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Electron transport and triplet formation in membranes of the photosynthetic bacterium *Heliobacterium chlorum*

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Key words: Electron transport; Iron-sulfur protein; Primary electron donor; Triplet formation; Bacterial photosynthesis; (*H. chlorum*)

The spectroscopic and thermodynamic properties of the electron-transport components of the photosynthetic bacterium *Heliobacterium chlorum* were studied by means of absorbance-difference spectroscopy. Upon flash illumination of membranes of *H. chlorum* photooxidation of the primary electron donor, P-798, was observed. In about 15% of the reaction centers P-798$^+$ was reduced by cytochrome *c*-553, while in the remaining reaction centers P-798$^+$ reduction occurred via a back reaction with a reduced electron acceptor. Titration experiments indicated a midpoint potential of −440 mV for the electron acceptor. At low redox potentials the formation of the triplet of P-798 was observed after a flash. The triplet was formed in about 30 ns by a back reaction with a reduced electron acceptor and decayed with a time constant of 35 μs. The yield of triplet formed in a flash was 30%. Upon continuous illumination at low redox potentials the accumulation in the reduced state of an electron acceptor was observed. The difference spectrum of this acceptor indicates that it is an iron-sulfur center. The yield of triplet formation was independent of the redox state of the iron-sulfur center, which indicates that the center is not located in the main electron-transport chain. A scheme with three acceptors in the main electron-transport chain is presented to accomodate our results and those of others.

Introduction

Heliobacterium chlorum, a strictly anaerobic photosynthetic bacterium, was discovered a few years ago by Gest and Favinger [1]. It is distinguished from other species of photosynthetic bacteria by the chemical nature of its major pigment, which is bacteriochlorophyll *g* [2]. The primary electron donor, P-798 [3], is probably likewise BChl *g*. Although *H. chlorum* does not possess chlorosomes [1], there is evidence that its electron-acceptor chain is similar to that of green sulfur bacteria [4,5]. Titration of its photochemical activity has shown that the acceptor chain operates at a low redox potential [4], and measurements of flash-induced absorbance changes in the subnanosecond region have indicated that the primary electron acceptor is spectroscopically similar to that of green sulfur bacteria [5], which was recently shown to be a special form of BChl *c* [6,7]. ESR studies indicated that the acceptor chain would contain one or more iron-sulfur centers [4,8], and also suggested the involvement of another electron acceptor with a quinone-like signal [8].

Abbreviations: BChl, bacteriochlorophyll; P-798, primary electron donor; PMS, *N*-methylphenazoniummethosulfate; PES, *N*-methylphenazoniumethosulfate.

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The present communication reports the results of a spectroscopic and kinetic study of electron transport and triplet formation in membranes of *H. chlorum* as a function of oxidation-reduction potential. Evidence will be reported for the existence of two different secondary electron acceptors. At low redox potential, the triplet of the primary electron donor, P-798, was formed upon illumination. The yield of triplet formation decreased in the presence of an external magnetic field, indicating that it is produced by the radical-pair mechanism. Photoaccumulation of a reduced iron-sulfur center could be demonstrated by optical spectroscopy. However, this iron-sulfur center does not seem to operate in the main electron-transport chain, since the yield of triplet formation reached its maximum value (approx. 30%) already at a redox potential where this center was still largely oxidized.

Materials and Methods

Heliobacterium chlorum was grown anaerobically in medium 112 of the American Culture Collection [1], containing 2.5 mM ascorbate. Cells were harvested by centrifugation and washed in a buffer (pH = 8.0) containing 10 mM Tris, 10 mM ascorbate and 2 mM dithiothreitol. Membrane fragments were prepared by sonication followed by centrifugation at $20\,000 \times g$ to remove large cell fragments. The membrane fragments were concentrated by 1 h centrifugation at $250\,000 \times g$ on a 40% sucrose layer and stored at $-20\,^{\circ}C$. Before use the sample was diluted with a buffer containing 10 mM Tris, 10 mM ascorbate and 2 mM dithiothreitol. Glucose, glucose oxidase and catalase were added to obtain anaerobic conditions.

