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An LD-ADMR study on reaction centers of the LH(L131) and LH(M160) hydrogen-bonding mutants of *Rhodobacter sphaeroides*

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Abstract

Reaction centers of the LH(L131) and LH(M160) mutants of *Rhodobacter sphaeroides*, which have a leucine near the primary donor, a bacteriochlorophyll dimer, changed into a histidine, have been investigated with linear-dichroic absorbance-detected magnetic resonance, and the results are compared with those obtained for native reaction centers. The microwave-induced triplet-minus-singlet absorbance-difference spectra of the three reaction centers upon triplet formation of the primary donor show small differences in band shifts in the Q_Y-region of the accessory bacteriochlorophylls. The orientations of the Q_Y-transition moments with respect to the dipolar axes of the triplet state of the primary donor were found to be equal for all reaction centers, indicating that the orientation of the triplet axes with respect to the reaction center coordinate frame is the same for all three reaction centers. From this we conclude that the electronic composition of the triplet state and the Q_Y transitions are basically the same for the mutant and native reaction centers.

Keywords: Absorbance-detected magnetic resonance; Photosynthesis; Mutant; Triplet state; Bacterial reaction center

1. Introduction

Charge separation in the reaction center (RC) of purple bacteria proceeds via a sequence of electron transfer steps whereby an electron is transferred from the singlet excited primary electron donor (P), a bacteriochlorophyll (BChl) dimer, to the primary acceptor, a bacteriopheophytin (BPh), and subsequently to the acceptor quinones. The structure of the RC, showing a near-C_2 symmetry, seemingly, allows for two possible routes for electron transfer [1–3], although only one is active [4]. The two C_2-symmetric branches are formed by the so-called L and M polypeptides, to which the chromophores are bound, namely the components of the dimer, D_L and D_M, two adjacent BChls, B_L and B_M, the BPhs, BPh_L and BPh_M, and a carotenoid. When electron transport is blocked, by removing or prereducing the first acceptor quinone, the triplet state of the primary donor (^3P) is formed via radical-pair recombination of the positive charge on P and the negative charge on the BPh_L primary acceptor, with a yield approaching unity at cryogenic temperatures.

The asymmetry in electron transport must be related to the inherent asymmetry of the RC, which deviates from a perfect C_2 symmetry [5–8]. The amino acids in the vicinity of the chromophores are thought to play an important role by influencing the electronic states of the chromophores. This influence may be altered using site-directed mutagenesis of the amino acids near the RC chromophores.

Mutations that involve the BChls of P are of special interest for studying the influence of the environment on the electronic states of the BChl-dimer and on its interaction with the accessory BChls. With absorbance-detected magnetic resonance (ADMR) triplet-minus-singlet (T − S) absorbance-difference spectra with high resolution can be recorded, which give information on the intermolecular interactions. The interactions between the singlet states of P and the accessory BChls are altered upon triplet formation on P, leading to changes in the absorption of the accessory BChls, which are observable as band shifts and intensity changes in the T − S spectrum [9,10]. In addition,

Abbreviations: (LD-)ADMR, (linear-dichroic) absorbance-detected magnetic resonance; T − S, triplet-minus-singlet; RC, reaction center; P, primary electron donor; BChl, bacteriochlorophyll; BPh, bacteriopheophytin.

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with linear-dichroic (LD)-ADMR the orientation of the optical transition moments contributing to the T − S spectrum with respect to the triplet axes can be obtained. In combination with the crystallographic data on the mutual orientation of the RC chromophores, this may provide an assignment of the absorption bands in the T − S spectra.

In this communication we present a comparative LD-ADMR study on two symmetry-related mutants of *Rhodobacter* (*Rb.*) *sphaeroides*, which involve the BChl-halves of the dimer. For one mutant, LH(M160), a leucine at site M160, close to D_M, is changed into a histidine. This mutation has been designed such that it may introduce a hydrogen bond with the 9-keto-group at ring V of the D_M-half of the dimer. (In contrast to leucine, histidine is able to form a hydrogen bond.) In the symmetry-related mutant, LH(L131), a leucine at site L131 is changed into a histidine, which may lead to formation of a hydrogen bond at the 9-keto-group of ring V of the D_L-half of the dimer [11]. Apart from being close to the dimer-halves, the L131 and M160 sites are close (within 5 Å) to the accessory BChls, B_L and B_M, respectively. The microwave-induced T − S absorbance-difference spectra of the RCs of the mutants, compared with those of native RCs, show differences in band shifts in the Q_Y-region of the accessory BChls, which are interpreted as small changes in mixing of the electronic states of the dimer-BChls and the accessory BChls. The orientations of the optical transition moments contributing to the T − S spectra with respect to the triplet *x*- and *y*-axes of ^3P do not change significantly upon mutation, indicating that the orientation of the triplet axes in the RC coordinate frame is the same for native and mutant RCs. From these observations, we conclude that the basic electronic composition of the Q_Y-transitions must be similar for the mutant and native RCs.

