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Charge separation and formation of bacteriochlorophyll triplets in *Heliobacterium chlorum*

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Triplet formation was observed upon excitation with a laser flash of membrane fragments of *Heliobacterium chlorum* by means of kinetic analysis of the absorbance difference spectra. At low redox potential charge recombination between the photooxidized primary donor P-798$^+$ and the reduced primary acceptor A_0^- yielded the triplet of P-798, both at room temperature and at 5 K. The exponential time constant for this recombination was found to be 17 ns at 300 K and 55 ns at 5 K. The yield of triplet formation from the state P-798$^+A_0^-$ was 20–25% and 25–35%, respectively. At low temperature the quantum yield for the formation of the primary radical pair was a factor of 2 lower in reaction centers in which the (first) secondary electron acceptor X was in the reduced state, than in reaction centers where X was in the oxidized state before illumination. Absorbance difference spectra were measured for the formation of the triplet of P-798, for P-798$^+A_0^-$ and for P-798$^+X^-$ formation. Electrochromic bandshifts of bacteriochlorophyll (BChl) *g* were only observed in the spectra of P-798$^+X^-$. In addition to the reaction center triplet an antenna triplet was observed at low temperature, located on the long wavelength absorbing BChl *g*-808. The quantum yield of formation of this triplet was approximately 1–2%, independent of the redox state of the reaction center.

Introduction

Triplet states of chlorophyll and bacteriochlorophyll in photosynthetic systems may be formed by two mechanisms, radical pair recombination in the reaction center and intersystem crossing from the singlet excited state (for a review, see Ref. 1). The first mechanism is based on a back reaction of the charge separated state in the reaction center. This reaction occurs when the secondary electron acceptors are either reduced or absent and results in the formation of the triplet state of the primary electron donor, P^T. This phenomenon has been observed in all photosynthetic systems studied so far: in Photosystem I [2–4] and Photosystem II [5–7] of plants, in reaction centers of purple bacteria [8–10] and green filamentous bacteria [11] and in preparations from green sulfur bacteria [12–14].

The second mechanism, intersystem crossing, has been studied less extensively in vivo. Monger et al. [15,16] observed the formation of BChl *a* triplets in the antenna of purple bacteria and provided evidence that this phenomenon did not require the presence of operational reaction centers. BChl *a* triplets have also been observed in the antenna of green sulfur bacteria, but the mechanism of their formation was not studied in detail [14]. It should be noted that in carotenoid-containing organisms radical pair recombination as well as intersystem crossing often results in the formation of carotenoid triplets by triplet energy transfer [15].

Triplet formation in membranes of heliobacteria was recently studied by Smit et al. [17,18]. The nature of the electron acceptor chain in these recently discovered bacteria is not known in detail, although there is evidence that a quinone and one or more iron-sulfur centers are involved [17,19–21]. The primary electron donor, P-798 [19,22], is probably a dimer of BChl *g* or its 13^2-epimer [23], while it has been demonstrated that the primary acceptor is a Chl *a* or BChl *c*-like pigment, absorbing at 670 nm [24,25]. In analogy to Photosystem I we will call this acceptor A_0. At room temperature, under reducing conditions, the formation of the triplet of BChl *g* was observed [17]. Evidence was obtained

Abbreviations: BChl, bacteriochlorophyll; P-798, primary electron donor; A_0, primary electron acceptor; X, secondary electron acceptor; PMS, *N*-methylphenazonium methosulphate.

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that the triplet is formed by the radical pair mechanism but the nature of the charge separated state involved could not be established in these studies. At liquid helium temperature two spectrally different triplets were observed, one of which was assigned to P-798, the other to antenna BChl *g* [18]. Triplet formation in the reaction center and possibly the antenna at low temperature was also observed by EPR and absorption detected magnetic resonance (see Ref. 21).

