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Exciton interactions in the light-harvesting antenna of photosynthetic bacteria studied with triplet-singlet spectroscopy and singlet-triplet annihilation on the B820 subunit form of *Rhodospirillum rubrum*

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The pigment-protein complex, B820, isolated from the long-wavelength antenna (LH-1) of the photosynthetic purple bacterium *Rhodospirillum rubrum* was studied with polarized nanosecond laser spectroscopy. The polarized triplet (T)-singlet (S) spectrum was obtained at 77 K. The spectrum is significantly different from the T-S spectrum of monomeric BChl *a*, and can be explained by assuming that upon excitation the absorption band at 825 nm, which is due to a dimeric pair of BChl *a* molecules, disappears from the absorption spectrum, and is replaced by a monomer absorption band peaking at 809 nm. From the energy-dependence of the triplet yield, the high polarization of the bleaching, and the absence of singlet-triplet quenching we conclude that the B820 complex contains only one dimer of interacting BChl *a* molecules.

Introduction

The light-harvesting antenna forms a major component of the photosynthetic apparatus of plants and photosynthetic bacteria. It performs the double task of absorbing light energy, and transporting this energy to the photosynthetic reaction center (RC), where it is efficiently used to produce a charge separation [1,2]. Whereas the crystal structure of the bacterial reaction center has been resolved, the structure of the light-harvesting antenna is still unknown. Information concerning the organization of the antenna is derived from spectroscopic studies [3–6], electron microscopy [7], biochemical studies of the protein structure [8,9], and from theoretical considerations about the process of energy transport [10,11]. The light-harvesting antenna of all the photosynthetic purple bacteria share a number of prominent features: In all cases a core antenna (LH-1) exists, closely interacting with the RC, and in several species a more peripheral antenna (LH-2) is present in addition. In all species the LH-1 antenna consists of a pair of pigment binding polypeptides, α and β. In the purple bacterium *Rhodospirillum rubrum*, subject of this study, the LH-1 polypeptides have been purified and biochemically characterized [12]. A heterodimer of α and β binds two BChl *a* molecules, probably by highly conserved histidine residues in the structure, and one molecule of spirilloxanthin.

The pigment-protein complexes can be isolated from the membrane using a variety of detergents. The resulting (detergent) pigment-protein complexes retain most of their spectroscopic features, but are large aggregates (typically 50–400 kDa) of a hypothetical $\alpha\beta$-heterodimer [13,14]. In all the pigment-protein complexes of purple bacteria the absorption maximum of the chromophores is strongly red-shifted when compared with

Abbreviations: BChl *a* Bacteriochlorophyll *a*; OG, *n*-Octyl β-D-glucopyranoside; B820, subunit form of light-harvesting complex 1 from *Rhodospirillum rubrum* obtained by OG titration of light-harvesting complex 1; RC, reaction center; LH-1, Light-harvesting complex type 1.

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the spectrum of the chromophore in organic solvents. LH-1 absorbs typically at 870–880 nm, the exact wavelength being slightly dependent on the species and on the presence of carotenoids. This red-shift is accompanied by a significant amount of hyperchromism of the near-IR transitions, indicating that exciton coupling may play an important role in these complexes [15]. Scherz and Parson [15,17] have shown that artificial aggregates of BChl *a* can be prepared with spectroscopic features strongly resembling those of the antenna pigments. These experiments suggested that most of the red-shift and hyperchromism could be due to exciton interactions. A model for the antenna complexes (B850, B875) was proposed in which the pigments are organized as dimers, similar to the special pair of the reaction center of purple bacteria [17,18]. On the other hand, the photooxidation experiments performed by Rafferty et al. [16] have indicated that exciton interactions might be responsible for only a minor part of the red-shift of the antenna-pigments.

The discussion about the extent to which exciton interactions contribute to the spectroscopic properties is not just important for a better understanding of the organization/structure of the antenna, it is also important for our understanding of the coupling between the antenna and the reaction center. The last step in the light-harvesting process, the trapping in the reaction center, is relatively slow [19], probably because of the large distance between the reaction center pigments and the antenna pigments. It has been proposed that the observed inhomogeneity of the LH-1 antenna, the B896, contributes to the high efficiency of the light-harvesting process [20,4], by means of concentrating/localizing the excitations close to the reaction center. No B896 pigment-protein complexes have been identified/isolated so far, and this spectral species could well be due to a specific exciton state of the LH-1 antenna.

The major problem in comparing the results from model systems with those obtained from isolated pigment-protein complexes has been that the latter are large aggregates containing at least 8–24 pigment molecules [13,14], in which the arrangement of the BChl molecules is strongly influenced by pigment–protein and protein–protein interactions. Recently, Miller et al. [21,22] were able to isolate a small pigment-protein complex (its near-IR absorption band is located at 820 nm at room temperature and it is therefore called the B820 complex) by titrating carotenoid depleted antenna complexes of *Rs. rubrum* with the detergent OG. This subunit form has an absorption spectrum that is intermediate between that of the native antenna and the absorption spectrum of BChl *a* in organic solvents. The detergent titration is reversible, lowering the detergent concentration results in the formation of the B875 form. The B820 complex consists of (and can be reconstituted from) the two types of short polypeptide α, β and BChl *a* in a molar ratio of 1:1:2 [21–23]. B820-like complexes can be isolated from a variety of photosynthetic purple bacteria, and they all share a variety of spectroscopic properties [24]. From gel-filtration experiments a mass of approx. 55 kDa was obtained for the B820 complex [23], The sedimentation experiments performed in Ref. 26 gave similar results. The amount of detergent bound to the complex is unknown and, consequently, the experimentally determined molecular weight gives only an upper bound of $(\alpha\beta)_3BChl_6$ for the size of the complex. The B820 complex aggregates into the B873 form as soon as the detergent concentration is lowered slightly below the critical micellar concentration, indicating that the B820 complex can not exist without certain detergent micelles. Thus, the amount of bound detergent could well be rather high, and consequently the lower bound for the particle size could be as low as $(\alpha\beta)BChl_2$. In Ref. 25 a model has been proposed for the pigment orientation of the B820 complex. In this model the bacteriochlorophyll molecules form a dimer in which the Q_y-transition dipoles of the two Bchl *a* molecules make an angle of approx. 30°.

