Reaction Center Photochemistry of *Heliobacterium chlorum*†

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ABSTRACT: Reaction center photochemistry in *Heliobacterium chlorum* has been investigated by using EPR and flash absorption spectroscopy at low temperatures. The following results were obtained. At 5 K, in the presence of ascorbate, continuous illumination resulted in the formation of P_{798}^+ and a reduced iron–sulfur center designated F_B (g_z = 2.07, g_y = 1.93, g_x = 1.89). This state was stable at low temperatures, but the yield for this reaction was low, and it was estimated that it occurred only in about 3% of the centers upon the first flash. After continuous illumination of a dilute sample for 10 min, still only half of the centers attained this state. In most centers, flash excitation at 5 K produced a state which recombined with time constants of 2.5 ms (≃80%) and 850 μs (≃20%). These two phases were differently influenced by the redox state of the reaction center, indicating that two different acceptors were involved in the recombination reactions. When continuous illumination was given at 200 K, a second center, designated F_A, was additionally reduced (g_z = 2.05, g_y = 1.95, g_x = 1.90). High concentrations of dithionite resulted in the chemical reduction of F_B and of most of F_A; illumination at 200 K resulted in the further reduction of F_A. Two triplet states were identified by EPR and optical spectroscopy. The amplitude of the narrower triplet ($|D|$ = 226 × 10^{-4} cm^{-1}) varied with the redox state of the iron–sulfur centers and was influenced by a component thought to be a quinone undergoing double reduction. It correlated with a triplet state observed by flash absorption spectroscopy showing a bleaching at 798 nm and is attributed to a triplet state formed by charge recombination in the reaction center. Its narrowness is taken as an indication of its origin on a pair of bacteriochlorophylls, and its orientation indicates an orientation of the chlorophyll ring plane perpendicular to the membrane plane. The second triplet had a wider splitting ($|D|$ = 242 × 10^{-4} cm^{-1}), did not vary systematically with redox conditions, corresponds to an optical spectrum with a maximum at 812 nm, and is not ordered in the membrane. It was thus attributed to a triplet located on a BChl *g* monomer in the antenna. The reaction center photochemistry in *H. chlorum* is comparable in many respects to that of photosystem I and green sulfur bacteria. Earlier contrasting conclusions are discussed and rationalized in light of the present results.

Until about 8 years ago, the reaction centers of bacterial photosynthesis could be classed into two types. First, purple bacteria and *Chloroflexus aurantiacus*, a green non-sulfur bacterium, contain a type of reaction center which is well characterized. The second type is that of the green sulfur bacteria, many basic details of which have only recently become apparent (Nitschke et al., 1990). In a sense, cyanobacteria can be viewed as containing both types of reaction centers, in that photosystem II can be regarded as a more sophisticated form of a purple bacterial reaction center (RC)[1] (Rutherford, 1989) whereas photosystem I seems to be an only slightly modified green sulfur bacterial RC (Nitschke et al., 1990).

With the discovery of the Heliobacteriaceae, a new photosynthetic actor entered the scene. The first species to be described was *Heliobacterium chlorum* [Gest & Favinger, 1983; see also Barber (1985)], followed by the isolation of *Heliobacillus mobilis* (Beer-Romero & Gest, 1987), *Heliobacterium gestii*, and *Heliobacterium fasciculum* (Ormerod et al., 1990). All members of the family of Heliobacteriaceae are characterized by the possession of BChl *g*, a hitherto unknown form of bacteriochlorophyll, which seems to be the major pigment in these organisms.

The chemical nature of BChl *g* has been elucidated by Brockmann and Lipinski (1983) and subsequently confirmed by Michalski et al. (1987).

According to phylogenetic trees based on 16S RNA comparisons, Heliobacteria cannot be classed with any of the other known photosynthetic organisms but rather seem to be more closely related to the (nonphotosynthetic) Gram-positive bacteria (Woese, 1987).

Since the discovery of *Heliobacterium chlorum*, research has focused mainly on the reaction center, and EPR, optical spectroscopy, and biochemical methods have been employed (Prince et al., 1985; Fuller et al., 1985; Smit et al., 1987, 1989; Brok et al., 1986; Vos et al., 1989; Trost & Blankenship, 1989; Van de Meent et al., 1990; Fischer, 1990). The midpoint potential of the primary donor was determined to be in the region of +225 to +250 mV (Prince et al., 1985; Brok et al., 1986), i.e., close to that found in green bacteria (Fowler et al., 1971; Prince & Olson, 1976; Knaff et al., 1979). For the terminal acceptor, a midpoint potential of −510 mV has been reported (Prince et al., 1985), which is much lower than that of purple bacteria but comes close to what is found in photosystem I (Golbeck, 1987) and green sulfur bacteria (Knaff et al., 1979; Nitschke et al., 1990). A relationship with reaction centers from green sulfur bacteria has therefore been

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[1] Abbreviations: RC, reaction center complex; BChl, bacteriochlorophyll; FeS center, iron–sulfur center; P_{798}, primary electron donor bacteriochlorophylls of *H. chlorum*; PS I, photosystem I; PS II, photosystem II; kDa, kilodalton(s).

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proposed (Prince et al., 1985; Amesz, 1989).

