Biochimica et Biophysica Acta 810 (1985) 94–105
Elsevier

BBA 41862

Singlet and triplet excited carotenoid and antenna bacteriochlorophyll of the photosynthetic purple bacterium *Rhodospirillum rubrum* as studied by picosecond absorbance difference spectroscopy

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(Received May 29th, 1985)

Key words: Singlet excited carotenoid; Carotenoid triplet formation; Singlet excited bacteriochlorophyll; Singlet–singlet annihilation; (*R. rubrum*)

Picosecond absorbance difference spectra at a number of delay times after a 35 ps excitation pulse and kinetics of absorbance changes were measured in chromatophores of the photosynthetic purple bacterium *Rhodospirillum rubrum* after chemical oxidation of the primary electron donor P-875. Kinetics and spectra were measured of the excited singlet states of carotenoid and bacteriochlorophyll *a* and also of the triplet state of the carotenoid. The excited singlet state of carotenoid, produced by direct excitation at 532 nm, is characterized by a bleaching of the ground state absorption bands in the region 450–490 nm and by an absorbance increase with a maximum near 570 nm. Its lifetime was calculated to be 0.6 ± 0.1 ps in vitro and less than 1 ps in vivo. The triplet state of carotenoid in vivo is formed within 100 ps after direct carotenoid excitation via a pathway that does not involve excited states of bacteriochlorophyll. Singlet excitation of a bacteriochlorophyll *a* molecule causes the bleaching of its Q_x and Q_y absorption bands, and is probably associated with blue shifts of the Q_y absorption band of about six neighboring bacteriochlorophyll molecules. Upon increasing the excitation density, the average lifetime of the singlet excitations on bacteriochlorophyll decreased from about 350 ps to about 10 ps or less. The results are in quantitative agreement with the known effect of singlet–singlet annihilation upon the fluorescence yield, and furthermore show that no bacteriochlorophyll or carotenoid triplet formation is associated with this annihilation.

Introduction

The photosystem of the purple bacterium *Rhodospirillum rubrum* consists of light-harvesting antenna complexes, denoted as B880 and of reaction center complexes, embedded in the B880 antenna [1]. B880 contains bacteriochlorophyll (BChl) *a* and carotenoid (Car) spirilloxanthin in a molecular ratio of 2:1 [2]. About 50 BChl *a* antenna molecules are present per reaction center, and together they constitute a photosynthetic unit [3].

Upon excitation of the carotenoid with a 532 nm laser pulse the excitations are either transferred to BChl and produce a bacteriochlorophyll excited singlet state (BChl*), or are lost by radiationless decay processes. The efficiency of the energy transfer is close to 30% [4]. The singlet excita-

Abbreviations: BChl, bacteriochlorophyll; B880, antenna complex with an absorption maximum at 880 nm; P-875, primary electron donor bacteriochlorophyll.

tions are transferred among the B-880 molecules. Most of them are finally trapped in the reaction centers, but some are lost by emission of fluorescence, by internal conversion or by intersystem crossing to the bacteriochlorophyll triplet state ($BChl^T$). With all the traps open (state P-875 Q) the sum of these loss processes constitutes about 7% of the total decay; with all the traps closed (state $P\text{-}875^+Q^-$) this sum is about 20% [5]. The remaining excitations are either trapped in the open reaction centers or quenched by $P\text{-}875^+$. In carotenoid containing purple bacteria $BChl^T$ has not been detected yet. Instead, a carotenoid triplet (Car^T) is usually observed, which is formed within about 20 ns, with $BChl^T$ as a likely intermediate [6]. In *R. rubrum* the Car^T yield proven to be higher upon direct carotenoid excitation than upon BChl excitation [7]. From a study of the influence of a magnetic field on the Car^T yield [8,7] and on the BChl emission yield [7,9] of several purple bacteria it was proposed that following direct carotenoid excitation singlet fission of the singlet excited carotenoid (Car*) state could take place, generating a triplet pair. From this pair a Car^T state could be formed without the need of a precursor BChl excited state. The rise time for these Car^T states has not been reported yet; an upper limit of 2 ns was given by Van Bochove et al. [10].

With all the reaction centers in the open state P-875 Q the low intensity B880 fluorescence lifetime ($1/e$) is about 60 ps, while in the state $P\text{-}875^+Q^-$ the lifetime increases to about 200 ps [11]. Upon excitation with an intense picosecond pulse a decrease is observed in several species of purple bacteria in both the fluorescence lifetime [12] and the fluorescence yield [13]. A general theory [14] describing the competition between trapping, loss and biexcitation annihilation explains the data quantitatively [13]. For chromatophores of *R. rubrum* it was concluded that excitation migration occurred over about 14–17 photosynthetic units, which together constitute a so-called 'domain'. The fluorescence lifetime with the traps in the state P-875 Q was estimated to be about 100 ps; with the traps in the state $P\text{-}875^+Q^-$ about 300–350 ps. These fluorescence lifetimes are markedly distinct from those obtained from a study utilizing a picosecond absorbance difference apparatus [15,16]. In that work BChl* was detected via the spectral changes in absorption in the near infrared and it was concluded that a minor antenna component existed, denoted as B905. This small pool of antenna BChl molecules was thought to funnel the excitation energy from the bulk B880 antenna to the reaction centers, and, to explain the irreversible energy transfer from B-880 to B905, charge transfer states were assumed to be involved. The lifetime of B905* was claimed to be 60 ps and for the decay of B880* two components were reported of 60 ps and less than 20 ps, at variance with the fluorescence data mentioned above. Moreover, the B905* lifetimes appeared to be independent of the pulse intensity [15] and the redox state of the traps [17].

