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Biochimica et Biophysica Acta 1276 (1996) 229–238

BBA Biochimica et Biophysica Acta

The triplet state of the primary donor in reaction centers of the HL(L173) and HL(M202) heterodimer mutants of *Rhodobacter sphaeroides*

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Received 24 October 1995; accepted 14 June 1996

Abstract

The triplet state of reaction centers of the HL(L173) and HL(M202) mutants of *Rhodobacter sphaeroides*, whose primary donor is a bacteriochlorophyll-bacteriopheophytin (BChl-BPh) heterodimer instead of a BChl-BChl homodimer, has been studied with absorbance-detected magnetic resonance in zero-magnetic field. The zero-field splitting parameters of the triplet state of the two heterodimers are close to those of monomeric ^{3}BChl *a*, indicating that the triplet state is largely localized on the BChl-half of the heterodimers, but not identical, demonstrating that the two BChls of the native primary donor are inequivalent. The microwave-induced triplet-minus-singlet absorbance-difference spectra of the two mutant reaction centers differ in the Q_Y-absorption region of the BChls that are located close to the dimer. The latter difference is attributed to differences in interaction between the dimer-BChls and the two adjacent BChls for the two mutants.

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

1. Introduction

The reaction center (RC) of purple bacteria contains two polypeptides, L and M, symmetrically arranged around a near-C_2 symmetry axis. Four bacteriochlorophylls (BChl), two bacteriopheophytins (BPh), two quinones (Q_A and Q_B, accepting an electron in that order) and a carotenoid are non-covalently bound to the L and M poplypeptides. Two BChls, denoted D_L and D_M, are closely spaced and approximately plane-parallel with overlapping π-electronic orbitals of the pyrrole rings I of the two BChls, forming a BChl-dimer [1–3]. Two BChls, B_L and B_M, are located close to the dimer, separating it from the two BPhs, BPh_L and BPh_M. The subscripts L and M refer to the polypeptides to which the chromophores are bound.

The dimer in the RC serves as primary electron donor, P, from which, after photo-excitation, an electron is transferred to the primary acceptor, BPh_L, leading to the charge-separated state $P^{\cdot+}BPh_L^{\cdot-}$. Subsequently, stabilization of charge separation occurs by electron transport to the secondary acceptors, the quinones, forming the relatively long-lived state $P^{\cdot+}Q_A^{\cdot-}$. Despite the near-C_2 symmetry of the RC, electron transport to BPh_L is much more efficient than that to the symmetry-related BPh_M chromophore [4], a phenomenon that has received much attention in the past few years.

Site-directed mutagenesis has proved to be a useful tool in studying the influence of the amino acids on the electron-transport properties of the RC [5]. Recently, with the aid of site-directed mutagenesis, either one of the two BChl-halves of the dimer in the RC of *Rb. sphaeroides* was changed into a BPh [6], by changing into a leucine one of the histidines binding to the central magnesium atom of a BChl on the opposite side of the dimer, His L173 for D_L and His M202 for D_M. The dimer in these mutants is a

Abbreviations: ADMR, absorbance-detected magnetic resonance; T–S, triplet-minus-singlet; RC, reaction center; P, primary electron donor; BChl, bacteriochlorophyll; BPh, bacteriopheophytin; zfs, zero-field splitting.

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PII S0005-2728(96)00082-5

BChl-BPh 'heterodimer', in contrast to the BChl-BChl 'homodimer' in native RCs. The two mutants, containing either a $D_M BPh_L$ or a $D_L BPh_M$ heterodimer, are denoted HL(L173) and HL(M202), respectively.

For both heterodimers, an enhanced asymmetry in the electronic states of the dimer compared to native RCs has been observed. The unpaired electron of $P^{\cdot+}$ was found to be localized on the BChl-half in both heterodimers [7], which can be understood because of the lower energy of the HOMO of BPh compared to that of BChl. The first excited state of the heterodimers presumably has a substantial intradimer charge-transfer character, as concluded from absorption and Stark spectroscopy [6,8], and from transient absorption measurements on a sub-picosecond time scale [9]. Despite the presence of an extreme asymmetry in the electron distribution over the dimer-halves, results of time-resolved absorbance-difference measurements indicated that for both heteromutants electron transport is still mediated via the L-branch [9].

For RCs of *Rb. sphaeroides* the $|D|$-value of the triplet state of P, ^3P, is reduced compared to that of BChl in vitro. This reduction was attributed to the admixture of intradimer triplet charge-transfer states, $^3(D_L^+ D_M^-)$ and $^3(D_L^- D_M^+)$, into ^3P [10]. With EPR, the zero-field splitting (zfs-) parameters of the triplet state of the heterodimer of the HL(M200) mutant of *Rb. capsulatus*, which is presumably similar to the HL(M202) mutant of *Rb. sphaeroides*, and those of the HL(L173) mutant of *Rb. sphaeroides*, were found to be equal, and close to those of the triplet state of monomeric BChl *a* [11,12]. The monomer-like $|D|$ and $|E|$ values for the heterodimer triplet states were explained by complete localization of the unpaired electron density of ^3P on the BChl-half of the corresponding heterodimers, without admixture of charge-transfer states, suggesting that in the triplet state of the heterodimers the intradimer charge delocalization has virtually vanished [11–13].

The absorbance-difference spectrum of RCs with and without ^3P, labeled the Triplet-minus-Singlet (T − S) spectrum, gives information on the interaction of P with its chromophore environment and on differences between the intradimer interactions in the triplet state and in the singlet ground state. For RCs of purple bacteria it has been shown that the interactions between P and the two accessory chromophores, B_L and B_M, are altered when P is excited to its triplet state, leading to features in the T − S spectra that have been interpreted as due to a change in dipole–dipole coupling between the Q_Y-transitions of the accessory BChls and those of the dimer-BChls upon ^3P formation [14–16]. Comparison of the T − S spectra of RCs of the heterodimer mutants with those of native RCs can give more information about these intermolecular interactions.

