transition dipoles of the B800's. These observations are in agreement with earlier linear dichroism (LD) measurements on the *Rsp. molischianum* B800-850 complexes.[16] CD measurements on *Rsp. molischianum* B800 have shown a large conservative signal.[16] The change in orientation of the Q_y and Q_x transition dipoles may explain this observation.[12]

In some purple bacteria apart form the B800-850 complex, a B800-820 complex is also found,[1,2] among others, in *Rps. acidophila*. Polarized spectroscopic features of the B850 and B820 bands are very similar as is the case for B800 in both species,[14] and therefore the overall structure of B800-820 is assumed to be closely related to the B800-850 structure. The protein sequences of the two LH2 types are also similar; however, there is a remarkable difference in the sequences of B800-850 of both *Rb. sphaeroides* and *Rps. acidophila* on one side and B800-820 of *Rps. acidophila* on the other. In B800-850 the two residues found at positions +13 and +14 on the α-polypeptide are two tyrosines, Tyr, Tyr (YY), for *Rb. sphaeroides* and a tyrosine tryptophan, Tyr Trp (YW), pair for *Rps. acidophila* (sequences given in Figure 1). Both of these amino acids are capable of forming hydrogen bonds. Hydrogen-bonding residues at these positions are often found in B800-850 of purple bacteria,[2] however not in *Rsp. molischianum*. In B800-820 of *Rps. acidophila* the Tyr and Trp, YW, were found to be replaced by respectively a phenylalanine (Phe, F) and a leucine (Leu, L), FL (Figure 1). These residues are not capable of forming hydrogen bonds. It was proposed that hydrogen bonds are responsible for the red shift of the B820 band to 850 nm in LH2. To check this hypothesis, a series of *Rb. sphaeroides* LH2 mutants were constructed in which the residues at α+13 and α+14 were altered.[11] It was indeed found that the B850 band in the mutants shifts to the blue, in analogy to B800-820 of *Rps. acidophila*. At 77 K B850 is found to shift from 856 nm for wild-type to 840 nm in the $\alpha Y_{+13}\rightarrow$F, FY, and to 824 nm in the $\alpha Y_{+13}\rightarrow$F and $\alpha Y_{+14}\rightarrow$L, FL, mutant.[11] Fourier transform (FT) and resonance Raman measurements showed that one and two hydrogen bonds were lost to C_2-acetyls of B850 in the FY and FL mutants, respectively.[12] FT-Raman measurements on *Rsp. acidophila* B800-850 and B800-820 confirm the absence of two hydrogen bonds to C_2-acetyls in the latter species and their presence in the former complex.[18] The energy transfer characteristics of the mutants are similar to wild-type (WT) as is concluded from fluorescence excitation,[11] picosecond pump−probe measurements,[19] and hole-burning spectroscopy.[20,21] The energy transfer rate from B800 to B850 was even found to increase in the blue-shifted mutants

due to the larger spectral overlap of the 800 nm emission and the wings of the 840 and 824 nm absorption bands of the FY and FL mutants, respectively, in comparison with the 856 nm band in WT.[19−21]

In LH2 of *Ectothiorhodospira sp.* the B850 band can be blue-shifted by increasing the detergent (LDAO) concentration.[22] FT-Raman experiments performed on these samples revealed that the detergent-induced blue shift of the absorbance band is accompanied by the loss of hydrogen-bonding interaction to C_2-acetyls of B850 molecules.[23]

Stark spectroscopy measures two molecular parameters: the difference dipole, $\Delta\boldsymbol{\mu}$, between ground and excited state and the difference polarizability, $\Delta\boldsymbol{\alpha}$.[24−27] The technique is sensitive to changes in electronic environment of the pigment molecules and the interactions between pigments.[24−31] Since hydrogen bonds affect the electronic environment, the breaking or formation of hydrogen bonds is expected to influence the Stark spectrum. An example of the strong influence that the protein environment may exert on the Stark parameters is found for the carotenoid sphaeroidene in *Rb. sphaeroides* LH2 as compared to solvents.[25,26,31] In solvents the Stark spectrum of the carotenoids is mainly due to a large $\Delta\boldsymbol{\alpha}$ whereas in LH2 the Stark effect is dominated by $\Delta\boldsymbol{\mu}$. This result was best explained by an induced dipole in the carotenoid due to an electric field arising from the protein surrounding of the pigment molecules.[25,26,31]

Apart from a protein effect on $\Delta\boldsymbol{\mu}$ and $\Delta\boldsymbol{\alpha}$, pigment-pigment interactions are also expected to influence the Stark spectra. For example, for the bacterial RC, it was observed that the low exciton state of P exhibited both a large $\Delta\boldsymbol{\mu}$ and large $\Delta\boldsymbol{\alpha}$ as compared to monomer Bchl *a*.[24,31]

Several antenna complexes have previously been characterized using Stark spectroscopy.[24,29] It was found that B850 of *Rb. sphaeroides* LH2 has reasonably large $\Delta\boldsymbol{\mu}$ and $\Delta\boldsymbol{\alpha}$ values.[24] The large $\Delta\boldsymbol{\alpha}$ in P, LH1, and LH2 is due to the mixing of an intradimer charge transfer (CT) state into the lowest exciton state;[27] the observed $\Delta\boldsymbol{\mu}$ is ascribed to a dipole induced by the electric field arising from (asymmetries in) the protein surrounding the pigments. The availability of the site-specific mutants and the species in which the B850 band is blue-shifted due to environmental effects provides a tool to further study the influence of the protein environment on the Stark parameters.