This paper describes a detailed study of the conditions that favor triplet formation in membranes of *Heliobacterium chlorum* and of the characteristics of the triplets formed. Evidence will be given that at low redox potential the triplet of P-798 is formed by a back reaction between P-798$^+$ and the reduced primary electron acceptor, both at room and low temperature and that at low temperature a triplet is formed of a long wavelength absorbing BChl *g*-species in the antenna. Formation of the latter triplet does not occur via the reaction center and is, thus, presumably due to intersystem crossing from singlet excited states.

Materials and Methods

Heliobacterium chlorum was grown and membrane fragments were prepared as described in Ref. 26.

Flash induced absorbance kinetics and difference spectra on μs and ms time scales were measured with the single beam spectrophotometer described in Ref. 17. Measurements with ns-resolution were performed with essentially the same optical arrangement but with a different detection and registration system. The measuring light was provided by a Xe-flashlamp (FWHM 15 μs) that was synchronized in such a manner that the excitation (laser) flash coincided with the middle of the Xe-flash. The light passing through the sample was detected with a fast photomultiplier (ITT F4102, rise-time 0.8 ns) and the signal was fed into a broadband linear amplifier (Comlinear CLC 104 AI, bandwidth 1.1 GHz). An electrical delay line (100–350 ns) was used to subtract the constant contribution by the measuring light and the resulting signal was registered on an oscilloscope (Tektronix 2467B, bandwidth 400 MHz). The trace on the scope screen was monitored with a CCD-camera (Philips, type 56470) and the video signal was fed into a home-built digitizer, connected to a personal computer. The overall response time of the system was approx. 1.5 ns, as determined from the response to a 35 ps laser pulse. Baseline corrections were made for the curvature of the Xe-flash shape and in some experiments fluorescence artifacts were recorded separately and then subtracted from the measurements. Recordings at single wavelengths were the average of 40–100 separate measurements.

In all experiments, excitation flashes were provided by the frequency doubled output (532 nm) of a Q-switched Nd-YAG laser (15 ns FWHM). The maximum incident energy density on the sample was approx. 5–10 mJ cm^{-2}. Neutral density filters were used to adjust the energy of the laser flash.

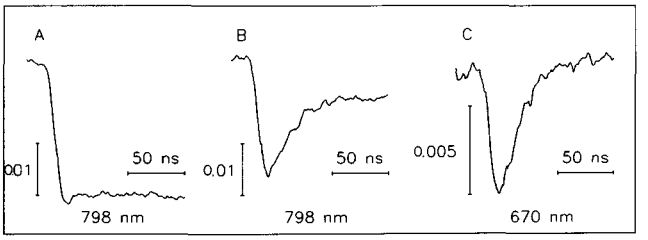

Fig. 1. Kinetics of absorbance changes induced by a 15 ns, 532 nm laser flash (incident energy approx. 1 mJ cm^{-2}) in membrane fragments of *H. chlorum* at room temperature. (A) In the presence of 50 mM Tris, 10 mM ascorbate and 40 μM PMS at pH 9.5 ($A_{788} = 0.46$). (B) Same sample as A with additional 20 mM dithionite. (C) Same as B; $A_{788} = 2.3$.

A helium flow cryostat was used for the low temperature measurements. Glycerol was added to the samples (66% v/v) in order to prevent crystallization upon cooling. For experiments in the presence of dithionite the sample was sealed with a layer of paraffin.

Results and Interpretation

Charge recombination in reduced reaction centers

Kinetics of absorbance changes induced by a 15 ns flash are shown in Fig. 1. At 798 nm, in the absence of dithionite, a rapid absorbance decrease was observed (Fig. 1A) which did not decay at the timescale of the experiment and which can be attributed to photooxidation of P-798 [17,19,22,24]. The electron is transferred to secondary acceptors, after which charge recombination takes place in the ms-region [17]. However, in the presence of dithionite (at pH 9.5), the absorbance decrease was followed by a rapid mono-exponential, partial recovery to a level which was constant on the time scale of the experiment (Fig. 1B). The time constant of this rapid component was 17 ± 2 ns. From measurements of the kinetics at an extended time scale (not shown) the decay time of the remaining absorbance decrease was determined at 35 ± 3 μs. This number is in good agreement with earlier measurements in the blue spectral region [17]. We conclude that the 35 μs-decay reflects the lifetime of the triplet of P-798 (P-798^T). This triplet is formed, with a time constant of 17 ns, by radical pair recombination in the reaction center when the first secondary electron acceptor X is reduced before the flash (perhaps to the doubly reduced state XH_2 [27,28]). We may assume that the 17 ns signal is the same as the approx. 30 ns component observed by Smit et al. [17] in the blue region with an apparatus with limited time resolution, and which was ascribed to a