A possible role of iron–sulfur centers as electron acceptors was proposed based on the presence of FeS centers with a very low redox potential in membrane preparations (Prince et al., 1985), but so far no stable low-temperature photoreduction of iron–sulfur centers has been demonstrated. However, the participation of an FeS center in physiological forward electron transfer has been questioned, because triplet formation in the reaction center did not correspond to the reduction state of an FeS center which can be observed optically in the reaction center (Smit & Amesz, 1988).

Recently the hypothesis has been put forward that the reaction center of *Heliobacterium chlorum* is substantially different from the reaction center of green sulfur bacteria and from photosystem I (Smit et al., 1987; Smit & Amesz, 1988; Van de Meent et al., 1990). In photosystem I, the reaction center is made up of two large subunits with an apparent molecular weight of 65K and additional subunits with lower molecular weight (Golbeck, 1987; Scheller et al., 1989; Ikeuchi et al., 1990; Wynn & Malkin, 1990). The 65-kDa subunits are also found in green sulfur bacteria (Hurt & Hauska, 1984), and there are strong indications for the presence of further subunits with lower molecular weight in these bacteria (Shirasawa & Sakurai, 1990; Nitschke et al., 1990). In contrast to this, results presented by Trost et al. (1989) and by Van de Meent et al. (1990) suggest that the reaction center in Heliobacteria is composed of protein subunits with an apparent molecular weight of 47K. Furthermore, stable low-temperature charge separation, which is characteristic for PS I (Evans, 1982; Malkin, 1982; Rutherford & Heathcote, 1985; Golbeck, 1987) and green sulfur bacteria (Jennings & Evans, 1977; Nitschke et al., 1990), could not be detected in Heliobacteria (Smit et al., 1989).

Recently, we showed that the reaction center of green sulfur bacteria is much more closely related to PS I than previously assumed. The electron acceptor side of the green sulfur bacterial RC is almost identical with that of PS I; however, the electron donor side of green sulfur bacteria was essentially the same as that in purple bacteria (Nitschke et al., 1990; Feiler et al., 1989). This let us to put forward the hypothesis of a common ancestor for all photosynthetic reaction centers and to gain new insight on the structure of photosystem I based on the detailed structural knowledge of the purple bacterial reaction center.

An understanding of the heliobacterial reaction center, a largely unknown and possibly rather different type of reaction center, could provide crucial evidence for or against this hypothesis.

In this paper, we show that, at least at the level of the chemical nature of the primary electron donor and the electron acceptor chain, the notion that Heliobacteria might contain a reaction center that is only very distantly related to other types of reaction centers is not justified. In contrast, the heliobacterial reaction center would appear to be classifiable between the green sulfur bacterial reaction center and photosystem I.

Experimental Procedures

Cultures of *H. chlorum* were grown as described by Liebl et al. (1990). The isolation of periplasmic membranes followed essentially the protocol described in this reference. However, in order to maintain the activity of the photosynthetic reaction center, it was crucial to perform all steps under anaerobic conditions. Therefore, all manipulations of material (i.e., filling the centrifuge tubes, resuspending, additions of reducing or oxidizing agents, loading of EPR tubes) were performed in a glovebox under an argon atmosphere using only degassed buffers.

The ratio of BChl *g* to the pigments absorbing at 670 nm, which consist of the primary electron acceptor (Nuijs et al., 1985a) and variable amounts of a degradation product of BChl *g*, was assayed by measuring A_{788nm}/A_{670nm}. This ratio was always higher than 7.

Oriented membranes were obtained by using essentially the technique of Blasie et al. (1978), i.e., drying of aqueous membrane suspensions on sheets of mylar in a 90% humidity argon atmosphere in darkness for about 2 days. Different degrees of reducing conditions in these oriented samples were produced as described (Nitschke et al., 1990).

EPR spectra were recorded on Bruker 200 and 300 X-band spectrometers fitted with Oxford Instruments cryostats and temperature control systems. Illumination in the EPR cavity was carried out using a 800-W tungsten projector lamp providing 16 000 $\mu Em^{-2}\ s^{-1}$ of white light at the EPR cavity window after being filtered through 2 cm of water and two Calflex filters (Balzers) to remove infrared radiation.

Flash-induced absorption changes with a microsecond time resolution were measured at 10 K using a cryostat (SMC, France) cooled with helium gas. The samples containing 80% glycerol were dark-adapted at room temperature and cooled in darkness to 10 K. The cuvette was oriented at 45° to the mutually perpendicular measuring and exciting beams. Excitation was provided by a YAG laser (532 nm, 20 ns, 50 mJ, repetition rate 0.5–1 Hz). A 2-fold attenuation of the beam intensity resulted in a 30% decrease of the signal size. The measuring light was provided by a 800-W tungsten–halogen lamp. Interference filters were inserted before and after the cuvette to select the measuring wavelength. A silicon photodiode was used as detector. The output signals were amplified (10 Hz–0.3 MHz) before digitization and transfer to a PC microcomputer. A kinetic analysis of the absorption transients was made with a Marquardt algorithm.

Results

Stable Low-Temperature Charge Separation and Characterization of the Terminal Acceptors by EPR. Isolated plasma membranes of *H. chlorum* taken to moderately low redox potentials (i.e., in the presence of ascorbate) and frozen in darkness showed the spectrum of the Rieske FeS center (Figure 1a, solid line) as already published (Liebl et al., 1990). Illumination of such a sample at 4 K resulted in the stable photoreduction of an additional FeS center (Figure 1a, dotted line, and Figure 2a) having $g_z = 2.07$, $g_y \simeq 1.93$, and $g_x = 1.89$ and the appearance of the signal of a radical at $g = 2.003$. Below about 80 K, both signals were stable; above 100 K, both spectral changes slowly reversed, and this is attributed to a recombination between the photooxidized primary donor P_{798}^+ and the reduced FeS center that acts as the terminal acceptor.