In this paper we have investigated the contribution of excitonic interactions to the absorption spectrum of the B820 complex. Using a short tunable laser-pulse, one of the pigments in the particle is converted into a triplet state. Consequently, the Q_y absorption is bleached and the influence on neighbors is strongly reduced. From the resulting transient absorption difference spectrum the contribution of exciton coupling to the absorption spectrum has been deduced. In a second experimental approach, the number of interacting pigments in the complex was deduced from the degree of singlet-triplet annihilation after an intense laser pulse.

Materials and Methods

n-Octyl β-D-glucopyranoside and BChl *a* were obtained from Sigma. *Rs. rubrum* B 820 complexes were prepared as described in Ref. 22 and stored in a dry state (lyophilized). For the 77 K measurements samples were prepared in 50 mM phosphate buffer (pH 7.8), 50% (v/v) glycerol, 1% OG. BChl *a* samples were prepared in 50 mM phosphate buffer (pH 7.8), 50% glycerol, 2% OG, by adding a small amount of concentrated BChl *a* (in acetone) to a 5% OG solution (other procedures tend to produce aggregates absorbing at longer wavelengths). For the low-temperature measurements the samples were cooled in an Oxford Instruments DN1704 liquid nitrogen cryostat, in acrylic cuvettes with a light-path of 1 cm. All low-temperature measurements were performed on crack-free samples.

Excitation light was produced by a short laser pulse

(FWHM 5–6 ns) from a Quanta Ray PDL2 dye-laser pumped by a Quanta Ray DCR2A Nd-YAG laser. To obtain homogeneous illumination of the sample, a diffuser plate was placed in the beam, 16 cm before the cuvette. For the polarized measurements a polarizer was placed between the diffuser and the cuvette, and in the measuring beam. The laser energy was measured using either a Gentec ED100 detector (single shot), or a Scientech 38-0101 energy monitor. Absorption and fluorescence were measured in a 90° setup. The light source used for the transient absorption measurement was a pulsed 150 W xenon arc lamp, which produced a light pulse with an electronically flattened top of approx. 300 μs [27]. Two 1/8 m monochromators were used, one between the lamp and the sample, the other between the sample and the detector. The spectral bandwidth of the transient-absorption measurements was 3 nm. The measuring light was detected by a Hamamatsu R928 photomultiplier. The signal from the photomultiplier was registered by a Gould 4500 transient recorder. The singlet excited state lifetime, and the pulse length/shape (used in the simulations) were measured with an avalanche-photodiode and a Tektronix 7912 AD transient recorder. Absorption spectra were recorded on a computer-linked Cary 219 spectrophotometer. Absorption spectra were recorded of the sample before and after the triplet measurements, and showed no detectable changes. Fits and simulations were performed on a Sun Sparc station 1.

Results

Fig. 1 shows the 77 K absorption spectrum of a B820 sample (lower trace), and of BChl *a* in 2% OG (upper trace). Under these conditions the BChl *a* is monomeric, as judged by the absorption spectrum, and

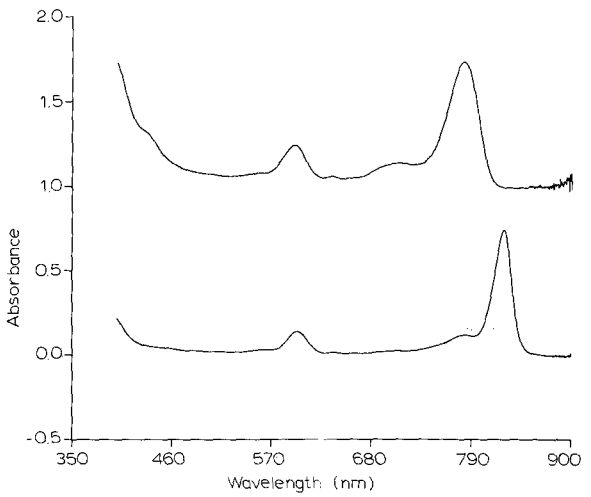

Fig. 1. 77 K Absorption spectrum of B820 in 1% OG, 50% glycerol (lower trace). Upper trace: offset by 1: 77 K absorption spectrum of BChl *a* in 2% OG, 50% glycerol (+sodium dithionite).

