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TRIPLET STATES OF BACTERIOCHLOROPHYLL AND CAROTENOIDS IN CHROMATOPHORES OF PHOTOSYNTHETIC BACTERIA

TERESA G. MONGER, RICHARD J. COGDELL* and WILLIAM W. PARSON

Department of Biochemistry, University of Washington, Seattle, Wash. 98195 (U.S.A.)

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SUMMARY

Chromatophores from photosynthetic bacteria were excited with flashes lasting approx. 15 ns. Transient optical absorbance changes not associated with the photochemical electron-transfer reactions were interpreted as reflecting the conversion of bacteriochlorophyll or carotenoids into triplet states. Triplet states of various carotenoids were detected in five strains of bacteria; triplet states of bacteriochlorophyll, in two strains that lack carotenoids. Triplet states of antenna pigments could be distinguished from those of pigments specifically associated with the photochemical reaction centers. Antenna pigments were converted into their triplet states if the photochemical apparatus was oversaturated with light, if the primary photochemical reaction was blocked by prior chemical oxidation of *P*-870 or reduction of the primary electron acceptor, or if the bacteria were genetically devoid of reaction centers. Only the reduction of the electron acceptor appeared to lead to the formation of triplet states in the reaction centers.

In the antenna bacteriochlorophyll, triplet states probably arise from excited singlet states by intersystem crossing. The antenna carotenoid triplets probably are formed by energy transfer from triplet antenna bacteriochlorophyll. The energy transfer process has a half time of approx. 20 ns, and is about 1×10^3 times more rapid than the reaction of the bacteriochlorophyll triplet states with O_2. This is consistent with a role of carotenoids in preventing the formation of singlet O_2 in vivo. In the absence of carotenoids and O_2, the decay half times of the triplet states are 70 μs for the antenna bacteriochlorophyll and 6–10 μs for the reaction center bacteriochlorophyll. The carotenoid triplets decay with half times of 2–8 μs.

With weak flashes, the quantum yields of the antenna triplet states are in the order of 0.02. The quantum yields decline severely after approximately one triplet state is formed per photosynthetic unit, so that even extremely strong flashes convert only a very small fraction of the antenna pigments into triplet states. The yield of fluorescence from the antenna bacteriochlorophyll declines similarly. These observations can be explained by the proposal that singlet-triplet fusion causes rapid quenching of excited singlet states in the antenna bacteriochlorophyll.

Abbreviation: BChl, bacteriochlorophyll.

* Present address: Department of Botany, University of Glasgow, Glasgow, U.K.

INTRODUCTION

The photosynthetic apparatus includes both reaction centers where the photochemical electron-transfer reactions occur, and an antenna system that absorbs light and directs energy to the reaction centers [1, 2]. In the photosynthetic bacteria, the antenna consists of an assembly of 40–200 bacteriochlorophyll (BChl) molecules per reaction center, and a somewhat smaller collection of various carotenoids [3]. The antenna pigments are associated noncovalently with hydrophobic proteins [4, 5], but little is known of their functional organization. Energy absorbed by the carotenoids is transferred to the BChl of the antenna with an efficiency of 30–90 %, depending on the species [6]. If the reaction center is in a photochemically active state, it traps energy from the antenna BChl extremely rapidly, probably in less than $1 \cdot 10^{-10}$ s [7].

This paper describes a study of transient states of the antenna pigments, following flash excitation of chromatophores under conditions that prevent the trapping of energy at the reaction center. The reaction center traps can be "closed" (or "filled") by chemical reduction of the primary electron acceptor (X) or oxidation of the electron donor (*P*-870). They can be filled photochemically by excitation with flashes that are supersaturating with respect to the photochemical reaction. Previous workers have observed the formation of metastable states in chromatophores under these conditions, and have afforded them at least four different interpretations. At redox potentials low enough to reduce X, flash excitation of chromatophores of *Rhodospirillum rubrum* [8] or *Chromatium vinosum* [9, 10] causes the formation of a transient state characterized by an absorption band near 420 nm. The formation of this state was confused initially with the photooxidation of *P*-870, which causes absorbance changes in the same region of the spectrum [8]. Seibert and coworkers [9, 10] later interpreted the transient state as reflecting a second photochemical system capable of operating at very low redox potentials. In chromatophores of *Rhodopseudomonas sphaeroides* strain Ga, excitation with very intense flashes causes the formation of a state with an absorption band near 510 nm [11]. This state can be seen whether or not the photooxidation of *P*-870 is blocked by the reduction of X. Leigh et al. [11] have interpreted the transient state as reflecting an electrochromic shift of a carotenoid absorption band. Similar metastable states have been seen in chloroplasts after excitation with supersaturating flashes [12–15] and in preparations of reaction centers from photosynthetic bacteria [16] after excitation at low redox potentials, and have been interpreted as triplet states of carotenoids. For reasons which we shall elaborate under Results, this last interpretation appears to be the most satisfactory. In very recent studies, Kung and DeVault [17] have come to the same conclusion.

Carotenoids have long been known to play an important role in protecting both photosynthetic and nonphotosynthetic organisms against destructive oxidation reactions which can occur in the combined presence of light and oxygen [18]. The destructive agent in these processes appears to be O_2 in its $^1\Delta g$ (singlet) state [18, 19]. Oxygen can be elevated to this state from its ground (triplet) state by energy transfer from the excited triplet states of many different sensitizers, including BChl. In photosynthetic bacteria, antenna BChl would have the opportunity of intersystem crossing to its triplet state if energy is not removed rapidly by trapping at the reaction

center. The absorption of light thus could have unfortunate consequences in the presence of O_2 if the reaction centers are closed. Carotenoids might intervene by quenching triplet BChl rapidly enough to prevent the formation of singlet O_2, or failing that, by quenching the singlet O_2. Both types of quenching occur at nearly diffusion-controlled rates in solution [19–21]. In either case, the quenching involves the transfer of energy to the carotenoid, with the promotion of the carotenoid to a triplet state.

MATERIALS AND METHODS

Cultures of *Rps. sphaeroides* strains 2.4.1, Ga and R-26, and *Rds. rubrum* strains S-1 and G-9 were obtained from Dr. R. K. Clayton. The nonphotosynthetic mutant *Rps. sphaeroides* strain PM-8 dpl was obtained from Dr. W. R. Sistrom. This strain is derived from strain Ga via an intermediate strain, PM-8. The absorbance spectra of chromatophores prepared from our cultures of strains PM-8 dpl and Ga were very similar. *C. vinosum* strain D was grown photoautotrophically as described elsewhere [22]. All other photosynthetic bacterial strains were grown photoheterotrophically under anaerobic conditions with succinate as the sole carbon source. Strain P-8 dpl was grown semiaerobically in the dark on a medium containing yeast extract, casamino acids and malate.

