

## Excited states and primary photochemical reactions in the photosynthetic bacterium *Heliobacterium chlorum*

(charge separation/bacterial photosynthesis/electron transfer/singlet state)

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**ABSTRACT** The charge separation and excited states of antenna bacteriochlorophyll in membrane fragments of the recently discovered photosynthetic bacterium *Heliobacterium chlorum* were studied by absorbance-difference spectroscopy. Formation of singlet excited states of bacteriochlorophyll *g* with a lifetime of 200 ps or less was observed as the disappearance of the ground state absorption bands. From the absorbance-difference spectra, it was concluded that the primary photochemical reaction consists of the transfer of an electron from the primary donor P-798 to a possibly bacteriochlorophyll *c*-like pigment absorbing at 670 nm. Electron transfer to the secondary acceptor occurred with a time constant of about 500 ps. The midpoint potential of this acceptor (between –450 and –560 mV) and the absence of significant absorbance changes in the near-infrared upon its reduction suggest that this acceptor is an iron-sulfur center. It is concluded that the primary photochemistry of *H. chlorum* is similar to that of green sulfur bacteria.

The recently discovered photosynthetic bacterium *Heliobacterium chlorum* (1) has an unusual pigment composition and structure. Instead of bacteriochlorophyll (BChl) *a* or *b*, it contains BChl *g* (2) and a pigment absorbing at 670 nm, and it does not appear to possess either chlorosomes, like green bacteria, or invaginations of the cytoplasmic membrane, like purple bacteria. Nothing is known about its mechanism of photosynthetic electron transport, except that illumination results in (i) the bleaching of a pigment absorbing at 798 nm that may serve as primary electron donor and (ii) the oxidation of a *c*-type cytochrome (3).

Recently we have shown that picosecond absorbance-difference spectroscopy of isolated membranes of the green sulfur bacterium *Prosthecochloris aestuarii* provides information not only on the properties of the excited states generated in the antenna but also on the components involved in the primary charge separation in this bacterium (4). In the present communication we report the results of a picosecond study of membranes of *H. chlorum*. Evidence will be presented that the primary photochemistry in this bacterium is similar to that in green sulfur bacteria.

### MATERIALS AND METHODS

*H. chlorum* was grown anaerobically in medium 112 of the American Type Culture Collection (1) containing 2.5 mM ascorbate. Cells were harvested by centrifugation and resuspended in a buffer of pH 8.0 containing 10 mM Tris, 10 mM ascorbate, and 2 mM dithiothreitol. Membrane fragments were prepared by sonication followed by centrifugation for 15 min at  $27,000 \times g$  to remove large cell fragments. The

preparation was stored in the dark at 5°C before use.

For measurements of absorption changes under continuous illumination, the samples were used without further additions. The absorbance changes were measured as in ref. 5. Suitable interference and absorbance filters were used to select the actinic illumination and to protect the photomultiplier from stray actinic light. The picosecond absorbance-difference measurements were performed with the apparatus briefly described in refs. 4 and 6. The samples were excited at 532 nm with a 35-ps pulse from a frequency-doubled mode-locked Nd-YAG (yttrium/aluminum garnet) laser (maximum excitation energy density,  $\approx 2.5$  mJ/cm<sup>2</sup>). The absorbance changes were measured with a 35-ps probe pulse (full width at half-maximum bandwidth = 3 nm), the wavelength of which was varied between 550 and 900 nm. Schott KV 550 filters were used to prevent stray excitation light from reaching the photodiodes. In some experiments a part of the excitation pulse was deflected by a beam splitter onto the sample via a shortcut to serve as a “preflash” to close most or all of the reaction centers before the arrival of the excitation pulse. All measurements were performed at room temperature.