In this paper we report a picoseond absorbance study of the singlet and triplet excited states of both carotenoid and BChl in the antenna of chromatophores of *R. rubum* following direct carotenoid excitation. The results confirm the existence of a pathway for Car^T formation which does not involve BChl excited states. The BChl* lifetimes obtained agree with the data from the fluorescence measurements [13]. Part of the results has been reported in a preliminary form elsewhere [18].

Materials and Methods

R. rubrum was grown anaerobically in a continuous culture as described in Ref. 7. Chromatophores were prepared using a French press and were diluted in a buffer medium containing 250 mM Tricine/5 mM KH_2PO_4/5 mM $MgCl_2$ (pH 8.0). 2 mM $K_3Fe(CN)_6$ was added prior to the measurements and continuous background illumination ($\lambda = 528$ nm) was given to keep the primary donor in the oxidized state. Spirilloxanthin was isolated from *R. rubrum* and dissolved in a medium of *n*-hexane and isopropanol (97 : 3, v/v). All measurements were performed at room temperature.

The picosecond absorbance measurements were performed by means of the apparatus shown in Fig. 1. The light source was a mode-locked Nd-YAG laser generating pulses at 1064 nm with a duration of 35 ps (see below). Part of the radiation was frequency-doubled to 532 nm to serve as an excitation pulse. The maximum incident excitation

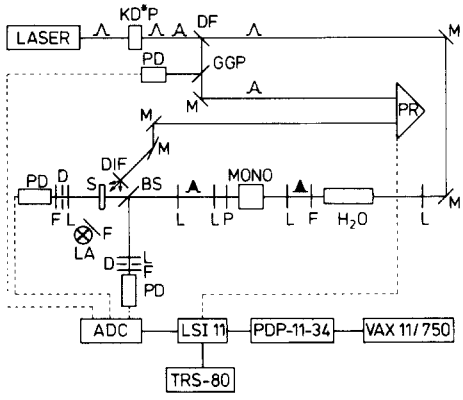

Fig. 1. Experimental set-up. LASER, 35 ps Nd-YAG laser; KD*P, frequency-doubling crystal; DF, dichroic filter; M, mirror; F, filter; BS, beam splitter; L, lens; D, diaphragm; P, polarizer; DIF, diffuser; S, sample; GGP, ground glass plate; MONO, monochromator; H_2O, water cell; PR, prism on variable delay line; PD, photodiode; ADC, analog-to-digital converter; LA, light source for continuous background illumination; A, 1064 nm; A, 532 nm; ▲, continuum pulse; A, probe pulse.

energy density was about 3 mJ/cm^2. The remaining infrared radiation was focused into a 15 cm cell filled with water in order to generate a broad wavelength continuum. The 35 ps measuring pulse with a bandwidth of 3 nm obtained from this continuum by means of a monochromator could be tuned between 450 and 910 nm. Part of the measuring pulse was deflected by a beamsplitter onto a photodiode to serve as a reference. The remaining pulse was transmitted through the sample and detected by a second photodiode. A time delay of maximally 4 ns between the excitation and probe pulses was obtained by means of a prism on a delay line. The position of the prism was controlled by a TRS-80 microcomputer. Stray excitation light and fluorescence were prevented from reaching the photodiode by diaphragms and suitably colored glass and interference filters. Absorbance changes were calculated by comparing the ratio of measuring and reference pulse intensities with the ratio obtained with a measuring pulse preceding the excitation pulse. By this method remaining effects of stray light and fluorescence, if any, were largely eliminated. The arrangement with the monochromator placed before the sample ensured that the probe could not become actinic. Since the area on the surface of the sample illuminated by the probe (about 2 mm^2) was small compared to that illuminated by the actinic beam (about 1 cm^2) a homogeneous excitation was provided. The incident excitation density was determined by means of a temperature-compensated radiometer (Laser Instrumentation LTD). The effect of chirping of the measuring pulse was determined not to exceed 15 ps over the spectral region from 400 to 900 nm. The excitation pulse was vertically polarized and the polarization of the measuring pulse could be set by means of a prism polarizer. The repetition rate of the pulses was 0.5 Hz. By averaging over about 25 laser flashes a resolution of about $(1-2)\cdot 10^{-3}$ units of absorbance was obtained. Data were stored in a main frame computer for further processing.

Nanosecond absorbance measurements were performed by means of the set-up described in Ref. 19. In some of these experiments the wavelength of the excitation pulse was Raman-shifted to 590 nm by focusing the 532 nm pulse in a 5 cm cell filled with perdeuterocyclohexane.

Results and Interpretation

Fig. 2A shows the absorbance difference spectrum of chromatophores of *R. rubrum* obtained during (solid circles) and 200 ps after (open circles) the 35 ps excitation pulse in the regions 450–490 nm and 550–650 nm. The spectrum at 200 ps has a maximum around 570 nm, and is similar in shape to the difference spectra observed on a microsecond timescale, which have been attributed to the formation of carotenoid triplets [6–8]. Since no reaction center carotenoid triplets are produced when the photochemistry in the reaction centers is blocked by oxidation of the primary donor [7], carotenoid triplet states observed in these experiments must be formed on antenna carotenoid molecules. The spectrum measured during the excitation pulse is clearly different from the 200 ps spectrum. It shows a bleaching in the region of carotenoid absorption (450–490 nm) and an absorbance increase between 550 and 650 nm with an apparent trough at about 595 nm. The kinetics of the absorbance changes at 470 and 610 nm are shown in Fig. 3 (circles and triangles, respectively). At both wavelengths the full width at half maxi-