2. Materials and methods

The procedures of site-directed mutagenesis of *Rb. sphaeroides* have been described [11]. The isolated reaction centers were prepared following the procedure in Ref. [12] with modifications as described in Ref. [13]. Chromatophores were suspended in Tris-buffer (pH = 8) containing 10 mM dithionite for prereducing the acceptor quinone. Isolated reaction centers were dissolved in Tris-LDAO buffer (pH = 8, 0.025% LDAO), containing 30 mM sodium-ascorbic acid. The latter samples were illuminated during cooling to prereduce the acceptor quinone. Glycerol (66% v/v) was added to prevent cracking of the sample upon freezing. The optical density was ~0.35 at the wavelength of maximum absorbance in the Q_Y-region (750–900 nm) for the samples containing isolated reaction centers and chromatophores.

All measurements were done at approx. 1.5 K with an LD-ADMR set-up similar to that described previously [9,14]. A beam of continuous, broad-band light was used for excitation and probing the transmittance. The transmitted light dispersed by a monochromator (resolution set at 3 nm), was detected with a silicon photodiode.

For the ADMR experiments microwaves were fed into a helix that was wound around a flat perspex cuvette with a path length of 2 mm. The microwaves were amplitude-modulated (312 Hz) and the spectra were recorded at a fixed detection wavelength, with the resolution of the monocromator set to 6 nm, while slowly sweeping the microwave frequency.

For recording the (LD-) T − S spectra, microwaves were fed into a loop-gap resonator, which was placed around the sample cuvette such that the direction of the microwave magnetic field was perpendicular to the light beam. A photo-elastic modulator, operating at 50 kHz, and a Glan-Thompson polarizer were placed between the sample and the monochromator, analyzing the transmitted light polarized parallel and perpendicular to the direction of the microwave magnetic field. Phase-sensitive detection of the change in transmitted light intensity (ΔI) caused by amplitude modulation of the microwaves, resulted in the absorbance-difference (ΔA) signal, where ΔA was taken proportional to $-\Delta I/I$. For the LD-absorbance-difference spectra the photodiode signal was doubly demodulated using two lock-in amplifiers, tuned at 100 kHz and 312 Hz, in series.

3. Results

3.1. Absorption spectra

The Q_Y- ($S_1 \leftarrow S_0$) region (730–960 nm) of the low-temperature (10 K) absorption spectra of RCs of *Rb. sphaeroides* wild-type and the LH(L131) and LH(M160) mutants are compared in Fig. 1. They are similar to those reported previously [11]. The long-wavelength band, as-

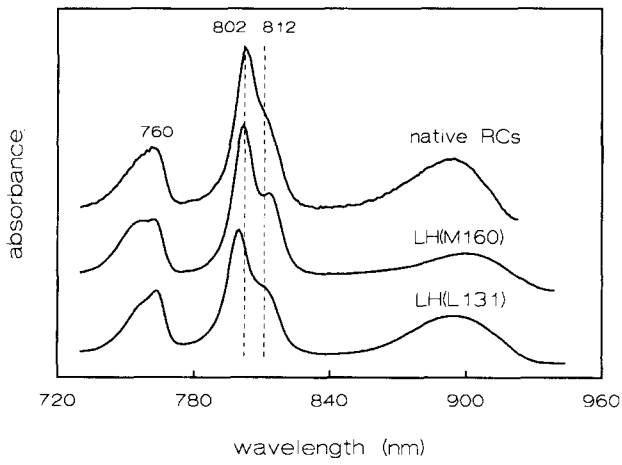

Fig. 1. The Q_Y-region of the absorption spectra of RCs of *Rb. sphaeroides* and of its LH(M160) and LH(L131) mutants. The spectra are normalized on the amplitude at 760 nm. Temperature 10 K.

cribed to the $S_1 \leftarrow S_0$ transition of P, centered at ~896 nm for native RCs, is shifted somewhat to lower energies, and is broadened for RCs of the LH(L131) and LH(M160) mutants.

The mutations also cause a change in the position of the absorption bands that are centered at 802 and 812 nm for native RCs. In the absorption spectrum of the LH(L131) mutant, the 802 nm absorption band is located at 800 nm, and for the LH(M160) mutant the 812 nm band is located at 814 nm. Both bands have a large contribution of the Q_Y-transitions of the accessory BChls, B_L and B_M. The Q_Y-absorption bands of the BPhs, located at ~750 and ~760 nm for BPh_M and BPh_L, respectively, are, apart from a small difference in intensity at 750 nm for LH(M160), similar for all three spectra. This is consistent with the relatively large distance between the mutation sites, L131 and M160, and the BPhs. Therefore, all absorption spectra were normalized on the absorption at 760 nm. The total integrated dipolar strengths in the Q_Y-region of the normalized spectra is practically the same for all three RCs, indicating that the mutations do not change the total dipolar strength in this spectral region.