Materials and Methods

The Stark spectroscopy setup is described in detail in ref 29. For randomly oriented and fixed molecules the Stark line shape is described by a sum of zeroth, first, and second derivative of the ground-state absorption spectrum.[27,29,30] The second-derivative contribution scales with the size of the difference in permanent dipole moment, $|\Delta\boldsymbol{\mu}|$, between the excited and ground state of the molecule. The first derivative yields Tr($\Delta\boldsymbol{\alpha}$), which is a measure of the difference polarizability between the excited and ground state, $\Delta\boldsymbol{\alpha}$. The zeroth derivative is a measure of the field-dependent changes of the oscillator strength of the optical transition and will be given in A_χ. Furthermore, the dependence of the signal on the angle, χ, between the applied electric field and the polarization direction of the light gives information of the orientation of $\Delta\boldsymbol{\mu}$ and $\Delta\boldsymbol{\alpha}$ with respect to the transition dipole, **p**. More detail can be found in refs 27 and 29.

The Stark results are thus best compared to the first and second derivative of the absorbance spectrum. In a detailed analysis the Stark and absorbance spectrum are fitted simultaneously using a nonlinear least-squares fitting program. The absorption spectrum was fitted with a number of (skewed) Gaussians and the Stark spectrum with the zeroth, first, and second derivative of these Gaussians. To limit the number of free parameters and to obtain realistic values for $|\Delta\boldsymbol{\mu}|$ and Tr($\Delta\boldsymbol{\alpha}$), the (skewed) Gaussians could be linked to act as a single absorption profile (for instance, a vibronic progression). Each absorption profile built up from a few Gaussians thus yields only one set of $|\Delta\boldsymbol{\mu}|$ and Tr($\Delta\boldsymbol{\alpha}$) (see also ref 29 for more details about the analysis). The molecules are embedded in a protein environment which may influence the value of the internal electric field, $\mathbf{F_{int}} = f^*\mathbf{F_{ext}}$.[30,32] Since f is a parameter of which it is hard to give a proper estimate, all values for $|\Delta\boldsymbol{\mu}|$ and Tr($\Delta\boldsymbol{\alpha}$) will be represented in terms of D/f and Å^3/f^2, respectively (1 D = 3.34×10^{-30} C m, 1 Å^3 = 1.113×10^{-40} C m^2/V).

Stark experiments were performed on eight different LH2 complexes. The LH2 mutants from *Rb. sphaeroides* were previously described in refs 11 and 17. The membranes contained only LH2 and were concentrated by Amicon centrifugation. The *Rps. acidophila* B800-850 strain 7050 and B800-850 strain 10050 were isolated from whole cells as previously described in ref 4. The preparation of the samples of *Ectothiorhodospira sp.* was described in refs 22 and 23, and the *Rsp. molischianum* B800-850 complexes were prepared as in refs 6 and 16. All experiments were performed at 77 K to obtain proper glasses all samples were mixed in a 50/50 v/v ratio with glycerol.

Results

Rps. acidophila. We have investigated both B800-820 and B800-850 LH2 complexes from *Rps. acidophila*. In Figure 2 the absorption spectrum and first and second derivative of the absorption spectrum and Stark spectrum of both types of LH2 complexes are shown. The absorption maxima of the spectral bands are shifted at low temperature as compared to the RT value, and the positions of the absorption bands as observed in these experiments are listed in Table 1. The Stark spectrum of B800-850 of *Rps. acidophila*, Figure 2A, strongly resembles the previously published Stark spectrum of B800-850 from *Rb. sphaeroides*.[24] The main characteristics of the spectra are the large signal around the B850 band and the small signal around 800 nm. It is furthermore apparent that the Stark spectrum in Figure 2A does not compare to a pure second or first derivative, but rather to a combination of the two. Interestingly, we observe a shoulder on the red side of the Stark spectrum similar to the one observed by Gottfried et al.[24] for isolated *Rb. sphaeroides* B800-850 complexes.

A simultaneous fit of the absorption and Stark spectra was performed as described above. The absorbance spectrum was approximated using two (or three) skewed Gaussian profiles per absorption band, which were linked in fitting the Stark spectrum. The fits to the absorption and Stark spectrum for the B850 band are shown in Figure 3. The obtained values for $|\Delta\boldsymbol{\mu}|$ and Tr($\Delta\boldsymbol{\alpha}$) are listed in Table 1.

In Figure 2B, the absorbance and Stark spectra of B800-820 of *Rps. acidophila* are shown. The shape of the Stark spectrum of this complex is similar to that recorded for B800-850 with only the B850 feature shifted to 820 nm and broadened. The intensity of the B820 Stark feature is smaller than in B850, but this is mainly due to the broadening of the absorption band. The shoulder prominently present in the Stark spectrum of B850 is also broadened and is not as prominent but seems to be present on the red flank the B820 Stark signal. Analysis of these spectra yielded comparable results for $|\Delta\boldsymbol{\mu}|$ and Tr($\Delta\boldsymbol{\alpha}$) for B800-820 as compared to B800-850, and the values are listed in Table 1.