Illumination at 200 K in the presence of ascorbate yielded a much bigger spectral change (Figure 1a, dashed line), and the "after illumination minus dark" difference spectrum shows that the g_y line is slightly shifted toward lower field (not shown). This spectrum was stable as long as the sample remained in the frozen state. Thawing and renewed freezing in darkness restored the initial spectrum.

The same illumination protocol at 200 K in the presence of low concentrations of dithionite at pH 6.8 instead of ascorbate further increased the size of the photoreduced signal by more than 2-fold [Figure 1b; note that the spectra in (b) and (c) are scaled down by 4 and 16, respectively, relative to (a)]. The difference spectrum of the light-induced spectral change (Figure 2b) is further shifted toward lower field.

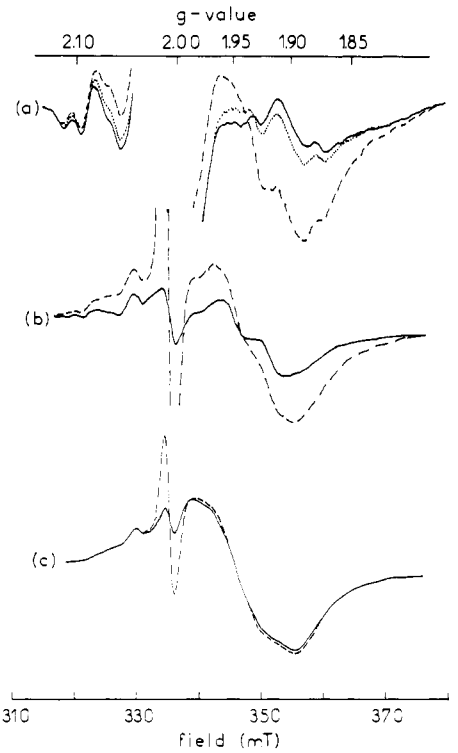


FIGURE 1: EPR spectra of isolated plasma membranes from *H. chlorum* (a) in the presence of 40 mM ascorbate/10 mM MOPS, pH 6.8, (b) in the presence of 2 mM dithionite at pH 6.8, and (c) in the presence of 200 mM dithionite/80 mM glycine, pH 11.0. Solid lines, dark-adapted sample (frozen after 3-min dark-adaptation at room temperature); dotted line [in (a)], after 10-min illumination at 4 K in the cavity; dashed lines, after 15-min illumination at 200 K in a quartz dewar containing a mixture of ethanol and dry ice. The spectra in (a) are expanded 16-fold; the spectra in (b) are expanded 4-fold compared to the spectra in (c). The shown spectra are averages over 16 [for (a)] and 8 [for (b) and (c)] scans. Instrument settings: temperature, 15 K; microwave power, 6.3 mW; frequency, 9.5 GHz; modulation amplitude, 1.6 mT.

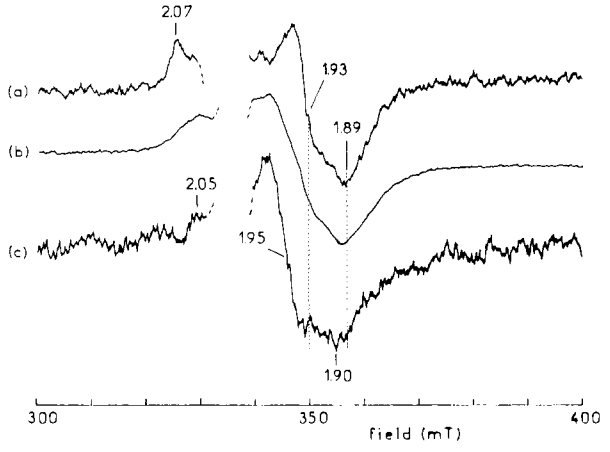


FIGURE 2: EPR difference spectra obtained by subtracting the spectra of the dark-adapted samples from those recorded after illumination in Figure 1. The reduction states of the samples for the differences (a) – (c) are as described in Figure 1. (a) shows the difference between the spectrum of the sample illuminated at 4 K and the "dark" spectrum, whereas in (b) and (c) the differences between the spectra of samples illuminated at 200 K and their respective dark spectra are shown. EPR conditions are as in Figure 1.

Figure 1c shows the spectrum of a sample which was prereduced by 200 mM dithionite at pH 11.0. Strong signals due to FeS centers could already be seen in the dark-adapted sample. Illumination at 4 K did not produce observable changes in this spectrum. Illumination at 200 K in the presence of dithionite, however, slightly further increased the size of the signals. The same difference spectrum [after illumination at 200 K minus dark] was reproducibly obtained and is characterized by the set of g values $g_z = 2.05$, $g_y = 1.95$, and $g_x = 1.90$ (Figure 2c). It arises from a center which is clearly different from that photoinduced at low temperature in the presence of ascorbate (Figure 2a). By comparing the g values of the g_y lines with the respective values in photosystem I (Rutherford & Heathcote, 1985; Golbeck, 1987) and green sulfur bacteria (Nitschke et al., 1990), we designate the center which can be photoinduced at 4 K in samples treated with ascorbate, center F_B (Figure 2a). The center which cannot be completely reduced by dithionite at pH 11.0 but only by illumination at 200 K in the presence of dithionite is designated center F_A (Figure 2c).