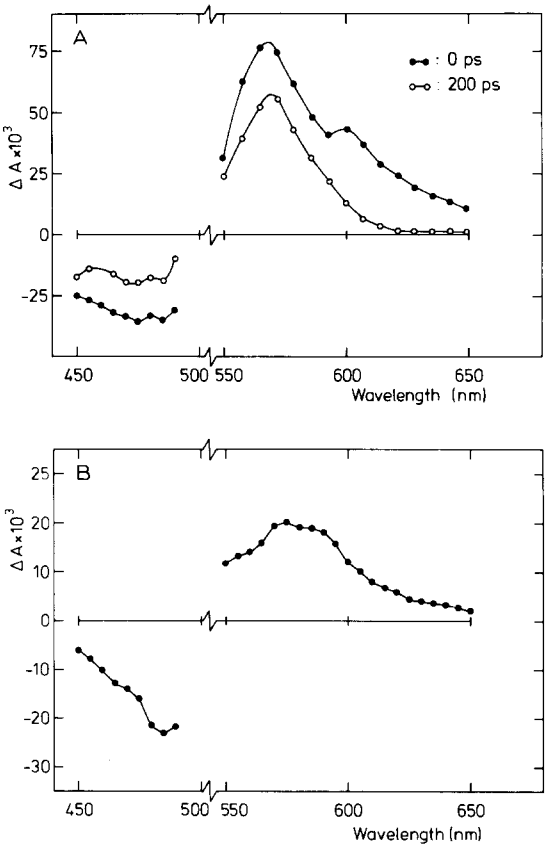

Fig. 2. (A) Absorbance difference spectra of chromatophores of *R. rubrum* at 0 ps (i.e., with coincident excitation and probe pulses) (●) and at 200 ps (○) after the excitation flash, in the presence of 2 mM $K_3Fe(CN)_6$ and with continuous background illumination at 528 nm. Parallel polarized excitation and probe pulses. The absorbance at 532 nm was 0.57 in a 2 mm cell. The incident excitation intensity was about 1.7 mJ/cm^2. (B) Absorbance difference spectrum of spirilloxanthin dissolved in a medium of *n*-hexane and isopropanol (97:3, v/v) at 0 ps. Parallel polarized excitation and probe pulses. The absorbance at 532 nm was 0.18 in a 2 mm cell. The incident excitation intensity was about 3.2 mJ/cm^2.

mum of the initial absorbance changes is about 50 ps, and is mainly determined by the width of the excitation and probe pulses which shows that the lifetime of the transient is short compared to the duration of the pulses. The bleaching remaining at 470 nm after the decay of the fast phase reflects the presence of the carotenoid triplet states, which thus must be formed within 100 ps upon direct carotenoid excitation.

The fast phase most likely also involves a

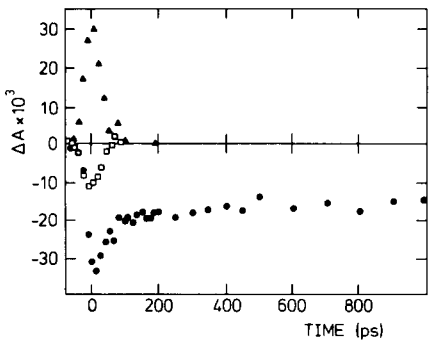

Fig. 3. Kinetics of absorbance changes (□) of spirilloxanthin at 470 nm; conditions as in Fig. 2B. Chromatophores of *R. rubrum* at 470 nm (●) and at 610 nm (▲). Conditions as in Fig. 2A.

short-lived carotenoid excited state, since the spectrum of the initial absorbance changes (Fig. 2A, closed circles) includes a pronounced bleaching in the region 450–490 nm. In order to identify this state, we have measured the absorbance difference spectrum of isolated spirilloxanthin in solution during the excitation pulse (Fig. 2B). The spectrum shows a bleaching in the region 450–490 nm and an absorbance increase with a maximum near 575 nm in the region 550–650 nm. The kinetics of the absorbance changes at 470 nm are shown in Fig. 3 (squares). The temporal profile is mainly determined by the width of the excitation and probe pulses, and thus points at the formation and decay of a state with a lifetime of not more than a few ps. Presumably this transient represents a singlet-excited carotenoid state (Car*). The absence of a long-lived bleaching at 470 nm after the decay of this state (Fig. 3, squares) implies that in solution no carotenoid triplets are formed by intersystem crossing from the excited singlet state, in agreement with earlier findings of Bensasson et al. [20]. A comparison of Figs. 2B and A (closed circles) indicates that the same transient as observed in isolated spirilloxanthin contributes to the absorbance changes observed in chromatophores during the pulse. The absorbance increase above 610 nm and part of the absorbance changes in the regions 450–490 nm and 550–610 nm are therefore presumably due to the formation of (singlet) Car*. As will be shown below, the lifetime of Car* in vitro is less than 1 ps. Since this lifetime is much

shorter than the duration of the pulses, the kinetics of Car* in vitro (Fig. 3, squares) are fully determined by the convolution of the exciting and probe pulses. If we assume, for the sake of simplicity, that the pulses are Gaussian, then the duration of the pulses is calculated to be 35 ps.