### RESULTS AND DISCUSSION

The absorbance difference spectrum of membranes of *H. chlorum* obtained upon illumination with continuous light is shown in Fig. 1. In the near-infrared region, the spectrum is very similar to that published recently (3). It shows a bleaching centered at 799 nm, which has been attributed to photooxidation of the primary electron donor P-798 (3). Other features of the spectrum include a positive band at 776 nm, which may be due to a blue shift of a neighboring BChl *g* molecule, and negative bands at 552 nm (3) and 418 nm due to photooxidation of a *c*-type cytochrome. The minimum at 595 nm is presumably due to bleaching of the Q<sub>x</sub> band of P-798. The weak positive and negative bands at 673 and near 660 nm, respectively, may be explained by a red shift of the pigment or pigments absorbing at 670 nm. The difference spectrum was obtained at a light intensity that was approximately two-thirds saturating for P-798 oxidation. If it is assumed that the *in vivo* extinction coefficient of BChl *g* in the absorption maximum at 788 nm and that of the bleaching of P-798 at 799 nm are equal, it can be calculated that there are about 30 BChl *g* molecules per reaction center.

Fig. 2 shows the absorbance-difference spectra of the membrane fragments in the region 550–900 nm at 1.5 ns and at 150 ps after a 35-ps excitation pulse at 532 nm. *N*-Methylphenazonium methosulfate (PMS) and ascorbate were added as electron donors to ensure complete rereduction of the primary electron donor P-798 between successive flashes. Above 550 nm, the 1.5-ns spectrum is similar to that obtained

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

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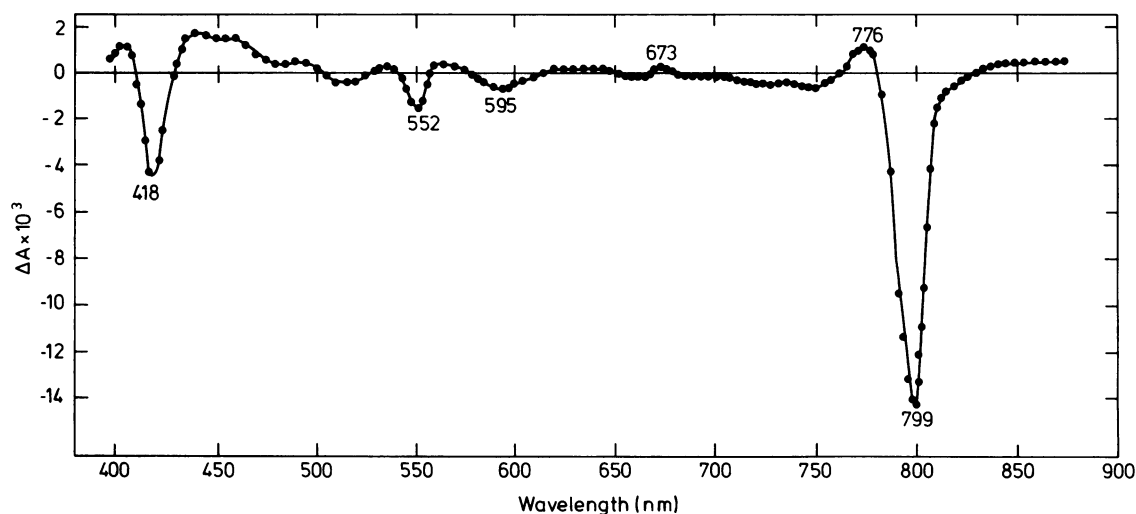


FIG. 1. Absorption-difference spectrum of membranes of *H. chlorum* upon illumination (10 s of light/100 s of darkness). The membranes were suspended in 10 mM Tris buffer (pH 8.0). The absorbance of the sample was 0.68 at 788 nm. Only the reversible part of the absorbance change was plotted at each wavelength. For the region 650–850 nm, illumination was provided through a Corning CS 4-96 and a Calflex C filter at an intensity of 50 mW/cm<sup>2</sup> and for the region 395–650 nm through a Schott RG 715 filter at an intensity of 100 mW/cm<sup>2</sup>. The vertical scale corresponds to the magnitude of the absorbance changes observed upon illumination with blue light.

with continuous illumination (Fig. 1) and reflects the oxidation of P-798. These same features can be discerned in the 150-ps spectrum. However, the latter spectrum shows additional absorbance changes in the region 630–740 nm and a stronger bleaching in the region 770–830 nm. As will be discussed below, the extra bleaching in the near-infrared can be ascribed to some residual singlet excitations on the BChl *g* of the antenna still remaining at 150 ps after the flash.