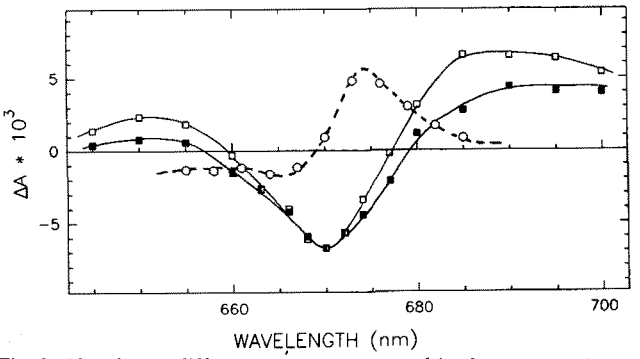

Fig. 2. Absorbance difference spectra measured in the presence (open squares) and in the absence (circles) of dithionite at 15 ns after the beginning of the flash. The closed squares indicate the spectrum of the 17 ns component, normalized at 670 nm. Conditions as for Fig. 1; $A_{788} = 2.3$.

back reaction of P-798$^+$ with an unknown reduced electron acceptor.

In order to obtain evidence about the identity of the electron acceptor involved in the recombination reaction we also measured absorbance changes at 670 nm (Fig. 1C). A rapid absorbance change was observed in the presence of dithionite, followed by a reversal which again could be fitted with a time constant of 17 ns. The spectra of the initial absorbance change and that of the 17 ns phase (Fig. 2) both showed a minimum at 670 nm. These spectra closely resemble the difference spectrum caused by reduction of the primary acceptor obtained in the absence of dithionite by Nuijs et al. [24] in ps time resolved measurements. We thus conclude that under reducing conditions P-798^T is formed by a back reaction with a time constant of 17 ns between P-798$^+$ and the reduced primary electron acceptor A_0^-. In the absence of dithionite, reduction of A_0 was not observed with the time resolution applied; the difference spectrum measured directly upon the 15 ns flash (Fig. 2, circles) showed the same features as that observed earlier in the μs region [17] and is thought to reflect the state P-798$^+X^-$, where X denotes the (first) secondary acceptor. The spectrum may be caused by an electrochromic shift of the absorption band of A_0, analogous to that of pheophytin *a* in Photosystem II [29].

From the amplitude of the absorbance changes at 798 nm due to the state P-798$^+X^-$ (in the absence of dithionite) and that of P-798^T the yield of triplet formation may be determined. However, the amount of P-798^T formed did not saturate at the same flash intensity as P-798$^+X^-$ formation. This effect may be explained by multiple turnovers of the reaction centers during the 15 ns flashes applied, resulting in an overestimation of the yield of triplet formation. Extrapolation to zero intensity flashes gave yields of 20% to 25%, depending on preparation, on the assumption that the differential extinction coefficients of P-798$^+$ and P-798^T are the same at 798 nm.