Flash-induced absorbance kinetics and difference spectra were measured with a single-beam spectrophotometer. Excitation flashes were provided by a Q-switched frequency-doubled Nd-YAG laser (15 ns halfwidth, 532 nm), by a rhodamine B dye laser pumped by the YAG laser (590 nm) or by a xenon flash tube (13 μs halfwidth). The measuring light, obtained from a 1000 W xenon lamp or a 250 W tungsten-halogen lamp, passed through a monochromator, a chopper, the sample and a second monochromator. The transmitted light was measured with a photomultiplier (EMI 9558 C), protected from stray actinic light by suitable optical filters, and the signal was fed into a transient digitizer (Biomation model 8100). The redox titrations were carried out in a anaerobic cell in the presence of sodium dithionite and redox mediators. When necessary, small aliquots of ferricyanide solution were added to obtain the desired redox potential. The redox potentials were measured with a Schott Pt61 electrode. The magnetic-field dependence of the absorbance changes was measured as in Ref. 9. Absorbance changes induced by continuous light were measured as in Ref. 10. The absorbances of the sample was approx. 1.1 at 788 nm, unless otherwise indicated.

Results

The absorbance difference spectrum of membranes of *H. chlorum*, obtained upon excitation with non-saturating 15 ns laser flashes is shown in Fig. 1. As judged from its similarity to spectra obtained by Fuller et al. [3] and Nuijs et al. [5] for the red and near-infrared region, and to that of Prince et al. [4] for the region 440–630 nm, the

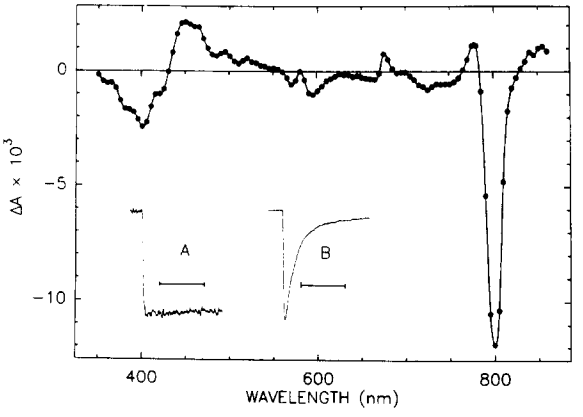

Fig. 1. Spectrum of the absorbance changes induced by 15 ns laser flashes in membranes of *H. chlorum*. Excitation at 590 nm for the region 500–550 nm and at 532 nm for the other wavelengths, at a repetition rate of 2.5 Hz. The membranes were suspended in 10 mM Tris, 10 mM ascorbate, 10% (w/v) sucrose and 2 mM dithiothreitol at pH = 8.0 under anaerobic conditions. Inset A: kinetics at 400 nm, the bar indicates 10 μs. Inset B: kinetics of absorbance changes at 800 nm induced by a 13 μs xenon flash, at a repetition rate of 0.8 Hz, the bar indicates 50 ms.

spectrum is largely due to photooxidation of the primary electron donor, P-798. In the Q_x region negative bands are observed at 595 and 570 nm; in the Soret region negative bands and shoulders at 380, 400 and 420 nm and positive bands at 450, 465 and 495 nm are seen.

The oxidation of P-798 was too fast to be resolved by our apparatus. During the first 20 μs after a flash the absorbance changes were essentially irreversible (Fig. 1, inset A), but measurements at longer time scales (Fig. 1, inset B) showed that the decay kinetics at 800 nm could be fitted with two exponential components of 6 ± 2 and 30 ± 3 ms, together with a minor contribution by a much slower component. The amplitudes of the 6 and 30 ms components had a ratio of 1 : 1, and together they comprised about 90% of the total decay. The same components were observed at 400 nm and at 450 nm.

The difference spectrum measured at 90 ms after a flash is shown in Fig. 2. This spectrum mainly represents the slowest component. The bleachings at 800 and 593 nm are presumably caused by P-798$^+$, but in the visible region the spectrum is clearly different from that of the initial absorbance changes (Fig. 1), with negative bands at 420, 552 and 515 nm, indicating the oxidation of cytochrome *c*-553 [3]. The amount of oxidized cytochrome *c*-553 observed in the difference spectrum is quite small. If an oxidized-minus-reduced extinction-difference coefficient $\Delta\epsilon_{553} - \Delta\epsilon_{540} = 20\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ for cytochrome *c*-553 and $\Delta\epsilon_{799} = 100\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$ for P-798 are assumed, it can be calculated that only in about 5% of the reaction centers cytochrome *c* oxidation could be detected.