Subtraction of the 1.5-ns spectrum from that at 150 ps in the region 630–740 nm results in the difference spectrum depicted in Fig. 2 *Inset*. It consists of absorbance increases at 630–650 nm and 690–740 nm and a bleaching centered at about 670 nm. These changes, which are spectrally similar to those caused by reduction of the primary electron acceptor, I, in membranes of the green sulfur bacterium *P. aestuarii* (4), can be ascribed also in the case of *H. chlorum* to reduction of I, as will be discussed below.

The kinetics of the absorbance changes at a number of wavelengths are depicted in Fig. 3. Fig. 3A shows the results obtained at 800 nm for two different excitation intensities. The development of the bleaching in both traces is mainly determined by the temporal profile of the 35-ps exciting and

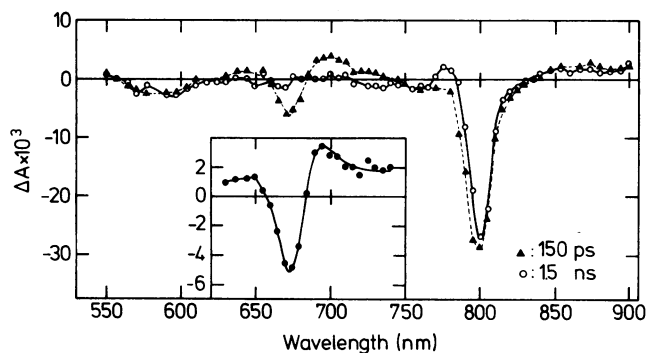


FIG. 2. Absorbance-difference spectra of membranes of *Helio-bacterium chlorum* in the presence of 10 mM ascorbate and 15  $\mu$ M PMS at 150 ps ( $\blacktriangle$ ) and 1.5 ns ( $\circ$ ) after a 35-ps excitation pulse at 532 nm. (*Inset*) Difference between the two spectra in the region 630–740 nm. The excitation density was about 2.8 mJ/cm<sup>2</sup>. The flash frequency was 0.5 Hz. The absorbance of the sample was 0.8 at 788 nm.

probing pulses. In the lower trace (solid circles), obtained at an intensity that was saturating with respect to photochemistry (see Fig. 4), a bleaching was observed, which decays within some tens of picoseconds to a level that was constant on our timescale. In the upper trace (open circles), a similar constant component can be discerned, but not the fast transient. The difference spectra of Fig. 2 indicate that the constant component reflects the presence of P-798<sup>+</sup>, whereas the transient can be ascribed to formation and decay of excited BChl *g* (BChl<sup>\*</sup>*g*) in the antenna (see below). Quite different kinetics were obtained at 670 and 695 nm (Fig. 3 *B* and *C*). At both of these wavelengths, the larger part of the absorbance change decayed with a time constant ( $1/e$ ) of 500  $\pm$  50 ps. From these observations, and from the difference between the 1.5-ns and 150-ps spectra (Fig. 2 *Inset*), we conclude that these absorbance changes reflect the rapid reduction and 500-ps reoxidation of a primary electron acceptor I with spectral and kinetic properties very similar to those of the primary electron acceptor in *P. aestuarii* (4).

A small contribution by a rapidly decaying component can be observed at 695 nm, which probably reflects the deactivation of an antenna excited state. If we assume that this state absorbs at 670 nm as well, its decay could account for the just discernable lag of about 100 ps in the formation of the bleaching at 670 nm (Fig. 3*B*).