***Rhodobacter sphaeroides*: Wild-Type and Mutants.** The absorbance and Stark spectra of membrane associated *Rb.*

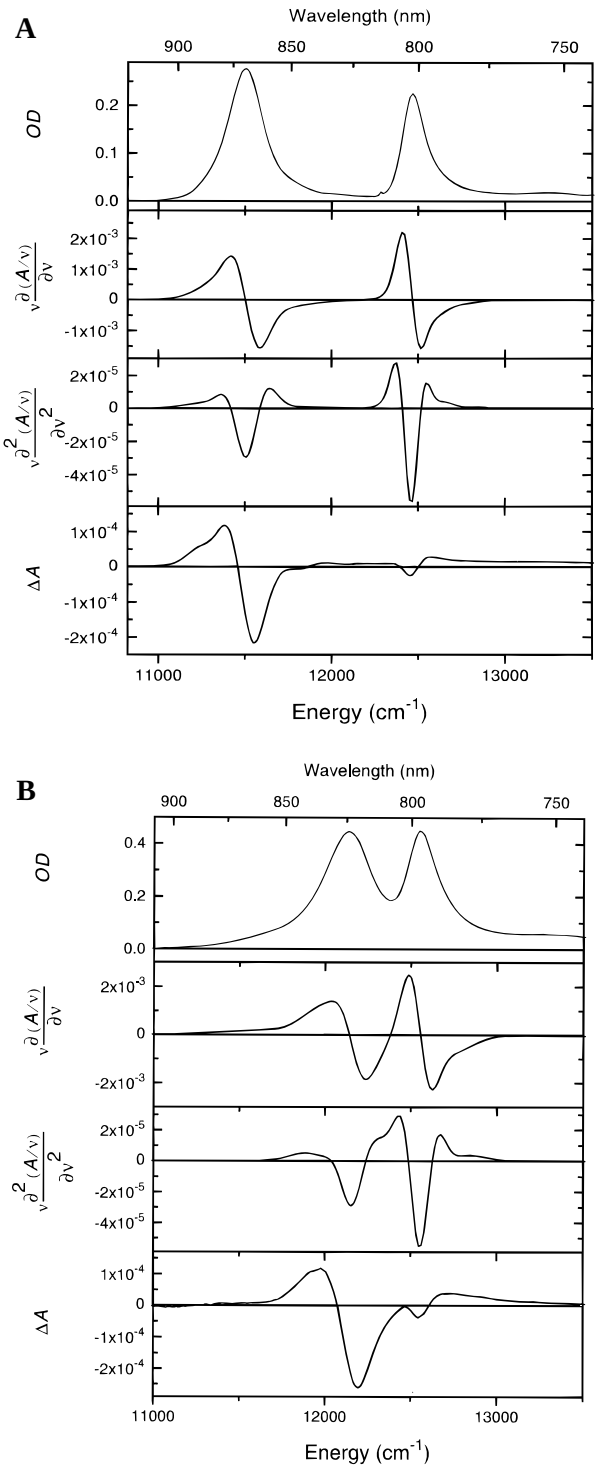

Figure 2. The 77 K absorbance, first-, and second-derivative, and Stark spectra of (A) *Rps. acidophila* B800-850 and (B) *Rps. acidophila* B800-820, both measured and magic angle and scaled to an electric field of $F = 1 \times 10^5$ V cm^{-1}.

sphaeroides LH2 complexes are shown in Figure 4. The only pigment−protein complex contained in these photosynthetic membranes is LH2, since the RC and LH1 were both genetically removed.[11,17] The similarity of these Stark spectra with those of the LH2 complexes from *Rps. acidophila* is striking, although B850 is much less red-shifted in the *Rb. sphaeroides* WT complex at 77 K as compared to the *Rps. acidophila* B800-850 (855 nm vs 869 nm). Note that furthermore the Stark spectra of the *Rb. sphaeroides* LH2 complexes do not exhibit the pronounced shoulder at the red edge of the B850 Stark signal, as was observed in detergent isolated LH2 complexes from both *Rps. acidophila* and *Rb. sphaeroides*.[24] The only indication of the presence of a long wavelength feature is the flat top of the positive lobe of the Stark spectrum (at 11 400 cm^{-1} (875 nm) for WT). The Stark parameters estimated for the WT B850 band are $|\Delta\boldsymbol{\mu}| = 4.2$ D/f and Tr($\Delta\boldsymbol{\alpha}$) = 620 Å^3/$f^2$. Again, the Stark signal around 800 nm is small, and the value of $|\Delta\boldsymbol{\mu}|$ is estimated to be about 1.1 D/f.

The absorption and Stark spectra of the *Rb. sphaeroides* mutants are shown normalized to the absorption of the B800 band, and the Stark spectra are scaled to an electric field strength of $F = 1 \times 10^5$ V/cm. The main observation is that the Stark spectrum follows the blue shift of B850 absorption band. The similarity of the Stark spectra of the "B850" bands in the *Rb. sphaeroides* LH2 mutants is even more pronounced than for the two LH2 types of *Rps. acidophila*. The shape of the B850 Stark signal hardly changes upon the blue shift, the only change being a broadening of the Stark signal; this follows the broadening of the absorption band, especially in the FL mutant, which reduces the size of the Stark signal. From the analysis of the B800 Stark signal an increase of $|\Delta\boldsymbol{\mu}|$ is found upon the spectral blue shift of the B850 band. From Figure 4, however, there is no clear evidence for a significant change in the B800 Stark spectrum. The increase in $|\Delta\boldsymbol{\mu}|$ obtained from the fitting procedure (Table 1) is probably an artifactual result due to two compensating contributions from the zeroth and second derivatives.

Ectothiorhodospira sp. In Figure 5 the spectra obtained for LH2 of *Ectothiorhodospira sp.*, with a low LDAO concentration (0.05%) and a high LDAO concentration (1%), are shown. Most striking in the Stark spectra of LH2 complexes from this species compared to the other systems is the large second-derivative line shape around 800 nm. The estimated $|\Delta\boldsymbol{\mu}|$ for this band in both *Ectothiorhodospira sp.* preparations is ∼2.7 D/f, compared to about 1.0−1.5 D/f for B800 in the other LH2 complexes. Furthermore, a Tr($\Delta\boldsymbol{\alpha}$) of about 100−240 Å^3/$f^2$ is present.