3.2. Zero-field splitting parameters

The microwave-swept ADMR spectra of the LH(L131) and LH(M160) mutants of *Rb. sphaeroides* and of the carotenoid-lacking mutant *Rb. sphaeroides* R26 are compared in Fig. 2. The detection wavelength was set at a wavelength within the $S_1 \leftarrow S_0$ absorption of the RC (900–915 nm), where only the absorption of P contributes. Sweeping the microwave frequency resulted in two observable zero-field frequencies for all RCs, attributed to the $|D|-|E|$ and $|D|+|E|$ transitions of ^{3}P. For chromatophores of the LH(L131) and LH(M160) mutants the bands in the ADMR spectra deviate considerably from single Gaussian bands, in contrast to the ADMR-bands for chromatophores of *Rb. sphaeroides* R26 (Fig. 2).

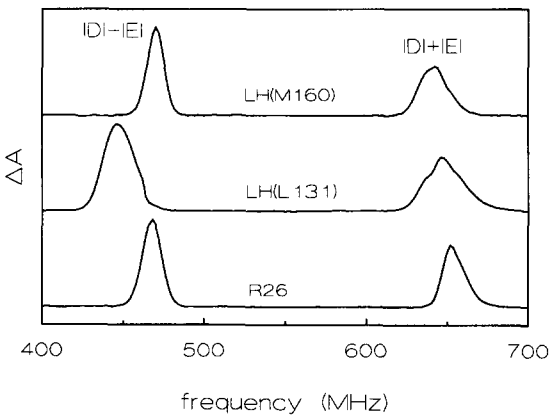

Fig. 2. ADMR spectra of chromatophores of *Rb. sphaeroides* R26 (a), LH(L131) (b) and LH(M160) (c) mutants of *Rb. sphaeroides*. The detection wavelength was set at 900 nm (a) and 915 nm (b,c).

Table 1
Zero-field splitting parameters (in MHz ± 0.5) of monomeric ^{3}BChl *a* [[22]], and of ^{3}P in chromatophores of *Rb. sphaeroides* R26 and the LH(L131) and LH(M160) mutants of *Rb. sphaeroides*

	$\|E\|$	$\|D\|$
R26 and native RCs	92	560
LH(M160)	86	555
LH(L131)	100	545
BChl *a*	165–210	680–700

For chromatophores the maximum frequencies changed less than 2 MHz when varying the wavelength within the Q_Y-region (750–930 MHz) (not shown). For isolated RCs of *Rb. sphaeroides* R26 and of the LH(L131) and LH(M160) mutants, the wavelength dependence of the zero-field transitions is much stronger than for chromatophores. Although the maximum ΔA signal was observed at approximately the same frequency in chromatophores and isolated RCs, for the latter the maximum of the bands varied about 10 MHz when changing the detection wavelength within the Q_Y-region (not shown). We attribute this variation to heterogeneity of the RCs, reflecting different conformations or environments of P, caused in part by the isolation procedure (G.J. Owen, J. Vrieze and A.J. Hoff, ms. in preparation).

On average, for both chromatophores and isolated RCs, the microwave transitions of the LH(M160) and LH(L131) mutants differ slightly from those of *Rb. sphaeroides* R26 (Table 1). The triplet state of P in native RCs of *Rb. sphaeroides* has the same zfs-parameters as in the carotenoid-less RCs of *Rb. sphaeroides* R26 (data not shown).

For both mutants we found a reproducible smaller signal intensity, with a factor of about 2, compared to that of native RCs of *Rb. sphaeroides*. The lower ADMR intensity has presumably the same origin as the ~50% decrease found for the quantum yield of initial charge separation for the mutants compared to native RCs [11].

3.3. Absorbance-difference spectra

The T − S absorbance-difference spectra of the native RCs and the mutants are compared in Fig. 3. The spectra were recorded at a resonant frequency within the $|D|-|E|$ zero-field transition of the triplet state of P, corresponding to the splitting between the *x*- and *z*-triplet sublevels of ^{3}P. The T − S spectra recorded at a frequency within the $|D|+|E|$ transition, the splitting between the *y*- and *z*-triplet sublevels, are practically identical (not shown). The T − S spectra were normalized on the amplitude of the long-wavelength absorption band in the absorption spectra of Fig. 1. As for the absorption spectra, this leads to a total integrated dipolar strength similar for all three T − S spectra.