The extent of FeS centers photoreduced varies considerably with the temperature of photoreduction and the reducing power of the exogenous reductant. However, in all samples examined, the maximal extent of photoreduced FeS centers at prolonged times at 200 K was always close to 20% of the total FeS signal in the dark in the presence of dithionite at high pH. This indicates that the dark spectrum obtained under very reducing conditions contains large contributions from other FeS cluster containing enzymes in addition to the FeS centers which are part of the photosynthetic reaction center.

Illumination at 4 K for 10 min of samples in the presence of ascorbate yielded only 8% of the maximum *photoreducible* signal, whereas the same treatment at 25 K resulted in the reduction of 11%. Prolonged illumination in both cases slowly increased the percentage of FeS centers reduced. However, even 200 K photoaccumulation for 15 min in the presence of ascorbate reduced only 36% of the photoreducible FeS centers (Figure 1a, dashed line). The maximum extent of photoreduced FeS centers was obtained with a prolonged illumination at 200 K in the presence of small concentrations of dithionite at pH 7.0 (Figure 1b) and was taken as the 100% reference for the photoinduced signals. This indicates that in *H. chlorum*, in contrast to the situation encountered in photosystem I and green sulfur bacteria, the *low-temperature* charge separation between P_{798} and the terminal acceptor ($P_{798}F_AF_B \rightarrow P_{789}^+F_AF_B^-$) is a process with a rather low yield. If the temperature is high enough (about 200 K) to allow for a rereduction of P_{798}^+ by exogenous reductants (i.e., ascorbate or dithionite), a further charge separation can occur: $P_{798}F_AF_B^- \rightarrow P_{798}^+F_A^-F_B^-$.

The almost complete absence of the g_z signal of F_B at $g = 2.07$ in the spectrum of the maximum change (Figure 2c) indicates that this signal is shifted to lower g values when both centers are reduced. This effect can be observed in photosystem I (Rutherford & Heathcote, 1985; Golbeck, 1987) and green sulfur bacteria (Nitschke et al., 1990) and is attributed to an exchange interaction between both centers. The g_x troughs of center F_B and F_A in *H. chlorum* are not sufficiently different to render this effect on g_x observable.

Characterization of the Low-Temperature Charge Separation by Time-Resolved Optical Spectroscopy. To check the low yield of stable charge separation further, we examined the optical changes in the visible and near-infrared region under conditions which are comparable to those used for the EPR experiments.

Figure 3 shows changes induced at 800 nm by single-turnover flashes in a sample containing 5 mM ascorbate. The kinetic trace depicted in Figure 3a represents the transient measured after the first flash after cooling the sample in darkness to 10 K. An initial bleaching, the rise time of which was not resolved by our apparatus, decays essentially with an

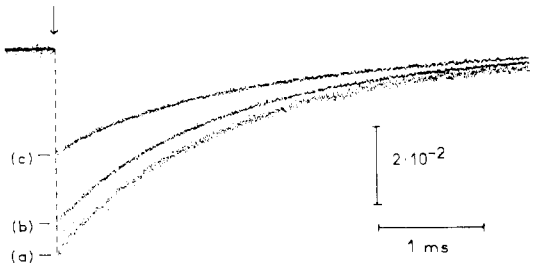


FIGURE 3: Absorption transients induced by laser flashes given at 10 K to a sample containing isolated membranes of *H. chlorum* (A_{780} = 0.7 at 45°) in the presence of 80% glycerol, 5 mM ascorbate, and 10 mM MOPS, pH 7.0. The sample was dark-adapted at room temperature before freezing in complete darkness. The transient induced by the first flash is shown in (a); (b) represents an average of 10 transients induced by the 301st to the 310th flash, and (c) shows the average of 10 transients after continuous illumination with white light at 10 K for 15 min.

average time constant of τ = 2 ms. The tenth flash gave almost identical kinetics, but with an amplitude which was decreased by 5% (not shown). After 300 flashes, the signal had fallen to 87% (Figure 3b), and after 15 min of continuous illumination with white light at 10 K, only 52% of the initial transient could be observed (Figure 3c). Prolonged illumination did not further decrease this amplitude. As in the case of the 4 K photoreduction of the FeS centers described above, this effect was reversed when the sample was thawed, dark-adapted, and refrozen.

A kinetic deconvolution of the measured transients showed that the kinetics induced by the first flash could *not* be fitted by only a monoexponential decay. Two different phases together with a constant negative offset of 3% were necessary for a satisfactory fit of the experimental data. The dominant phase with a τ of 2.5 ms makes up 80% of the total transient, whereas a smaller contribution (20%) is provided by a phase with τ = 850 μs.

The kinetic trace induced by a flash after the continuous illumination at low temperature (Figure 3c) only contains these two phases; a negative offset is not needed; i.e., the transient decayed fully to the level measured prior to the flash.

At wavelengths at and above 810 nm, an additional phase with a lifetime of 350 μs contributed considerably to the absorption change (see below).