In this work we present an investigation of the triplet states and the T − S absorbance-difference spectra of the HL(L173) and HL(M202) heterodimer mutants of *Rb. sphaeroides* with the aid of Absorbance-Detected Magnetic Resonance (ADMR) in zero-magnetic field. With this double-resonance method the triplet state of the RCs of the mutants could be unequivocally assigned to the primary donor. The zfs-parameters of the two mutants differ significantly, indicating that the two dimer-halves, D_L and D_M, are not equivalent. From a comparison of the microwave-induced T − S absorbance-difference spectra of the heterodimer mutants, we conclude that the two dimer-halves have a different interaction with B_M and B_L.

2. Materials and methods

The procedure for constructing the mutants and the procedure of isolation of the reaction centers have been described elsewhere [6]. Glycerol (65% v/v) and sodium ascorbate (10 mM) were added to the reaction center solutions. The optical density of the mutant and native reaction center samples was 0.1–0.2 and 0.3, respectively, measured at room temperature at 800 nm in a 2-mm perspex cuvette. The samples were illuminated during freezing for prereducing the first acceptor quinone. All experiments were carried out at approx. 1.5 K.

The ADMR set-up was similar to that described previously [17]. The resolution of the monochromator was set at 3–4 nm. Microwaves from a microwave sweep oscillator (HP8350B with HP8352A plug-in) were fed into a helix placed around the sample cuvette. The change in transmittance, caused by amplitude modulation (311 Hz) of the microwaves, was phase-sensitively detected by a lock-in amplifier (Stanford SR 510). The transmittance itself was simultaneously recorded. For enhancing the signal-to-noise ratio when recording the microwave-induced absorbance-difference spectra of the heterodimer mutants, a frequency modulation of about 100 kHz was used for sweeping rapidly through the entire ADMR line.

3. Results

3.1. Low-temperature absorption spectra

The Q_Y-($S_1 \leftarrow S_0$) absorption spectra of RCs of the HL(M202) and HL(L173) mutants are compared with that of the native RCs in Fig. 1. The spectra are normalized on the amplitude of the absorption at 750–760 nm. The absorption spectra of the mutants are similar to those reported previously [6,8]. For the RCs of the mutants, the long-wavelength band (~ 830–950 nm), attributed to the $S_1 \leftarrow S_0$ absorption of P, shows a considerable broadening, compared to the absorption band of native RCs. The broadening is strongest for RCs of the HL(M202) mutant, for which the long-wavelength absorption extends to about 1000 nm (not shown, see Ref. [8]).

In the 780–820 nm region of the absorption spectra of the three RCs, where the Q_Y-absorptions of B_L and B_M

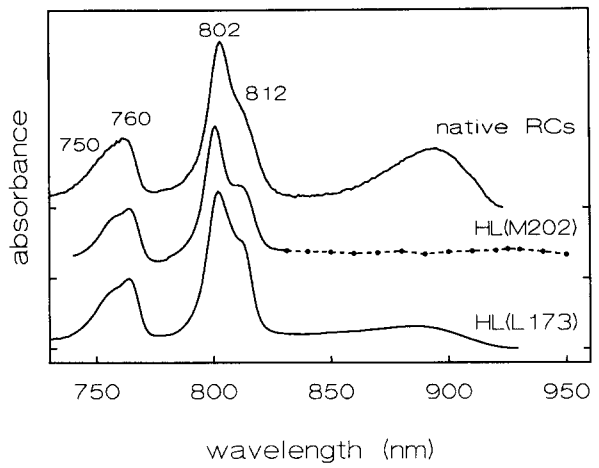

Fig. 1. The Q_Y-region of the absorption spectra of reaction centers of *Rb. sphaeroides* and of the HL(M202) and HL(L173) mutants. In the 730–830 nm region, the spectrum of HL(M202) was sampled with a step size of 1 nm, whereas in the 830–960 nm region a step size of 10 nm was used (indicated by the dots).

contribute, small differences are observed in the relative intensities of the 802 and 812 nm bands, and in their wavelength of maximum absorption (the 812 nm band shows up as a shoulder of the 802 nm band).

Despite replacement of a BChl by a BPh in the RCs of the heterodimer mutants, the shape of the Q_Y-absorptions of the BPhs, BPh_L and BPh_M, in the 730-770 nm region has not changed significantly, indicating that for the mutants no observable extra BPh-absorption is present in this region compared to native RCs.

In the Q_X-($S_2 \leftarrow S_0$) region (500–700 nm), the intensity of the 610 nm shoulder is decreased for RCs of the HL(L173) mutant compared to native RCs (Fig. 2). In addition, there is an absorption band at 557 nm that is absent in the absorption spectrum of native RCs, and is well-separated from the peaks attributed to the Q_X-absorptions of BPh_L (545 nm) and BPh_M (535 nm). The absorption at 610 nm of native RCs has been ascribed to the Q_X-transitions of P and of B_M [18]. Its lower intensity for the HL(L173) mutant, and the concomitant appearance of the 557 nm band, suggests that the absorption at 557 nm is due to the Q_X-transition of the BPh of the HL(L173) heterodimer. Moreover, its transition energy is close to that of the Q_X-transition of BPh in vitro and in vivo (530–550 nm).

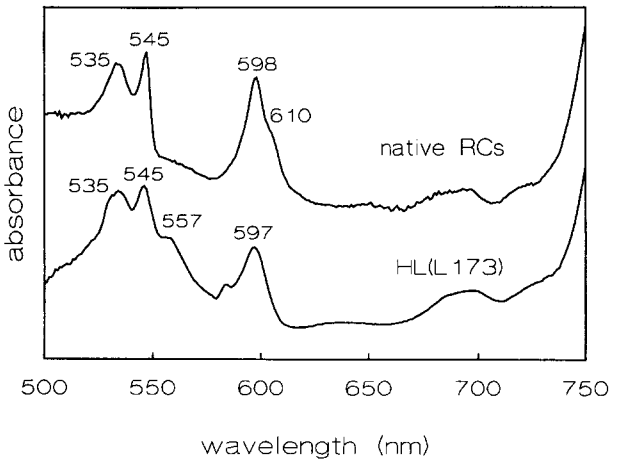

Fig. 2. The Q_X-region of the absorption spectra of native reaction centers of *Rb. sphaeroides* and the HL(L173) mutant.