The yield of P-798<sup>+</sup> formation as a function of the flash energy density is shown in Fig. 4. It can be seen that the absorbance changes are fully saturated at the maximum excitation density used. A comparison of the maximum amount of P-798<sup>+</sup> with that observed under continuous illumination indicates that, in contrast to what was observed with *Rhodospirillum rubrum* (7), even with such a short flash all of the reaction centers can be closed. This suggests that, in *H. chlorum*, trapping by the reaction centers competes efficiently with the loss of singlet excitations because of singlet-singlet annihilation.

From the fluorescence excitation spectrum, measured at 4 K (unpublished data), it is estimated that excitations absorbed at 532 nm are transferred to BChl *g* with an efficiency of 65–70%. From the absorption of the sample at 532 nm and the incident energy density, it then follows that the quantum yield of trapping by the reaction centers of singlet excitations on BChl *g* is close to 100% in the linear region of the saturation curve. The assumptions are made here that the

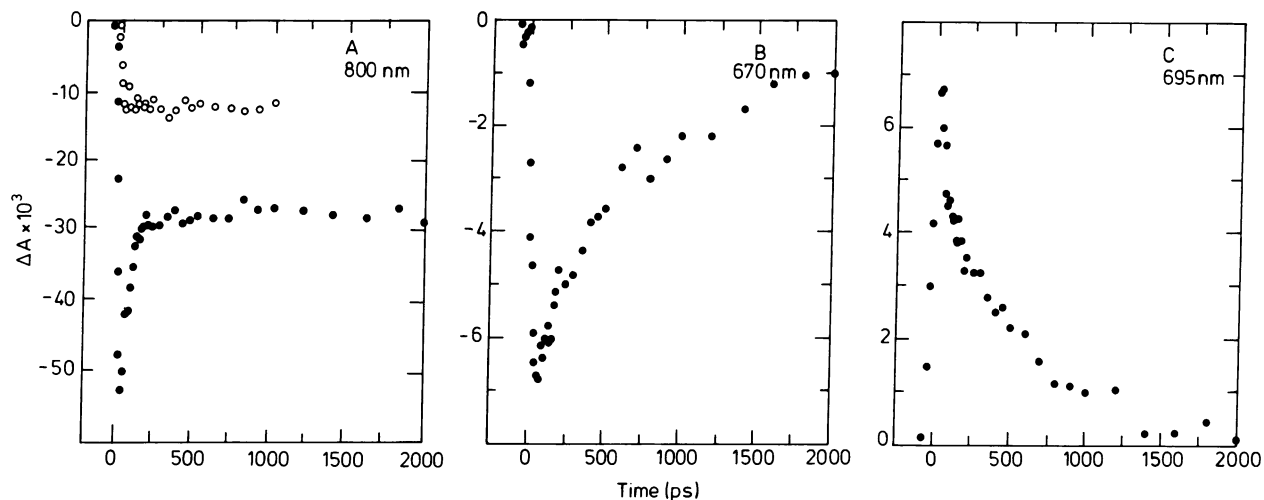


FIG. 3. Kinetics of absorbance changes at 800 (A), 670 (B), and 695 nm (C). Conditions were as in Fig. 2, except in A (open circles), where the excitation intensity was 0.22 mJ/cm<sup>2</sup>. Zero picoseconds on the time scale corresponds to coincident excitation and probe pulses.

same transfer yield applies at room temperature and that the differential extinction coefficient at 798 nm for the oxidation of the primary electron donor P-798 is about 100 mM<sup>-1</sup>·cm<sup>-1</sup>.

The solid circles in Fig. 5 show the absorbance-difference spectrum in the region 550–900 nm obtained with simultaneous excitation and probe pulses. The spectrum was recorded at roughly the same energy density as that used for the high-intensity kinetics of Fig. 3A (solid circles). The spectrum shows a bleaching around 800 nm that is about twice as large as the absorbance decrease due to formation of P-798<sup>+</sup> as shown in Fig. 2 (open circles). A bleaching in this wavelength region was also observed with simultaneous excitation and probe pulses when the reaction centers had been closed by an additional saturating flash given about 5 ns before the actual measurement (Fig. 5, open circles). Under these conditions, no P-798 oxidation occurred, and only a rapidly decaying absorbance change was visible (Fig. 5 Inset). We conclude that this absorbance change is caused by the formation of BChl\**g* and subsequent reversal to the ground state. Similar absorbance changes have been observed in the antenna systems of *R. rubrum* (8) and *P. aestuarii* (4). The rise and the first part of the decay (Fig. 5 Inset) are distorted because of convolution with the temporal profile of the pulses. The tail

of the decay indicates a lifetime of the excitations of 200 ± 50 ps.