The spectrum of Fig. 2 was obtained with a series of flashes spaced at 1.2-s intervals. Fig. 3A shows the kinetics of cytochrome oxidation measured at 553 nm with 10 s dark times between the flashes. It can be seen (Fig. 3B) that the amount of cytochrome *c* oxidized by a flash increased with increasing dark time with a half time of about 1.8 s and reached a plateau at approx. 10 s. With a dark time of 1 s the amount of cytochrome oxidized was only one-third, while at 400 ms hardly any cytochrome oxidation was observed. This indicates that the re-reduction of oxidized cytochrome *c*-553 was very slow, but even with a dark time of

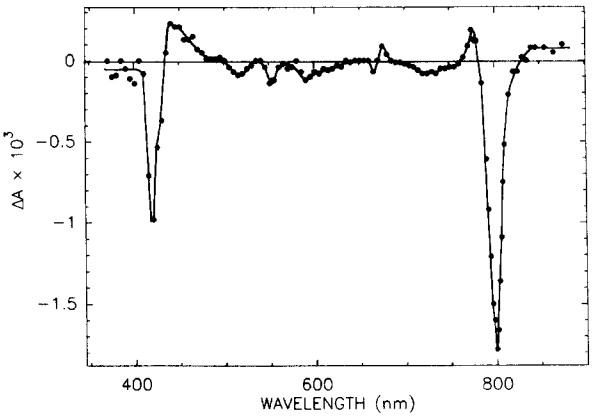

Fig. 2. Spectrum of the 'irreversible' component of the absorbance changes shown in Fig. 1B, measured 90 ms after the flash.

10 s cytochrome oxidation in a flash occurred in only about 15% of the reaction centers, in contrast to what was observed in continuous light [5]. Thus we conclude that in our preparation most of the P-798$^+$ formed in a flash decays by a back reaction with one or more reduced electron acceptors. The time constant for cytochrome *c* oxidation was approx. 5 ms, i.e., the same as that of the rapid phase of P-798$^+$ re-reduction. The small amount of cytochrome oxidized, even after a long dark time, may be explained by a low rate of reaction with P-798$^+$, so that the time constant for the 6 ms decay phase of P-798$^+$ (Fig. 1B) is largely determined by that for the back reaction. This reasoning implies the existence of two types of reaction center. Cytochrome oxidation only occurs in a fraction of those reaction centers that show a 6 ms decay of P-798$^+$; the absence of time constants other than 6 ms indicates that cytochrome oxidation does not occur in the reaction centers that show a 30 ms decay. It is of interest to note here that similarly small amounts of oxidized cytochrome *c* were also generated upon flash illumination of intact cells, even when the flashes were given at 10 s intervals (not shown). The time constant for cytochrome oxidation in intact cells was approx. 8 ms.

The difference spectrum of Fig. 1 in principle should contain contributions due to both photooxidation of P-798 and photoreduction of one or more components of the electron-acceptor chain.