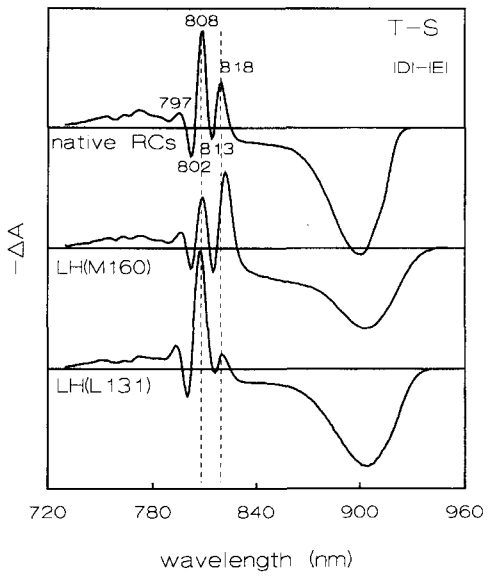

Fig. 3. The microwave-induced T – S spectra in the Q_Y-region. The microwave frequency was set at 467 MHz (native reaction centers), 470 MHz (LH(M160)) and 445 MHz (LH(L131)), corresponding with the $|D|-|E|$ transitions. The microwaves were amplitude-modulated at 312 Hz for all T – S spectra.

The T – S spectra are dominated by the broad, negative Q_Y-absorption of P, reflecting an increase in its ground-state absorption due to microwave irradiation. For all three T – S spectra the width of the long-wavelength absorption band is somewhat reduced and the maximum shifted to longer wavelengths compared to Fig. 1. This happens because the microwaves select a sub-population of the RCs with a specific resonant microwave transition, and because the RCs absorbing at a longer wavelength possibly have a somewhat higher triplet yield.

In the T – S spectrum of native RCs, the 780–820 nm region shows two relatively strong positive bands, centered at 808 and 818 nm, which are overlapping with negative bands, centered at 802 and 812 nm. The latter two peaks reflect the absorption bands that are present in the ground-state absorption spectra (cf. Fig. 1). The bands with positive amplitude in this region of the T – S spectra represent the absorption of the RC with the triplet state localized on P, that is, the absorptions of those chromophores that are not in the triplet state, and the spectrum of which is shifted or altered in intensity due to a change in interaction with P upon ^{3}P formation. In addition, triplet-triplet absorptions of the lowest triplet excited state of P to higher excited triplet states may contribute. Because the latter absorptions are usually very broad, it is unlikely that they are responsible for the sharp bands at 808 and 818 nm. The main difference between the three T – S spectra in Fig. 3 is a difference in relative intensities of the positive 808 and 818 nm bands.

3.4. Linear-dichroic T – S absorbance-difference spectra

To determine the orientation of the optical Q_Y-transition moment of P with respect to its triplet axes frame, the LD-(T – S) and T – S signals were recorded at the $|D|-|E|$ and $|D|+|E|$ zero-field transitions at a fixed wavelength within the long-wavelength band (910 nm). Applying resonant microwaves with a frequency within, for instance, the $|D|-|E|$ (x-z) transitions, molecules that have their triplet y-axis more or less parallel to the direction of the microwave magnetic field are preferentially selected. We assume a positive sign of D and E for ^{3}P, as for monomeric ^{3}BChl a [15,16], so that the $|D|-|E|$ and the $|D|+|E|$ transitions are polarized along the triplet y-