Washed cells were disrupted by passage through a French pressure cell (or, on rare occasions, by sonic oscillation) and crude chromatophores were prepared as described by Clayton and Clayton [4]. Chromatophore preparations were further clarified using sucrose density gradient centrifugation or sonic oscillation, if required. Chromatophores were stored at −5 °C in 40 % ethylene glycol 60 % Tris · HCl (0.1 M, pH 7.4) for periods up to several months. Reaction centers from *Rps. sphaeroides* 2.4.1 and R-26 were isolated as described previously [16].

Photosynthetic unit concentrations in chromatophore suspensions were measured from flash-induced absorbance changes at 605 nm reflecting *P*-870 photooxidation [23]. (We use the term "photosynthetic unit" in an operational sense to indicate a reaction center with its associated antenna. The term is not intended to imply a structural model of the photosynthetic apparatus.) Chromatophores from *Rps. sphaeroides* strain PM-8 dpl were shown to lack functional reaction centers by this criterion. The concentrations of purified reaction centers were measured from the absorbance at 802 nm [24]. Bacteriochlorophyll concentrations were measured from the absorbance spectra of the intact chromatophores, using the extinction coefficients given by Clayton [25].

For study, chromatophores were suspended in solutions containing 10 mM Tris · HCl (pH 7.6) and 100 mM KCl, transferred to an anaerobic cuvette with a 1-cm light path, and bubbled gently with N_2 for approx. 5 min to remove O_2. Bubbling with N_2 was continued for 1.5 h to insure more rigorous anaerobiosis for measurements of the triplet decay kinetics. When specified, the redox potential was monitored with Pt and calomel electrodes as described elsewhere [22]. For most of the experiments at low redox potentials, the potential was lowered by the addition of excess solid $Na_2S_2O_4$, and the potential was not monitored. In the figure legends and text, "moderate" potential indicates a potential of about +250 mV, which is optimal for *P*-870 photooxidation.

Absorbance changes following laser-flash excitation were measured essentially as described previously [26, 27]. Laser flashes of both 694 and 834 nm were used; their width at half-maximum amplitude generally was approx. 15 ns. For most of the measurements in the μs time range, the preamplified signal from the measuring photomultiplier was digitized for signal averaging with a computer of average transients. Two systems were used for analog-to-digital conversion. One employed a Biomation transient recorder (model 610 or 802); the other was a home-built instrument employing a video camera, similar to that described by Den Haan et al. [28]. Averaging usually was performed with four flashes, spaced 1 min apart.

For fluorescence measurements, the chromatophore samples were held in the same 1-cm cuvettes that were used for the absorbance measurements. Fluorescence emitted at 90° to the excitation path passed through a monochromator (910 nm), a 910 nm interference filter and three Corning 2600 glass filters, and was detected with an RCA 7102 photomultiplier. The photomultiplier anode was connected via 50 Ω cable to a Tektronix 7903 oscilloscope equipped with a 7A19 vertical amplifier and a camera. Before the laser excitation flash reached the sample, a beam splitter directed a small portion of the flash to a calibrated photodiode and integrator [26]. The integrated photodiode signal was digitized and stored with the Biomation 610 transient recorder to provide a record of the flash strength. Neutral density filters calibrated at 694 nm were placed between the beam splitter and the sample to adjust the incident flash intensity, and filters calibrated at 910 nm were placed between the sample and the monochromator to adjust the sensitivity of the detection system. The latter filters were used to maintain the fluorescence intensity in a range over which the photomultiplier's response was linear. Linearity was checked by measurements of scattered laser light, after appropriate modification of the monochromator and the auxilliary filters.

For both fluorescence and absorbance experiments, the amount of laser light absorbed was calculated from the incident irradiance, as measured with a ballistic thermopile, and the absorbance of the sample, as measured in an integrating sphere with continuous light at the laser's wavelength. This calculation would overestimate the amount of light absorbed, if the flash were to deplete the population of the ground state BChl. However, the lifetime of the excited singlet state of the antenna BChl probably is short enough with respect to the width of the flash so that such depletion is not significant in the present experiments (see Discussion).

RESULTS

I. The carotenoid metastable state

When chromatophores are illuminated with a laser flash of relatively low intensity, the well-known absorbance changes generated as a result of electron transfer in the reaction center can be observed. These include absorbance changes due to the photooxidation of *P*-870, and changes due to a small bathychromic shift in the absorption bands of antenna carotenoids. Fig. 1A shows the increase in absorbance at 535 nm so generated in *Rps. sphaeroides* 2.4.1 chromatophores. Absorbance changes due to *P*-870 are small at this wavelength, and they do not decay significantly during the μs timescale of the figure. If the same chromatophores are illuminated with a laser flash of higher intensity, additional absorbance changes

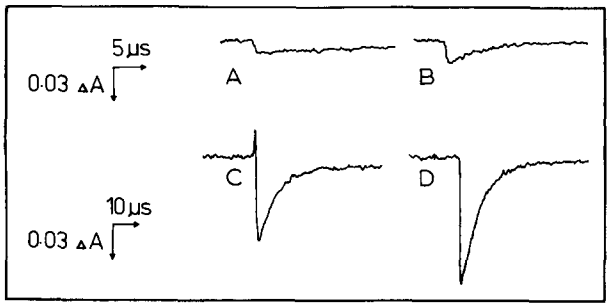

Fig. 1. Flash-induced absorbance changes at 535 nm in chromatophores from *Rps. sphaeroides* 2.4.1. A downward deflection is an absorbance increase. (A) moderate redox potential (about +250 mV); 47 μM BChl (0.40 μM photosynthetic unit); actinic wavelength, 694 nm; flash strength about 4 neinsteins absorbed · cm^{-2} (10 quanta per photosynthetic unit). (B) as A except low redox potential (excess $Na_2S_2O_4$ added prior to the flash). (C) as A except 49 μM BChl (0.43 μM photosynthetic unit) and flash strength about 700 neinsteins absorbed · cm^{-2} (1630 quanta per photosynthetic unit). The upward spike in trace C is a flash artifact. (D) as C except low potential.

with shorter decay times can be observed (Fig. 1C). Similar transient absorbance changes can be seen after flash excitation when the photooxidation of *P*-870 is blocked by chemical reduction of the primary electron acceptor (Fig. 1B and D). In fact, the amplitude of the short-lived absorbance changes is greater under these conditions than it is at higher redox potentials. There is no decrease in the amplitude of the absorbance changes, even at potentials as low as −500 mV. The transient absorbance changes also can be seen when photochemistry is blocked by the chemical oxidation of *P*-870. At a redox potential of +500 mV, well above the +450 mV midpoint potential of *P*-870, the amplitude of the absorbance changes is essentially the same as it is at moderate potentials when the photooxidation of *P*-870 can occur. (At potentials much above +500 mV, oxidation of the antenna BChl occurs, and the amplitude of the flash-induced absorbance changes does decline.) From these observations, it is clear that the transient absorbance changes do not result from the normal electron-transfer reactions of photosynthesis, but rather reflect a process that occurs when the normal reactions are blocked or saturated. Additional evidence for this conclusion was obtained by examining chromatophores of *Rps. sphaeroides* strain PM-8 dpl, which lacks the photosynthetic reaction center. Transient absorbance changes of the same type occur in the PM-8 dpl chromatophores.