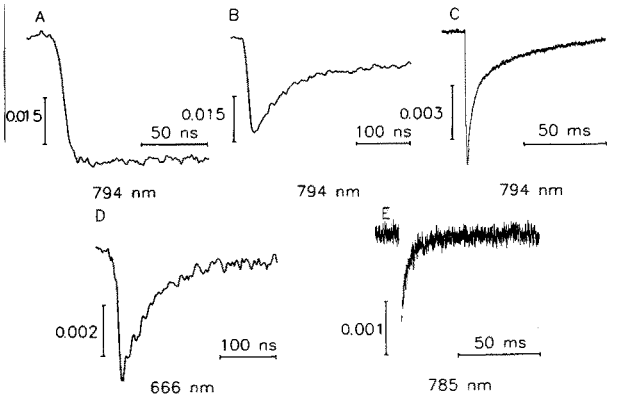

Fig. 3. Kinetics of absorbance changes induced by a 15 ns, 532 nm laser flash (incident energy approx. 1 mJ cm^{-2}) in membrane fragments of *H. chlorum* at 5 K ($A_{788} = 0.44$ at room temperature). (A) In the presence of 10 mM Tris and 10 mM ascorbate at pH 8.0. (B–E) In the presence of 50 mM Tris, 10 mM ascorbate, 40 μM PMS and 20 mM dithionite at pH 9.5. (D,E) Cooled in the dark ($A_{788} = 0.73$). All samples contained glycerol (66% v/v).

Triplet formation was also observed at low temperature. Fig. 3 shows kinetics of absorbance changes at 5 K in the absence and presence of dithionite. Without dithionite, no decay could be seen at 794 nm in the sub-μs region after the initial flash-induced absorbance decrease. With dithionite, however, a rapidly decaying signal with an exponential time constant of 55 ± 8 ns was observed, both at 794 nm and at 666 nm. The amplitude of this component increased if we cooled the samples under continuous illumination (Fig. 3B). Unless otherwise indicated, this procedure was therefore used for the experiments to be described below. Like at room temperature, the difference spectrum in the red region (Fig. 4) was similar to that observed with ps resolution without dithionite [25], indicating that the 55 ns decay was caused by a reversal of the primary charge

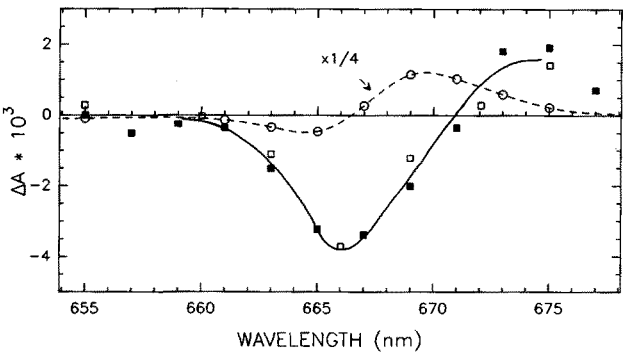

Fig. 4. Solid line: absorbance difference spectrum of the formation of P-798$^+A_0^-$ in the presence of dithionite; conditions as in Fig. 3. Solid squares: obtained from the 55 ns decay component extrapolated to 15 ns after the beginning of the flash (sample cooled in the dark). Open squares: measured at 15 ns after the beginning of the flash; spectrum normalized at 666 nm. Broken line: absorbance difference spectrum of the formation of P-798$^+X^-$, represented by the 2.3 ms component observed in the absence of dithionite; conditions as in Fig. 3A.

separation. In the absence of dithionite a bandshift was observed around 666 nm, similar to that at room temperature.

At longer timescale (Fig. 3C) a biphasic decay was observed in the presence of dithionite with a component of 500 ± 50 μs and one with a time constant in the ms-range. This indicates that reversal of the primary charge separation did not occur in all reaction centers; the slow component (which had a time constant of 3.6 ms and 2.3 ms for samples cooled in the light and in the dark, respectively) may be attributed to a rereduction of P-798$^+$ by the reduced secondary acceptor X$^-$ [18]. At 785 nm, an isosbestic point for P-798$^+$X$^-$ formation (see below), only the 500 μs component was observed (Fig. 3E). We ascribe this component to the decay of P-798^T. Control experiments at room temperature indicated that the incomplete reduction of X by dithionite was at least partly due to the presence of glycerol.

Comparison of the amplitudes of the decay components at 794 nm indicated that radical pair recombination occurred in 90% of the reaction centers. In dark-cooled samples samples this number was 60%.