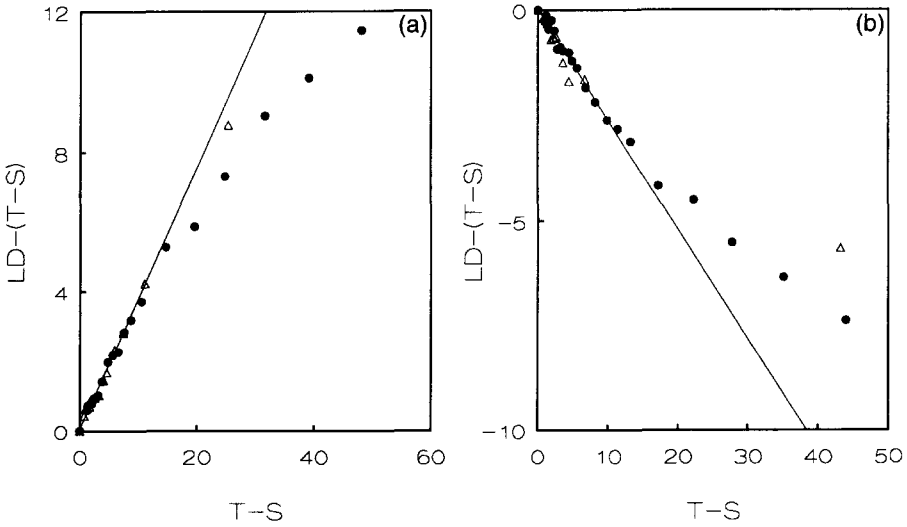

Fig. 4. The LD-(T – S) signal amplitude plotted against that of the T – S signal for LH(M160) (triangles) and LH(L131) (dots) recorded at a detection wavelength of 910 nm as a function of the microwave power (0.01–1 mW at the microwave source). The microwave frequency was set at 470 (LH(M160)) and 445 MHz (LH(L131)) (a), and at 645 (LH(M160)) and 640 MHz (LH(L131)) (b). The R-value for vanishing microwave power is given by the slope of the straight line, using a linear least-square fit through the data points obtained for low microwave powers.

and x-axis, respectively. The angle α_i between the optical transition moment and the triplet axis i can be obtained via the relation [14,17,18]:

$$R_i = \frac{LD(T-S)}{T-S} = \frac{\Delta A_{\parallel} - \Delta A_{\perp}}{\Delta A_{\parallel} + \Delta A_{\perp}}$$

$$= \frac{(3\cos^2\alpha_i - 1)}{(3 + \cos^2\alpha_i)} \quad i = x, y, z \qquad (1)$$

where $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ refer to the absorbance-difference signals polarized parallel and perpendicular to the direction of the microwave magnetic field. Eq. (1) holds for randomly oriented molecules. Since at high microwave power saturation effects occur, the LD-(T − S) and T − S signals are extrapolated to zero-microwave power to obtain the value of $\alpha_{x,y}$. The experiment was performed at a wavelength within the long-wavelength band of P, both at the $|D| - |E|$ and $|D| + |E|$ transition. In Fig. 4 the LD-(T − S) signal amplitude is plotted against the T − S signal amplitude, both recorded as a function of the microwave power. The values of $\alpha_{x,y}$ were obtained by a linear least-square fit through the data points at low microwave power, where within the scatter of the data points the R-value does not change with the microwave power. In this way, we found for both mutant RCs an orientation of the transition moment of P (at 910 nm) of 17 ± 3° and 80 ± 5° with respect to the triplet y- and x-axis, respectively. These values are equal to those of native RCs and the carotenoidless RCs of *Rb. sphaeroides* R26 [13,19,20].

The LD-(T − S) spectra recorded at the $|D| - |E|$ and $|D| + |E|$ zero-field transitions, simultaneously with the corresponding T − S spectra, are shown in Fig. 5. The LD(T − S) spectra were normalized on the corresponding R-values at the long-wavelength band, to obtain the orientations of the other optical transition moments with respect to the triplet axes of ^{3}P. For the three most intense absorption bands in the T − S spectra, centered at ~ 808, ~ 818 and ~ 900 nm (cf. Fig. 3), the ratio of the LD-(T − S) signal to the T − S signal, and thus the orientation of the corresponding optical transition moments with respect to the triplet axes of ^{3}P, is approximately equal for the three RCs. From the near equality of all orientations we conclude that the mutations do not cause a significant change in the orientation of the triplet axes with respect to the RC coordinate system.

4. Discussion

4.1. The triplet state of the primary donor

For both mutants, involving a substitution of the leucine either at site L131 or at site M160, the zfs-parameters have changed compared to native RCs. Apparently, in the triplet state the two dimer-halves are to some extent coupled. The

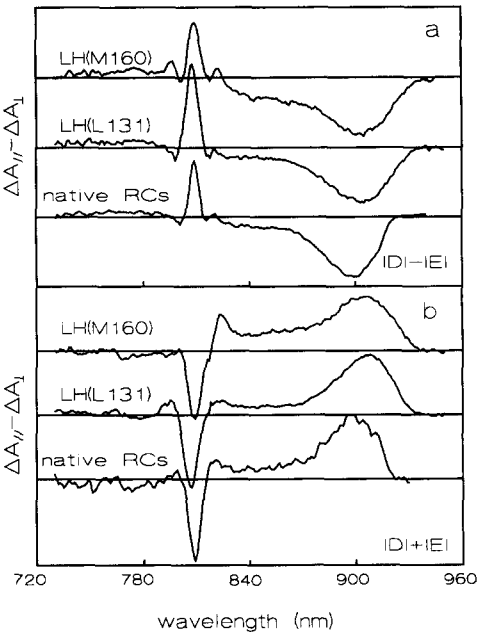

Fig. 5. LD-(T − S) spectra of reaction centers of the LH(M160) and LH(L131) mutants and of native reaction centers, recorded at a frequency within the $|D| - |E|$ (a) and $|D| + |E|$ (b) bands. See caption of Fig. 3 for the microwave frequencies chosen. For native reaction centers the microwave frequency was set at 467 (a) and 658 MHz (b).

small changes in zfs-parameters of ^{3}P of the mutants compared to those of native RCs are probably caused by the influence of the direct environment of P on the electronic structure of the triplet state. It has been reported by Nabredyk et al. [21] and Mattioli et al. [22] that the LH(L131) and LH(M160) mutants selectively produce a shift of the carbonyl frequencies of D_L and D_M, respectively. Apart from the small differences observed, the zfs-parameters of all RCs studied here are quite similar, and differ by practically the same ratio from those of monomeric ^{3}BChl *a* (Table 1).