Fig. 2 shows difference spectra for the transient absorbance changes generated by flash excitation of chromatophores of three carotenoid-containing strains of *Rps. sphaeroides*, including PM-8 dpl, and one strain each of *Rds. rubrum* and *C. vinosum*. For all of these measurements, except those with *Rps. sphaeroides* PM-8 dpl, the redox potential was low enough to block *P*-870 photooxidation. Essentially identical difference spectra are generated by strong flashes at higher redox potentials, if absorbance changes due to photochemical processes are discounted. (As expected, discounting other absorbance changes was not necessary with strain PM-8 dpl, and in this case the amplitude of the absorbance changes was not affected by lowering the redox potential.) Absorbance changes of the type illustrated in Fig. 2 were not seen in two strains which lack carotenoids (*Rps. sphaeroides* R-26 and *Rds. rubrum*

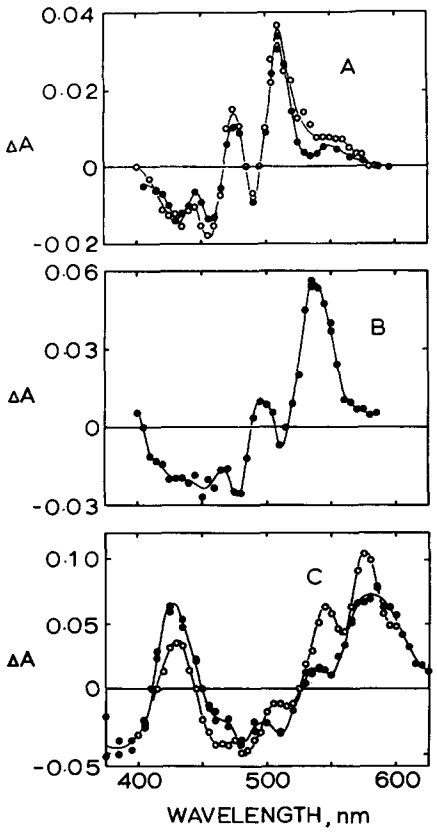

Fig. 2. Flash-induced difference spectra for the formation of Car^T states in chromatophores from five carotenoid-containing strains. Actinic wavelength, 694 nm; flash strength, 600–900 neinsteins absorbed · cm^{-2}. (A) ○, *Rps. sphaeroides* Ga; 17 μM BChl; low redox potential; ●, *Rps. sphaeroides* PM-8 dpl; 19 μM BChl; moderate potential. (B) *Rps. sphaeroides* 2.4.1; 22 μM BChl; low potential. (C) ○, *C. vinosum*, 26 μM BChl, low potential; ●, *Rds. rubrum* S-1; 20 μM BChl; low potential.

G-9), but were replaced by absorbance changes which were spectrally and kinetically distinct (see below).

The difference spectra shown in Fig. 2 include a bleaching of carotenoid ground-state absorption bands, and the development of strong, new bands to the red of the original bands. Comparison of the spectra obtained with the five different strains shows that the new bands lie progressively farther to the red, in the sequence *Rps. sphaeroides* PM-8 dpl ≈ Ga < 2.4.1 < *Rds. rubrum* S-1 ≈ *C. vinosum* D. This sequence parallels a change in the nature of the carotenoids present in chromatophores of the different strains. *Rps. sphaeroides* PM-8 dpl and Ga are defective in carotenoid biosynthesis and contain predominantly neurosporene and chloroxanthin, with nine conjugated double bonds; *Rps. sphaeroides* 2.4.1, the wild type, contains principally spheroidene, with 10; and *Rds. rubrum* S-1 and *C. vinosum* D contain spirilloxanthin, with 13 [29–31]. The positions of the ground-state carotenoid absorption bands follow the same sequence. The difference spectra for the formation of

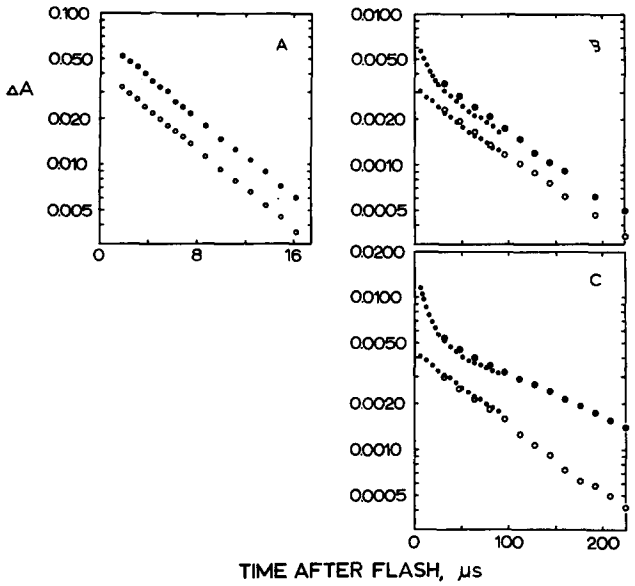

Fig. 3. Decay kinetics of flash-induced absorbance changes due to Car^T and $BChl^T$ in chromatophores. The abscissa scales are logarithmic. All samples were bubbled extensively with N_2 to remove O_2. (A) *Rps. sphaeroides* 2.4.1 chromatophores; photosynthetic unit concentration, 0.24 μM; ΔA due to Car^T measured at 535 nm; actinic wavelength, 694 nm; flash strength, about 250 neinsteins absorbed · cm^{-2}. ○, moderate redox potential; ●, low potential. (B) *Rps. sphaeroides* R-26 chromatophores; photosynthetic unit concentration, 0.59 μM; ΔA due to $BChl^T$ measured at 510 nm; actinic wavelength, 834 nm; flash strength, about 300 neinsteins absorbed · cm^{-2}. ○, moderate potential; ●, low potential. Different sized symbols indicate separate measurements with different instrumental timescales. (C) as B except *Rds. rubrum* G-9 chromatophores; photosynthetic unit concentration, 0.89 μM.

the transient states are similar to the spectra that are expected for the conversion of carotenoids to their lowest excited triplet states. Such spectra have been obtained for many different polyenes in solution, and it has been shown that the triplet-triplet absorption bands and the singlet-singlet bands move in parallel to progressively lower energies as the length of the conjugated chain increases [16, 32, 33]. In the spectra for *Rds. rubrum* S-1 and *C. vinosum* D, the triplet-triplet absorption bands lie at sufficiently low energies that a second strong band becomes apparent near 420 nm (Fig. 2C). It apparently was this second band which was detected in the earlier work of Parson [8] and Seibert et al. [9, 10].