Absorbance difference spectra

Difference spectra in the near-infrared region of the various kinetic phases are shown in Figs. 5 and 6. Difference spectra for the formation of P-798^T are given by the triangles. The low temperature difference spectrum (Fig. 6), obtained by plotting the 500 μs component in the presence of dithionite, showed bleaching bands in the Q_y and Q_x regions of BChl *g*, and, apart from a narrowing of the bands, had similar characteristics as the spectrum of P-798^T formation at room temperature (Fig. 5, triangles, see also Ref. 17). The maxima of the bleachings are located at 573 nm and 794 nm, with a broad absorbance increase in the region between 600 and 700 nm.

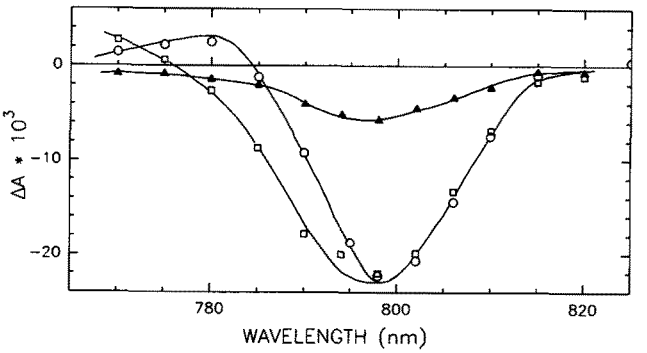

Fig. 5. Room temperature absorbance difference spectra of the formation of P-798$^+$A$_0^-$ (squares) and of P-798^T (triangles) measured at 15 and 100 ns after the beginning of the flash, respectively, in the presence of dithionite. Circles: P-798$^+$X$^-$ spectrum measured at 15 ns in the absence of dithionite. All conditions as in Fig. 1A,B. The scale refers to the spectra with the circles and triangles; the spectrum with the squares was normalized at the maximum bleaching.

As noted earlier [17,18], the absorbance difference spectrum of P-798^T showed some significant differences from that of P-798$^+$X$^-$ (circles). At room temperature the maximum bleaching was shifted by approx. 2 nm to the blue. Moreover, the positive band near 780 nm was missing at room temperature as well as at 5 K. These differences can be explained by a blue shift of a BChl *g* species absorbing near 790 nm in the P-798$^+$X$^-$ spectrum, due to an electric field induced by the charge separation in the reaction center. The same reasoning applies to the red spectral region, where the positive and negative band around 666 nm are present in the P-798$^+$X$^-$ spectrum, but absent in the spectrum of P-798^T. It is of interest to note, however, that the positive band at 780 nm also appeared to be absent in the state P-798$^+$A$_0^-$ (Figs. 5 and 6, squares). This indicates that the field is only observed by the 790 nm pigment when

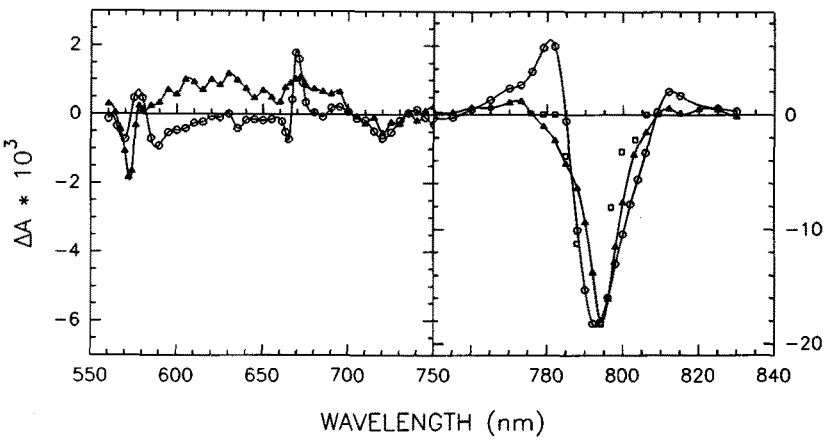

Fig. 6. Circles: absorbance difference spectrum of P-798$^+$X$^-$ formation, obtained from the 2.3 ms component observed at 5 K in the presence of dithionite (sample cooled in the dark). Triangles: difference spectrum of P-798^T, obtained from the 500 μs component. Squares: absorbance difference spectrum of the formation of P-798$^+$A$_0^-$, measured at 15 ns after the beginning of the flash. Conditions as for Fig. 3. Note the different scales for the two parts of the figure. The vertical scales apply to the spectrum with the circles; the other spectra were normalized at 794 nm.