For the HL(M200) heterodimer mutant of *Rb. capsulatus*, which is the analogue of the HL(M202) mutant of *Rb. sphaeroides* studied here, the Q_X-absorption of the BPh of the heterodimer has been observed at 548 nm [19]. The differences in position of the Q_X-transitions of the BPhs of the two heterodimers, $D_L BPh_M$ and $D_M BPh_L$, may reflect an electronic asymmetry or structural differences of the two dimer (BPh) halves. Similar differences have been observed for the transition energy of the heterodimer BPh-anion band of the two heterodimer mutants of *Rb. sphaeroides*. This band appears within 0.3 ps after excitation of P, and has a lower transition energy for HL(L173) than for HL(M202) [9].

3.2. Zero-field splitting parameters of ^3P

For determining the zfs-parameters of the triplet state in RCs of the mutants and the native RCs, the transmittance was recorded as a function of the microwave frequency at a detection wavelength within the long-wavelength absorption band of P (Fig. 3).

For RCs of the HL(M202) mutant, zero-field transitions were found at 421 MHz and around 800 MHz at a detection wavelength of 930 nm, and are ascribed to the $|D|-|E|$ and $|D|+|E|$ transitions, respectively. The $|D|+$

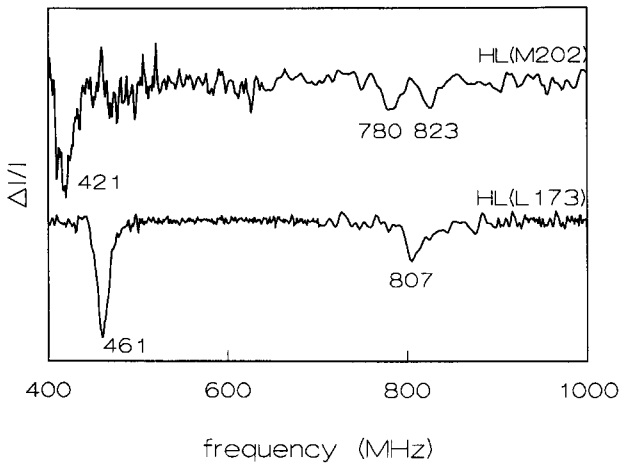

Fig. 3. The change in transmittance as a function of the microwave frequency for reaction centers of the HL(M202) and HL(L173) mutants of *Rb. sphaeroides*. The detection wavelength was set at 930 and 875 nm, respectively. The step size was 1 MHz (400–700 MHz) and 2 MHz (700–1000 MHz). The spectra of HL(L173) were averaged 5 times, whereas the spectra of HL(M202) were averaged 8 (400–700 MHz) and 16 (700–1000 MHz) times.

$|E|$ transition seems to be split into two transitions, centered at 780 and 823 MHz, but the signal-to-noise ratio was too low, even after extensive signal averaging, for drawing a definitive conclusion. The amplitude of the absorbance-difference signal was approx. 40–50 times lower than for the native RCs, after correction for the difference in optical density.

The zero-field transitions of RCs of the HL(L173) mutant, recorded at a detection wavelength of 875 nm, are centered at 461 MHz ($|D| - |E|$) and 807 MHz ($|D| + |E|$). For this mutant the amplitude of the absorbance-difference signal was significantly higher than for the HL(M202) mutant, yet a factor of about 20 lower compared with native RCs.

The resonance frequencies observed for the mutants did not change within the experimental error when varying the detection wavelength inside the long-wavelength absorption band (820–950 nm). Because for the RCs of the two mutants, just as for native RCs, a difference in transmittance is observed at the absorption of P, the above-mentioned zero-field transitions can be attributed unequivocally to the triplet state of P of the heterodimer mutants. The present $|D|$ and $|E|$ values are close to those determined by EPR in an external magnetic field for the heterodimer mutants HL(M200) of *Rb. capsulatus* [12] and HL(L173) of *Rb. sphaeroides* [11], viz. 630 and 180 MHz, respectively, for both mutants. With ADMR in zero-field, however, we observe a significant difference between the zfs-parameters of the two mutants of *Rb. sphaeroides*. The $|D|$ and $|E|$ values are collected, with those of ^{3}P in native RCs and monomeric ^{3}BChl *a* and ^{3}BPh *a* in vitro, in Table 1.

3.3. Microwave-induced T − S spectra

For the RCs studied, the application of resonant microwaves decreases the triplet lifetime, resulting in an enhanced singlet ground-state population. The absorbance-difference spectrum, obtained by modulation of the microwaves set at a resonant frequency of ^{3}P, reflects therefore the absorption of the RCs with P in its triplet state minus that with P in its singlet ground state (T − S spectrum).

Table 1
Zero-field splitting parameters (in MHz) of ^{3}P in the reaction center and monomeric ^{3}BChl *a* and ^{3}BPh *a* in vitro

	$\|E\|$	$\|D\|$	
Native RCs	96	563	this work
HL(M202)	191 ± 10	611 ± 10	this work
HL(L173)	173 ± 3	633 ± 3	this work
BChl *a*	165	680	[20] [a]
	207	696	[21] [b]
PhB *a*	138	795	[21] [b]

[a] In toluene/pyridine.
[b] In a biosynthesis precursor protein.

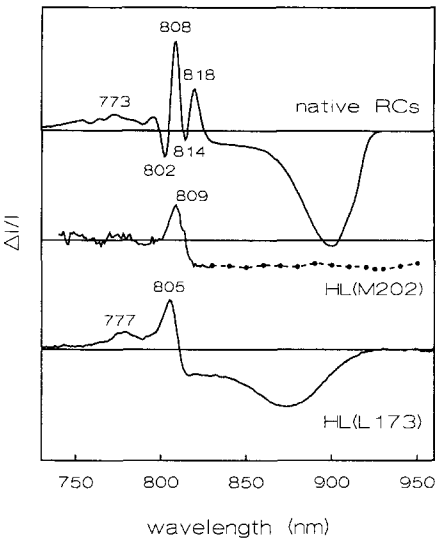

Fig. 4. Microwave-induced absorbance-difference spectra in the Q_Y-region. The microwave frequency was set at 467 MHz (native RCs), 420 MHz with a width of 7 MHz (HL(M202)) and 461 MHz with a width of 7 MHz (HL(L173)). For the dots, see caption Fig. 1.