For LH(M160) the unpaired electron of the cationic state P^+ was found to be localized for 85% on the D_L-half of the dimer, while for the LH(L131) mutant it was almost symmetrically delocalized [23]. If a similar effect of (de)localization over the BChl-halves of P would occur for the triplet state, a large change of the D and E values would be expected. Especially, the E value (xy-splitting) is sensitive to triplet delocalization. For example, a completely localized triplet state is expected to have an E value close to that of monomeric ^{3}BChl *a* in glassy solution (165–210 MHz), which is approximately twice the E values reported here for the RCs. Indeed, for the HL(L173) and HL(M202) heterodimer mutants of *Rb. sphaeroides*, which contain a BChl-BPh heterodimer, the values of E are almost twice that of native RCs, which phenomenon has been explained by complete localization of the triplet state on the BChl-half of the heterodimers [22,24,25]. Therefore, because the values of E of the mutants presently considered differ little from that of

native RCs, we conclude that the degree of triplet delocalization is almost equal for all RCs studied here. Similarly, results from a FTIR study by Nabredyk et al. [21] on the LH(L131) and LH(M160) mutants indicate that the dimeric character of the cationic state $P^{\cdot+}$ is conserved for both mutants.

4.2. The Q_Y-absorption region of B_L and B_M (780–820 nm)

When comparing the absorption spectra of the mutants and the native RCs a difference in transition energy from ~ 802 nm to ~ 800 is observed for the LH(L131) mutant, whereas for the LH(M160) mutant the transition energy of the 812 nm band shifts to ~ 814 nm. These slight changes in transition energy are ascribed to a change in the environment of the accessory B_L and B_M chromophores resulting from the mutation. Because the mutation sites M160 and L131 are close (within 5 Å) to B_M and B_L, respectively, mutation of site L131 is likely to cause a change in transition energy of B_L, whereas for a mutation of site M160 the Q_Y-transition of B_M may alter. Therefore, a mutation-induced change in transition energy of the accessory BChls is plausible if the 802 and 812 nm transitions have contributions of the Q_Y-transitions of B_L and B_M, respectively, which is in agreement with previous reports [26,27]. An interaction between the dimer BChls and the accessory BChls has to be taken into account to explain the band shifts in the T − S spectra and the orientation of the 812 nm transition (see below and Refs. [19,20]). Therefore the 812 nm transition, and likely also the 802 nm transition in the absorption spectrum, has a contribution from a transition of P. The couplings between P and $B_{L,M}$, however, are small compared to the intradimer interaction, and the largest contribution to the 812 and the 802 nm transitions are those of the B_M and B_L transitions, respectively.

A quantitative comparison of the T − S spectra of the mutant and native RCs, as regards the intensities and positions of the positive bands in the 780–820 nm region, cannot be made directly from the T − S spectra, since the positive bands are overlapping with the negative 802 and 812 nm ($S_1 \leftarrow S_0$) absorption bands, the latter being centered at slightly different wavelengths for the three RCs (Fig. 1). In principle, for obtaining the intensities and positions of the positive T − S signals, the positive part of the T − S spectrum may be deconvoluted with Gaussian bands while keeping the fit parameters of the negative part, i.e., of the singlet ground-state absorption spectrum, fixed, and normalizing the T − S spectrum on the amplitude of the long-wavelength band of the corresponding absorption spectrum. A deconvolution of the T − S spectrum of all three RCs, similar to that for the T − S spectrum of *Rb. sphaeroides* R26 [19,20], then contains three positive bands in the 780–820 nm region, located at ~ 818, ~ 808 and ~ 800 nm (not shown).

Table 2
The positions (in nm ± 0.1) of the absorption bands contributing to the T − S spectra and the angles (in degrees) between the corresponding Q_Y-transition moments and the x- and y-triplet axes of ^3P

Native RCs	LH(M160)	LH(L131)	T − S sign [1]	$\|\alpha_x\|$	$\|\alpha_y\|$
900	910	910	−	17 ± 3	80 ± 5
818	821	818	+	45 ± 5	45 ± 5
812	814	812	−	45 ± 5	45 ± 5
808	808	808	+	25 ± 5	70 ± 5
802	802	800	−	35 ± 10	65 ± 10
800	800	798	+	30 ± 10	65 ± 10

[1] The sign, + (or −), corresponds with a positive (or negative) signal in the T − S spectrum.