Upon the addition of LDAO the B850 band of *Ectothiorhodospira sp.* shows both a broadening and blue shift, reminiscent of what was observed for the other LH2 complexes where the blue shift was induced by a mutation resulting in the loss of a hydrogen bond. However, for *Ectothiorhodospira sp.* with LDAO the Stark spectrum shifts less to the blue than the absorption maximum, contrary to what was observed in the other blue-shifted complexes. The analysis of the two spectral forms shows that upon addition of LDAO Tr($\Delta\boldsymbol{\alpha}$) is significantly decreased and $|\Delta\boldsymbol{\mu}|$ increases slightly (see Table 1).

Rhodospirillum molischianum. In Figure 6 data are shown measured for B800-850 of *Rsp. molischianum*. For this sample the B850 band compares well to that of *Rps. acidophila*, whereas the B800 band shows an almost identical behavior as observed for *Ectothiorhodospira sp.* Furthermore, in this Figure the results of the simultaneous fitting of the absorbance and Stark spectrum are shown.

Discussion

Stark spectra were recorded for LH2 complexes from four different photosynthetic purple bacteria. For all complexes, B850 (and the blue-shifted B820) gives rise to an intense Stark signal which has contributions from the zeroth, first, and second derivative of the absorption line shape. $|\Delta\boldsymbol{\mu}|$ values are on the order of 3−4.5 D/f, and Tr($\Delta\boldsymbol{\alpha}$) values were found in the range 600−1400 Å^3/f^2. The Stark signal of B800 is much weaker for *Rb. sphaeroides* and *Rps. acidophila* LH2 complexes. However, in *Ectothiorhodospira sp.* and *Rsp. molischianum* the B800 Stark signal is found to be of similar size as the B850 signal and is dominated by the second derivative of the

TABLE 1: Values of $|\Delta\mu|$ and Tr($\Delta\alpha$) for All Studied LH2 Complexes[a]

species	LH2 type	band position (nm)	$\|\Delta\mu\|$ (D/*f*)	Tr($\Delta\alpha$)(Å^3/*f*^{2})	A_χ (cm^2/kV^2)
Rb. sphaeroides	WT	855	4.2(0.1)	619(13)	$-113(4) \times 10^{-10}$
		797	1.1(0.1)	5(10)	$38(8) \times 10^{-10}$
	FY	840	4.3(0.1)	578(17)	$-32(7) \times 10^{-10}$
		798	1.5(0.1)	20(30)	$85(11) \times 10^{-10}$
	FL	826	3.8(0.1)	610(12)	$-79(4) \times 10^{-10}$
		796	1.5(0.1)	42(8)	$159(13) \times 10^{-10}$
Rps. acidophila	B800-850	869	3.2(1)	1250(10)	$-152(11) \times 10^{-10}$
		802	1.5(0.1)		$180(30) \times 10^{-10}$
	B800-820	824	3.6(0.1)	1302(30)	$-195(5) \times 10^{-10}$
		769	1.4(0.1)		
Ectothiorhodospira sp.	low LDAO	867	4.25(0.1)	950(30)	$-110(10) \times 10^{-10}$
		794	2.8(0.1)	240(20)	$109(17) \times 10^{-10}$
	high LDAO	846	5.6(0.1)	270(30)	$-95(7) \times 10^{-10}$
		794	2.6(0.1)	100(10)	$31(9) \times 10^{-10}$
Rsp. molischianum	B800-850	866	3.2(0.1)	1420(20)	$-110(10) \times 10^{-10}$
		799	2.8(0.1)	290(20)	$220(10) \times 10^{-10}$

[a] The values were obtained by simultaneous fitting the absorbance and Stark spectrum measured with χ being (approximately) the magic angle. The wavelengths given in the column "band" indicate the position at which the fitted Gaussian profile was found; the position may differ slightly from the position observed in the absorbance spectrum.

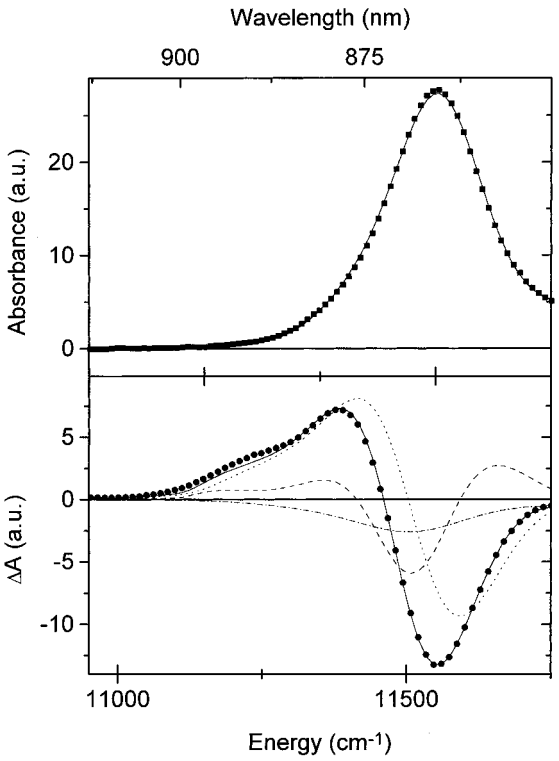

Figure 3. Simultaneous fit to the B850 band of *Rps. acidophila*. Two linked skewed Gaussian profiles were used to fit the absorption and Stark spectrum. The dots represent the data points and the drawn lines the fit. In the Stark spectrum the second (dash), first (dot), and zeroth (dash-dot) derivative contributions are shown. The values for $|\Delta\mu|$ and Tr($\Delta\alpha$) are listed in Table 1.