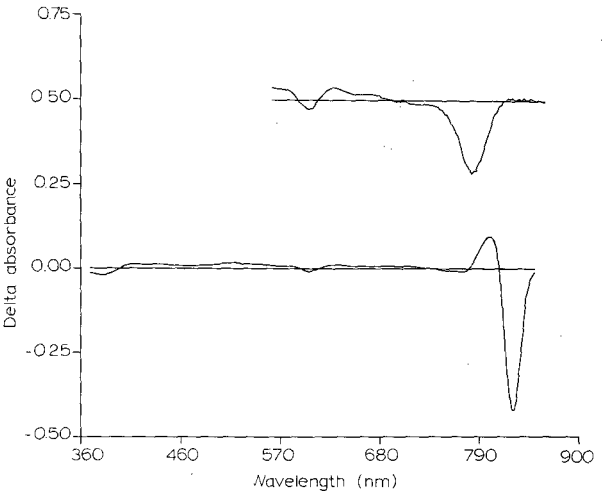

Fig. 2. Absorption difference spectrum of B820 after a 20 ml 5 ns laser flash at 609 nm, absorption spectrum as in Fig. 1, $A_{825} = 1.5$. The spectrum was measured with a resolution of 2.5 nm. Upper trace: absorption difference spectrum (multiplied with a factor of 5, offset by 0.5) of BChl *a* in 2% OG obtained with a 12 ml 5 ns laser flash at 605 nm, absorption spectrum as in Fig. 1, $A_{780} = 0.3$.

the polarization of the fluorescence [25]. The B820 near-IR absorption band, peaking at 825.5 nm, is significantly sharpened (FWHM 320 cm^{-1}), when compared with the spectrum of BChl *a* in 2% OG (FWHM 660 cm^{-1}). The B820 band is also sharper than that of the native LH-1 antenna [4]. Apart from the main 825 nm band there is a minor band, located at approx. 780 nm. In this particular sample, the 780 nm absorption is less intense than is typically observed in these preparations. As was shown in Ref. 25, this band is composed of some contamination with the fully dissociated 777 form, vibrational bands of the main 825 nm band, and a minor contribution of what is thought to be the high-energy exciton component of the dimer, located at about 795 nm.

Fig. 2 shows the absorption difference spectrum of the B820 complex recorded after a 5–6 ns, 605 nm laser flash at 77 K (lower trace). The difference spectrum is due to formation of the B820 triplet state. The amplitude of the difference spectrum was obtained by fitting the absorption transient from 20 to 150–300 μs after the laser-flash with a single exponential decay, and extrapolating the amplitude to time zero.

In the upper trace the same spectrum is shown of BChl *a* in 2% OG. Also in this case the changes are due to the formation of the BChl *a* triplet state [28–30], the lifetime of the BChl *a* triplet state was 135 μs (77 K, degassed samples).

In the near-IR region of the BChl *a* difference spectrum all that is observed is the bleaching of the 780 nm band. No triplet absorption bands are observed, which is in agreement with results obtained at room temperature in Ref. 28. In the visible region there is

some 'triplet' absorption, and a bleached band in the Q_x region. The bleached 780 nm band is notably sharper than that of the absorption spectrum, and slightly red-shifted. This is probably due to the fact that the excitation was performed at the (extreme) red side of the BChl *a* Q_x band. This is an indication that some of the broadening of the absorption band is due to the variability of the OG-micelles surrounding the pigments.

The near-IR region of the B820 difference spectrum is very different from that of BChl *a*, the main difference being the induced absorption band peaking at approx. 802.5 nm. The bleached band at approx. 827 nm appears to be caused by the bleaching of the 825 nm band. The minimum in the B820 difference spectrum is shifted to a slightly longer wavelength due to the appearance of the positive band. Around 780 nm the difference spectrum is slightly negative, probably due to triplet formation in the fully dissociated 777 form that is also excited by the laser-flash. In all experiments both the bleaching and the induced absorption band decay with a single exponential lifetime of 125–150 μs. All absorption changes were fully reversible at 77 K, the absorption spectra before and after recording the difference spectrum were identical. At room temperature significant sample degradation was observed, even in degassed samples.

In Fig. 3 the near-IR region of the B820 difference spectrum is shown, this time with excitation at 825 nm. The major difference between these data and those obtained after excitation at 605 nm is that in this case the absorption changes in the 700–780 nm region are absent, showing that these features are indeed due to a pigment that is not excited at 825 nm, most likely the 777 form.

The absorption transients shown in Fig. 3B demonstrate the high polarization of the bleaching of the 825 nm band. The curves obtained using high-energy excitation (0.38 mJ/cm^2) have a *P*-value of 0.27; from the low energy (0.012 mJ/cm^2) traces a *P*-value of 0.41 ($\pm$0.02) was calculated.

In order to be able to measure polarized absorption changes quantitatively, the laser pulse must be of low intensity and homogeneous, to prevent (local) saturation effects. Other factors that can disturb polarization measurements are: depolarization by scattering in the sample, (strain)birefringence of the cryostat/cuvette windows, imperfect overlap between the laser pulse and the measuring beam, and (at very high intensities) two-photon processes. To test our experimental set up we measured the polarization of the bleaching of BChl *a* in 2% OG. The obtained value ($P = 0.44 \pm 0.04$) is slightly lower than the value of 0.5 reported for the polarization of the fluorescence [25], but note that all the above-mentioned sources of experimental error cause a decrease in polarization (see the Discussion).