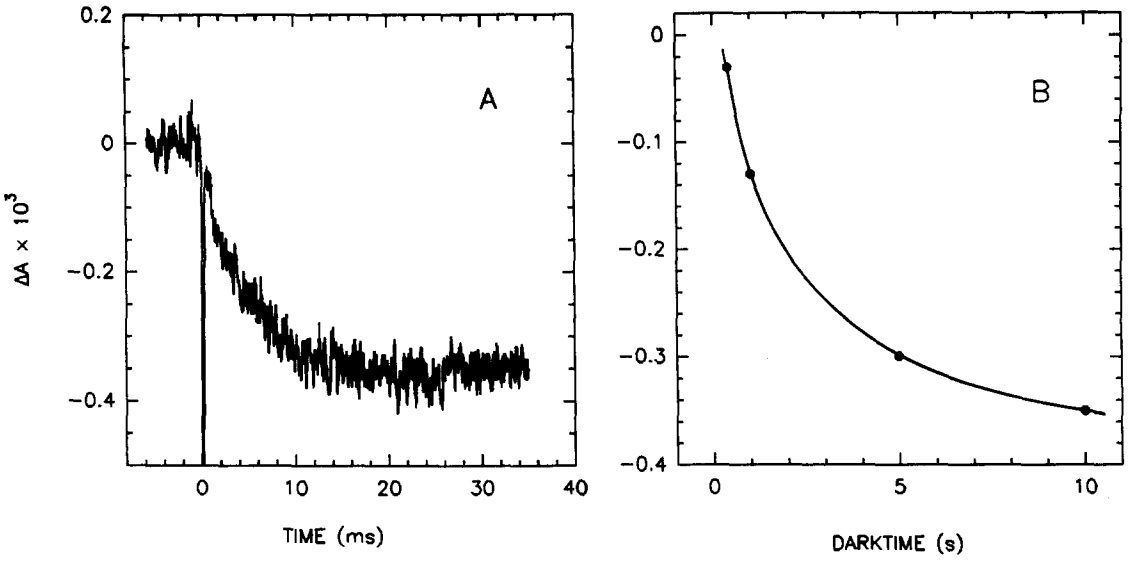

Fig. 3. (A) Kinetics of cytochrome oxidation measured at 553 nm with a 15 ns, 532 nm laser flash. Dark time between flashes: 10 s. The spike at 0 μs is an artefact caused by the laser flash. (B) Amplitude of the absorbance changes as a function of dark time between flashes. Other conditions as for Fig. 1.

In the presence of 20 μM *N*-methylphenazonium methosulfate (PMS) or *N*-methylphenazonium ethosulfate (PES) the absorbance change at 800 nm showed a mono-exponential decay of 6 ± 1 ms. The difference spectrum of the 6-ms component was very similar to that of Fig. 1. Assuming that PES and PMS directly reduce P-798$^+$ we thus conclude that the spectrum of Fig. 1 is almost entirely due to photooxidation of P-798, with only minor contributions of reduced acceptors.

In the presence of PMS and dithionite at an apparent redox potential of −480 mV, we observed absorbance changes upon continuous illumination which could be attributed to the photoaccumulation of an electron acceptor in the reduced state. The kinetics, together with the difference spectrum of the reversible part of the absorbance changes, are shown in Fig. 4. In the blue region this spectrum shows a broad absorbance decrease with minima at 400, 430 and 460 nm, which suggests that it is due to reduction of an iron-sulfur center. Similar spectra have been obtained with the green sulfur bacterium *Prosthecochloris aestuarii* [11] and with Photosystem I preparations from cyanobacteria and higher plants [12,13]. From the amplitude of the absorbance change at 460 nm a differential extinction coefficient of 6 $mM^{-1} \cdot cm^{-1}$ per reaction center can be calculated. This value is in the same range as those obtained for *P. aestuarii* [11] and for Photosystem I [12,13], indicating that one iron-sulfur center was

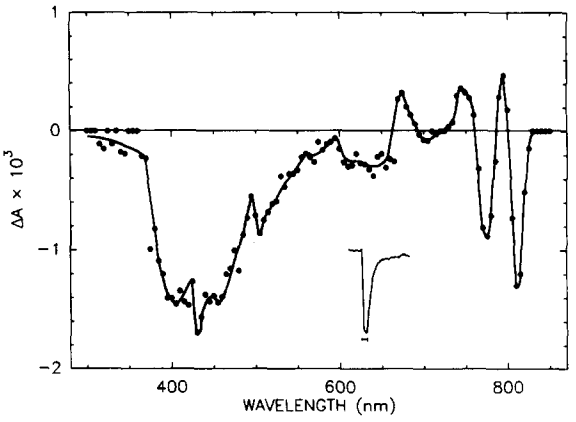

Fig. 4. Absorbance difference spectrum of membranes of *H. chlorum* obtained upon illumination with strong continuous light under reducing conditions. The sample contained 10 mM Tris (pH = 8.0), 10 mM ascorbate, 10% (w/v) sucrose, 15 mM dithionite and 20 μM PMS under anaerobic conditions. The absorbance at 788 nm was 0.9. Illumination with Schott BG 12 for measurements above 600 nm, with Schott RG 715 for the region 370–600 nm and with Corning CS 496 and Schott GG 545 below 370 nm. Inset: kinetics at 450 nm. The bar indicates the 3 s illumination period.

reduced per reaction center in our preparation. Between 320 and 380 nm the absorbance changes were very small. This indicates that reduced quinones were not accumulated under the conditions applied.