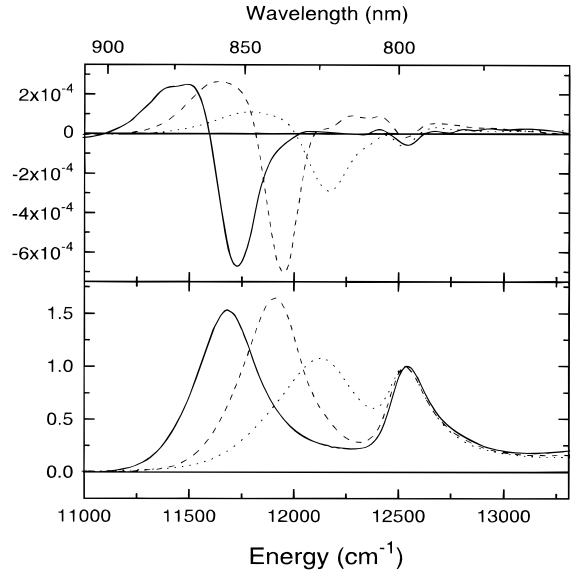

Figure 4. Absorbance and Stark spectra of the *Rb. sphaeroides* mutant LH2 complexes. The absorption and Stark spectra (lower panel) are normalized on the 800 nm absorption band. The wild-type species is given by the dashed curves, the α_{+13}Y→F, FY is shown as the dashed curves, and the α_{+13}Y→F and α_{+14}Y→L, FL is given by the dotted curves. The Stark spectra are scaled to an electric field of $F = 1 \times 10^5$ V cm^{-1} and were all measured at the magic angle.

absorption spectrum. The results will be discussed in terms of the LH2 structures reported for *Rps. acidophila*[4,5] and *Rsp. molischianum*.[6]

B850 Band. The Stark data on B850 will be compared to LH1 and P of the bacterial RC, because the Stark results for these bands yield similar $|\Delta\mu|$ and Tr($\Delta\alpha$) values.[24,27,29] Stark spectra of B850 of different purple bacteria are very similar and are characterized by an S-shaped spectrum which is positive on the red side. This indicates that, as for LH1, a large polarizability change is observed upon optical excitation. However, the ratio of the positive and negative peaks is about 1:2−3, which implies that the symmetric first derivative of the absorption bands cannot solely describe the Stark spectrum. There is clearly a requirement for a second derivative and may be even some zeroth-derivative contribution.

The Tr($\Delta\alpha$) values found for B850 in LH2 are all large and of the same order of magnitude, although generally smaller, as those obtained for LH1 (600−1400 Å^3/*f*^{2} vs ∼1300−1800 Å^3/*f*^{2}) as is also the case for the $|\Delta\mu|$'s (3−5 D/*f* vs 3.5−4.0 D/*f*). From the LH1 data it was suggested that the basic spectral unit, possibly a dimer, is highly polarizable, due to the mixing of exciton states with CT states. Overlapping electron densities of the exciton coupled molecules are important in decreasing CT state energies. In this picture $\Delta\boldsymbol{\mu}$ arises from a matrix field, $\mathbf{F}_{\mathbf{matrix}}$, due to the protein environment: $\Delta\boldsymbol{\mu} = \Delta\boldsymbol{\alpha}\cdot\mathbf{F}$. A similar model was put forward for P[27] and may also hold for B850 in LH2.

In both *Rb. sphaeroides* and *Rps. acidophila* specific replacement of hydrogen-bonding residues by non-hydrogen-bonding ones alters the direct protein environment of B850 and leads to