The near-IR region of the B820 difference spectrum is due to overlapping positive and negative bands. We tried to separate it into two components by successive additions of the 825 nm band of the absorption spectrum to the difference spectrum, with the aim of obtaining a difference spectrum with no bleaching for wavelengths longer than 830 nm. Fig. 4 shows that the difference spectrum obtained upon formation of the B820 triplet state is best explained by the disappearance of the band at 825 nm, and the formation of a

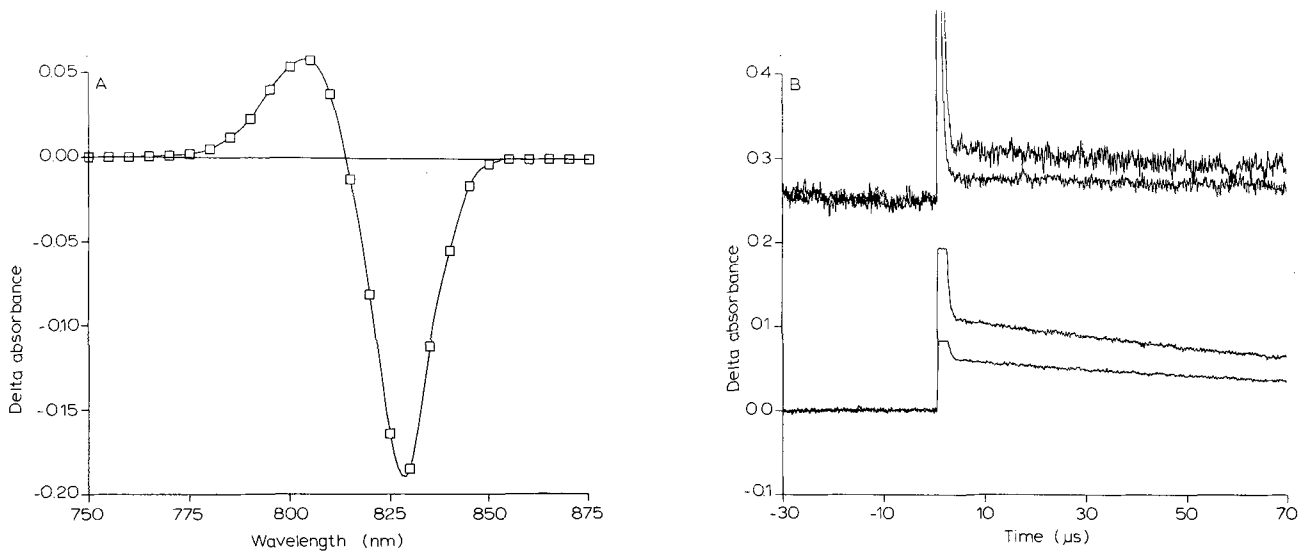

Fig. 3. Absorption difference spectrum of B820 in 1% OG, 50% glycerol, after a 0.5 mJ/cm^2 5 ns laser flash (polarization vertical) at 825 nm. A_{825} 0.5. Absorption measurements performed with the same polarization as the laser flash. (B) Polarized absorption transients measured at 827 nm. Lower curves: excitation with a 0.38 mJ/cm^2 5 ns pulse (each curve averaged 10 times). Upper curves: excitation with a 0.012 mJ/cm^2, multiplied by a factor of 10, offset by 0.25 (each curve averaged 100 times).

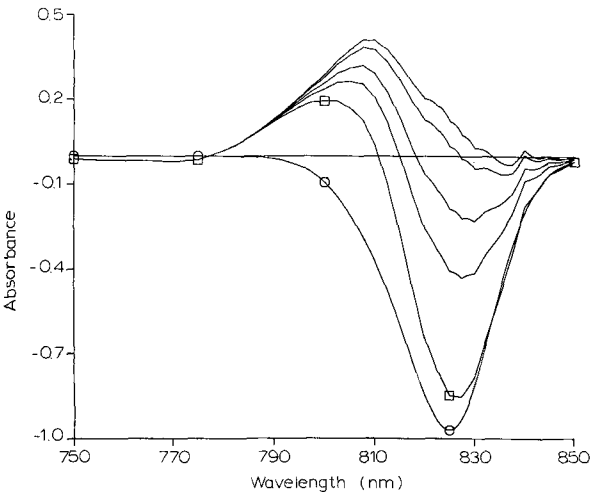

Fig. 4. Deconvolution of the 'monomer' band by adding variable amounts of the 825 nm band from the 77K B820 absorption spectrum from Fig. 1 (circles) to the difference spectrum from Fig. 2. (squares).

new band at 809 nm. The new absorption band is broader than the original 825 nm band, 390–420 cm^{-1} vs. 340 cm^{-1}. The 'monomer' band is slightly perturbed by the 780 nm bleaching, so the true width is slightly higher. By integrating the deconvoluted bands, the relative dipole strengths of the 'monomer' and 'dimer' transitions are obtained, which relate as 1:2.1 ($\pm$0.1). The simplest interpretation of these results is that the B820 complex is indeed a dimer of interacting BChl *a* molecules, and when it is excited to a triplet state, the 825 nm absorption band disappears, and a monomer band is formed.