The absorbance changes at 740–825 nm in the spectrum of Fig. 4 may be ascribed to band shifts of BChl *g*. Changes in pigment absorption accompanying the reduction of electron acceptors have also been observed in preparations from purple [14] and green [11,15] photosynthetic bacteria, and may ascribed to the transfer of a negative charge to the acceptor. The band shift centered at 670 nm may represent a red shift of the primary electron acceptor; a similar band shift is also seen in the difference spectrum of P-798 (Fig. 1).

The 6 ms decay component observed in the presence of PMS after a flash almost completely disappeared at low redox potential and was replaced by faster components. Fig. 5 shows the kinetics observed under these conditons. A rapid absorption change was followed by a biphasic decay. The time constant of the rapid decay component was close to the resolution of the apparatus and was found to be approx. 30 ns; the slower component had a time constant of 35 μs. The spectrum of the 35 μs component is given in Fig. 6 and showed a bleaching centered at 793 nm. We conclude that the spectrum is due to the formation of the triplet of P-798 and is formed by P-798$^+$ in

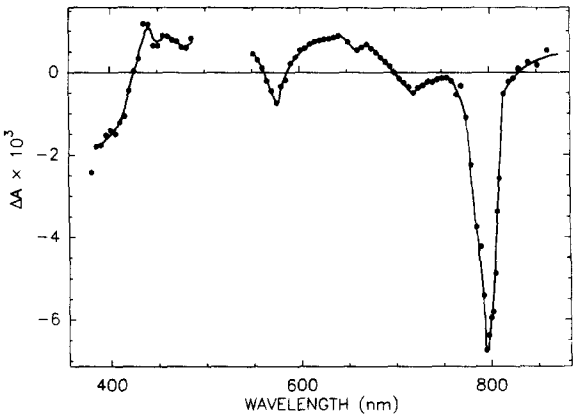

Fig. 6. Absorbance difference spectrum of the formation of the triplet of P-798, obtained by plotting the amplitude of the 35 μs component shown in Fig. 5C as a function of wavelength.

a back reaction with a reduced electron acceptor, as will be discussed below. The spectrum shows significant differences compared to the difference spectrum of P-798$^+$. The bleaching band in the near infrared is blue-shifted and broader than that of P-798$^+$, which can be explained by the absence of the band shift that causes the maximum at 776 nm in the P-798$^+$ spectrum (Fig. 1). A similar broadening was observed in the triplet spectrum of P-700 in Photosystem I particles [16]. It is interesting to note that in the Q_x region the spectrum, in contrast to that of P-798$^+$ (Fig. 1), shows a single bleaching band at 575 nm. This indicates