The decay kinetics of the transient states also are similar to the decay kinetics which have been reported [32, 33] for the triplet states of carotenoids in solution. The kinetics are first order, at both moderate and low redox potentials (Fig. 3A). In the absence of O_2 they have rate constants that range from 0.8×10^5 s^{-1} for *Rps. sphaeroides* PM-8 dpl and Ga to 1.4×10^5 s^{-1} for *Rps. sphaeroides* 2.4.1 and 2.5×10^5 s^{-1} for *Rds. rubrum* S-1 and *C. vinosum* D. The variation of the decay rate constant with polyene chain length is expected from the fact that as the number of conjugated double bonds increases, the triplet state energy decreases and the possibilities for vibrational relaxation increase. Both of these effects can facilitate faster radiationless decay of the triplet state.

The triplet nature of the transient states is supported further by the observation that O_2 markedly accelerates the decay of the absorbance changes. In chromatophores of *Rps. sphaeroides* PM-8 dpl, for example, the decay halftime is 8.6 μs in the absence of O_2. If the solution is saturated with O_2 at 1 atmosphere (giving approx. 1.2 mM O_2 in solution), the decay halftime decreases to 1.2 μs. The initial extent of the absorbance change is unaltered, and the decay kinetics remain pseudo-first-order. Samples in equilibrium with air (240 μM O_2 in solution) give a decay halftime of 3.0 μs. These data give a second order rate constant of approx. 5×10^8 $M^{-1} \cdot s^{-1}$ for the quenching of the transient state by O_2. Essentially the same rate constant is obtained with the other bacterial strains.

For the reasons that are stated above, we conclude that the transient absorbance changes reflect the excitation of carotenoids to their lowest triplet states. We shall refer to these states collectively as Car^T states.

The question that arises next is to what extent the Car^T states form in the carotenoids of the antenna system, and to what extent they represent carotenoids that are specifically associated with the reaction center. Preparations of purified reaction centers from carotenoid-containing strains contain close to one equivalent of a specially bound carotenoid which can be excited to a Car^T state if the photooxidation of *P*-870 is blocked by chemical reduction of X [16, 34]. The spectral and kinetic properties of the triplet states of the reaction center carotenoids are similar to those that are described above. It is clear, however, that the Car^T states seen in chromatophores under certain conditions must be due to antenna carotenoids rather than to the reaction center pigments. The Car^T states in chromatophores of *Rps. sphaeroides* PM-8 dpl must fall in this class, because this strain lacks reaction centers. So must the Car^T states that are generated in any of the strains at moderate redox potentials, or at potentials that are high enough to oxidize *P*-870 chemically before the flash. Car^T states of the reaction center carotenoids do not form under these conditions [16]. In agreement with this, the amount of Car^T that strong flashes generate in chromatophores of *Rps. sphaeroides* Ga at moderate potentials is essentially identical with the amount that they generate in strain PM-8 dpl. Fig. 4A demonstrates this point by comparing the absorbance changes that occur in the two strains, as a function of the intensity of the laser flash.

At very low redox potentials, on the other hand, one might expect to generate Car^T states in the reaction centers of chromatophores, as well as in the antenna.This could account for the observation that, in all of the strains other than PM-8 dpl, the Car^T absorbance changes are larger at low redox potentials than they are at moderate or high potentials (Figs. 1 and 3A). Fig. 4B shows a more extensive comparison of the absorbance changes that accompany the formation of Car^T states in *Rps. sphaeroides* 2.4.1 chromatophores at low and moderate redox potentials, plotted as a function of the flash intensity. At all flash intensities, the absorbance changes at low potentials are greater than those at moderate potentials, but the difference between the two reaches a maximum with relatively weak flashes.

To consider whether the additional absorbance changes that occur at low redox potentials are appropriate in magnitude for a contribution from reaction centers, one needs to know the differential extinction coefficient, $\Delta\varepsilon$, for the formation of Car^T. A reexamination of reaction centers isolated from *Rps. sphaeroides* 2.4.1 gave a value of 0.085 $\mu M^{-1} \cdot cm^{-1}$, on the assumption that saturating excitation

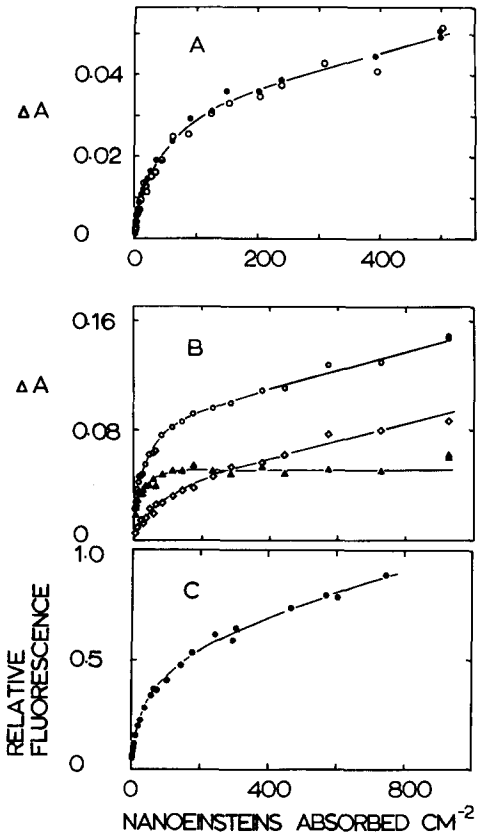

Fig. 4. Formation of the Car^T states and BChl fluorescence in *Rps. sphaeroides* chromatophores, as a function of the strength of the actinic flash. Actinic wavelength, 694 nm. (A) ○, *Rps. sphaeroides* Ga; 20 μM BChl; moderate redox potential; ΔA measured at 510 nm; ●, as ○, except *Rps. sphaeroides* PM-8 dpl and 19 μM BChl. (B) ◇, *Rps. sphaeroides* 2.4.1; 47 μM BChl (0.40 μM photosynthetic units); moderate redox potential; ΔA measured at 535 nm; ○, as ◇, except low redox potential; ▲, difference between ○ and ◇. (C) as B ◇, except fluorescence measured at 910 nm.

generates' one Car^T per reaction center at low redox potential [40]. This value is somewhat larger than values that can be calculated from data in ref. 16, because of a more accurate calibration of the absorbance changes. It is smaller than the $\Delta\varepsilon$ values of 0.15–0.20 $\mu M^{-1} \cdot cm^{-1}$ that have been reported for carotenoids in solution [33]. Taking 0.1 $\mu M^{-1} \cdot cm^{-1}$ as a rough estimate of $\Delta\varepsilon$, the difference between the amounts of Car^T formed at low and moderate redox potentials in chromatophores (Figs. 1, 3A and 4B) corresponds to 1.1–1.3 mol Car^T per mol of phosphosynthetic units. This seems consistent with the conclusion that the additional component is due mainly to the reaction centers, considering the uncertainties inherent in the determination of $\Delta\varepsilon$. Measurements with chromatophores of *Rps. sphaeroides* Ga and *Rds. rubrum* S-1 gave similar results: the amount of Car^T formed at low redox potentials was greater than that formed at moderate potentials, by approximately the amount that one would expect the reaction centers to contribute. It is possible, however, that lowering the redox potential also influences the amount of Car^T that

is formed in the antenna, and that this accounts for part of the additional Car^T.