It was found that the difference between the three T − S spectra could be accounted for by differences in band shifts only, the relative intensities of the positive 818 and 808 nm bands being similar to those of the 812 and 802 nm bands in the absorption spectra, respectively. In this interpretation, we find for LH(M160) and LH(L131) a relatively large red shift of the 812 and 802 nm bands, respectively, compared to native RCs. Irrespective of the details of the deconvolution, the analysis confirms that for LH(L131) there is mainly a change in the 802/808 nm absorbance-difference signal, whereas for LH(M160) the main change takes place at 812/818, compared to native RCs. Previously, the 790–820 nm region of the T − S spectrum of *Rb. sphaeroides* was interpreted to reflect a shift upon ^3P formation to lower energies of the 812 and 802 nm transitions, to 818 and 808 nm, respectively [19,20]. The T − S spectra of the LH(M160) and LH(L131) mutants presented here confirm this interpretation.

For the Q_Y-bands in the 780–820 nm region, the values of $\alpha_{x,y}$ were obtained by a fit of the LD-(T − S) spectra using the fit parameters (position and width) used in the deconvolution of the corresponding T − S spectra. The results, which are essentially the same for the three species and rather insensitive to details of the deconvolution, are listed in Table 2. The orientations of the optical transition moments with respect to the triplet axes of ^3P are the same as obtained for *Rb. sphaeroides* R26 [19,20]. For all three RCs, we found with LD-ADMR that the positive 818 nm transition in the T − S spectrum has the same polarization as that of the 812 nm band in the absorption spectrum. Similarly, the polarization of the positive 808 nm band equals that of the negative 802 nm band. From the similarity in polarization, and taking into account the results from single-crystal EPR [23] and LD-absorption spectroscopy [26], we have concluded that the positive 818 and 808 nm bands in the T − S spectrum of *Rb. sphaeroides* R26 represent a shift to lower energies of the 812 and 802 nm bands upon ^3P formation [19,20]. Accepting the orientation of the triplet axes in the RC coordinate frame as found by Norris et al. [23], the polarization of the 812 and 818 nm transitions (~ 45° with respect to the in-plane triplet axes)

does not agree with that of an uncoupled Q_Y-transition of any of the BChls in the RC, which all are expected to be oriented at 20–30° and 70–60° with respect to the triplet y- and x-axis of ^3P. Therefore, to explain the orientations of both the 812 and the 818 nm transitions with respect to the triplet axes, we must assume that they are equally mixed with one or more other transition(s). It is unlikely that the higher-energy exciton transition of P is important in the admixture, as this transition is bleached upon ^3P formation, and would therefore give rise to a change in polarization of the positive 818 nm band, contrary to observation.

4.3. The effect of the mutations on the T − S spectrum

Because the positive signals at 808 and 818 nm are most likely caused by band shifts of the 802 and 812 nm absorptions, respectively, which must be largely ascribed to the accessory BChls, the T − S spectrum is not that of an isolated dimer, and reflects the changes in coupling between the dimer states and the accessory BChls going from a singlet-excited to a triplet-excited dimer.

In the following we assume that the mutations considered in this work affect the energy levels corresponding to the optical transitions of the chromophores by promoting hydrogen bonding to the 9-keto group [20] and/or by structural changes. Then, the changes in band shifts can be ascribed to a change in coupling of the electronic states of the dimer with those of the accessory BChls, due to a shift of the energy levels of the dimer, or to a direct influence of the mutation on the energy levels of the accessory BChls. If the dimer and the accessory BChls interact, both effects may cause a change in electronic composition of the Q_Y-transitions of the accessory BChls and the dimer-BChls, and lead to different band shifts in the T − S spectra of the different RCs. We do not consider second-order effects due to the possible admixture of oxidized and/or reduced states.

Comparing the T − S spectra of LH(M160) and LH(L131) with the spectrum of native RCs, the first mutant shows a 7 nm shift of the 814 nm band to 821 nm, accompanied by a shift of 6 nm of the 802 nm band to 808 nm, whereas for the latter mutant the 812 nm band shifts to 818 nm and the 800 nm band shifts to 808 nm. Accepting that the 802 (and 808) and 814 (and 821) nm transitions are mixed 'B_M' and 'B_L' transitions, respectively, mixed with P-transitions, the T − S spectrum of LH(M160) might be explained to first order by a mutation-induced change in energy of the electronic states of B_M, resulting in a different coupling of the electronic states of B_M to ^1P and ^3P compared to native RCs. The mutation at site L131 may cause a similar effect on B_L.