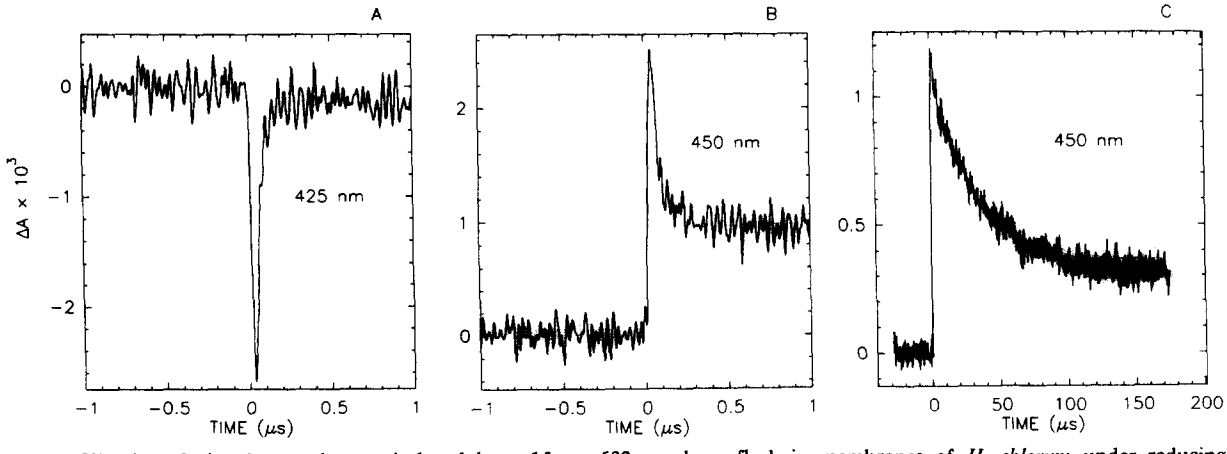

Fig. 5. Kinetics of absorbance changes induced by a 15 ns, 532 nm laser flash in membranes of *H. chlorum* under reducing conditions. The sample contained 10 mM Tris (pH = 9.5), 10 mM ascorbate, 10 mM dithionite and 20 μM PMS under anaerobic conditions. Tracing C was recorded with a 1 MHz high-frequency cut-off filter, which removed the rapid initial rise and decay component.

that the difference spectrum for P-798 oxidation in this region (see Fig. 1) results from a superposition of a bandshift of a neighboring pigment on the bleaching of the Q_x band of P-798. The amplitude of the band at 793 nm indicates that the triplet was formed with a yield of 30% in a flash at room temperature. The overall time constant of 30 ns for the decay of P-798$^+$ presumably results from the combined rates of processes which produce the singlet as well as the triplet states of P-798. Together with a triplet yield of 30%, this indicates that the effective time constant for triplet formation is about 100 ns.

The amount of triplet formed in a flash decreased in the presence of a magnetic field as is shown in Fig. 7. The maximum value of the depression of the triplet yield was 50%. The magnetic field strength at which half the effect occurred was about 30 mT. Similar magnetic field effects have been seen in reaction centers of purple bactria and have been discussed in terms of the radical-pair mechanism [17–20]. We therefore conclude that the triplet of P-798 is formed via the recombination of a radical pair.

The spectrum of the initial absorbance changes during the 15 ns laser flash at low redox potential is shown in Fig. 8. This spectrum is essentially the same as that of Fig. 1, except that the band at 450 nm appears to be missing. This indicates that the spectrum of Fig. 8 is due to formation of P-798$^+$,

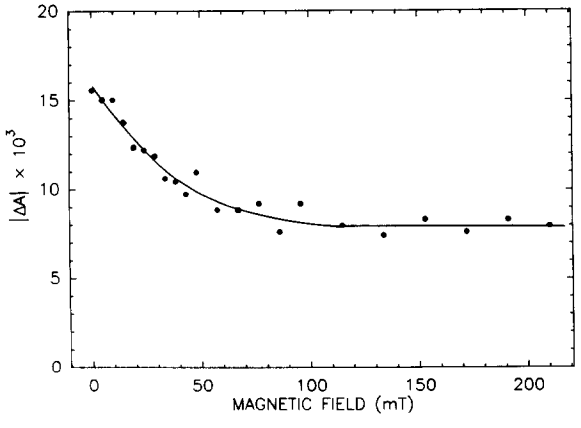

Fig. 7. The yield of P-798 triplet formation, measured by the amplitude of the 35 μs component at 800 nm, as a function of the magnetic-field strength. The absorbance at 788 nm was 2.2, other conditions as for Fig. 5.

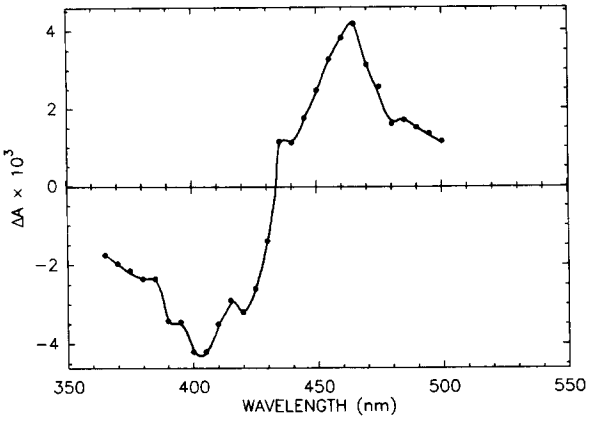

Fig. 8. Difference spectrum of the initial absorbance change obtained with a 15 ns laser flash (see Fig. 5A and B). Conditions as for Fig. 5.