The bleaching, which is centered at 800 nm, is shifted with respect to the absorption maximum at 788 nm. From an analysis of the shape of the near-infrared absorption band at 4 K, it was concluded that the Q<sub>y</sub> band consists of at least three spectroscopically different forms of BChl *g* (BChl *g* 778, BChl *g* 793, and BChl *g* 808), present in relative amounts of 11:6:1.4, respectively (unpublished data). If we assume that at room temperature the position of these bands are the same and that thermal equilibrium exists, then it can be calculated that the bulk of the singlet excitations are shared equally between the BChl *g* 793 and BChl *g* 808 components, resulting in a maximal bleaching in the Q<sub>y</sub> region of BChl *g* at 800 nm. Thus, our results indicate that thermal equilibrium was reached already during the 35-ps pulse, implying a very fast rate of energy transfer between the various forms of BChl *g*.

The spectrum with active reaction centers (Fig. 5, solid circles) also shows the bleaching at 673 nm and the absorption increase at 695 nm characteristic for the formation of I<sup>-</sup>. The bleaching at about 580 nm is probably due to disappearance

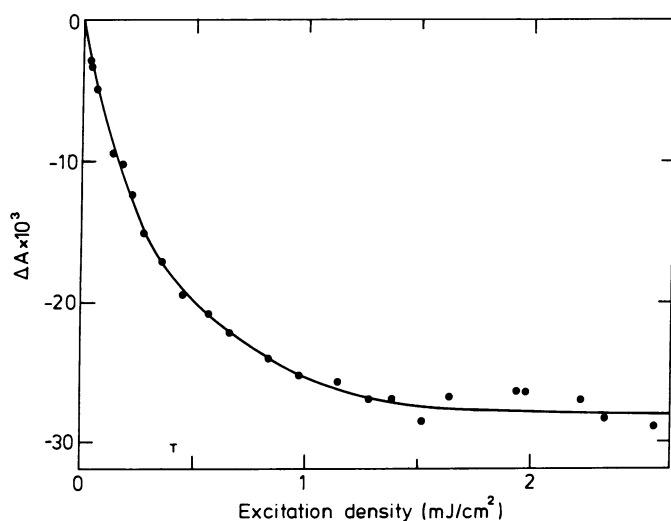


FIG. 4. Amplitude of the absorbance change at 800 nm at 500 ps after a flash as a function of the flash energy density. Conditions were as for Fig. 2.

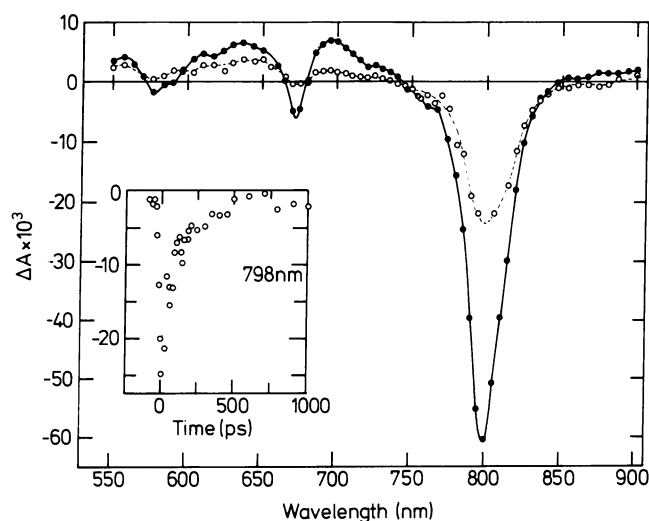


FIG. 5. Absorbance difference spectra with simultaneous excitation and probe pulses. ●, Conditions as in Fig. 2; ○, about 5 ns after a 35-ps "preflash," in the presence of 10 mM ascorbate. (Inset) Kinetics with a preflash at 798 nm.