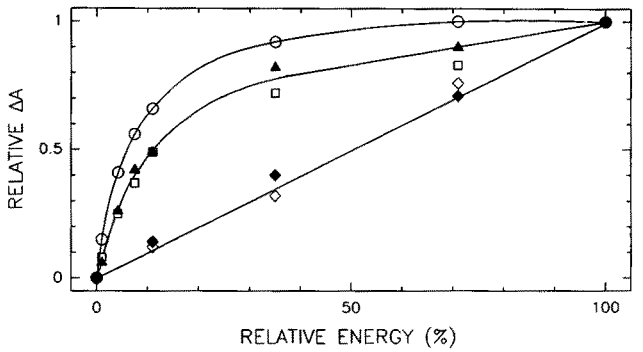

Fig. 7. Absorbance changes at 5 K as a function of excitation energy, normalized at 100% of the available laser energy (approx. 5–10 mJ cm^{-2}). Circles: 2.3 ms component at 794 nm ($P\text{-}798^+X^-$) in the absence of dithionite. Triangles: 500 μs component ($P\text{-}798^T$) in the presence of dithionite. Squares: absorbance differences at 15 ns after beginning of flash ($P\text{-}798^+A_0^-$) in the presence of dithionite. Open diamonds: 400 μs component at 809 nm (antenna triplet) in the absence of dithionite. Solid diamonds: same component in the presence of dithionite.

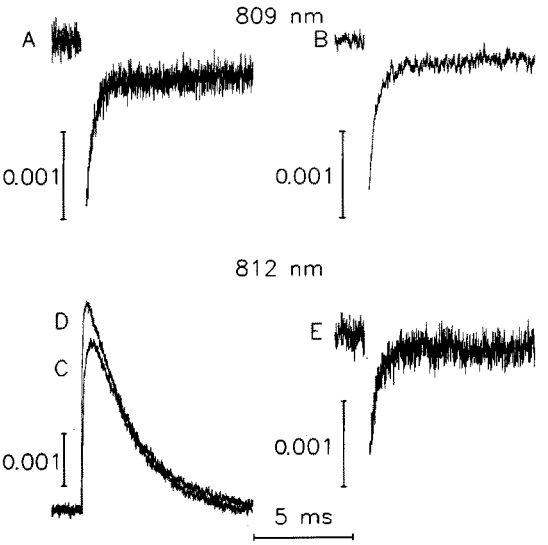

Fig. 8. Kinetics of absorbance changes associated with the antenna triplet. $A_{788} = 0.67$, other conditions as in Fig. 3. (A) 100% laser energy in the absence of dithionite. (B) 100% laser energy in the presence of dithionite. (C) 100% and (D) 30% laser energy in the absence of dithionite. Tracing (E) shows the difference between recordings A and B.

the positive and negative charges are separated at a sufficiently large distance.

Efficiency of charge separation

The dependence of the light induced processes at 5 K on the laser flash energy is shown in Fig. 7. For $P\text{-}798^+X^-$ formation, represented by the 2.3 ms decay component, 65% saturation was already obtained at 10% of the available laser energy (circles). However, formation of the state $P\text{-}798^+A_0^-$ in reaction centers where X had been reduced by dithionite saturated only at approx. 2-times higher intensities (Fig. 7, squares). The same intensity dependence was obtained for triplet formation (Fig. 7, triangles). These results indicate that the yield of the primary charge separation is approx. 2-times lower in reaction centers where X was in the reduced state before the flash than in those where X and possibly other acceptors were oxidized.

Calculation of the yield of triplet formation by comparing the amount of $P\text{-}798^T$ formed with that of $P\text{-}798^+A_0^-$, as measured immediately after the flash, gave a number of 25–35%, depending on the sample. It was assumed that the differential extinction coefficients at 794 nm were the same for both processes.