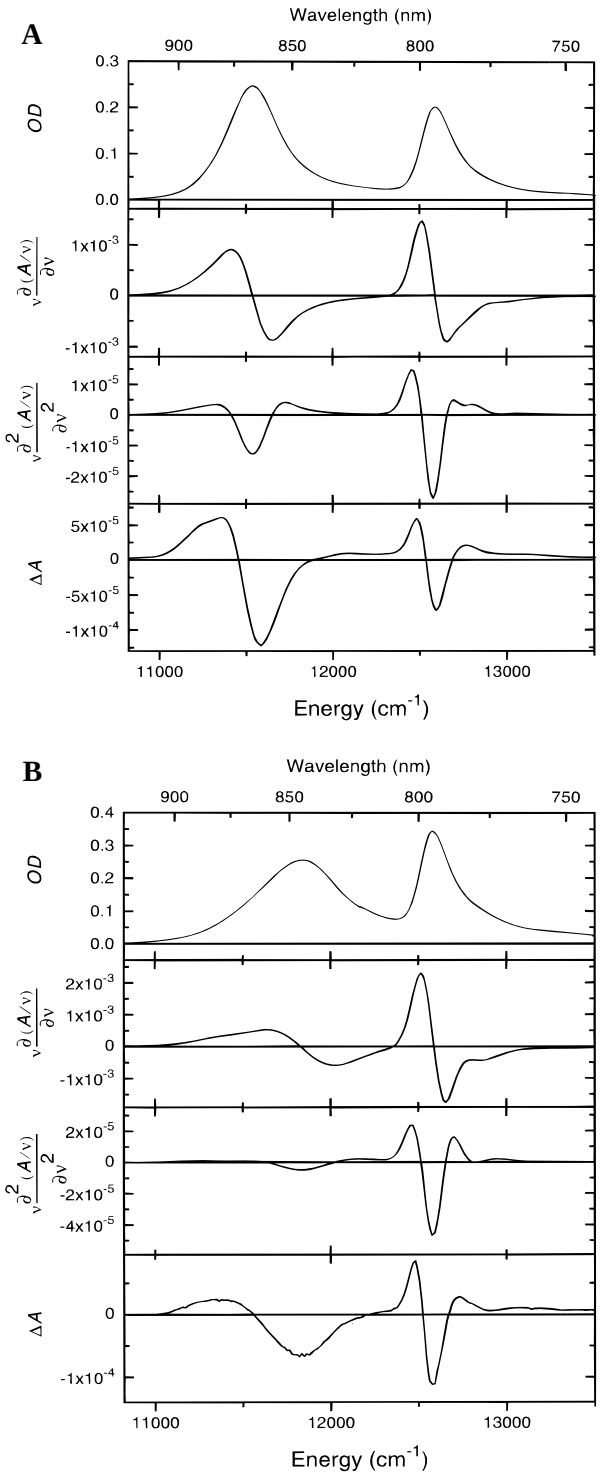

Figure 5. The 77 K absorbance, first-, and second-derivative, and Stark spectra of *Ectothiorhodospira sp:* (A) low LDAO (B800-850) complex, (B) high LDAO (B800-820), measured at the magic angle and scaled to the electric field strength of $F = 1 \times 10^5$ V cm^{-1}.

a blue shift of B850. However, in Figure 4, which displays the Stark and OD spectra of the mutant LH2 complexes from *Rb. sphaeroides*, one of the most striking features is the similarity of the B850 Stark line shapes. Apart from the expected blue shift and a slight broadening, no differences are observed. To a somewhat lesser extent, this is also true for B800-820 of *Rps. acidophila*. Note, however, that in this case more amino acid residues have changed as compared to B800-850 (see Figure 1). The $|\Delta\boldsymbol{\mu}|$ values obtained for the *Rb. sphaeroides* and *Rps. acidophila* complexes are similar, although the Tr($\Delta\alpha$) values seem so be a factor of 2 smaller in *Rb. sphaeroides*. This will be further discussed below.

The line shapes hardly change upon the spectral blue shift, and also $|\Delta\boldsymbol{\mu}|$ and Tr($\Delta\alpha$) are hardly affected. This means that although the spectral shift is induced by the loss of hydrogen bonds, there is hardly any change in the polarizability and the protein matrix field. Apparently, breakage of the H bonds has a direct effect on the excited-state energy of the Bchl's but only a minor effect on the electric properties of the binding pocket. The overall matrix field sensed by B850 is rather insensitive to the mutation of a single amino acid and seems to be a more global protein property. A further reason for the relatively small influence of single mutations may be the symmetric nature of the structure. Each mutation on a $\alpha\beta$-subunit is accompanied by the same mutation on the neighboring subunits, and this may have a reducing effect on possible changes in the protein matrix field.

In *Ectothiorhodospira sp.* the LDAO induced blue shift is accompanied by large changes in the Stark parameters; in particular, the polarizability drops almost by a factor of 4. In this system, however, the loss of the hydrogen bonds is not as well defined as in *Rb. sphaeroides* and *Rps. acidophila*. LDAO shifts the B850 band, but whether it is by breaking all hydrogen bonds to the C_2-acetyl carbonyls of the Bchl's in the $(\alpha\beta)_8$ ring or only to a few is unknown. Furthermore, LDAO may also have direct interactions with the Bchl *a* molecules and change the overall structure slightly. This relatively ill defined, and the broad blue-shifted B850 band in *Ectothiorhodospira sp.* may thus be composed of a series of spectral bands each with its own Stark parameters. Energy-selective fluorescence polarization experiments also indicated a strong heterogeneity in these samples.[23]

Shoulder on the "B850" Band. The shoulder on the red edge of the Stark spectrum, as observed for isolated B800-850 of *Rb. sphaeroides*,[24] *Rps. acidophila*, and *Rsp. molischianum* and somewhat less apparent in B800-850 of *Ectothiorhodospira sp.* and membrane bound *Rb. sphaeroides* B800-850 may have a few origins. With the exception of the membrane bound B800-850, the shoulder could be due to contamination of the samples with RCs or LH1. These complexes are expected to give rise to Stark signals red-shifted from the 850 nm band with a similar sized Stark effect. The contamination of the samples is not impossible, but the shoulder also seems to be present in B800-820 of *Rps. acidophila* and blue-shifted with respect to the position where contaminations of LH1 or RC should be present.

Furthermore, the insensitivity of the shoulder to treatment with ferricyanide rules out a possibility of a contamination of the sample with RCs.[24] It is significant that Stark spectra obtained from membrane bound LH2-only mutants of *Rb. sphaeroides* also show the shoulder, although this is not as apparent as in the case of complexes isolated from *Rb. sphaeroides*,[24] *Rps. acidophila*, and *Rsp. molischianum.* These *Rb. sphaeroides* complexes are certainly free from RCs and LH1 since these complexes were genetically removed. Therefore, we suggest that the pronounced feature observed in the Stark spectrum of *Rps. acidophila* is most probably not due to a contamination with RC's or LH1.

The shoulder could be a result of a low-lying state as suggested by Gottfried et al.[24] This state could be due to closely interacting LH2 rings. Ring−ring contacts may have increased in these experiments since highly concentrated samples are required. For the membrane bound complexes the ring−ring contacts are determined by their positions in the membrane. To