The dependence of the amounts of Car* and Car^T produced on the excitation intensity is shown in Fig. 4. The saturation behavior for the two processes is about equal, which indicates that the amount of Car^T is directly proportional to the amount of Car*. In order to obtain quantitative information it is necessary to correct for photodichroism, since both the excitation and probe pulses are polarized. If $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ are the absorbance changes parallel and perpendicular, respectively, to the vertical polarization direction of the excitation pulse, then the ratio of $\Delta A_{\parallel}/\Delta A_{\perp}$ was 2.9, 2.2 and 1.8 for Car* in vitro (at 590 nm), Car* in vivo (at 610 nm), and Car^T in vivo (at 570 nm after 1 ns), respectively (data not shown). In view of the long, chain-like structure of the carotenoid molecules, the transition moments of the excited states will probably be parallel to the ground state transition moment, and therefore the value of 2.9 for Car* in vitro is close to the ratio of 3.0 expected for isolated molecules [21]. The lower value for Car^T in vivo indicates that some depolarization occurs (see Discussion). The value for Car* in vivo should be taken with caution, since during the pulse a small contribution by BChl* to the absorbance increase at 610 nm could be present. However, since the lifetime of BChl* is longer than that of Car*, the kinetics at 610 nm (Fig. 3, triangles) exclude a significant contribution of BChl*. Corrected for photodichroism the Car^T yield in the linear region of the saturation curve of Fig. 4 is calculated to be 0.08, when a differential extinction coefficient of 92 $mM^{-1} \cdot cm^{-1}$ at 570 nm for the carotenoid triplet-minus-ground state absorbance is used [27]. The lifetime of Car* in vitro is too short to be measured directly, but can be estimated from the amplitude of the bleaching in the spectrum of Fig. 2B. Taking a value of 152 $mM^{-1} \cdot cm^{-1}$ for the extinction coefficient of ground state carotenoid at 485 nm [28], the amount of Car* observed can be compared with that excited during the flash, as calculated from the absorbance of the sample at 532 nm and the incident excitation intensity. Simulation of the absorbance changes at 485 nm by convolution of a mono-exponential decay with 35 ps Gaussian excitation and probe pulses then results in a lifetime of Car* in vitro of 0.6 ± 0.1 ps. This value is in agreement with the upper limit of 1 ps for the lifetime of the excited singlet state of β-carotene as estimated from resonance Raman spectroscopy [29]. A value for the decay time of Car* in vivo can be obtained from the spectrum of Fig. 2A measured during the flash. Assuming that the bleaching at 485 nm is solely due to the formation of Car*, a lifetime of 1 ps is calculated. If Car^T states contribute to the initial spectrum of Fig. 2A, the calculated lifetime of Car* will be shorter. The value of 1 ps or less for the decay time of Car* in *R. rubrum* is about the same as that of Car* in membranes of the green sulfur bacterium *Prosthecochloris aestuarii* [22].

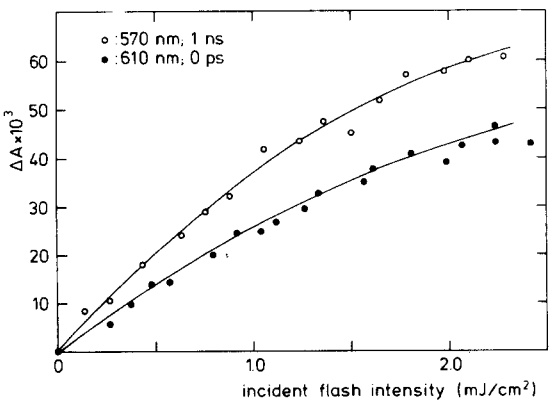

Fig. 4. Absorbance changes of chromatophores of *R. rubrum* as a function of the incident excitation energy density. (●) 610 nm at 0 ps, and (○) 570 nm at 1 ns. Conditions as in Fig. 2A.

Within their lifetime, about 30% of the Car* molecules transfer their excitation energy to BChl molecules [4]. Intersystem crossing to the triplet state of BChl ($BChl^T$) then may take place, followed by transfer of the triplet energy to carotenoid, generating a Car^T state [6,7]. The rise-time of 20 ns reported by Monger et al. [6] for the formation of the Car^T state after BChl excitation with a 15 ns pulse is remarkably different from the upper limit of 100 ps for the generation of a triplet state after direct carotenoid excitation with a 35 ps pulse estimated in this work. To investigate if in

our experiments a 20 ns component in Car^T formation can be observed after excitation with a picosecond pulse we have recorded the kinetics at 580 nm on a longer timescale for two different wavelengths of excitation with a 35 ps pulse. The results are shown in Fig. 5. Obviously, rapid formation of Car^T states (i.e., within 2 ns) only takes place upon direct carotenoid excitation. The absence of a 20 ns component in the traces of Fig. 5 indicates that formation of BChl*, either by direct excitation at 590 nm, or by energy transfer from Car* does not give rise to a measurable Car^T formation in these experiments. The negative peak in trace B is due to actinic light. The most likely explanation for the absence of Car^T formation upon excitation at 590 nm is the efficient excitation annihilation within BChl *a*, which causes a low fluorescence and triplet yield upon excitation with a picosecond flash (see Discussion).

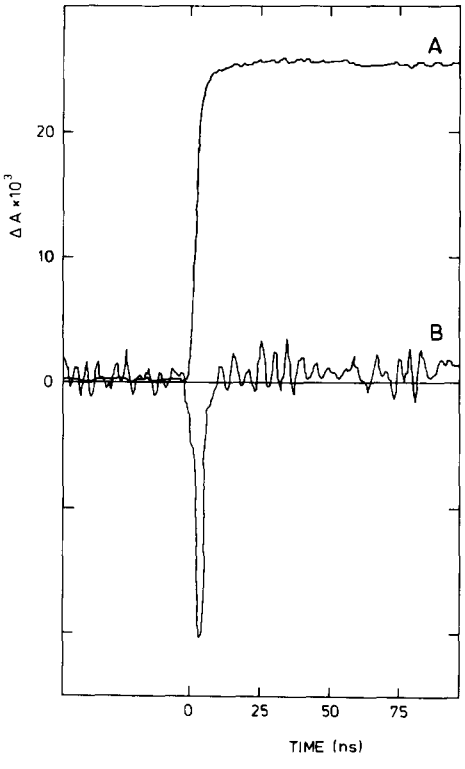

Fig. 5. Kinetics of the absorbance changes in chromatophores of *R. rubrum* at 570 nm for 532 nm excitation (A) and 590 nm excitation (B). The absorbance at 532 nm was 0.67 in a 2 mm cell.