The spectrum of the absorption change of the residual signal after continuous illumination at 10 K is depicted in Figure 4. Spectrum a in Figure 4 (continuous line) was obtained by subtraction of the absorbance change after 400 μs from that at very short times (20 μs after the flash). It is compared to spectrum b in Figure 4 (dotted line) measured after 2 ms. In spectrum a, the 850-μs component contributes 40% and the 2.5-ms phase contributes 60% to the total change at 800 nm, whereas in spectrum b the contribution from the 2.5-ms phase is higher than 90%; i.e., the dotted line represents an almost pure spectrum of the 2.5-ms phase. In Figure 4, both these spectra have been normalized to equal amplitudes at 790 nm to visualize differences between them. The most obvious difference occurs at 810 nm, the only wavelength where the 350-μs phase is the only observable phase.

Below 800 nm, both spectra resemble that published by Smit et al. (1989) for the difference spectrum P_{798}^{+}/P_{798} at 5 K. These authors determined a lifetime of about 2.3 ms and attributed the phase to a back-reaction. They furthermore attributed a 350-μs phase having a maximal bleaching at 812 nm to antenna triplet since this wavelength corresponds roughly to that of the antenna pigment BChl *g* 808 (Van Dorssen et al., 1985). A further 350-μs phase with a bleaching

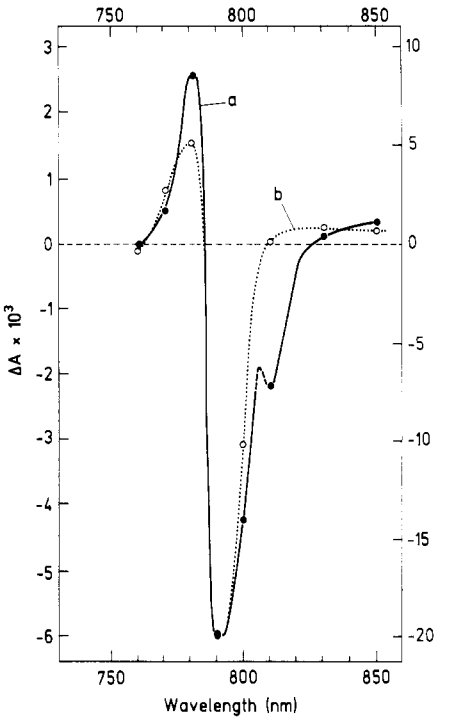


FIGURE 4: Normalized spectra of flash-induced absorption changes (ΔA) at 10 K after illumination with white light for 15 min at 10 K. Spectrum a (continuous line): The ΔA at 400 μs after the flash is subtracted from the ΔA at 20 μs after the flash. The transients at each wavelength are averages over 10 flashes. The region between 800 and 810 nm (dashed line) is interpolated according to the spectrum reported by Smit et al. (1989), which was determined at higher resolution in wavelength. Spectrum b (dotted line): ΔA at 2 ms after the flash. The left-hand scale applies to spectrum a, and the right-hand scale applies to spectrum b.

at 795 nm was initially attributed to reaction center triplet (Smit et al., 1989); however, it was later reinterpreted as triplet formed on an antenna pigment (Kleinherenbrink & Amesz, 1990). In our experiments, we were unable to see a component at 795 nm having a time constant of 350 μs. At present, we cannot see any obvious explanation for this discrepancy. The 850-μs phase had also been mentioned by Kleinherenbrink and Amesz (1989) and had initially been attributed to triplet formed within the reaction center by radical pair recombination. The 2.3-ms recombination phase seen by Smit et al. (1989) is undoubtedly equivalent to the 2.5-ms phase reported here. As noted earlier, there is a strong positive component at 780 nm (see Figure 4) which is attributed to an electrochromic shift associated with charge separation. An electrochromic shift is not expected to be associated with triplet decay, and in fact, the absorbance increase at 780 nm is completely absent in reduced samples (see Figure 7). Nevertheless, the positive change around 780 nm is even more pronounced in the spectrum representing predominantly short-lived components (Figure 4a). Since the 350-μs component contributes only very weakly (with a negative transient) at this wavelength, then clearly an electrochromic shift giving rise to a positive change at 780 nm must be a feature not only of the 2.5-ms but also of the 850-μs phase. This indicates that the 850-μs phase arises from charge recombination rather than reaction center triplet decay.

Another argument that the 850-μs phase reflects charge recombination rather than triplet decay comes from measurements at 670 nm. At this wavelength, electrochromic shifts of the primary acceptor, a BChl molecule, are observed (Nuijs et al., 1985a). Here again, the short-lived phases show a 1.5-fold absorption increase compared to the slow 2.5-ms phase

(not shown), instead of being smaller, which would be expected if the fast phases were all due to triplet.

Our data on the 350-μs phase with a maximal bleaching at 812 nm agree with the earlier attribution as an antenna triplet (Smit et al., 1989).

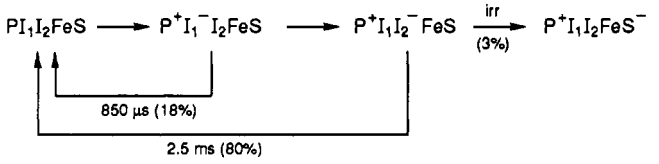
Since our spectral resolution does not allow for a determination of peak and trough wavelengths at the accuracy of 1 nm, we adopt the more accurate wavelengths determined by Smit et al. (1989) to denote the respective phases in our experiments.