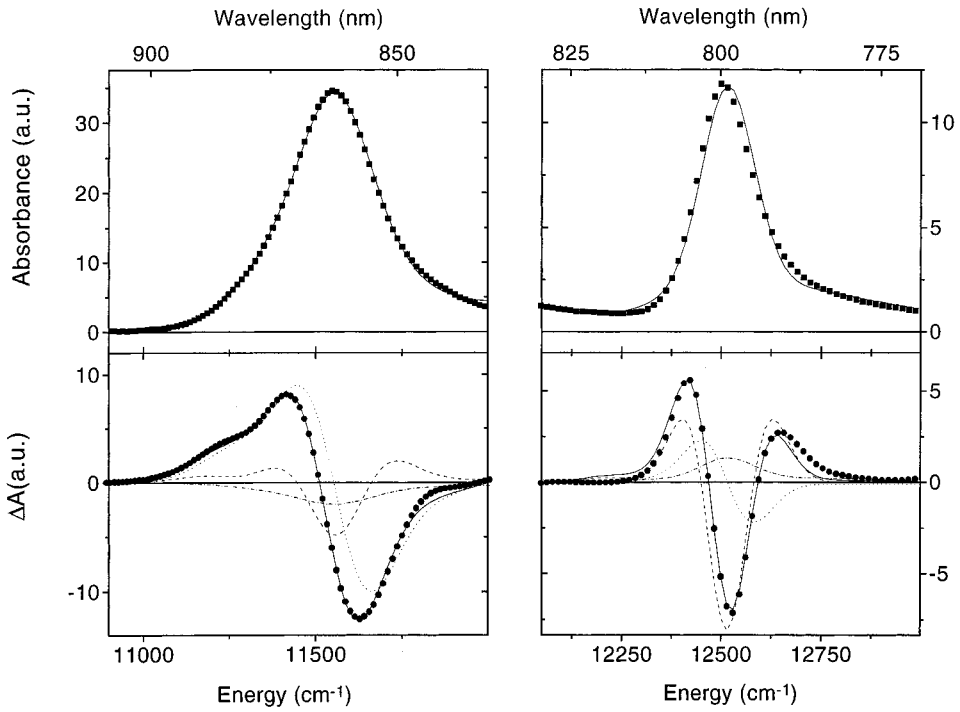

Figure 6. Absorption (top) and Stark (bottom) spectra of the B850 (left) and B800 (right) bands of *Rsp. molischianum* LH2 measured at 77 K are shown together with the simultaneous fits. A sum of two skewed Gaussian profiles was used to fit the absorption and Stark spectra in both spectral regions. The dots represent the data points, and the drawn lines represent the fitted spectra. In the bottom pictures apart from the Stark spectra also the second (dash), first (dot), and zeroth (dash dot) derivative contributions to the Stark spectra are shown. Values for $|\Delta\boldsymbol{\mu}|$ and Tr($\Delta\boldsymbol{\alpha}$) are listed in Table 1.

investigate these effects, the dependence of the shoulder on the concentration of LH2 and detergent should be studied.

Differences in $\Delta\boldsymbol{\alpha}$. The LH2's can be categorized in two groups based on their Tr($\Delta\boldsymbol{\alpha}$): *Rps. acidophila* to *Ectothiorhodospira sp.* and *Rsp. molischianum* having values larger than 1000 Å^3/f^2 and *Rb. sphaeroides* with values on the order of 600 Å^3/f^2. One possible correlation is with the estimated ring sizes. LH2 of *Rb. sphaeroides* was estimated to be $(\alpha\beta)_6$,[15] while the others were crystallized as $(\alpha\beta)_9$[4,5] or $(\alpha\beta)_8$.[6,33] Furthermore, LH1, with a Tr($\Delta\boldsymbol{\alpha}$) of 1300−1800 Å^3/$f^2$, has an estimated ring size of $(\alpha\beta)_{16}$.[34] A smaller ring may result in a change in pigment−pigment interactions, resulting in, for instance, a decreased electron density overlap that could affect the polarizability. A similar explanation was given for the low Tr($\Delta\boldsymbol{\alpha}$) observed in B820.[29] However, the observed effect is rather large; the room temperature spectra of all the LH2's are essentially the same, and consequently we do not believe this to be the major cause.

A second correlation of the size of Tr($\Delta\boldsymbol{\alpha}$) is with the observed red shift of the B850 Q_y transition upon cooling, which for *Rb. sphaeroides* LH2 is about 1−2 nm compared with 10 nm for *Rps. acidophila* and the other two LH2's and 11 nm for LH1. From solvent shift studies it was found that the shift of the optical transition of a pigment with the refractive index depends linearly on $\Delta\boldsymbol{\alpha}$.[35] Assuming that due to cooling a change in the refractive index of the protein surrounding B850 occurs, this would induce a smaller shift in LH2 of *Rb. sphaeroides* compared with the other LH2's simply because the latter $\Delta\boldsymbol{\alpha}$ is larger. This explanation suggests that the contribution of the protein solvent to shifting the band to 850 nm at room temperature is rather small. In this respect it should be interesting to compare the response of LH2 complexes to increases in pressure.[36]

Assuming that $\Delta\boldsymbol{\alpha}$ arises as a result of the mixing of CT states with the exciton states, the size of $\Delta\boldsymbol{\alpha}$ is largely determined by the energy level of the relevant CT states which can be stabilized by the protein matrix field. Thus, differences in the protein matrix near B850 in *Rb. sphaeroides* compared to the other complexes could also be responsible for the difference in $\Delta\boldsymbol{\alpha}$ values. On the α-polypeptide a polar serine residue is found at position −4, αSer$_{-4}$ (Figure 1), which is about one turn of the α-helix further into the membrane. This residue is replaced by an alanine in the other LH2 complexes. And the polar βMet$_{-5}$ as found in *Rb. sphaeroides* is replaced by the nonpolar leucine or isoleucine in the other complexes (Figure 1). These differences in the protein matrix could be responsible for the observed difference in $\Delta\boldsymbol{\alpha}$. However, also aromatic amino acids may increase $\Delta\boldsymbol{\alpha}$. A possible candidate could be at position β_{+4}, where an alanine in *Rb. sphaeroides* is replaced by an aromatic residue, Tyr or Trp, in *Rps. acidophila* and *Rsp. molischianum*. Finally, the distortion of the macrocycles of the Bchl's as observed in the structure of *Rps. acidophila*[5] may also stabilize specific CT states. If this would be the case, this would suggest that the Bchl's are less distorted in LH2 of *Rb. sphaeroides*.