The T − S spectra of native RCs and the two heterodimer mutants, recorded at a frequency within the $|D| - |E|$ transition, are depicted in Fig. 4. The T − S spectra at the $|D| + |E|$ transition are similar, with a lower signal-to-noise ratio (not shown). For all three RCs the long-wavelength band shows up as a negative signal in the T − S spectra (increase in ground-state absorption), having approximately the same shape as the long-wavelength band in the corresponding ground-state absorption spectra (see Fig. 1).

The T − S spectra contain positive signals in the wavelength region of the Q_Y-absorption of the accessory BChls (780–820 nm). For native RCs, sharp positive signals are centered at 808 and 818 nm, overlapping with negative signals at 802 and 814 nm. These features have been interpreted as band shifts [22,23]. For the HL(L173) mutant, a positive signal is centered at 805 nm, whereas the T − S spectrum of the HL(M202) mutant shows a positive signal at 809 nm. To parametrize the changes in the 780–820 nm region occurring upon ^{3}P formation with respect to the ground-state absorption spectrum (Fig. 1), first the absorption spectrum of each species was deconvoluted with Gaussian bands (not shown). The resulting parameters (band position, intensity and width) then were used to represent the 'singlet' contribution to the T − S spectrum (which, apart from the sign, equals the absorption spectrum). The 'triplet' contribution to the T − S spectrum (its positive part in Fig. 4) was similarly deconvoluted, the absorption and T − S spectrum being normalized on the intensity of the long-wavelength band. Such Gaussian deconvolutions of the 770–830 nm region of the T − S spectra are shown in Fig. 5, and the fit parameters are listed in Table 2.

Table 2
Fit parameters of a Gaussian deconvolution of the absorption T − S spectrum in the 770-830 nm region of native-, HL(L173) and HL(M202) reaction centers

Native reaction centers			HL(M202)			HL(L173)		
12233 (817.5) [a]	10.9 [b]	140 [c]	12336 (810.6)	14.1	150	12321 (811.6)	12.9	150
12277 (814.5)	−10.9	140	12324 (811.4)	−11.0	150	12318 (811.8)	−12.7	150
12391 (807.0)	23.0	140	12501 (799.9)	22.2	150	12457 (802.8)	23.0	150
12437 (804.1)	−23.0	140	12502 (799.9)	−20.9	150	12468 (802.1)	−19.7	150
12486 (800.9)	9.3	140				12550 (796.8)	3.1	150
12706 (787.0)	1.3	200	12720 (786.2)	0.6	200	12730 (785.5)	1.5	200
12915 (774.3)	1.6	200	12930 (773.4)	0.8	200	12897 (775.4)	1.8	200

[a] Position of the Gaussian band in cm^{-1} (in nm).
[b] Relative amplitude.
[c] Widths (FWHM) in cm^{-1}.

The Gaussian deconvolution of the T − S spectrum of native RCs (Fig. 5a) shows three positive bands at ~ 818, ~ 807 and ~ 801 nm, and two negative bands at ~ 804 and ~ 814 nm corresponding to the ground-state absorption. The widths of the three Gaussian bands in the 'triplet' part of the T − S spectrum were assumed to be equal to the (identical) widths of the two Gaussian bands in the 'singlet' part (the absorption spectrum). In other words, the positive bands are regarded as band shifts, a notion that is supported by the linear-dichroic T − S spectrum of *Rb. sphaeroides* [22,23]. In the 740–790 nm region four positive bands are needed for a good fit, but these relatively weak features, composed of the BPh-absorptions, vibronic transitions and triplet-triplet transitions, will not be discussed further.

For the HL(M202) mutant, the 790–820 nm region of the T − S spectrum does not show features comparable to the band shifts in the T − S spectrum of native RCs (Fig. 5b). In contrast, upon ^3P formation there is a sizeable increase of dipolar strength at ~ 811 nm, and some increase at 800 nm, with respect to the ground-state absorption (Table 2). For the HL(L173) mutant, a similar deconvolution is depicted in Fig. 5c, showing that also for this mutant, the 802 and 812 nm bands do not shift appreciably upon triplet formation. An increase of absorption at 803 nm and at shorter wavelength (about 797 nm) is observed. The band at about 797 nm could represent a non-Gaussian shape of the positive 803 nm band, or it may be the analogue of the positive 801 nm band in our fit of the T − S spectrum of native RCs. The 812 nm band in the absorption spectrum is almost completely cancelled by a positive band, indicating that the 812 nm band does not shift, and that its intensity does not change, upon triplet formation.

For all three T − S spectra, the negative intensity at lower energies is due to the high-energy wing of the long-wavelength band. It is represented by a very broad Gaussian profile.

The region of the Q_X-absorption of BChl and BPh (500–750 nm) in the T − S spectrum of the HL(L173) mutant is compared with that of native RCs in Fig. 6. For RCs of the HL(M202) mutant, we were not able to observe an absorbance-difference signal in this wavelength region owing to the very low triplet concentration, resulting in a poor signal-to-noise ratio. For the HL(L173) mutant the increase in ground-state Q_X-absorption between 600–650 nm (observed as a dip in a broad positive absorption) is smaller, and its width larger, than for native RCs. The negative signal at 557 nm corresponds to the shoulder at 557 nm in the absorption spectrum. Since it appears upon modulation of the population of ^3P with resonant microwaves, this signal must be due to a BPh that is relatively close to the triplet-carrying molecule. Because for both RCs the BPhs with Q_X-absorption bands at 535 (BPh_M) and 545 nm (BPh_L) in the absorption spectrum show no absorbance difference in the T − S spectrum, the 557 nm band in the T − S spectrum of HL(L173), and therefore also that in its absorption spectrum, can be attributed with certainty to the BPh-half of the heterodimer. By comparing the amplitude of the 557 nm band to that of the signal of the long-wavelength band at 875 nm, a net decrease of oscillator strength upon ^3P formation of about 10% was estimated.