We conclude that following a single-turnover flash at low temperature in the majority of centers (80%) the photooxidized donor recombines with an electron from a secondary acceptor with a time constant of 2.5 ms. Furthermore, it seems likely that the 850-μs phase represents a recombination reaction of P^+ with an electron acceptor which is possibly different or modified compared to that involved in the 2.5-ms phase.

The absorption changes measured at 430 nm (not shown) suggest that the electron acceptors involved in the 850-μs and the 2.5-ms recombination reactions could be different species. However, these absorption changes exhibit a poor signal to noise ratio and therefore this suggestion needs further assessment.

In agreement with the low yield of stable low-temperature photoreduction deduced from the EPR experiments, only a small fraction of centers (about 3%) are competent on a single flash in electron transfer to the terminal electron acceptor, which is the FeS center F_B (see above). This charge separation between the primary donor and the terminal acceptor is irreversible at helium temperatures. Due to this irreversibility, there is an accumulation of the stable charge separation after many flashes or continuous illumination. However, even after prolonged illumination, a fraction of centers (40–50%) remains, which is not capable of transferring electrons to the terminal FeS acceptors.

Thus, the processes induced by the first flash can be schematically depicted as follows:



In about 50% of the centers, the irreversible reduction of the FeS centers does not occur at all.

This value of about 50% of the centers which can perform stable charge separation at helium temperatures in the presence of ascorbate is higher than the about 20% photoreduced center F_B (equivalent to the $\simeq$10% of the total photoreducible FeS centers) seen in the EPR experiment under conditions comparable with respect to reduction state of the sample as well as to temperature and duration of illumination. However, these two experiments differ considerably in the optical properties of the sample. Clearly, in the optical cuvette with a 1-mm light path containing a rather dilute and glassy (due to the presence of glycerol) sample, light saturation will be reached much more rapidly than in an EPR tube containing a very concentrated sample without glycerol. In the latter experiment, an outer surface layer of the sample will be saturated rather easily whereas, due to the high absorption, it will take considerably longer to have enough photons penetrate to the core region of such a sample. This is in line with the observation in the EPR experiment that a level of approximately 10% reduced FeS centers was reached after several minutes, whereas prolonged illumination continuously but only very slowly increased the size of the signal.

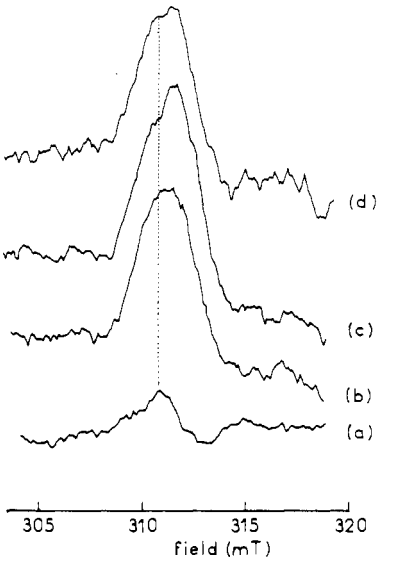


FIGURE 5: Light-induced EPR spectra of triplet states in isolated membranes from *H. chlorum* (only the low-field *z* peaks are shown). (a) In the presence of 40 mM ascorbate/10 mM MOPS, pH 6.8; (b) in the presence of 200 mM dithionite/80 mM glycine, pH 11.0; (c) under the same conditions as in (b), but after 10-min illumination at 200 K; (d) under the same conditions as (b), but in the additional presence of 40 μM methyl viologen and after reoxidation of the FeS centers by bubbling air through the sample. The spectra drawn are differences of spectra recorded under illumination and spectra taken in the dark. Each spectrum is an average over four scans. Instrument settings: temperature, 4 K; microwave power, 630 μW; frequency, 9.45 GHz; modulation amplitude, 1.6 mT.

Triplet States in H. chlorum As Observed by EPR. In whole cells of *H. chlorum* under reducing conditions, we recorded a triplet spectrum which closely resembles that published in reduced membrane fragments by Fischer (1990). The spectrum shows only *z* peaks; however, these peaks contain additional structure, i.e., a slight shoulder on the inner wings of the peaks (not shown). Fischer (1990) deconvoluted this peak into two triplets having different zero-field splitting parameters, $|D|$ (240×10^{-4} and 226×10^{-4} cm^{-1}). These values came from unpublished ADMR data (Lous & Hoff) cited in Fischer (1990).

The following results support the presence of variable stoichiometries of two overlapping triplet spectra with slightly different $|D|$ parameters.

From our spectrum, a composite value of 242×10^{-4} cm^{-1} for this parameter was calculated without attempting to attribute a separate $|D|$ to the minor component with narrower splitting (the $|D|$ values can be determined from our spectra to an accuracy of $\pm 2 \times 10^{-4}$ cm^{-1}).

Membrane samples reduced by 20 mM ascorbate, which are capable of low-temperature photoreduction of center F_B, also showed the spectrum of a triplet state (Figure 5a). Again, only the *z* peaks could be observed, but now the splitting parameter $|D|$ was 238×10^{-4} cm^{-1}. This decreased value is attributed to an increase in the contribution of the narrower of the two triplets. Photoaccumulation of reduced acceptors by illumination at 200 K in the presence of ascorbate (cf. Figure 1) does not alter the triplet spectrum in membrane samples.

Addition of 200 mM dithionite at pH 11.0, however, increased the signal by a factor of 3 (Figure 5b) and decreased the apparent $|D|$ to 229×10^{-4} cm^{-1}. Again, this is attributed to a further, very pronounced increase of the narrow triplet. Further illumination of this sample at 200 K resulted in a slight further increase (by a factor of 1.3; Figure 5c).