together with the reduction of an electron acceptor which shows only small absorbance changes in the blue region reduction. The differences in the region 430–500 nm between the spectra of Fig. 8 and Fig. 1 are presumably caused by the reduction of different electron acceptors, which we call X_1 and X_2. X_1^- and X_2^- react with P-798$^+$ in approx. 30 ns and in the millisecond region, respectively.

The redox titration curve of the fraction of P-798$^+$ which decays on a millisecond time scale is shown in Fig. 9 (circles). The data can be fitted by a one-electron Nernst curve with a midpoint potential of −440 mV. This value is significantly higher than that of −510 mV obtained by Prince et al. [4] under similar conditions. The reason for this discrepancy is not known. Fig. 9 also shows the titration curve for the formation of the triplet of P-798. At redox potentials above −430 mV this curve (open squares) was complementary to that of P-798$^+$, but at lower redox potentials the yield of triplet formation did not increase above about 10% in the presence of redox mediators. A 'normal' yield of 30% was only obtained in the absence of redox mediators (solid square), but under these conditions a proper titration was not possible due to imperfect equilibrium at the electrode. We checked that the decrease of the triplet yield in the presence of mediators was not due to an incomplete charge separation. The rate of triplet formation appeared to be the same, but part of P-798$^+$ now decayed at a much slower time scale.

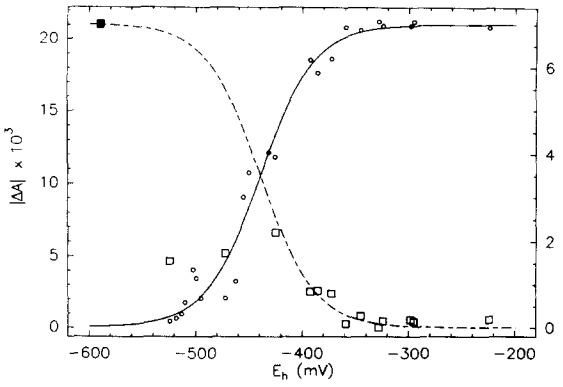

Fig. 9. Titration curves of P-798$^+$ reduction and of triplet formation measured at 800 nm. Circles, left hand scale: amplitude of the absorbance changes that decay on a ms time scale. Squares, right hand scale: amplitude of the 35 μs decay component of the triplet of P-798. Membranes were suspended in 10 mM Tris, 10 mM ascorbate, pH = 9.5. For the open symbols the following redox mediators (20 μM) were used: methyl viologen, benzylviologen, 2-hydroxy-1,4-naphthoquinone, PMS, neutral red.

The reason for this effect is not clear, perhaps an alternative electron acceptor became accessible under these conditions. Nevertheless, our results indicate that the disappearance or the 6 ms decay of P-798$^+$ is correlated with the formation of the P-798 triplet. We conclude that the electron acceptor for X_1 has a midpoint potential of -440 mV, considerably higher than that of the iron-sulfur protein which can be photoreduced in continuous light. The yield of triplet formation was independent of the redox state of the iron-sulfur center: when the iron-sulfur center was reduced before the flash by the application of continuous background illumination at low redox potential, the same kinetics after a laser flash were obtained with the same triplet yield as in the absence of background light. These results indicate that neither X_1 nor its electron acceptor are identical to the iron-sulfur center and make it difficult to assign to this center a role in the main electron-acceptor chain.

Discussion

The results presented here indicate that in addition to the primary electron acceptor I [5] at least two other components are present in the acceptor chain of *H. chlorum*. A scheme that could accomodate our results and those of others is shown in Fig. 10.