In addition, the bands that appear to be shifted in the absorption spectrum (812 to 814 nm for LH(M160), 802 to 800 nm for LH(L131)) are shifted somewhat more in the T − S spectrum than the corresponding bands for native RCs, viz. 7 and 8 nm for the mutants, and 6 nm for the native RCs. Although small, these differences in shift are outside the limits of error, and well reproducible. We attribute the additional small shift to an effect of the mutation on the energies of the dimer states. Such an effect is not unlikely, as ENDOR [28], FTIR [21] and resonance Raman [22] studies show changes in the properties of P for the two mutants. A change in the coupling of the states of the dimer-halves leads to a change in the degree of delocalization of the singlet/triplet excitations over the dimer-halves. Provided the interactions of the individual dimer-halves with the accessory BChls are different, this may lead to a change in band shifts of the 'B_L' and 'B_M' transitions in the T − S spectra. From an ADMR study on the 'heterodimer' mutants of *Rb. sphaeroides*, HL(M202) and HL(L173) [19,28], we have concluded that the B_M-D_L interaction is stronger than the B_L-D_L interaction, and that the B_L-D_M interaction is stronger than the B_M-D_M interaction. A somewhat larger shift of the 814 nm 'B_M' transition is expected if the triplet state is predominantly localized on D_L, whereas a larger band shift of the 804 nm 'B_L' transition is expected if the triplet state is predominantly localized on D_M, compared to the shifts of the 812 and 802 nm bands in native RCs. In this interpretation of the optical spectra, the triplet state of LH(M160) and LH(L131) is somewhat more localized on D_L and D_M, respectively, compared to native RCs. Such a change in triplet delocalization parallels qualitatively the degree of localization of the cationic state P^+ of the same mutants found with ENDOR spectroscopy by Rautter et al. [28]. These authors have concluded that for LH(M160) the unpaired spin density was almost completely localized on D_L, whereas for LH(L131) it was found to be symmetrically delocalized on both dimer-halves. Native RCs exhibit an intermediate case, both for the triplet state (as the T − S spectra suggest) and for the cationic state [28].

The relatively small difference in the above shifts for mutant and native RCs agrees with the comparable zero-field splitting parameters [19] and the LD-ADMR results for all RCs considered here. The equal polarizations of all optical transitions (Table 2) indicate that the mutations do not cause a significant change in the orientation of the triplet axes with respect to the RC coordinate system. Furthermore, the T − S spectrum of the LH(M160) mutant, for which it has been concluded that the cationic state is largely localized on D_L, is quite different from that of the heterodimer mutant HL(M202), which contains a $D_L BPh_M$ dimer instead of a BChl-dimer, for which the triplet state is almost certainly localized on the D_L-half of the dimer [29]. This spectral information and the observation of the same orientations of the optical transition moments (and of the triplet axes) leads us to conclude that the electronic compositions of the singlet states of the RC and ^3P are almost equal for the three RCs studied here, allowing for only small differences in coupling of the electronic (triplet and singlet) states, and in triplet delocalization.

Norris et al. [24] proposed that the EPR linewidth of $P^{\cdot+}$ is governed by the same factors that determine the *D* value of ^3P, in other words, that the degree of delocalization of the cation resembles that of the triplet state. However, the *D* values reported here are quite similar, whereas the ENDOR results on $P^{\cdot+}$ of the mutants indicate considerable difference in cation delocalization [27]. Accepting the conclusion of Rautter et al. [27], that the mutation at site M160 provokes a large change in delocalization of the unpaired electron of $P^{\cdot+}$ over D_L and D_M, these observations imply that the electronic composition of the triplet state and the cationic state is quite different.

5. Conclusions

For the LH(M160) and LH(L131) mutants of *Rb. sphaeroides*, we conclude from the small change in zero-field splitting parameters that the mutations cause no appreciable change in the electronic configuration of the triplet state compared to native RCs. From a comparison of the T − S and LD-(T − S) spectra, we conclude that the mutations cause only a small change in the coupling of the electronic states of the dimer-halves and the accessory BChls, and that the basic electronic composition of the singlet states and ^3P is the same for RCs of the LH(L131) and LH(M160) mutants and native RCs. The small differences in band shifts of the transitions in the Q_Y-region of the accessory BChls may be explained by only slight changes in triplet delocalization over the dimer-halves. The T − S spectra of the mutants confirm our earlier interpretation of the (LD-) T − S spectra of *Rb. sphaeroides* R26, where the positive bands in the Q_Y-region of the accessory BChls have been ascribed to shifts of transitions to lower energy upon ^3P formation [19,20].

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