Fig. 6 shows the absorbance difference spectra in the region 550–910 nm of oxidized chromatophores at a number of delay times with respect to the excitation pulse. Apart from the absorbance changes due to excited carotenoid, the spectrum at 0 ps (solid circles) shows large changes in the region 750–910 nm. The bleaching with a maximum around 888 nm most likely must be attributed to the disappearance of BChl ground states due to singlet excitation of the antenna molecules. In addition, a broad absorbance increase in the region 750–870 nm, with a maximum at about 855 nm, is observed. The already mentioned trough at 595 nm may represent the bleaching of the Q_x band of BChl. The spectrum recorded after 300 ps (crosses) in the region 800–910 nm is similar in shape to the spectrum at 0 ps. The absence of absorbance changes in this region after 1 ns (open circles) indicates that the BChl singlet excitations have decayed and that no detectable amount of $BChl^T$ is present. No polarization in the region 750–910 nm was observed at 0 ps, probably because of depolarizing steps of the excitations among the BChl molecules.

The kinetics of the absorbance changes at 898 nm are shown in Fig. 7 for a number of excitation intensities. In all cases the initial increase of the bleaching is determined by the temporal profile of the excitation and probe pulses. The decay of the

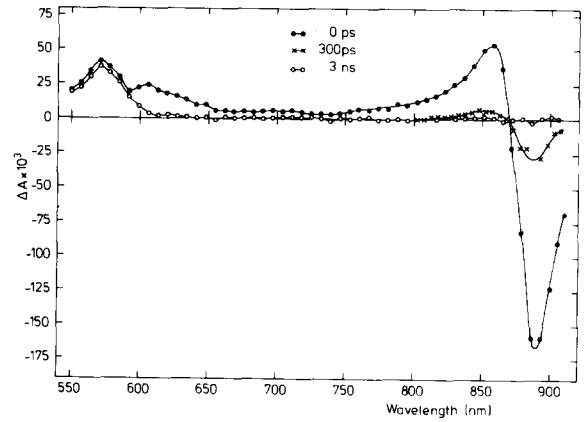

Fig. 6. Absorbance difference spectra at 0 ps (●), 300 ps (×) and 3 ns (○) after the 532 nm flash in chromatophores of *R. rubrum*. Perpendicularly polarized excitation and probe pulses. Further conditions as in Fig. 2A. The incident excitation intensity was about 1.3 mJ/cm^2.

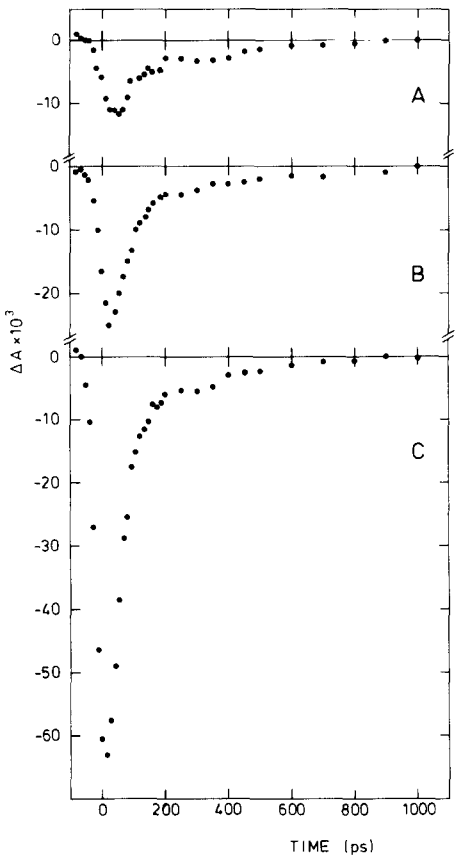

Fig. 7. Kinetics of absorbance changes in chromatophores of *R. rubrum* at 898 nm for different incident energy densities. (A) 0.031 mJ/cm^2; (B) 0.081 mJ/cm^2; (C) 0.34 mJ/cm^2. Conditions as in Fig. 6.

bleaching is not mono-exponential. BChl* remaining at 200 ps after the flash seems to disappear with a decay time ($1/e$) of 300–400 ps. The first 200 ps of the decay contain phases with much shorter lifetimes; this is especially noticeable in trace c. Accurate values for the decay times of these phases cannot be extracted from the figure, since these fast kinetics are strongly distorted by convolution with the shapes of the excitation and probe pulses. We ascribe the 300–400 ps decay component to the mono-excitation deactivation of BChl* when all the traps are closed. It compares reasonably well with the 200 ps decay observed in the fluorescence with the reaction centers in the state P-875$^+$Q$^-$ [11]. The occurrence of the fast phases in the initial decay of BChl* is probably due to singlet-singlet annihilation, which is known to shorten the fluorescence decay time when intense picosecond excitation pulses are used [12]. This is confirmed by the observation that the contribution of the 300–400 ps tail in the kinetics to the total decay increases with decreasing excitation energy (Fig. 7). The absorbance difference spectra of the fast and slow components are similar (Fig. 6, solid circles and crosses, respectively). The amplitude of the bleaching at 890 nm measured at 0 ps as a function of excitation intensity is shown in Fig. 8. The apparent saturation behavior is strikingly distinct from that of the formation of Car* (Fig. 4, solid circles). It will be shown in the Discussion section that the apparent saturation behavior of BChl* formation is due to a distortion of the curve at high intensities: because of efficient singlet–singlet annihilation, the average lifetime of an excitation decreases with increasing intensity which reduces the measured amplitude of the absorbance changes when this average lifetime approaches and becomes less than the duration of the pulses.