Fig. 5 shows the time-dependence of the fluorescence detected at 850–860 nm (the extreme red side of the emission spectrum, to avoid the interference with scattered light), from the B820 complex at 77 K, upon excitation at 815 nm using a depolarized laser pulse. The pulse-profile (instrumental response curve) was recorded using a scattering solution of latex spheres, and is also shown in Fig. 5. From the deconvolution we calculated a fluorescence lifetime of 1.05 ns. Fig. 5B shows the fit and the residual curve. The residual curve is non-zero in the region of the pulse due to small fluctuations in the laser pulse. The B820 excited-state lifetime is only slightly longer than that observed at room temperature in Ref. 31.

In the following we shall investigate further whether the B820 complex should be considered as a true dimer, or whether it represents a more complicated aggregate of BChl *a* molecules. Excitation annihilation is a technique that is extensively used for the study of the number of chromophores in a pigment system coupled via efficient excitation energy transfer [32,13]. In order to measure the functional size of the B820 complex, we looked for annihilation effects in two different ways. Firstly, the energy dependence of the intensity of the difference spectrum was measured, and secondly we measured the relative emission quantum yield of a xenon flash fired after the laser flash as a function of the laser-induced triplet concentration. Since BChl *a* triplets are very efficient quenchers of singlet excited states [32], it is to be expected that the quantum yield of fluorescence (and triplet generation) of a complex containing a triplet will be extremely low.

In Fig. 6 the bleaching at 825 nm is plotted as a function of the energy density of the laser pulse. The energy was varied in the experiment by attenuating the laser-pulse using calibrated neutral-density filters. The laser flash and the measuring beam were vertically polarized. Each energy was averaged 20–100 times, the

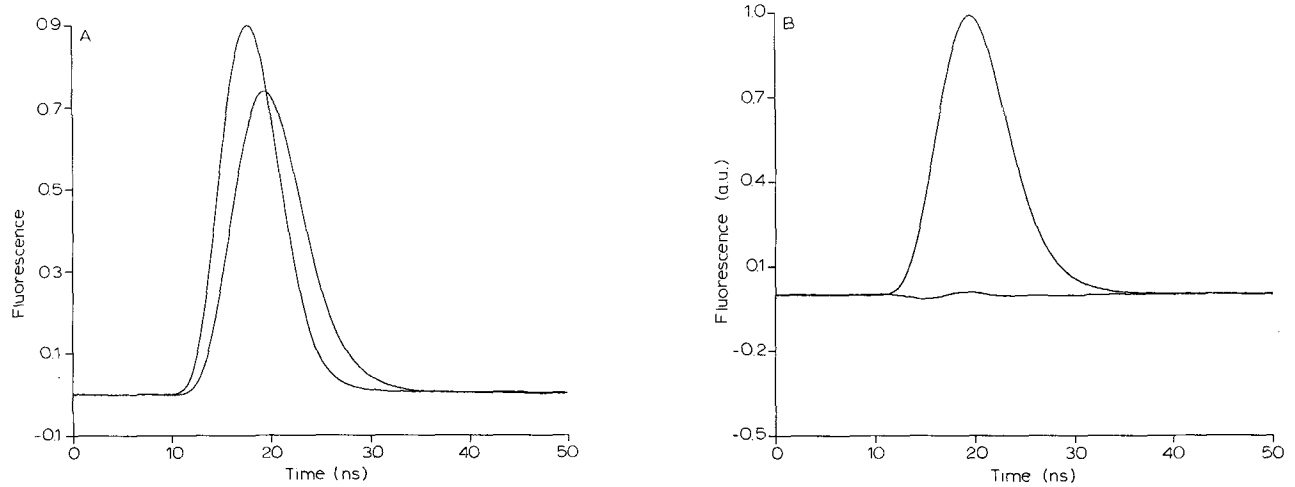

Fig. 5. (A) Fluorescence lifetime measurement, measured through a RG850 filter combined with an AL861 interference filter. The excitation wavelength was 815 nm, (energy < 0.005 mJ) The instrumental response curve (squares) obtained by measuring the light scattering from a solution of latex particles. Both curves were averaged 72 times, a baseline (no flash) was subtracted from both curves. (B) Non-linear least squares fit (+ residual curve) of the fluorescence signal with a convolution of the scatter function and a single exponential decay.

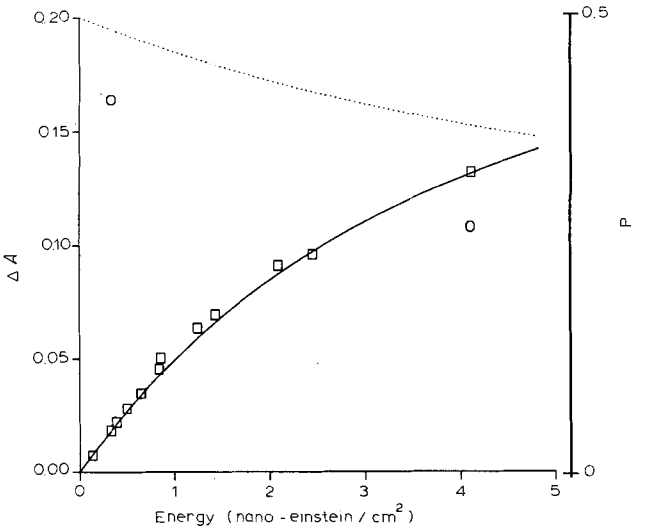

Fig. 6. Energy dependence of the amplitude of the 825 nm bleaching. Absorption measurements (squares) were made with polarization parallel to the exciting laser flash. The circles represent two polarization measurements (right scale). The curve through the squares, and the dotted curve, representing the polarization of the bleaching, were calculated as described in the text. The values of the parameters were: $\epsilon_{825} = 3 \cdot 10^5$ M^{-1} cm^{-1} (77 K, dimer extinction coefficient), singlet excited state lifetime = 1.05 ns, triplet quantum yield = 0.16. The laser pulse was approximated by a gaussian with FWHM = 5 ns.

pulse to pulse variation of the laser energy was approx. 10%. The absolute accuracy of the energy scale was estimated to be 15%, depending mainly on the calibration of the ED_{100}, and the corrections for the reflections from the cryostat windows.