The formation of the antenna Car^T in chromatophores depends on the flash intensity in a complicated manner (Fig. 4A and B). The initial slope of a plot of the absorbance changes as a function of the flash intensity provides a measure of the quantum yield of Car^T. Taking $\Delta\varepsilon$ as 0.1 $\mu M^{-1} \cdot cm^{-1}$, the yield is approximately 0.02 in *Rps. sphaeroides* PM-8 dpl. (The yields of the antenna carotenoid triplets are similar to this in the strains that contain functional reaction centers, but they are more difficult to measure unambiguously, because of the effects of the competing reactions that occur at the reaction centers.) As one increases the flash intensity, the amount of Car^T that is formed increases steadily until the flashes are intense enough to provide approx. 200 quanta per photosynthetic unit. At this point, slightly less than 1 mol of Car^T is formed per mol of photosynthetic units at moderate redox potentials (and about 2 mol at low potentials in the strains that contain reaction centers). With stronger flashes, the amount of Car^T formed continues to increase indefinitely with flash intensity, but the quantum yield drops significantly. Mathis [35] has reported similar observations on the formation of carotenoid triplets in broken chloroplasts.

Fig. 4C shows parallel measurements of the amount of fluorescence emitted by the antenna BChl, as a function of the flash intensity. These measurements employed *Rps. sphaeroides* 2.4.1 chromatophores at moderate redox potentials. Fluorescence parallels Car^T in its dependence on flash intensity, declining in quantum yield as the flashes increase in strength. The parallel behavior of fluorescence and Car^T suggests that the decline in the yield of Car^T at very high flash strengths is due to an increasingly rapid quenching of the excited singlet state of the antenna BChl.

To place the measurements of Fig. 4 in perspective, Fig. 5 shows measurements of the relative quantum yield of fluorescence over a much wider range of flash intensities, including flashes that were weak enough so that they did not saturate photochemistry at the reaction centers. The fluorescence yield is maximal for flashes that provide approx. 3 quanta per photosynthetic unit. Separate measurements of *P*-870 photooxidation showed that such flashes were approx. 80 % saturating with respect to photochemistry. With weaker flashes, photochemistry provides an increasingly

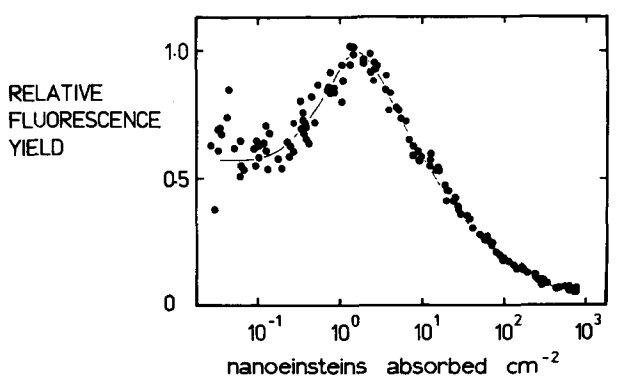

Fig. 5. Relative yield of BChl fluorescence (fluorescence intensity/flash intensity), as a function of the strength of the excitation flash. *Rps. sphaeroides* 2.4.1 chromatophores; 47 μM BChl (0.40 μM photosynthetic units); moderate redox potential.

important quenching path for excited BChl molecules in the antenna, and the fluorescence yield declines. The behavior of the fluorescence yield with very weak flashes has been described previously for *C. vinosum* chromatophores [26]. Mauzerall [36] and Campillo et al. [37] recently have described fluorescence quenching with very strong flashes in studies of the alga *Chlorella pyrenoidosa*. Using flashes of 7 ns or 20 ps duration, Mauzerall [36] and Campillo et al. [37] found the fluorescence yield to decrease with increasing flash intensity. The decline in yield that they observed was somewhat more gradual than that shown in Fig. 5. The mechanism of the quenching will be considered in the Discussion.

Fig. 6 shows the kinetics of the formation of the Car^T states under various conditions. At low redox potentials, and with relatively weak flashes, the rise half-times are approx. 30 ns for *Rps. sphaeroides* 2.4.1 (Fig. 6A) and Ga (not shown), 45 ns for *Rds. rubrum* S-1 (Fig. 6B), and 25 ns for *C. vinosum* (not shown). These measurements probably reflect the formation of Car^T in both the reaction centers and the antenna, with the contributions from the reaction centers predominating.