In Fig. 9 we have plotted the absorbance difference spectra in the region 800–910 nm measured during the pulse for a number of excitation

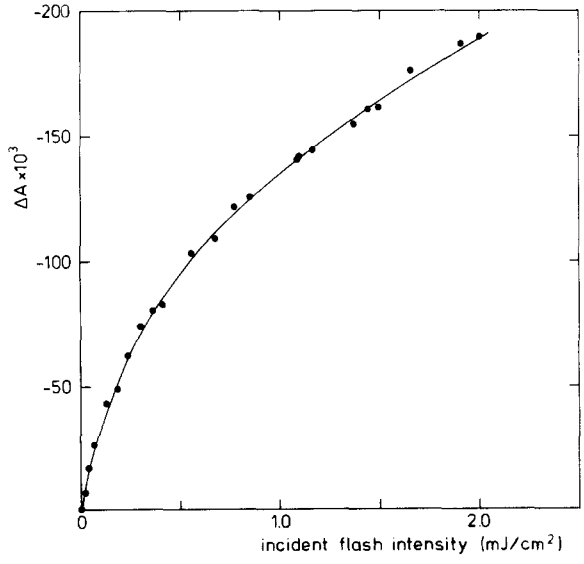

Fig. 8. Absorbance change at 890 nm in chromatophores of *R. rubrum* vs. the incident excitation energy density, with coincident excitation and probe pulses. Conditions as in Fig. 6.

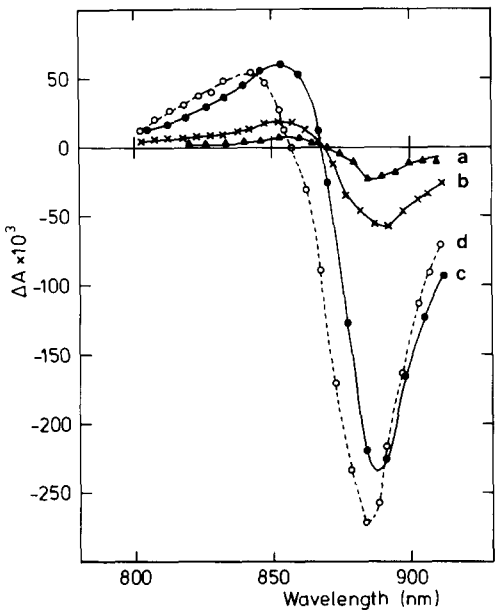

Fig. 9. Absorbance difference spectra in chromatophores of *R. rubrum* at 0 ps for different excitation energy density. (▲) 0.050 mJ/cm^2; (×) 0.250 mJ/cm^2; (●) 2.5 mJ/cm^2. Conditions as in Fig. 6. (○) more than 40 mJ/cm^2, the absorbance at 532 nm was 0.31; further conditions as in Fig. 6.

energies. Spectra a, b, and c, recorded at 0.05, 0.25 and 2.5 mJ/cm^2, respectively (i.e., $1.4 \cdot 10^{14}$, $6.8 \cdot 10^{14}$ and $6.8 \cdot 10^{15}$ photons per cm^2, or about 0.2, 1 and 1.0 photons absorbed per reaction center, respectively) are similar in shape, with the maximum of the bleaching at 888 nm. Spectrum d, obtained with focused laser excitation (10^{17} photons per cm^2 or more) shows a maximum bleaching at 885 nm. The wavelength at which ΔA is zero is shifted from 868 to 857 nm. The maximum absorbance increase at the blue side of the bleaching is also blue-shifted as compared to the other spectra. An analysis of these spectra will be given in the Discussion.

Discussion

The application of picosecond time-resolved absorbance difference spectroscopy to antenna complexes gives information about the processes of formation and decay of excited states of antenna pigments. The absorbance changes brought about by a 35 ps, 532 nm laser flash in chromatophores of *R. rubrum* are due to three different phenomena: (a) formation of singlet excited BChl *a* and carotenoid; (2) carotenoid triplet formation; and (3) a band shift of the absorption of BChl *a*. Direct excitation of the carotenoid spirilloxanthin with the 532 nm flash generates Car* which has a lifetime of 0.6 ± 0.1 ps in vitro and of less than 1 ps in vivo. Upon direct carotenoid excitation, triplets of antenna carotenoid are formed within 100 ps with a relatively high yield. These triplets are probably not formed by intersystem crossing of Car*, since the yield of Car^T by this process is very low in vitro (Fig. 3, and Ref. 20). Two other possible mechanisms may account for the observed Car^T yield. The first, intersystem crossing of BChl* to BChl^T and subsequent triplet transfer to Car, with a halftime of about 20 ns [6], is unlikely in view of the rapid rise time of less than 100 ps and the fact that no significant amount of Car^T can be detected upon direct excitation of BChl *a* (Fig. 5). The second mechanism is the fission of Car* into a triplet pair ^{T}[Car.X]^T, in which X may be a second carotenoid molecule (homofission) or a BChl molecule (heterofission). This process has been proposed to occur in photosynthetic purple bacteria on the basis of magnetic field effects on Car^T formation and BChl emission [7–9]. Upon inspection of the saturation curves of Car* and Car^T formation (Fig. 4) it is seen that the amount of Car^T is directly proportional to the amount of Car*, which indicates that in the pathway from Car* to Car^T to non-linear losses occur. Since non-linear processes take place in the decay of BChl* (see below), it thus follows that BChl* does not act as an intermediate in the rapid formation of Car^T, which supports the singlet fission mechanism. Moreover, the still appreciable dichroism of the absorbance change due to Car^T seems to exclude the involvement of many depolarizing steps, such as transfer among BChl molecules, in the formation of Car^T. We therefore conclude that fission of Car* is the most likely source for the generation of Car^T in these experiments. Our data are consistent with the homofission process, since BChl^T states are not detected.