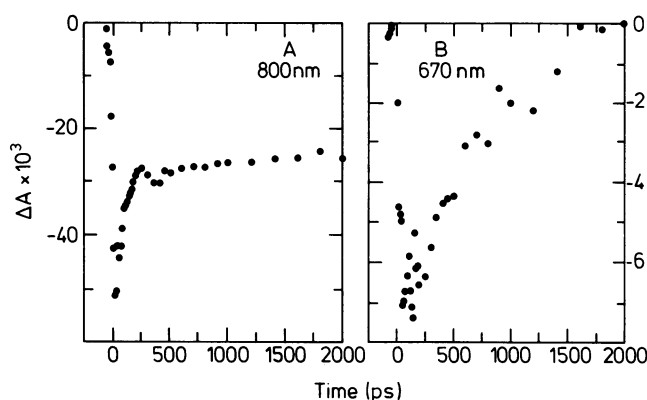
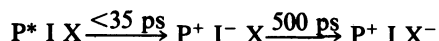


FIG. 6. Kinetics of absorbance changes at 800 (A) and 670 nm (B) at an ambient redox potential,  $E_h = -450$  mV, in the presence of 20 mM Tris, pH 8.0/10 mM sodium dithionite/100  $\mu$ M methylviologen/15  $\mu$ M PMS with catalase, glucose, and glucose oxidase to maintain anaerobiosis.

of the  $Q_x$  band of P-798. Similar, but weaker bands in the spectrum obtained with closed reaction centers (Fig. 5, open circles) may be ascribed to excited states of BChl *g* and of the pigment absorbing at 670 nm.

Fig. 6 shows the kinetics of the absorbance changes at 800 nm (Fig. 6A) and 670 nm (Fig. 6B) at an ambient redox potential of  $-450$  mV in the presence of dithionite and methylviologen. They show that the charge separation between P-798 and I was not affected and that electron transfer from  $I^-$  was not inhibited at this redox potential. Flash-induced charge separation also was observed at pH 9.5 along with a redox potential of about  $-560$  mV, but under these conditions the kinetics at 670 and 695 nm indicated that the reoxidation of  $I^-$  was slowed down to about 2 ns. The kinetics at 800 nm indicated that this 2-ns decay could not be explained by a charge recombination between P-798 $^+$  and  $I^-$ . This may suggest a reaction between  $I^-$  and an "alternative" electron acceptor, the normal secondary electron acceptor, X, being reduced under these conditions.

In summary, we conclude that the primary steps in the electron transport of *H. chlorum* may be represented by the scheme (where P denotes P-798):



These processes appear to be very similar to those in the green sulfur bacterium *P. aestuarii* (4): I is a pigment with an absorption band near 670 nm and a midpoint potential below  $-560$  mV and is probably similar or even identical to the primary acceptor of *P. aestuarii*, which was suggested to be bacteriopheophytin *c* (9). However, recent experiments by T. Braumann and H. Vasmel (personal communication) have shown (i) the virtual absence of bacteriopheophytin *c* in the reaction center containing preparations of *P. aestuarii* but also (ii) the presence of a BChl *c*-like pigment, different from one of the known forms of BChl *c*, that is responsible for the absorbance band at 670 nm. Thus, contrary to earlier suggestions, it must be concluded that the primary electron acceptor in green sulfur bacteria, and perhaps by analogy in *H. chlorum*, is a BChl *c*-like pigment. The midpoint potential of the secondary electron acceptor X appears to be between  $-450$  and  $-560$  mV. This rules out a quinone as secondary electron acceptor. No absorbance changes in the region around 800 nm were observed that could be ascribed to reduction of X, indicating that X is not a BChl-like pigment. Thus, we conclude that X may conceivably be an iron-sulfur center, as in green sulfur bacteria.

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