4. Discussion

4.1. The absorption of the heterodimers

The optical transitions of the BChl-dimer in native RCs are usually described in terms of basis states composed of localized $\pi\pi^*$ and charge-transfer excitations (Fig. 7). In this description linear combinations of the Q_Y-($\pi\pi^*$) excitations, the so-called exciton transitions P(−) and P(+), give rise to transitions located at ~ 900 and ~ 812 nm, respectively [15,24]. (The nomenclature expresses that for two equivalent dimer-halves the two excited states would correspond to the antisymmetric and symmetric linear combinations.) To the pure exciton states, however, some 20% intradimer charge-transfer character, $D_L^+D_M^-$ and $D_L^-D_M^+$, is probably admixed [25–27]. The energy of $D_L^+D_M^-$ is thought to be lower than that of $D_L^-D_M^+$, and its

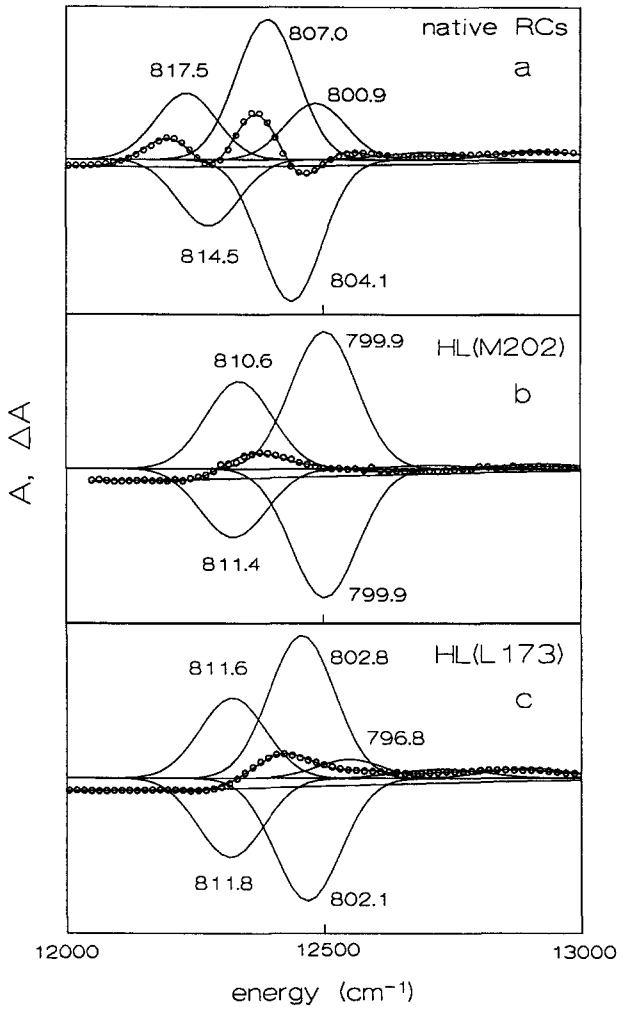

Fig. 5. Gaussian deconvolution of the microwave-induced T−S absorbance-difference spectra in the 770–830 nm region. A broad negative band, centered at 12000 cm^{-1} for each spectrum, represents the tail of the long-wavelength band. Each T−S spectrum is normalized at the intensity of the long-wavelength band of the corresponding absorption spectrum in Fig. 1. The scale of the vertical axis is equal for the three T−S spectra. The positions of the Gaussians in nm are indicated.

contribution to the P(−) transition accordingly larger than that of the latter state [28].

The broadening of the Q_Y-absorption of P observed when a BChl homodimer is changed into a BChl-BPh heterodimer, has been ascribed to enhanced electron-phonon coupling. This coupling becomes stronger with the increase of intradimer charge-transfer $BChl^+BPh^-$ contribution to S_1, which results from the lower HOMO and LUMO energy levels of BPh compared to those of BChl [29]. The difference between the $D_L BPh_M$ (HL(M202)) and $D_M BPh_L$ (HL(L173)) heterodimers, concerning the shape and position of their long-wavelength absorption, is presumably caused by an energy of the charge-transfer configuration $D_L^+ BPh_M^-$ lower than that of $D_M^+ BPh_L^-$ [28]. For a further discussion on the relative energies of the charge-transfer and localized $\pi\pi^*$ states we refer to Refs. [8,28,30].

In the heterodimers, as compared to native RCs, the contribution of exciton transitions to the absorption is thought to be diminished relative to the $BChl^+BPh^-$ charge-transfer contribution. From the linear- and circular-dichroic absorption spectra of the HL(M200) heterodimer mutant of *Rb. capsulatus*, it has been concluded that the contribution of the P(+) transition to the absorption at 812 nm is substantially lower than in native RCs, or even absent [19]. In view of the relatively high electronic asymmetry observed for all heterodimers, we may expect a similar decrease of the P(+) contribution for the heterodimer mutants of *Rb. sphaeroides* studied here.

In the absorption spectrum of both mutants, the Q_Y-region of the BPhs shows no evidence for an isolated BPh-absorption of the heterodimer. Similarly, in the T − S spectra no negative signal attributable to BPh is observed in the BPh Q_Y-region. This implies that the BPh-half of the heterodimer, when in its first excited singlet state, must interact strongly with the BChl-half of the heterodimer, leading to the absence of a pure BPh Q_Y-transition. The Q_X-absorption of the BPh-half of the heterodimer of the HL(L173) mutant (at 557 nm) and of the heterodimer of the HL(M200) mutant of *Rb. capsulatus* [19] are sharp and well-resolved, indicating that the electronic states giving rise to the Q_X-transitions are more weakly coupled. This observation is supported by the relatively small change in intensity at 557 nm upon ^3P formation.

The similarity between the zfs-parameters of the mutants and of monomeric BChl *a* in vitro, suggests that the triplet state of the heterodimer is largely localized on the BChl-half of the dimer, and that charge-transfer contributions are much smaller than in ^3P of native RCs [11,12] (see also Section 4.2). The absence of charge-transfer

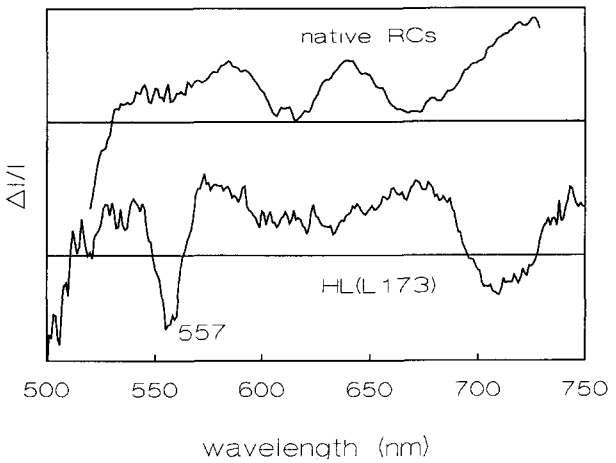

Fig. 6. Microwave-induced absorbance-difference spectra in the Q_X-region of native reaction centers and HL(L173). See also the caption of Fig. 4.