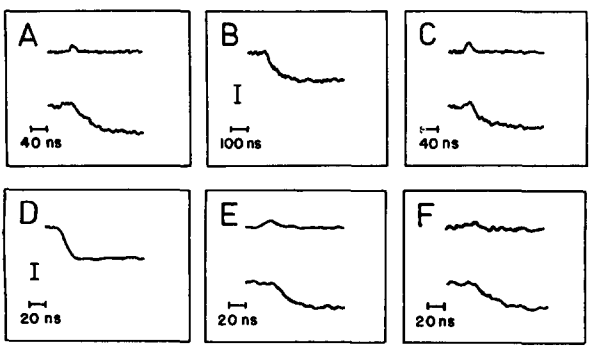

Fig. 6. Rise kinetics of flash-induced absorbance changes reflecting the formation of Car^T states in chromatophores from four carotenoid-containing strains. Actinic wavelength, 834 nm in all cases. The vertical scale markers in B and D indicate absorbance changes of 0.04, and apply to all of the measurements in the figure. A downward deflection is an absorbance increase. (A) *Rps. sphaeroides* 2.4.1 chromatophores; 58 μM BChl; low potential; ΔA measured at 535 nm. (B) *Rds. rubrum* S-1 chromatophores; 36 μM BChl; ΔA measured at 425 nm; low potential. (C) *Rps. sphaeroides* PM-8 dpl chromatophores; 36 μM BChl; moderate potential; ΔA measured at 510 nm. (D) purified reaction centers of *Rps. sphaeroides* R-26; 4.3 μM reaction centers; 50 mM Tris · HCl, pH 7.5/0.05 % Triton X-100; moderate potential; ΔA measured at 430 nm. This trace shows the absorbance change due to *P*-870 photooxidation, a process that is very fast on the time scale of the present measurements. It is included to show the instrumental response time of approx. 6 ns. (E) *Rps. sphaeroides* Ga chromatophores; 72 μM BChl; moderate potential; ΔA measured at 510 nm. (F) *Rps. sphaeroides* 2.4.1 chromatophores; 97 μM BChl; moderate potential; ΔA measured at 535 nm. In A, C, E and F, the upper traces show the responses of the apparatus to laser flashes in the absence of the measuring light. These traces give an indication of the sizes of flash artifacts that can complicate the measurements in the lower traces. They also indicate the timing and duration of the flashes. Flash artifacts were undetectably small in the measurements of B and D. Risetimes given in the text are averages of approx. 5 measurements. They were obtained from semilog plots of the absorbance changes, starting from the point at which the flash artifact became negligible; they are corrected for the instrumental response time if necessary. To minimize flash artifacts, all of the measurements were made with relatively weak flashes (in the order of 50 neinsteins absorbed · cm^{-2}). The photooxidation of *P*-870 contributes 10–20 % of the absorbance changes in E and F. (The chromatophore samples contained approx. 0.7 and 1.0 μM photosynthetic units, and $\Delta\varepsilon$ for the photooxidation of *P*-870 is approx. 0.01 $\mu M^{-1} \cdot cm^{-1}$ at both 510 and 535 nm.)

The halftime listed for *C. vinosum* is somewhat less than the halftime Seibert and DeVault [10] have reported, but those for the other strains are slightly longer than the halftimes that we have measured in isolated reaction centers [16]. In the antenna carotenoids of *Rps. sphaeroides* PM-8 dpl chromatophores, the rise halftime is about 25 ns (Fig. 6C). In chromatophores of *Rps. sphaeroides* strains 2.4.1 and Ga, measurements at moderate redox potentials indicate halftimes of approx. 12 ns (Fig. 6E and F). The risetime of the Car^T states in the antenna carotenoids of strains 2.4.1 and Ga probably is slightly longer than 12 ns, because the photooxidation of *P*-870 makes a small but fast contribution to the absorbance changes shown in the figure.

II. The bacteriochlorophyll metastable state

Carotenoids in solution generally do not undergo intersystem crossing from their excited singlet states to give triplet states in significant yields [32]. Instead, their triplet states usually arise by the transfer of energy from donors in triplet excited states. Triplet BChl can act as such a donor in solution [39], and one would expect it to play a similar role in the formation of the Car^T states of chromatophores. In accord with this view, the 694 nm and 834 nm light that we have used for excitation of the chromatophores is absorbed by the BChl, but not by the carotenoids themselves. In isolated reaction centers, Car^T states arise by energy transfer from an excited state called P^R which appears to be a triplet state of the reaction center BChl [16, 27, 40]. Presumably, the same state is a precursor of the Car^T states that form in the reaction centers of intact chromatophores. However, P^R cannot be a precursor of the Car^T states which form in the antenna carotenoids. These can arise when the reaction center BChl complex is in a chemically oxidized state (P^+-870), preventing the formation of P^R. To search for potential precursors in the antenna BChl, we therefore examined chromatophores of two carotenoidless strains, *Rps. sphaeroides* R-26 and *Rds. rubrum* G-9.

Fig. 7A shows the absorbance change that occurs at 510 nm, following excitation of *Rps. sphaeroides* R-26 chromatophores with a flash of relatively low intensity. Under the conditions used for the measurement, the absorbance change is due mainly to the photooxidation of *P*-870; its decay is relatively slow. If the same sample of chromatophores is illuminated with a flash of high intensity, additional absorbance

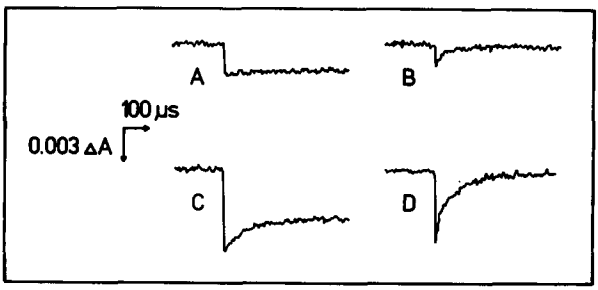

Fig. 7. Flash-induced absorbance changes at 510 nm in chromatophores from *Rps. sphaeroides* R-26. A downward deflection is an absorbance increase. Photosynthetic unit concentration, 0.59 µM. (A) moderate redox potential; flash strength, about 30 neinsteins absorbed · cm^{-2} (834 nm). (B) as A, except low potential. (C) moderate potential; flash strength, about 300 neinsteins absorbed · cm^{-2}. (D) as C, except low potential.

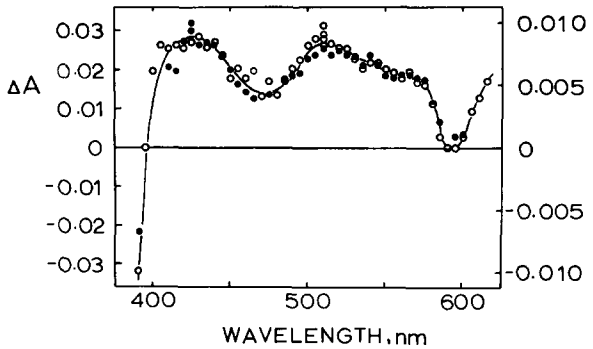

Fig. 8. Flash-induced difference spectra for the formation of $BChl^T$ states in chromatophores from two carotenoidless strains. Low redox potential; actinic wavelength, 834 nm. ○, chromatophores of *Rps. sphaeroides* R-26; 34 μM BChl; ΔA scale on right; ●, chromatophores of *Rds. rubrum* G-9; 122 μM BChl; ΔA scale on left.

changes with short decay times occur, superimposed on the absorbance changes due to *P*-870 (Fig. 7C). Short-lived absorbance changes can be seen with both low- and high-intensity flashes, if the photooxidation of *P*-870 is blocked by chemical reduction of X (Figs. 7B and D). Similar results are obtained with *Rds. rubrum* G-9 chromatophores.