Antenna triplets

Smit et al. [18], when plotting the difference spectrum of a rapidly decaying component in the low temperature difference spectrum of *H. chlorum* membranes in the absence of dithionite, observed a negative band near 812 nm which could not be attributed to the triplet of P-798. Fig. 7 (open diamonds) shows the intensity dependence of this signal. The measurements were done at 809 nm, which is an isosbestic point for $P\text{-}798^+X^-$ formation (Fig. 6). In contrast to the other phenomena described here, the absorbance change showed a linear dependence over the whole range of laser energies used. This indicates that it originates from the antenna rather than from the reaction center. In order to study this phenomenon more closely, its kinetics were separated from those of the reaction center processes by subtraction of the flash induced kinetics obtained at 30% laser energy from those at 100% energy. Fig. 8C and D shows the result of this operation at 812 nm. The kinetics upon subtraction, like those at 809 nm, after an initial laser artifact showed a monophasic decay with a time constant of 400 ± 50 μs. The amplitude of the 400 μs component was essentially the same with and without dithionite, again indicating that it was not due to a

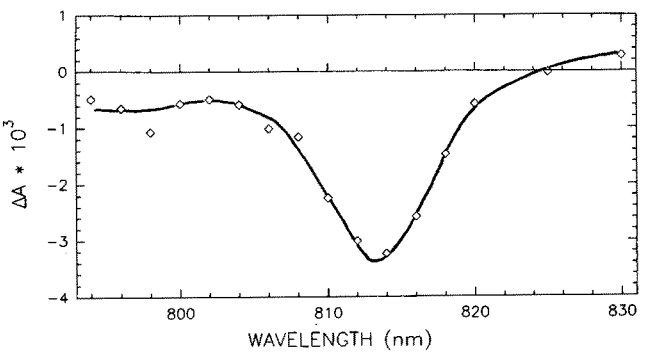

Fig. 9. Absorbance difference spectrum of the 400 μs antenna triplet signal at 100% laser energy as obtained with the sample of Fig. 8 in the absence of dithionite (see text).

reaction center process (Fig. 7, solid diamonds, and Fig. 8B).

The spectrum of the 400 μs component is given in Fig. 9. It shows a minimum at 813 nm and resembles the difference spectrum of the rapidly decaying signal observed by Van Kan et al. [25] at 15 K in the ps-region. The latter spectrum was ascribed to formation and decay of the excited singlet state of the long wavelength absorbing form of BChl *g*, BChl *g*-808. We thus conclude that the absorbance decrease at 813 nm is due to the formation of the triplet state of BChl *g*-808, presumably by intersystem crossing from the singlet excited state. A similar antenna triplet was not observed at room temperature, either at 813 nm or at shorter wavelengths. It must be noted that the spectrum ascribed to P-798^T (Fig. 6, triangles) does not contain a significant contribution of the antenna triplet because of the relatively weak flashes that were used.

The quantum yield of formation of the BChl *g*-808 triplet was estimated by comparing the number of triplets formed with the number of photons absorbed at 532 nm, assuming a differential extinction coefficient of 100 $mM^{-1}\ cm^{-1}$ at 813 nm. Taking into account that the efficiency of energy transfer to BChl *g*-808 is approx. 70% for quanta absorbed at 532 nm [26,30], we found an efficiency of triplet formation from the excited singlet state of 1–2%, depending on the sample used. This yield did not decrease with increasing laser flash energy (Fig. 7), indicating the absence of singlet-triplet quenching.