The curve through the points was calculated by numerically integrating the rate equations for the transitions between ground state, excited singlet state, and triplet state. The integration was performed over time, to account for the shape of the laser-pulse, and over the orientational distribution of the pigments (described by the angle between the Q_y transition dipoles and the polarization vector of the laser pulse), to account for the energy dependence of the polarization of the bleaching. The details of the calculation are given in Appendix A. In the simulation it is assumed that the bleached dimer has no absorption at 825 nm and that the polarization of the bleaching at low excitation energy equals 0.5. In the low-energy part of the curve no saturation effects are to be expected, so from the tangent of the curve at zero energy the triplet yield can be calculated (here the high polarization of the bleaching has to be taken into account, since it increases the vertical absorption by a factor of 9/5 compared with an isotropically bleached sample). The extinction coefficient was calculated from the room-temperature values in [31] and corrected for the effect of cooling to 77 K on the absorption spectrum. For the rate-constant for stimulated emission the value for stimulated absorption was used. The intensity dependence was calculated assuming that for each triplet formed a pair of BChl molecules disappears from the B820 spectrum (or in other words, B820 is a dimer). The calculated curve clearly describes the energy dependence of the bleaching rather well, which proves that indeed no quenching of singlet excitation by triplets takes place. This limits the size of the B820 complex to only one dimer of BChl *a*.

In addition, the polarization of the bleaching was measured at low (0.2 nE/cm²) and high (4.0 nE/cm²) laser energy. Fig. 6 (circles) shows that at high energy the polarization decreases due to saturation effects. A fit based on the expressions given in Appendix A and assuming a low energy polarization of the bleaching of 0.5 yields a similar change in the polarization over this intensity range. The fit suggests that the true initial polarization is in fact lower than 0.5, and this is further discussed below (see Discussion).

Fig. 7 shows the effect of quenching of the fluorescence of a B820 sample by laser-induced triplets at 77 K. The fluorescence was excited with a weak xenon flash (λ_{exc} = 814.5 nm) fired 50 μs after an intense 825 nm laser flash. The fluorescence was measured at 860 nm, outside the region of the absorption changes, to eliminate all effects due to changes in the self-absorption of the sample. The fluorescence induced by the xenon flash fired after the laser flash (Fig. 7, lower curve) is 12% lower as compared with the signal obtained without a preceding laser flash (Fig.7, upper curve). From the amplitude of the bleaching at 814.5 nm a 10% decrease of the fluorescence signal is pre-

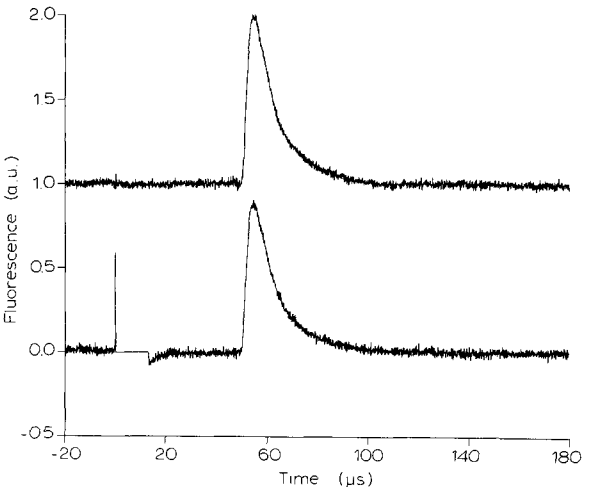

Fig. 7. Relative quantum yield of the fluorescence of a xenon flash fired 50 μs after an intense laser flash (lower trace), and without a preceding laser-flash (upper trace). The polarization of laser and xenon flash was vertical. The fluorescence was detected through an 860 nm interference filter combined with a RG830 filter. The xenon flash was fired through a double 814.5 nm interference filter. In the lower trace the signal from a laser flash without a xenon flash was subtracted from the signal.

dicted, assuming singlet-triplet annihilation to be absent. The difference between these two numbers is most likely due to the absorption at 814.5 nm of the induced monomer band, which will cause a further (slight) reduction of fluorescence signal. The result again indicates that B820 is a dimer, uncoupled from other B820 dimers in the sample.

Discussion

Based on polarized fluorescence and CD measurements, a model has been proposed for the B820 complex [25]. In this model the spectroscopic features are accounted for by a single dimer of BChl *a* in a configuration in which the Q_y transition dipoles make an angle of approx. 30°. Upon bleaching one of the pigments of such a dimer, one expects to observe a complete bleaching of the dimer band, accompanied by the creation of a monomer band with 50% of the 'intensity' of the dimer band (apart from hyper/hypochromism effects), and a FWHM that is increased by a factor of $\sqrt{2}$ when compared with the dimer band [33]. As Fig. 3b shows, our results are in excellent agreement with these predictions.