B800 Band. In contrast to B850, where a large spectral shift is accompanied by almost no change in the Stark properties of the optical transition, B800 shows large differences in the Stark properties that are not accompanied by a large shift or change in shape of the B800 absorbance spectrum. The Stark parameters of *Rb. sphaeroides* and *Rps. acidophila* are small and comparable to monomeric Bchl *a*, whereas *Ectothiorhodospira sp.* and *Rsp. molischianum* exhibit large Stark parameters, without an apparent effect on the absorption band. An explanation of the increase in Stark parameters is probably best sought in differences between the structures of *Rps. acidophila* and *Rsp. molischianum*,[4−6] and we will focus the discussion mainly on differences in the two LH2 structures around B800.

To explain the Stark effect of the B800 band, Bchl−Bchl interactions can be ruled out because of the large distances (∼20 Å). Indeed, for *Rps. acidophila* a monomeric behavior of B800 is found,[24,31] in contrast to the large $|\Delta\boldsymbol{\mu}|$ = 2.8 D/f and Tr-

$(\Delta\alpha) = 290$ Å$^3/f^2$ for *Rsp. molischianum* B800. Important is the fact that both $\Delta\mu$ and $\Delta\alpha$ increase with respect to monomer Bchl *a*. This indicates that similarly to P, LH1, and B850, a (pigment) molecule is probably interacting strongly with B800 of *Rsp. molischianum*, with interpigment CT states probably playing a role. In both LH2 structures a carotenoid is found to be in close contact with the B800 porphyrin macrocycle. In *Rps. acidophila*, however, the carotenoid is oriented perpendicular to the macrocycle whereas in *Rsp. molischianum* an angle between the two of ~38° is observed.

For P, LH1, and B850 the CT states are believed to mix into the exciton states and give rise to $\Delta\alpha$ and $\Delta\mu$ character. However, in the case of the interaction between Bchl *a* and carotenoids the energy difference between the optical transitions is too large to be able to observe an effect of dipole−dipole interactions on the spectra. However, it is also possible that exchange interactions between the two pigments play a role. For exchange interactions close contacts are required as are found in the LH2 structures. The excitation transfer between the 2^1A_g carotenoid state to Bchl *a* was proposed to occur via an exchange mechanism.[37] Furthermore, for B800-850 from *Rps. palustris* which, like *Rsp. molischianum*, has a large B800 CD spectrum,[38] a large effect is found in the microwave-induced spectrum resulting from carotenoid triplets.[39] In *Rps. acidophila* and *Rb. sphaeroides* this effect is negligible.[39] If the large CD in *Rps. palustris* has a similar origin as was proposed for *Rsp. molischianum*, that is an orientation differing from that found in *Rps. acidophila*, the same change in orientation increases the exchange interactions between B800 and carotenoid molecules, as is indicated by the effect of the carotenoid triplet on the B800.

A similar result was arrived at from model calculation on the Bchl−carotenoid excitation energy transfer process.[40] In this study both the resonance interaction and exchange interactions are considered for the S_1 and S_2 states of both molecules. It was found that for all interactions a perpendicular arrangement of the dipole moments is unfavorable. This would thus also indicate that the exchange interactions have increased in *Rsp. molischianum* as compared to *Rps. acidophila*. Due to increased exchange interactions CT states may be significantly lowered in energy and mixed with the optical transition of B800, which may lead to the observed large $\Delta\mu$ and $\Delta\alpha$. Since $\Delta\mu$ and $\Delta\alpha$ parameters of carotenoid molecules are generally large, a small change in the exchange interactions between the carotenoid and B800 may already lead to significant influence on $\Delta\mu$ and $\Delta\alpha$ of the B800 pigments.

As previously observed for the carotenoids in LH2, a strong protein matrix field is present. The large $|\Delta\mu|$ for B800 indicates that a protein matrix field also lowers specific carotenoid−B800 CT states. The protein environment of B800 is polar, much more polar than the environment of B850,[4,6] and several charged residues are found in the vicinity of B800. This directly leads to an other striking difference between *Rps. acidophila* and *Rsp. molischianum*. In the former the central magnesium of B800 is ligated by a formyl-methionine,[4] which is the last residue on the α-polypeptide (Figure 1). In *Rsp. molischianum* the ligation of the Mg is by an aspartate, αAsp_{-28}, D, which is a charged residue.[6] This may be one of the residues determining the matrix field around the B800. In *Rb. sphaeroides* and *Rps. acidophila* a charged arginine (Arg, R) residue is found at position −10 on the β-polypeptide, βArg_{-10}, which is replaced by a polar serine in *Rsp. molischianum*. Furthermore, near B800 also a pair of charged residues is found which are found to form a salt bridge in the LH2 structure,[6] βAsp_{-17} and βLys_{-14}, lysine, K (Figure 1). A salt bridge is expected to be important in determining the protein matrix field. Other charged residues may play a role, but from the structural information that can be obtained from the publication on the *Rsp. molischianum* structure[6] it is not possible to say much more concerning the matrix field. It seems as if the matrix field is indeed pointed toward the membrane surface. There are thus enough charged residues close to B800 that may be responsible for the electric matrix field as we suggest based on the Stark data.

The similarity of *Ectothiorhodospira sp.* and *Rsp. molischianum* in terms of our Stark measurements indicates that there is also a high degree of similarity at the level of the protein sequences. It would be especially interesting to see whether *Ectothiorhodospira sp.* also has an Asp that ligates B800 and/or the above-mentioned salt bridge, βAsp_{18}, βLys_{21}.

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