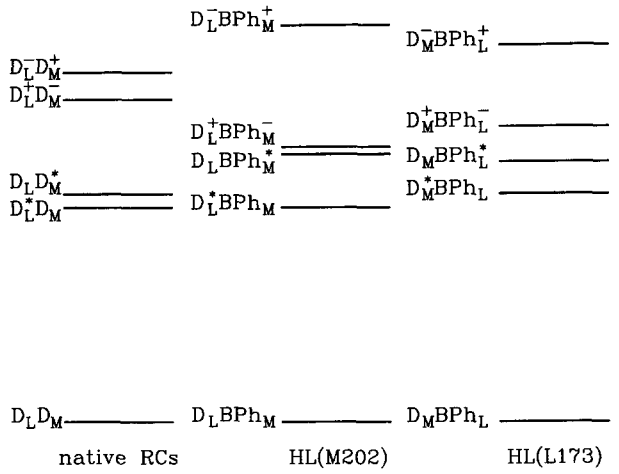

Fig. 7. Schematic energy level diagram of the excited configurations contributing to the S_1-state of the dimer, representing qualitatively the difference between $D_L D_M$, $D_L BPh_M$ and $BPh_L D_M$. Linear combinations of the $\pi\pi^*$ configurations, $D_L^* D_M$ and $D_L D_M^*$ etc., yield the P(+) and P(−) transitions. The energy of $D_L^+ BPh_M^-$ for HL(M202) might even be lower than that of the $\pi\pi^*$ states [28].

and/or excitonic coupling in the triplet state, would be expected to result in the appearance in the T − S spectrum of a positive absorption band of the monomeric BPh-half of the dimer, located in the 730–780 nm and the 530–550 nm region for the Q_Y- and the Q_X-transitions, respectively. In the T − S spectra of the HL(L173) and HL(M202) mutants we do not observe a strong positive absorption in the Q_Y-region of BPh. Upon ^3P formation the increase of intensity in the total 770–820 nm region is $20 \pm 5\%$ and $30 \pm 5\%$ for RCs of the HL(M202) and HL(L173) mutants, respectively, which is slightly less than the value of $\sim 35\%$ for native RCs. Thus, as in the ground-state absorption spectrum, the heterodimer BPh absorption is not, or only weakly, in evidence in the T − S spectra of the heterodimer mutants, perhaps because it is severely broadened.

From a recent X-ray study of both heterodimer mutants, Chirino et al. [31] have concluded that there are no significant changes in the positions of the BPhs of the heterodimers, compared to the corresponding BChls. Therefore, it seems unlikely that a loosening of the dimer structure or a heterogeneity in the protein environment of the heterodimer-BPh leads to a broadening of its absorption such that it is rendered unobservable. Moreover, this should lead to a broadening of the BPh Q_X-transition, contrary to observation.

If a significant electronic interaction between the BChl and the BPh of the heterodimers still exists, then broadening of the BPh Q_Y-absorption (and of all dimer Q_Y-transitions) may yet occur due to charge-transfer contributions to the triplet state. However, a comparison of the $|D|$ values of the heterodimer mutants with that of BChl *a* in organic solvents (Table 1) suggests that in the mutants the charge-transfer contribution to the lowest excited triplet state is at most 10% when using the dimer geometry of native RCs. This is probably much less than for the excited singlet state of the heterodimer mutants [28], and a broadening similar to that observed for the long-wavelength band in the singlet absorption spectrum seems therefore unlikely.

In any case, either the absence or the severe broadening of a BPh Q_Y-absorption in the 'triplet' part of the T − S spectrum can be explained only if there remains an interaction between the dimer-halves when the BChl is in the triplet state. In native RCs a similar problem arises. For RCs of *Rb. sphaeroides* and *Rps. viridis* a dimer Q_Y (in case of a localized triplet state), or two dimer Q_Y (triplet delocalization over the dimer-halves) $\pi\pi^*$ excitations are expected to appear in the 'triplet' part of the T − S spectrum between 775–830 nm [15,16], but such a transition, however, is not observed [22,23]. The nature of the interaction between the dimer-halves of ^3P is presently not understood [22,23].

The 660–680 nm band in the absorption spectrum of native RCs has been ascribed to the low-energy exciton Q_X-absorption, possibly with charge-transfer contribution to explain its large red-shift [24,25]. Following this interpretation, the broad signal between 600–650 nm in the T − S spectrum of Fig. 6 is attributed to a similar transition; its larger width is probably caused by a relatively large charge-transfer contribution. The shift to shorter wavelengths compared to native RCs, closer to the monomeric wavelength of BChl, may be caused by a weaker interaction between the electronic states of the dimer-halves compared to native RCs, leading to a large asymmetry in the contribution of BChl and BPh to the Q_X-components. Accepting the interpretation of the 660–680 nm band for native RCs, the negative 700–710 nm absorption in the T − S spectrum of HL(L173) is not related to this band. Possibly, it is due to the 1200 cm^{-1} Q_Y-vibronic transition of the BPh of the heterodimer, which is only weakly coupled to the BChl Q_Y-transition.

4.2. The zero-field splitting parameters and the yield of ^3P

The $|D|$ and $|E|$ parameters of ^3P of the heterodimer mutants differ considerably from those found for the native RC, and are close to those found for monomeric ^{3}BChl *a* in organic solution. The zfs-parameters are characteristic for the triplet state of BChl *a* rather than for that of BPh *a*, whose $|D|$ value is considerably higher than that of BChl *a* (Table 1). In agreement with the interpretation of EPR results [11,12], we conclude therefore that for both mutants the triplet state is largely localized on the BChl-half of the heterodimer, irrespective of which side of the dimer has been changed into a BPh. With ENDOR spectroscopy, a similar localization as reported here for the triplet state has been observed for the unpaired electron in $P^{\cdot+}$ of the heterodimers [7].