Discussion

The experiments reported in this paper provide strong evidence that the triplet state of P-798, P-798^T, is formed in membranes of *Heliobacterium chlorum* by a reversal of the charge separation, in a back reaction between P-798$^+$ and the reduced primary electron acceptor A_0^-. This reaction has a time constant of 17 ns at room temperature and of 55 ns at 5 K, i.e., in the same range as reported for various other systems [4,6,7,9,13]. The yield of formation of P-798^T from the state P-798$^+A_0^-$ increased only slightly, from 20–25% to 25–35% upon cooling to 5 K. An increase in the triplet yield like in purple bacteria, where it approaches unity at low temperature [9], was not observed. Evidence for the involvement of the primary electron acceptor A_0, which has been identified as a Chl *a*- or BChl *c*-like pigment [24,25] was obtained from the kinetics of the absorbance changes near 670 nm: at room temperature as well as at 5 K the rate of A_0^- reoxidation was the same as that of triplet formation. Earlier measurements suggesting that the reoxidation rate of A_0^- in the presence of dithionite was considerably faster [24] may have been due to the establishment of an insufficiently low redox potential in those experiments. In our experience sufficiently strong and stable reducing conditions could only be obtained at high buffer concentration and after sealing of the sample with paraffin.

The use of dithionite to block electron transfer from A_0^- to X allows one to measure the absorbance difference spectrum of the formation of the primary radical pair P-798$^+A_0^-$ without interference by excited states in the antenna as in the earlier measurements of Nuijs [24] and Van Kan [25] in the ps-region. Comparison with the spectrum of P-798$^+X^-$ shows the virtual absence of field induced absorbance changes, indicating that the electrical field generated by the primary charge separation is not observed by the pigments responsible for these changes. A similar observation was made by Van Bochove et al. [13] in an antenna reaction center complex from the green sulfur bacterium *Prosthecochloris aestuarii*.

The experiments illustrated in Fig. 7 indicate that the efficiency of the primary charge separation at low temperature is significantly reduced when the first secondary electron acceptor X (and possibly other acceptors) are in the reduced state prior to flash illumination. To our knowledge this is the first demonstration of such an effect in bacterial systems. Similar observations were reported for Photosystem II preparations at room temperature [7,28], where a decrease of the yield of charge separation was observed when the first acceptor Q_A was in the reduced state. The effect may be tentatively explained by the electrostatic effect of a negative charge or charges at the acceptor side [31]. In reaction centers of purple bacteria a lower yield of triplet formation was observed in the presence of Q_A^- than when Q_A had been removed, but in this case the effect was explained by a lowering of the yield of triplet formation from the charge separated state [10]. Inspection of the kinetics of Fig. 1 suggests that in *H. chlorum* at 300 K the effect, if it exists at all, is less than at 5 K, but because of the enhanced rate of back reaction at 300 K an accurate estimation was not possible with the 15 ns flashes applied in our measurements.

The absorbance decrease at 813 nm observed at low temperature after a flash is probably due to the triplet state of the long wavelength absorbing BChl *g*-808, which may be formed by intersystem crossing from the excited singlet state. The lifetime of the excited singlet state at 15 K was found to be 200 ps by Van Kan et al. [25]. Together with the triplet yield of 1–2% reported here, this gives a rate constant for intersystem crossing of $(5–10)\cdot 10^7\ s^{-1}$. This is the same as the rate of intersystem crossing for Chl *a* in solution, which is approx. $10^8\ s^{-1}$, as calculated from its excitation decay time and triplet yield (see Ref. 32). The much lower fluorescence yield [30] and shorter lifetime [33] of BChl *g* in vivo at 300 K than at 5 K explains our inability to observe antenna triplets at room temperature. A yield of 2% was reported by Monger et al. [15] for the

formation of BChl *a* antenna triplets in chromatophores of purple bacteria, in agreement with the much longer lifetimes of fluorescence at room temperature in these species [34]. It should be noted that, in contrast to the observations of Monger et al. [15] in purple bacteria, we did not observe singlet-triplet quenching in *H. chlorum*. This can be explained by the small amount of triplet formed (approx. one per 15 reaction centers) even by the strongest flashes applied in our experiments. There is evidence that the antenna of *H. chlorum* is organized in separate photosynthetic units, each containing one reaction center, between which no transfer of singlet excitations takes place (G. Deinum, personal communication). Therefore, in our conditions at most 6% singlet-triplet quenching would be expected to take place.

Acknowledgments

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