The results show that biexcitation annihilation processes contribute to the decay of BChl*, in agreement with earlier work [13]. Due to this process the yield and lifetime of the BChl fluorescence

decrease strongly [12,13]. In our experiments this is manifested by the absence of Car^T formation upon direct BChl excitation with a relatively intense picosecond flash (Fig. 5B). This is contrast to BChl excitation with a nanosecond pulse of similar energy that leads to a Car^T yield of 0.2 [7] (or rather 0.1, as recalculated with a differential extinction coefficient for the carotenoid triplet-minus-ground state absorbance of 92 $mM^{-1} \cdot cm^{-1}$ [27] instead of 43 $mM^{-1} \cdot cm^{-1}$ [20]). The Car^T formation via BChl* intersystem crossing constitutes about 20–40% of the total amount of Car^T formed following nanosecond excitation at 532 nm [7] and therefore a higher Car^T yield of 0.3 (or rather 0.14 as recalculated) is observed under these conditions as compared to the yield of 0.08 obtained with a 35 ps, 532 nm excitation flash. From the kinetics of Figs. 5 and 7 it can be seen that biexcitation annihilation of BChl* is not accompanied by the formation of triplets of BChl *a* or carotenoid. This indicates that the annihilation can probably be described by the process BChl* + BChl* → BChl* + BChl + heat.

The effect of biexcitation annihilation on the fluorescence yield in *R. rubrum* was studied in detail by Bakker et al. [13]. The fluorescence yield as a function of the laser energy as measured by these authors is shown in Fig. 10 (solid circles and drawn curve). Because it is important to establish whether or not fluorescence and absorbance measurements reflect the same properties of a photosynthetic system, we have compared the absorbance data obtained in this work with the results from the fluorescence study. This was done in the following two ways.

(i) The area under the kinetics of Fig. 7 divided by the excitation energy (the 'ΔA yield') should be proportional to the fluorescence yield. The ΔA yields are represented in Fig. 10 (open circles). (Note that the corresponding kinetics at 0.75 mJ/cm^2 are not shown in Fig. 7). The ΔA yields were normalized to the fluorescence yields at a flash energy of 0.031 mJ/cm^2 (Fig. 7A).

(ii) The apparent saturation behavior of BChl* formation as a function of the excitation energy (Fig. 8) can be explained by assuming that the decay of BChl* becomes relatively fast at high excitation energies. When the decay time (or part of the decay) becomes of the order of the duration

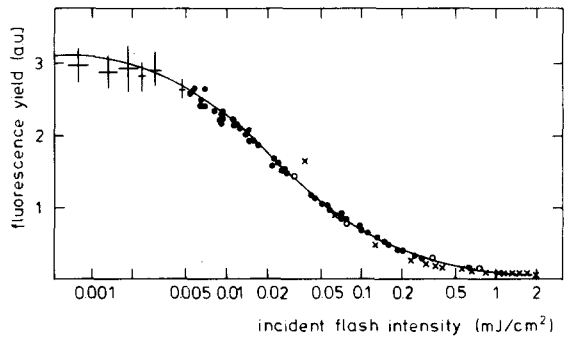

Fig. 10. *R. rubrum* chromatophores. The following quantities are plotted as a function of the intensity of a picosecond flash: (●) Fluorescence yield [13] (the uncertainties in the low-energy region are indicated by the error bars). (○), Relative yield of the absorbance change due to formation of BChl*. (×) The fluorescence yield as calculated from the saturation of the absorbance change. See text for details.

of the laser pulse, the measured amplitude of the absorbance change will be decreased. If we make the simplifying assumption that BChl* decays monoexponentially with lifetime τ, then a convolution procedure of this decay with 35 ps Gaussian excitation and probe pulses yields a normalized absorbance change at 0 ps as a function of τ. From a comparison with Fig. 8 it is then possible to assign an average lifetime to each of the measured points. If the additional assumption is made that τ equals 350 ps at low excitation energy (i.e., in the absence of biexcitation annihilation) then the expected fluorescence yield as a function of the laser energy can be calculated from the data of Fig. 8. The results are shown in Fig. 10 (crosses). The fluorescence yield corresponding to a decay of 350 ps was set to 3.1 for normalization purposes.

From inspection of the figure it is seeen that the results obtained by picosecond pulse-induced fluorescence and absorbance spectroscopy agree very well.

From the above described convolution procedure it followed that the average lifetime of BChl* with all the traps closed decreases from 350 ps at low excitation energy to 10 ps at the highest excitation energy at which about 10 BChl* are formed per reaction center. Since the loss rates of excitations due to fluorescence, triplet formation and internal conversion is about $5 \cdot 10^8$ s^{-1} [13], a lifetime of 300–400 ps corresponds to a loss yield

of 15–20%, in agreement with results by Kingma et al. [5]. The estimated lifetime of BChl* with all the traps open will then be of the order of 100 ps, assuming a loss yield of 7% [5]. In a subsequent paper we shall show that the experimentally obtained low-intensity lifetime under these conditions is close to 80 ps. These data correspond reasonably well with the fluorescence lifetimes of 200 ps and 60 ps obtained for closed and open traps, respectively, by Freiberg et al. [11], but are markedly different from the results obtained by Borisov et al. in an experiment similar to ours. The latter authors reported a decay time of BChl* of 60 ± 15 ps at 905 nm, independent of the excitation energy and the state of the traps [17], and two components of 60 ps and less than 20 ps at 880 nm [15,16]. The inconsistency with our data is presumably due to the fact that their kinetics only ranged to about 300 ps after the pulse and to a neglect of the distortion of the kinetics by convolution with the shape of the pulses.