Fig. 8 shows difference spectra of the absorbance changes caused by exciting each of the carotenoidless strains with strong flashes at low redox potentials. Similar spectra are generated at higher redox potentials, if absorbance changes due to *P*-870 and cytochromes are discounted. The difference spectra show a bleaching of absorption bands near 590 nm and below 400 nm, and the development of very broad new absorption bands extending from 400 to 580 nm. They are similar to the difference spectra associated with the conversion of BChl into its lowest excited triplet state in solution [27, 39, 41]. They are essentially identical to the spectrum associated with the conversion of the reaction center BChl into state P^R, which has been identified tentatively as the lowest triplet state [16, 27]. We therefore interpret the

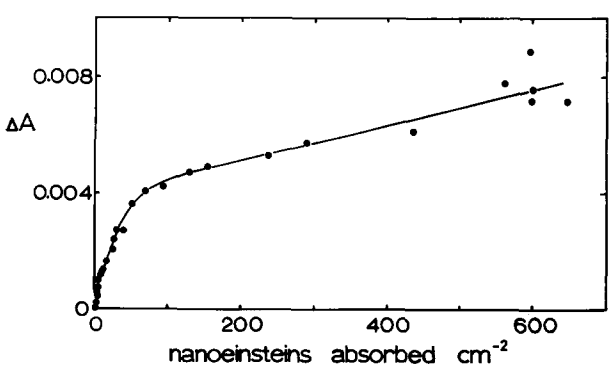

Fig. 9. Formation of the $BChl^T$ state in chromatophores from *Rps. sphaeroides* R-26, as a function of the strength of the actinic flash. Actinic wavelength, 834 nm; potential, $+413 \pm 5$ mV; photosynthetic unit concentration, 0.46 μM; ΔA measured at 510 nm.

transient absorbance changes in chromatophores as reflecting the formation of a BChl triplet state, $BChl^T$.

Fig. 9 shows the amount of the $BChl^T$ state that is formed at moderate redox potentials, as a function of the flash intensity. Like Car^T, $BChl^T$ exhibits a multiphasic dependence on the flash strength. A pronounced drop in the yield occurs as the flashes become strong enough to provide approximately 200 quanta per photosynthetic unit. Using the value of $\Delta\varepsilon$ estimated (27) for the triplet state P^R in isolated reaction centers (0.011 $\mu M^{-1} \cdot cm^{-1}$ at 510 nm), approx. 0.8 $BChl^T$ is formed per photosynthetic unit at this point. With weak flashes, the quantum yield is in the order of 0.02.

As with Car^T, the $BChl^T$ states of chromatophores probably can arise both in the reaction centers and in the antenna. Judging from the behavior of isolated reaction centers [16, 27], one would expect to observe the triplet state of the reaction center BChl, state P^R, after flash excitation at low redox potentials, but not at moderate or high potentials. In agreement with this expectation, the $BChl^T$ absorbance changes in chromatophores are larger at low redox potentials than they are at moderate potentials (Figs. 3B, C, and 7).

The additional absorbance changes that occur at low potentials differ in their decay kinetics from the absorbance changes that occur at moderate potentials (Fig. 3B and C). At moderate potentials, the decay can be fit reasonably well by a single first-order process, with a half-time of about 70 μs for both *Rps. sphaeroides* R-26 and *Rds. rubrum* G-9. Bacteriochlorophyll triplet states in solution have a similar first-order decay rate, but the kinetics in solution also include a second-order term that probably results from triplet-triplet annihilation [39, 41]. (In *Rps. sphaeroides* R-26 chromatophores, small deviations from first-order decay kinetics were sometimes observed at moderate redox potentials. The deviations did not appear to represent a second-order decay process, because they did not vary significantly with changes in the amount of $BChl^T$ that was present.) Lowering the redox potential introduces a major new component with a decay half-time of approx. 6 μs in *Rps. sphaeroides* R-26 (Fig. 3B); we view this component as reflecting the reaction center triplet. Its decay kinetics are essentially identical with those of state P^R in isolated reaction centers of this species [27]. Assuming $\Delta\varepsilon_{510\,nm} \approx 0.011\ \mu M^{-1} \cdot cm^{-1}$, the initial amplitude of the 6-μs component would imply the formation of approx. 0.6 mol of P^R per mol of reaction centers in the chromatophores. This is close to the amount of P^R that is obtained in isolated reaction centers [27].

In *Rds. rubrum* G-9 chromatophores also, lowering the potential introduces a new $BChl^T$ component with a fast decay (Fig. 3C). The amplitude of the short-lived component would imply the formation of 1.0 mol of P^R per mol of photosynthetic units, if one uses the extinction coefficient obtained in the studies of *Rps. sphaeroides* R-26 reaction centers. The decay half time of the short-lived component is approx. 10 μs. Although this is shorter than the half-time of 50 μs that we have measured [16] for P^R in isolated reaction centers of *Rds. rubrum* G-9, we tentatively interpret the 10 μs component as being due mainly to P^R.

For reasons that are not clear, lowering the redox potential also affects the longer-lived component that we attribute to antenna $BChl^T$. In *Rps. sphaeroides* R-26 chromatophores, lowering the potential increases the amount of this component, without changing its decay kinetics (Fig. 3B). In *Rds. rubrum* G-9, lowering

the potential has little effect on the amplitude of the longer-lived component, but it appears to slow its decay (Fig. 3C).

At moderate redox potentials, O_2 accelerates the decay of the $BChl^T$ states by about an order of magnitude in both species. Suspensions of chromatophores in equilibrium with air give pseudo first-order decay rate constants of approx. 3×10^4 s^{-1}. With 1.2 mM O_2, the pseudo first-order constant is 1×10^5 s^{-1}. These data give a second-order quenching constant of approximately 0.8×10^8 $M^{-1} \cdot s^{-1}$. This is somewhat smaller than the constant for the quenching of the Car^T states by O_2.

DISCUSSION

When the photooxidation of *P*-870 is blocked or saturated, excitation of the antenna BChl results in the formation of two types of metastable species. In chromatophores that lack carotenoids, the transient species appears to be a triplet state of BChl. In those that contain carotenoids, carotenoid triplet states replace the BChl triplet. In both cases, one can distinguish triplet states of the antenna pigments from those of the reaction center. The latter arise only if the photooxidation of *P*-870 is blocked by the chemical reduction of X, whereas triplet states of the antenna pigments can form if *P*-870 is in the oxidized state as well.

In both *Rps. sphaeroides* R-26 and *Rds. rubrum* G-9, the reaction center BChl triplet appears to decay more rapidly than do triplet states in the antenna BChl. This could be a heavy-atom effect of the Fe that is a component of the reaction center.

It seems more than likely that BChl triplet states are precursors of the antenna Car^T states in the carotenoid-containing chromatophores. This assumption would be in accord with the finding that the formation of the Car^T states occurs with half-times of 12–25 ns after the flash, long after the excited singlet state of the antenna BChl has decayed (see below). It would be in accord with the fact [39] that energy transfer from triplet BChl to β-carotene occurs at a high rate in solution. Further, Breton and Mathis [38] have provided kinetic evidence for the sequential formation of the triplet states of chlorophyll *a* and β-carotene in chloroplasts. We were not able to detect the transient formation of $BChl^T$ in any of the chromatophores that contained carotenoids, but to do so would be technically difficult because $\Delta\varepsilon$ for the formation of $BChl^T$ is much smaller than that for Car^T.