The small differences in the $|D|$ and $|E|$ values for the two heterodimer mutants might reflect differences in the protein environment in the vicinity of the BChl molecules of the dimer or structural differences between the BChl-halves of the dimer [32,33].

On the other hand, the lower $|D|$ value of the triplet state of the HL(M202) mutant may also be due to a relatively larger charge-transfer contribution, compared to the triplet state of HL(L173). In fact, the $|D|$ value of the triplet state in RCs of HL(M202) is lower than ever reported for monomeric ^{3}BChl *a*, whereas the $|D|$ value of the triplet state of HL(L173) is closer to that of ^{3}BChl *a* in vitro. The triplet state of the HL(M202) mutant then is expected to have some intradimer charge-transfer $^3(D_L^+BPh_M^-)$ character, and that of the HL(L173) mutant some $^3(D_M^+BPh_L^-)$ character. The values of $|D|$ observed for the mutants suggest that the participation of $^3(D_L^+BPh_M^-)$ in ^3D_L exceeds that of $^3(D_M^+BPh_L^-)$ in ^3D_M; accordingly, the energy of the former charge-transfer configuration should be lower than that of the latter. This explanation parallels that given for the differences between the absorption spectra of the two heterodimer mutants, which have been interpreted by assuming that the energy of $D_L^+BPh_M^-$ is lower than that of $D_M^+BPh_L^-$ (Fig. 7). Likewise, it has been suggested that the decrease in $|D|$ value of ^{3}P in native RCs of *Rb. sphaeroides* and *Rps. viridis* compared to the corresponding values for the triplet state of monomeric ^{3}BChl *a* in organic solution, is (partially) due to charge-transfer contribution to the triplet state [10]. As the zfs-parameters of the heterodimer mutants are closer to those found for monomeric ^{3}BChl *a* than the zfs-parameters of the native RC, we may conclude that the contribution of charge-transfer states to the triplet state of P in the heterodimer mutants is less than for the native RC (Table 1), in contrast to the corresponding contributions suggested for the singlet states [28]. Norris et al. [13] proposed that the heterodimer of the HL(M200) mutant of *Rb. capsulatus* in the triplet state, in contrast to its singlet state, behaves as a pure monomer without any charge-transfer contribution to the triplet state. However, the absence of a well-defined absorption of the BPh-half of the heterodimer in the T − S spectra does not support this interpretation, as discussed in Section 4.1.

Repeated measurements showed that the T − S absorbance-difference signal of the mutants is drastically decreased compared to the that of native RCs. A lower signal intensity, by a factor of 12, has also been observed in EPR experiments on the HL(L173) mutant [11]. Probably, the triplet yield for the heterodimer mutants is much lower than for native RCs. The yield of initial charge separation is decreased by a factor of about two [9], but obviously this cannot explain the decrease in the ADMR signal by a factor of 20–50. In principle, a lower ADMR signal might be caused by a different population distribution over the triplet sublevels caused by differences in triplet sublevel populating probabilities and/or decay rates. The triplet decay rates of BChl *a* in organic solvents and in a number of RC and antenna complexes, however, do not differ significantly [34]. We may assume, therefore, that the triplet-sublevel decay rates to the ground state are similar for the mutants and the native RC. Because the high-field spin-polarization pattern for the triplet state of the heterodimer mutants has not changed drastically compared to that of native RCs, also the populating probabilities of the individual triplet sublevels should not differ much from those of native RCs [11,12], so that the above explanation for the low ADMR intensity seems unlikely. Possibly, for the heterodimer mutants in which the first acceptor quinone is prereduced, recombination to the ground state of the initially formed charge-separated state is much faster than the rate of triplet formation. Alternatively, the singlet-triplet conversion may be less efficient for the heterodimer mutants than for native RCs. Clearly, for resolving this problem additional experiments would be required, in which the triplet yield of the RCs of the mutants and the yield and lifetime of the radical pair precursor state are determined.

4.3. The coupling between the dimer and the accessory BChls

The absorption spectra of the native and the mutant RCs all show transitions at ~ 812 nm and ~ 802 nm. In view of the difference in transition energy and in dipolar strength normally found for BChl and BPh, and because of the difference in charge-transfer contribution (Section 4.1), it seems unlikely that the high-energy exciton P(+) transition for the heterodimers would absorb at the same wavelength (812 nm) and with a comparable intensity as in the absorption spectrum of native RCs. Therefore, the main contribution to this transition, and also that to the 802 nm transition, must be the Q_Y-transition of an accessory BChl. Previously, it has been concluded from photo-accumulation experiments on native RCs that the absorption at 802 and at 812 nm is dominated by the Q_Y-transitions of B_L and B_M, respectively [35]. This conclusion also holds for chemically modified RCs [36,37]. Some participation of a P transition in the 812 nm absorption has to be taken into account to explain the linear- and circular-dichroic absorption spectra of native RCs [15]. Such a participation is supported by the polarization of the 812 nm band in the absorption spectrum of the HL(M200) heterodimer mutant of *Rb. capsulatus*, which has changed drastically compared to that of native RCs [19]. The above results suggest that this participation does not lead to appreciable shifts and that the (uncoupled) Q_Y-transitions of B_M and B_L are located close to 812 and 802 nm, respectively.

The features in the 790–820 nm region of the T − S spectra reflect interactions between the triplet-carrying chromophore(s) of P and the accessory BChls. First we compare the T − S spectra of the two heterodimer mutants. The most prominent difference in the 800 nm region is the

change in absorbance at ~811 nm and ~802 nm for the HL(M202) and HL(L173) mutants, respectively (Fig. 5, Table 2). Because the 812 nm band in the absorption spectrum of native RCs is ascribed to B_M, not B_L, we ascribe the increase at 811 nm in the T − S spectrum of HL(M202) to an increase in dipolar strength of B_M only. This implies that there is an interaction between the singlet states of B_M and D_L, the latter being the triplet-carrying chromophore, which is apparently weakened upon triplet formation on D_L. Because the absorption of B_L at 802 nm changes relatively less than that of B_M, the interaction between D_L and B_L seems to be somewhat weaker than that between D_L and B_M. For RCs of the HL(L173) mutant we observe only a change in dipolar strength at 795–803 nm, indicating that the interaction between B_L and D_M, the triplet-carrying chromophore in this heterodimer, is stronger than the interaction between B_M and D_M.