The polarization of the bleaching of BChl *a* in 2% OG, $P = 0.44$, was slightly lower than the value of 0.5 expected for a monomeric pigment. Since the polarization of the fluorescence measured under identical conditions was reported to be close to 0.5 [25], we must assume that the deviation from 0.5 is at least for a large part due to instrumental errors and noise. With regard to the latter, the error in the polarization measurements is significant because the P values are calculated from low-energy excitation traces (to avoid saturation effects). The polarization of the bleaching of the 825 nm band is also high ($P = 0.41 \pm 0.02$), and the true value is probably between 0.4 and 0.5. In this respect we note that also in the high-energy region of Fig. 6, where the error in the measured polarization values is much lower, the observed polarization is 25% lower than the simulation predicts. We conclude that the polarization of the bleaching of the B820 complex is significantly lower than that measured for BChl *a* in 2% OG. Also, the polarization of the bleaching is identical (within experimental error) to the measured values of the fluorescence polarization of the B820 complex at 77 K [25]. The observed exciton splitting of the B820 absorption band puts the coupling of the pigments inbetween the cases of strong and weak coupling. The fact that the polarization of the bleaching of the 825 nm band is not maximal indicates that the B820 complex is not a strongly coupled dimer, since in that case we would have expected to find a P value of 0.5. In the limit of weak coupling the observed P values of bleaching and fluorescence correspond with an angle of about 30° between the two monomer transition-dipoles. It should be noted that also non-degenerate excited-state interactions between the two BChls *a* in the B820 complex contribute to the spectrum. The B820 band is hyperchromic [25] and the CD spectrum is clearly non-conservative [21,22], indicating that the near-IR absorption band contains contributions from Q_x, B_x, and B_y excited states [15].

Efficient triplet formation occurs in the B820-complex and the triplet quantum-yield of B820 is comparable to that of BChl *a*. Thus, we can explain the process of B820 triplet formation by intersystem-crossing and no charge-transfer states/charge-recombination processes have to be invoked. The data presented in Figs. 6 and 7 show that singlet-triplet annihilation is absent in the B820 preparations. It could be argued, however, that the inclusion of stimulated emission in the simulation of Fig. 6 to some extent hides any annihilation effects. In this respect the delayed flash experiment of Fig. 7 is more revealing. Let us assume for the moment that the B820 complex does contain two dimers of BChl *a*, which exchange their excitation energy. At the moment of the xenon flash 12% of the population that is excited by the polarized flash is bleached. Since we know that BChl a^T is a very efficient quencher of excited singlet states [32], it is to be expected that the number of triplets per complex is not described by a Poisson distribution, the case of two triplets per complex being very unlikely. This effectively means that 24% of the complexes in the population under observation contains a triplet state, which should have given a more dramatic decrease of the fluorescence of the xenon flash than was observed in Fig. 7. This apparent absence of quenching by the triplet states is a strong indication that the B820 complex contains only one dimer of BChl *a*.

Our and other measurements have shown that the B820 complex is an interesting particle for studying pigment–pigment and pigment–protein interactions, but how relevant is it for our ideas about the in vivo pigment organization in the light-harvesting antenna of photosynthetic purple bacteria? The reconstitution experiments performed by the group of Loach have clearly demonstrated that the B820 complex is not a 'random' aggregate. All its components are necessary for the formation of the B820 complex. The binding of the BChl *a* is rather specific, since only minor chemical modifications of the BChl *a* are allowed for a successful formation of 'B820' [35]. The LH-1 α and β polypeptides from different purple bacteria are very similar [8]. Nevertheless, the combination of α and β polypeptides from different bacteria does not in all cases lead to a stable B820 complex [35–37], suggesting that the two polypeptides have specific interactions. This specificity is an indication that what is isolated is a particle which has retained many of the properties

essential for its structure/function in the intact LH-1 antenna.

What is the relation between 'B820' and intact LH-1, absorbing at 870–880 nm? Stopped-flow spectral kinetics indicate that two B820's are required to form the 870–880 nm absorbing species (Van Mourik et al., unpublished data). Excited state difference spectra may reveal the true number of interacting pigments and resolve the nature of the interaction as demonstrated in this work for the B820-complex. In the past, T-S difference spectra were recorded for the larger B850-complex from *Rhodobacter sphaeroides* R26 [30]. The spectra were interpreted as reflecting a transition from a strongly coupled Bchl *a* dimer to a non-interacting pair BChl *a* + BChl a^T. However, it is clear that the T-S spectrum of B820 is much simpler than that obtained for B850 (Fig. 5B, Ref. 30) and in fact we suggest a rather different interpretation of the latter spectrum. Our measurements show that BChl a^T bound to the protein has no significant absorption bands in the 800–900 nm region of the spectrum. This implies that all the induced absorption bands in the B850 T-S difference spectrum are due to the transition (BChl $a)_n \rightarrow$ (BChl $a)_{n-1}$. The observed B850 difference spectrum, which apparently contains at least two positive contributions, suggests that $n \geqslant 4$. We are currently performing T-S absorption and annihilation experiments on B875 and B850, to test these ideas.