Reoxidation of the FeS centers by bubbling air through the sample reversed the effects of dithionite and illumination and resulted in a spectrum which was almost identical with the initial spectrum obtained in the presence of ascorbate. Renewed illumination at 200 K or addition of reductant resulted in the reduction of the FeS centers and restored the ability to photoinduce the narrow triplet to the extents previously observed.

When methyl viologen was added together with dithionite in the dark, the narrow triplet signal was obtained, but with an amplitude similar to that observed in Figure 5c, i.e., as after 200 K illumination in the presence of dithionite. However, the signal decreased by only 20% when the FeS centers were reoxidized by air (Figure 5d). It is of note that under these circumstances the degree of reoxidation was sufficient to completely oxidize centers F_A and F_B.

To further investigate the origins of the two apparently different triplet signals, we decided to look for the triplet in more purified material. To this end, two different reaction center preparations were used, one of which followed a procedure published by Trost et al. (1989). The other used a method developed for the isolation of the green sulfur bacterial reaction center (U. Feiler, unpublished results).

In all samples examined, the amount of the triplet with the greater splitting ($|D| = 242 \times 10^{-4}$ cm^{-1}), which was present already with ascorbate, seemed uncorrelated to the redox state of the reaction center components and sometimes even was present when P_{798} was oxidized (not shown). In contrast, in all types of samples, reduction by dithionite at high pH and/or methyl viologen plus dithionite resulted in the appearance of the narrow triplet ($|D| = 226 \times 10^{-4}$ cm^{-1}).

In the preparation following the *Chlorobium* protocol, x peaks could be observed yielding an $|E|$ value of $(60 \pm 10) \times 10^{-4}$ cm^{-1}. This value is much higher than that reported for BPheo g (Michalski et al., 1987) but comes close to the ADMR results for BChl g cited in Fischer (1990).

In oriented membranes, the z peaks ($|D| = 226 \times 10^{-4}$ cm^{-1}) were maximal when the magnetic field was parallel to the membrane (Figure 6a). However, the orientation dependence was rather peculiar in that the signal intensity did not decrease continuously when the sample was rotated from 0° to 90°. It seemed as if a signal with no detectable orientation was superimposed on the oriented signal. This might be explained by a high degree of disorder in these oriented membranes. However, this seems unlikely because the signal of the Rieske center was well-ordered in the same membrane samples (Liebl et al., 1990). Thus, we assume that a second triplet is present which is located on molecules with no well-defined orientation in the membrane. A direct comparison of spectra taken at 90° and at 0° indicated that the unoriented triplet had a larger splitting (not shown) but the low signal intensity of the unoriented signal made the comparison difficult. Summing up all orientations using appropriate weighing factors in order to produce a powderlike spectrum [see Nitschke et al. (1990)], however, yielded a triplet spectrum with a $|D|$ of 240×10^{-4} cm^{-1} and a shoulder on the inner wings (Figure 6b). By contrast, the triplet spectrum measured when the magnetic field is parallel to the membrane is narrower, having a splitting of $|D| = 230 \times 10^{-4}$ cm^{-1}.

Thus, the triplet spectrum we observed in oriented membranes consists of two different components, just as was found in the unoriented samples. The triplet having the wider splitting seems to be randomly oriented, whereas the z direction of the narrower triplet was maximal when the magnetic field was parallel to the membrane plane, suggesting that this triplet

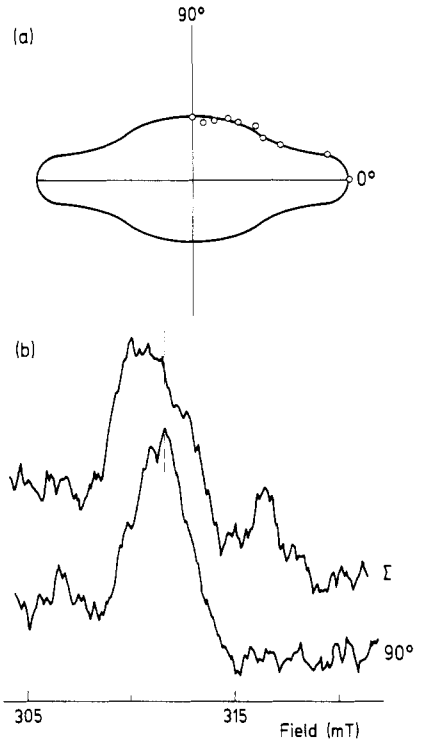


FIGURE 6: Orientation dependence of the light-induced triplet z peaks in oriented samples from *H. chlorum*. (a) Angles are measured between the direction of the magnetic field and the membrane plane. The data points are an average of the signal intensities of the high-field and the low-field z peaks. (b) The spectrum marked $\sum$ is a weighted sum of spectra taken at orientations between 0° and 90°, whereas that labeled 90° is the spectrum at this single orientation. For comparison, the two spectra are scaled to about equal signal amplitudes. All spectra at orientations between 0° and 90° are averages over four scans. EPR conditions are as in Figure 5.

is located on a BChl g molecule which is oriented with its ring plane perpendicular to the membrane plane.