The observation that D_M has a relatively strong interaction with B_L, and D_L with B_M, agrees with the structure of the RC. When considering dipole–dipole coupling between the Q_Y-transitions, relatively strong D_L-B_M and D_M-B_L interactions may result from the almost parallel alignment of the two Q_Y-transition moments of each pair. Additionally, the relative strength of the interactions may be influenced by direct D_L-B_M orbital overlap [38]. In any case, for observing an absorbance-difference signal in the T − S spectrum, the relevant energy levels of the different chromophores should be close enough to allow coupling of different excited configurations, since the interactions between the dimer-BChls and the accessory BChls are thought to be weak compared to the intradimer interaction. Because upon triplet formation the absorptions of the accessory BChls show up at approximately the same transition energy as in the ground-state absorption spectrum, participation of the excited configurations of the BPh of the heterodimer in the excited state concerned, presumably, is small.

A dipolar interaction between the BPh of the heterodimers and the accessory BChls might be thought to be the cause of the 'missing' positive BPh Q_Y-absorption (cf. Section 4.1). When considering the mutual orientation of the Q_Y-transition dipoles of the RC chromophores, a simple dipole–dipole coupling model predicts that a large positive B_M-absorption at ~812 nm appears in the T − S spectrum of the HL(L173) mutant upon triplet formation on the BChl-half (D_M) of the heterodimer, caused by intensity-borrowing of the Q_Y-transition of B_M from the Q_Y-transition of the heterodimer BPh-half. By analogy, the T − S spectrum of HL(M202) would show a large positive B_L-absorption at ~802 nm (due to a relatively strong dipole–dipole interaction with the BPh-half at the M-side of the heterodimer). This is entirely opposite to our observations, and we conclude that dipole–dipole interactions between the heterodimer-BPh and the accessory BChls are unimportant.

On comparing the 780–820 nm region of the T − S spectrum of native RCs with the corresponding region for the mutants, the former reveals more drastic changes upon excitation to the triplet state. Red-shifts of transition energies of ~45 cm^{-1}, perhaps in combination with changes in dipolar strength, take place upon ^3P formation (for a further discussion on the T − S spectrum of native RCs we refer to [22,23]. From a straightforward comparison with the heterodimer mutants, we might merely expect an increase in absorption at 802 and/or 812 nm for the T − S spectrum of native RCs, not a shift. The relative amplitude of the increase at 802 and 812 nm would then depend on the degree of delocalization of the triplet state over the dimer-halves. However, such an expectation implicitly rests on the assumption that the coupling of the excited singlet and triplet configurations of the dimer-BChls with the singlet configurations of the accessory BChls is comparable for the heterodimer mutants and native RCs. This assumption is probably not valid, since the coupling of the $\pi\pi^*$ and charge-transfer configurations of the dimer in the homodimer is undoubtedly stronger than that in the heterodimers because the energy levels of the two uncoupled dimer-chromophores are energetically closer for the homodimer. A change in the interaction between the dimer configurations may lead, indirectly, to a difference in character of all (singlet and triplet) transitions in the 780–820 nm region in which excited configurations of $B_{L,M}$ and $D_{L,M}$ participate. This seems to be supported by the changes in the linear- and circular-dichroic absorption spectra of the HL(M200) mutant of *Rb. capsulatus* [19] compared to native RCs. The circular-dichroic absorption spectrum in the 780–820 nm region suggests that the coupling between the excited states of the dimer and the accessory BChls is weaker for the heterodimer RCs. Also, the absence of band shifts in the T − S spectrum suggests a weaker coupling of the excited configurations of the RC chromophores compared to native RCs. A difference in the structure of the singlet ground state of P in the heterodimer mutants and native RCs is suggested by results of FTIR spectroscopy, which have shown that the vibrational frequencies of the heterodimers are different from those of the homodimer, and that the homodimer spectrum cannot be constructed from the two heterodimers [39].

5. Concluding remarks

From a comparison of the zfs-parameters of the HL(L173) and HL(M202) heterodimer mutants, which contain a BChl-BPh heterodimer, with those of native RCs, we conclude that in both heterodimers the triplet state is localized on the BChl-half of the dimer. The small differences in triplet zfs-parameters for the two heterodimer RCs may be due to variations in the charge-transfer contribution to the triplet state, reminiscent of what has been suggested for the differences in transition energy of the $S_1 \leftarrow S_0$ dimer transition [9].

Neither the absorption nor the T − S spectra of the heterodimer mutant RCs show a Q_Y-absorption of the BPh-half of the heterodimers, possibly because of charge-transfer coupling between the BPh and BChl. The absence of a separate BPh Q_Y-absorption of the heterodimer upon triplet formation on the BChl-half, however, remains puzzling, because one expects triplet formation on one dimer-half to abolish the charge-transfer interaction. It follows that the T − S spectra of the heterodimer mutants cannot be interpreted in terms of a simple model considering a delocalized singlet excitation and a localized triplet state, and that there still exists an interaction between the dimer-halves in the triplet state. A similar problem arises for the description of the T − S spectrum of native RCs in which there is no evidence for isolated dimer absorption upon ^3P formation, and only band shifts of transitions in the region of the accessory BChls occur [22,23].

The 802 and 812 nm bands in the mutants and in the native RCs are attributed mainly to transitions of the accessory BChls. The T − S spectra of the two heterodimer mutants show that B_L and B_M interact differently with the BChl-halves of the dimer, B_L having a stronger interaction with D_M than with D_L, and B_M a stronger interaction with D_L than with D_M. This difference in interaction between the two accessory BChls and the two dimer-halves could (partly) cause the differences in charge separation rates found for the two heterodimer mutants [9].

Acknowledgements

We thank Prof. J.H. van de Waals for his interest and helpful suggestions. This work was supported by the Foundation for Chemical Research (SON) financed by the Netherlands Organization for Scientific Research (NWO).

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