The system studied in this work is considerably less complicated than the bacterial reaction center. However, it might serve as a useful model system to interpret the spectral properties of the reaction center special pair. Singlet-triplet spectra have been measured for the primary donor of bacterial photosynthetic RCs [38]. The spectra were explained by a bleaching of the long-wavelength dimer band (the low-energy exciton component), the formation of a new monomer band in the region of BChl-monomer absorption, and the formation of a triplet-triplet absorption band. In fact, our spectra are more consistent with the appearance of a new monomer band in between the two exciton states and, as stated above, do not hint at the presence of a significant triplet-triplet absorption band.

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Appendix

The energy dependence data displayed in Fig. 6 depend on the extinction coefficient of the B820 complex (actually ($\Delta\epsilon$), the intersystem-crossing rate, the fluorescence lifetime of the complex, the polarization of the bleaching, and on the pulse length. No analytical expression is available to describe this curve, so we numerically integrated the rate constants of the system, as described in the following.

The saturation effects that are observed are partly due to a saturation of the polarization of the bleaching, so we have to take the orientation of the pigments into account. The geometry, of the experiment and simulation is displayed in Fig. 8. Since the excitation is always vertically polarized, the photo-selection is symmetric around the *z*-axis. The important angle for describing the orientational distribution of the pigments (dimers) is the angle θ between the Q_y (dimer 825 nm) transition dipole of the pigment and the experimental *z*-axis. Before the laser flash, at $t = 0$, the population of the ground ($G(\theta, t = 0)$), excited singlet ($S(\theta, t = 0)$), and triplet states ($T(\theta, t = 0)$) is:

$$S(\theta, 0) = T(\theta, 0) = 0 \tag{1a}$$

$$G(\theta, 0)\, \mathrm{d}\theta\, \mathrm{d}\phi = \frac{\sin(\theta)\, \mathrm{d}\theta\, \mathrm{d}\phi}{\int_0^{2\pi} \mathrm{d}\phi \int_0^{\pi} \sin(\theta)\, \mathrm{d}\theta} \times C$$

$$= \frac{C}{4\pi} \sin(\theta) \mathrm{d}\theta\, \mathrm{d}\phi \tag{1b}$$

where C is the pigment concentration. We look only at the bleaching of the dimer band, and assume that the produced triplet and singlet excited state do not absorb at the wavelengths of interest. The vertically and horizontally polarized absorption of the sample at any time is:

$$A_{v,t} = 3\epsilon \int_0^{2\pi} \mathrm{d}\phi \int_0^{\pi} \cos^2(\theta)(G(\theta, t) - \alpha S(\theta, t))\, \mathrm{d}\theta \tag{2a}$$

$$A_{h,t} = 3\epsilon \int_0^{2\pi} \int_0^{\pi} \cos^2(\phi) \sin^2(\theta)(G(\theta, t) - \alpha S(\theta, t))\, \mathrm{d}\theta\, \mathrm{d}\phi$$

$$= 3\pi\epsilon \int_0^{\pi} \sin^2(\theta)(G(\theta, t) - \alpha S(\theta, t))\, \mathrm{d}\theta \tag{2b}$$

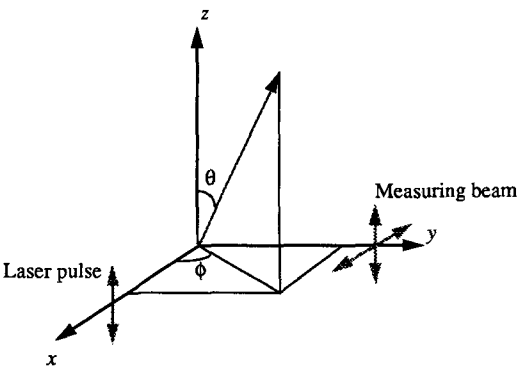

Fig. 8. The geometry of the polarized bleaching experiment and simulation.

Here it is assumed that the polarization of the bleaching at very low excitation densities is 0.5. The singlet term enters the absorption-equations to take stimulated emission into account, in all calculations $\alpha = 1$. From the polarized absorption the number of absorbed photons in a time interval can be calculated, the produced excited states are distributed over the pigments by the weighing-factor $(G(\theta, t) - S(\theta, t)) \cos^2(\theta)$
The master equations for the system are:

$$\frac{d}{dt}G(\theta, t) = -P(t)(G(\theta, t) - S(\theta, t)) \cos^2(\theta) + K_{ST}S(\theta, t) + K_{TG}T(\theta, t) \quad (3a)$$

$$\frac{d}{dt}S(\theta, t) = P(t)(G(\theta, t) - S(\theta, t)) \cos^2(\theta) - K_{ST}S(\theta, t) - K_{SG}S(\theta, t) \quad (3b)$$

$$\frac{d}{dt}T(\theta, t) = K_{ST}S(\theta, t) - K_{TG}T(\theta, t) \quad (3c)$$

Where $P(t)$ contains the pulse shape and energy, K_{ST}, K_{SG}, K_{TG} are the inter-system crossing rate constant, the sum of the singlet radiative and non-radiative decay rate constants, and the triplet decay rate-constant. The master equations were numerically integrated. In the calculations the decay of the triplet was neglected: $K_{TG} = 0$.
From the measured fluorescence lifetime $K_{ST} + K_{SG}$ is obtained, the triplet yield $K_{ST}/(K_{SG} + K_{ST})$ obtained from the initial slope of the energy-dependence curve gives a value for K_{ST}.

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