The shape of the absorbance difference spectra due to BChl* formation is independent of the excitation energy in the interval from $1 \cdot 10^{14}$–$7 \cdot 10^{15}$ photons per cm^2 and shows a maximal bleaching at 888 nm. At a flash energy of more than approx. 10^{17} photons per cm^2 the maximum of the bleaching is shifted to 885 nm (Fig. 9). These results are again in contrast to those obtained by Borisov et al. [15], who found the maximal bleaching at 905 nm for excitation energies of less than 10^{16} photons per cm^2 and at 880 nm for an energy of $4 \cdot 10^{17}$ photons per cm^2 (although it was later reported that the low-intensity peak was in fact between 895 and 900 nm [private communication]). The reason for this discrepancy is not clear. These observations led Borisov et al. to the hypothesis that the bulk B880 antenna and the reaction center are coupled via a minor antenna pigment B905 on which at low excitation energies all the excitations are concentrated.

Recently, low temperature spectroscopy has shown that the B880 antenna of all purple bacteria including *R. rubrum* is indeed inhomogeneous [23]. About 10–20% of the BChl molecules were found to absorb at longer wavelength (about 12 nm) compared to the main fraction. This inhomogeneity may contribute to the observed spectral effects on picosecond pulse excitation (Fig. 9), but in view of the small energy difference between the two BChl species and the relatively low concentration of the long-wavelength absorbing BChl, we do not expect a large concentration of excitations on this latter pigment at room temperature.

We therefore propose a different interpretation for the shape of the difference spectrum after a picosecond excitation pulse. If we assume that the difference spectrum is mainly composed of a bleaching of B880 molecules and an absorbance increase solely due to the formation of BChl*, then the shape of the spectrum should be independent of the excitation intensity, in contrast to our observations (Fig. 9). Therefore the absorbance increase around 850 nm in the low-intensity spectra cannot be due to BChl* only. Alternatively, we can ascribe the absorbance increase to a blue shift of the remaining BChl ground state absorption that results from the excitation of a neighboring BChl. Then the integral over the difference spectra of Figs. 9a, b or c should yield the area of the bleaching only. From this area and the fraction of excited molecules, as calculated from the incident excitation intensity, from the absorbance of the sample at 532 nm and with a transfer efficiency from Car* to BChl of 30%, a value of about 180 $mM^{-1} \cdot cm^{-1}$ for the extinction coefficient of BChl in its Q_y band is calculated. This value compares reasonably well with that of 140 $mM^{-1} \cdot cm^{-1}$ given by Clayton et al. [24], and we conclude that a single BChl molecule is bleached per excitation in the B880 antenna.

Fig. 11 shows an absorbance difference spectrum and its decomposition into a bleaching and a blue shift. The shape of the spectrum of the bleaching is assumed to be identical to that of the absorption spectrum; the shift is calculated by subtracting the bleaching from the measured spectrum. If it is assumed that the molecules partaking in the shift originally all absorbed at 880 nm and shift equally, then from the wavelength of the zero transition (877 nm) it follows that the blue shift occurs over 6 nm. It is then easily calculated that about six BChl *a* molecules take part in the shift upon excitation of one BChl molecules. It is noteworthy at this point that the B880 complex may be composed of units of 6–8 BChl molecules [25]. Our results then indicate that the presence of one excited BChl molecule induces a blue shift of

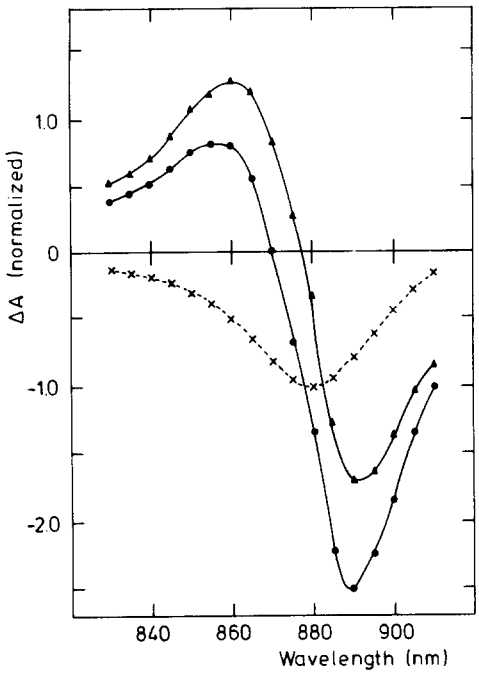

Fig. 11. Decomposition of the absorbance difference spectrum (●) induced in chromatophores of *R. rubrum* upon excitation with a picosecond flash at 0 ps, into a bleaching (×) and a shift (▲) of the original absorption spectrum. See text for details.

the whole unit. The shift of the molecules could result from their release from the exciton interaction with the molecule that had been excited. A similar blue shift was observed in the B800–850 complex of *Rhodopseudomonas sphaeroides* upon triplet formation by Shuvalov and Parson [26] and interpreted on basis of change of interaction between two molecules, and upon formation of the singlet excited state (Nuijs, A.M. et al., unpublished results). Upon increasing the excitation intensity, one would expect the other bacteriochlorophyll molecules to bleach when more than one excitation is generated per unit. This is in agreement with Fig. 9d. The amplitude of the shift is reduced relative to that of the bleaching, and as a result the minimum in the difference spectrum is shifted from 888 to 885 nm when more than about 20% of the BChl molecules are excited.

In conclusion, the picosecond difference spectra induced in *R. rubrum* chromatophores can be explained by assuming a bleaching of one BChl molecule per B880 unit coupled to a blue shift of the absorption of the remaining bacteriochlorophyll. It is not necessary to invoke the fast and irreversible concentration of all the excitations on a special long-wavelength antenna pigment [15,16].

Acknowledgements

The authors are indebted to Dr. M.J.M. van de Ven for valuable help and to Prof. Dr. J. Amesz for reading the manuscript. The investigation was supported by the Netherlands Foundation for Biophysics, financed by the Netherlands Organization for the Advancement of Pure Research (ZWO).

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