In the strains that contain carotenoids, the carotenoids remove energy from the antenna BChl with rate constants that are on the order of 3×10^7 s^{-1}. One can compare this number with the pseudo first-order rate constant of 3×10^4 s^{-1} with which $BChl^T$ reacts with O_2 in chromatophore suspensions that are in equilibrium with air. A large part of the quenching of $BChl^T$ by O_2 probably results in the excitation of the O_2 to the reactive $^1\Delta g$ state. The 1000-fold difference between the rates of transfer of energy to the carotenoids and to O_2 would appear to account in large measure for the successfulness of carotenoids in preventing the formation of singlet O_2 in vivo. The quenching of the Car^T states themselves by O_2 presumably reflects mainly facilitated intersystem crossing, which does not result in the generation of singlet O_2.

The risetimes that we have measured for the Car^T states are similar to those

that have been reported previously for isolated reaction centers [16], broken spinach chloroplasts [38], and *C. vinosum* chromatophores [10]. Energy transfer from $BChl^T$ to carotenoids ($BChl^T + Car \rightarrow BChl + Car^T$) is allowed by exchange interaction, although it is doubly forbidden by dipole-dipole interaction [42]. In optimal cases, exchange interaction of this type can occur in less than 10^{-10} s, but the rate falls off very rapidly with increasing distance between the donor and acceptor molecules [43, 44]. The formation of the Car^T states, though rapid compared to the rate at which O_2 can attack $BChl^T$, is relatively slow on this time scale. This would suggest that the distance between the $BChl^T$ and the carotenoid is great enough to limit the rate of energy transfer. Energy transfer to the BChl from carotenoids in their excited singlet states could still occur rapidly via dipole-dipole interaction, which is not such a critical function of distance.

In contrast to our observations, Leigh et al. [11] have found that absorbance changes similar to those accompanying the formation of Car^T states appear within 10 ps, if chromatophores are excited with very intense 530 nm flashes lasting a few ps. Although Leigh et al. [11] originally offered a different interpretation, subsequent work has suggested that the absorbance changes caused by ps excitation reflect the formation of triplet states similar to the ones we have described here (Dutton, P. L. and Kaufman, K. J., personal communication). It is not clear, however, how the carotenoids are converted to triplet states so much more rapidly by ps excitation. Conceivably, processes involving direct excitation of the carotenoids become important at the extremely high light intensities that the ps flashes provide.

There remains for discussion the quenching process that limits the formation of the antenna Car^T and $BChl^T$ states at high flash intensities (Figs. 4, 5 and 9). As one increases the flash strength, the quantum yield falls to a very low value before more than one or two percent of the antenna pigments have been converted to the triplet state. Because the quantum yield of fluorescence shows the same decrease with increasing flash strength, the decrease in the triplet yield appears to result from quenching processes that limit the lifetime of the excited singlet state of the antenna BChl, BChl*. Several investigators* have pointed out recently that at least two such processes are expected to become increasingly important at very high flash intensities. These are singlet-singlet fusion ($BChl^* + BChl^* \rightarrow BChl + BChl^*$) and singlet-triplet fusion ($BChl^* + BChl^T \rightarrow BChl + BChl^T$). In singlet-singlet fusion, energy transfer from one BChl* to another promotes the recipient to a higher excited singlet state. This is followed by rapid radiationless relaxation of the acceptor back to its lowest excited singlet state. Single-triplet fusion involves energy transfer from BChl* to a triplet acceptor, followed by radiationless relaxation of the triplet back to the lowest excited triplet state. Both of the initial energy-transfer processes are allowed by dipole-dipole interaction and both can be extremely rapid [45, 46]. The importance of the two processes will depend on the effective concentrations of BChl* and $BChl^T$, and on the values of the rate constants for energy transfer to the two acceptors. The rate constants can be estimated from the overlap of the emission spectrum of BChl* with the absorption spectra of BChl* and $BChl^T$ [45, 46]. From recent work on bacteriopheophytin [47], it is likely that the absorption spectra of BChl* and $BChl^T$ are sufficiently similar so that the two rate constants will be of the same order of

* Refs. 36, 37 and 45 and Campillo, A. J., Kollman, V. H. and Shapiro, S. L. (1976) submitted.

magnitude. If this is correct, the relative importance of singlet-singlet and singlet-triplet fusion would depend mainly on the concentrations of BChl* and $BChl^T$. Campillo et al. (submitted) have emphasized that singlet-singlet fusion will be particularly important during excitation with single, very short flashes, and singlet-triplet fusion during excitation with longer flashes or trains of flashes that allow the accumulation of a significant population of triplet species.

In the experiments of Figs. 4 and 9, quenching becomes severe when the flash intensity is high enough to excite about 200 BChl molecules per photosynthetic unit, over the course of the 15 ns flash. (Because the quenching increases progressively with flash intensity, the selection of the number 200 is rather arbitrary; we use it only to give an order of magnitude for purposes of discussion.) To calculate the instantaneous concentration of BChl* at any given flash intensity, one must know the lifetime of the excited state. Recent measurements of the fluorescence yields and lifetimes after excitation with ps flashes indicate that the lifetime of BChl* in *Rps. sphaeroides* 2.4.1 chromatophores would be less than 20 ps under the conditions of interest here (Campillo, A. J., Shapiro, S. L., Monger, T. G. and Parson, W. W., to be published). The steady-state concentration of BChl* during the 15 ns flash thus would be less than 0.3 per photosynthetic unit ($200 \cdot 2 \cdot 10^{-11}/1.5 \cdot 10^{-8}$). Under the same conditions, the concentration of $BChl^T$ is approximately one per photosynthetic unit. It appears, therefore, that singlet-triplet fusion could be the predominant quenching process under these conditions. Rapid quenching of BChl* by $BChl^T$ would provide a simple explanation for the observation that the formation of further $BChl^T$ states (or their descendent Car^T states) becomes severely limited after the formation of one triplet per photosynthetic unit. Quenching by chlorophyll triplets could explain Mauzerall's [48] observation that the fluorescence yield in *Chl. pyrenoidosa* increases with a half-time of 25 ns after excitation with a strong flash. The kinetics of the increase in the fluorescence yield match the kinetics [38] with which β-carotene quenches the chlorophyll triplet in spinach chloroplasts. A more refined analysis of the singlet-triplet and singlet-singlet quenching processes in chromatophores should provide information on the functional organization of the antenna system, and work is in progress toward this end.

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