The chemical identity of the electron acceptors X_1 and X_2 in the scheme is not known. Their existence in the electron acceptor chain is based on the measurements of the kinetics of P-798$^+$ re-reduction and triplet formation as a function of redox potential. It cannot be excluded, however, that X_1 is the substance that produces the quinone-like ESR signal observed by Brok et al. [8] at low temperatures. We assume that the electron acceptor for X_1, which has a midpoint potential of -440 mV, is identical to X_2, which recombines with P-798$^+$ in the ms region. The biphasic kinetics of the reduction of P-798$^+$ might be taken as evidence that X_2 in our scheme represents two different electron acceptors. However, in view of the evidence discussed above for the existence of two types of reaction centers, we assume that X_2 is a single substance which shows somewhat different rates of back reaction in these centers.

Under reducing conditions the triplet state of P-798 was formed in a flash with a yield of about 30%. It is interesting to note that, as in the green sulfur bacterium *P. aestuarii*, no energy transfer from the triplet of P-798 to carotenoid occurs. The magnetic-field dependence of the triplet yield indicates that it is formed by a radical pair recombination [17–20]. The difference spectrum of this radical pair ($P^+X_1^-$, see Fig. 8) suggests that X_1 is not identical to the primary electron acceptor I. Flash

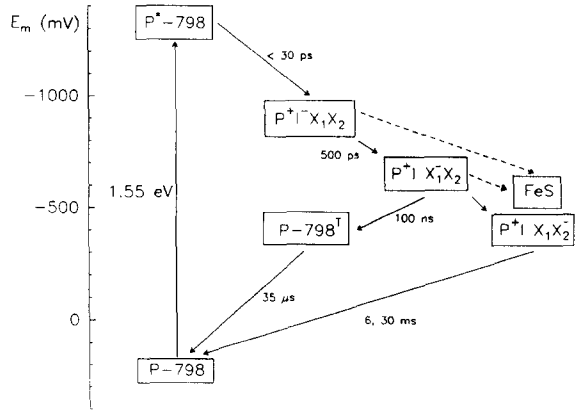

Fig. 10. Proposed scheme for the electron transport in membranes of *H. chlorum*. The rate constants are based on our own measurements and those by Nuijs et al. [5].

spectroscopy in the picosecond region [5] has indicated that I is a BChl *c* like pigment and the spectrum of BChl *c* reduction in vitro [21] indicates that a larger contribution by the acceptor should be expected in the blue region if the difference spectrum of Fig. 8 would be caused by reduction of BChl *c* together with oxidation of P-798. This conclusion is in agreement with the data of Nuijs et al. [5] who observed that the decay rates of $P\text{-}798^+$ and I^- did not match at low redox potentials: I^- was reoxidized in about 2 ns while the re-reduction of $P\text{-}798^+$ took more than 5 ns, indicating the absence of a direct back reaction between these two compounds.

A puzzling phenomenon is not only the low rate of cytochrome oxidation (see also Ref. 4) but also the small amount of oxidized cytochrome *c*-553 observed in our experiments, even after a long dark time between the flashes. The amount is clearly less than observed by Prince et al. [4] with similar preparations, and by Nuijs et al. [5] in continuous light, although it should be noted that also in those experiments less than one oxidized cytochrome per reaction center was produced. With isolated membranes our results could be explained by a competing back reaction, as discussed above, but such an explanation is unattractive to explain the low yield of cytochrome oxidation in a flash observed by us in intact cells. Further experiments will be needed to investigate this point.

The role of the iron-sulfur center that could be photoaccumulated at low redox potentials is uncertain. It may be identical to the iron-sulfur center observed in the ESR spectrum at low temperature, but is not identical to either X_1 or its electron acceptor because the yield of triplet formation was independent of the redox state of the iron-sulfur center.

Our results, and those of others [8] would seem to indicate that there are some significant differences between the electron-acceptor chain in *H. chlorum* and the currently accepted scheme for green sulfur bacteria [11,22]. However, it should be kept in mind that kinetics of flash-induced absorbance changes at low redox potential are not available for green sulfur bacteria. Thus the evidence for the role of a low potential iron-sulfur-center in the main electron-acceptor chain in these bacteria [11] is only indirect, whereas triplet formation has only been studied in preparations that were deficient in the electron-acceptor chain [23–25]. Titration experiments like those presented here for *H. chlorum* will be needed to obtain more conclusive evidence.

Acknowledgements

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