Optical Changes Observed under Reducing Conditions. Flash-induced absorbance changes at 10 K were measured in the wavelength region between 760 and 850 nm under strongly reducing conditions in the presence of methyl viologen. At all wavelengths, the kinetics were essentially monophasic in the observed time range with a lifetime of 400 μs.

A spectrum of this optical change was obtained by plotting the initial amplitudes immediately after the flash (Figure 7). It shows a bleaching which is maximal at around 795 nm. The positive absorption change at 780 nm, which is attributed to a band shift induced by charge separation (see Figure 4), is completely absent. The wavelength of maximal bleaching is close to that of P_{798}^+ as seen under mildly reducing conditions and is clearly different from that attributed to the antenna triplet (812-nm bleaching, $\tau = 350$ μs; see Figure 4a) in an ascorbate-reduced sample.

The bleaching at 812 nm, attributed to the antenna triplet, could still be observed under these very reducing conditions. Due to the fact that the bleaching attributed to the reaction center triplet at around 800 nm was shifted to higher wavelengths compared to the formation of P_{798}^+ [with a bleaching maximum at 792 nm at low temperature; see Smit and Amesz (1989) and Figure 4], the 812-nm bleaching was less well resolved and showed up as a shoulder on the main bleaching.

By correlating the redox behavior of the EPR triplets with the optical changes, we identify the triplet bleaching at 812 nm in optical studies as being the wider (randomly oriented) triplet spectrum seen in EPR and attribute it to a triplet state formed in the antenna. On the same basis, the narrow, or-

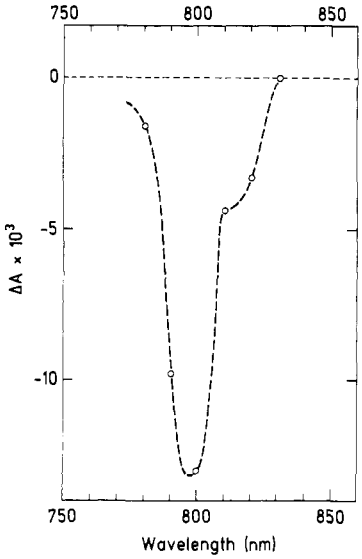


FIGURE 7: Spectrum of the initial flash-induced absorption changes measured at 10 K in isolated membranes of *H. chlorum*. The sample was prepared in the presence of 80% glycerol, 50 mM dithionite, 1 μM methyl viologen, and 200 mM glycine, pH 11.0, and illuminated for 7 min with white light when lowering the temperature from 250 K down to 180 K. The transients at each wavelength are averaged over 10 or 20 flashes.

ienten triplet and the 400-μs component having a maximal bleaching around 795 nm are attributed to a triplet state formed in the reaction center via radical pair recombination. Both triplets have rather similar decay times (350–400 μs). Kleinherenbrink and Amesz (1990) observed a 500-μs component which made up 10–30% of the absorption change at 794 nm under mildly reducing conditions and attributed it to triplet formed in the reaction center. We assume that this phase corresponds to the triplet formed by radical pair recombination which we described above.

Under strongly reducing conditions in the *absence* of methyl viologen, we found the triplet described above as the major contribution. However, a minor recombination reaction with a time constant of 850 μs (as also seen under mildly reducing conditions; see above) could still be observed. These observations are in agreement with the large but still less than 100% triplet signal seen in the EPR spectra under comparable conditions (Figure 5c vs Figure 5b). The 2.5-ms phase has greatly diminished relative to the 850-μs phase. This suggests that the two phases possibly arise from recombination reactions of P_{798}^+ with two electron acceptors having different redox potentials.

Discussion

Terminal Electron Acceptors. In *H. chlorum*, FeS centers act as electron acceptors during light-induced charge separation at liquid helium temperatures, just as is found in photosystem I and the reaction center from green sulfur bacteria. This charge separation is stable at liquid helium temperatures when the light is switched off. Two different FeS centers could be distinguished which, according to their *g* values, are thought to correspond to centers F_B and F_A. Center F_B seems to be less negative in redox potential than center F_A, contrary to what is found in photosystem I from most sources (Rutherford & Heathcote, 1985; Golbeck, 1987) but just as is the case in green sulfur bacteria (Nitschke et al., 1990).

Indications that these two centers interact magnetically argue in favor of a close proximity, i.e., possibly in favor of them both being located on a small protein subunit, as is the case in PS I (Golbeck, 1987) and probably also in green sulfur bacteria (Shirozawa & Sakurai, 1990; Nitschke et al., 1990).

The result that stable low-temperature charge separation occurs in Heliobacteria is in conflict with data presented by Smit et al. (1989), showing that the 2.3-ms kinetics at 790 nm essentially fully decayed to the level prior to the flash (some irreversible cytochrome oxidation was observed but no stable P_{798}^+). Our experiments demonstrate that there is, in fact, stable charge separation at helium temperatures, however, with only a very low yield per flash (a few percent of the centers are capable of performing reduction of F_A/F_B). Therefore, with many flashes or continuous illumination, a considerable fraction of the centers can be taken to the stable charge-separated state. Apparently, at 15 K, recombination between P_{798}^+ and the electron on a reduced acceptor preceding the FeS centers greatly outcompetes forward electron transfer to the terminal acceptors. From the given percentages and lifetimes for the back-reaction, a time constant of ≥80 ms can be estimated for the forward reaction to the FeS centers. Whether this slow forward reaction is an effect of the unphysiological temperature or whether it is due to damage of the reaction center cannot be decided at present. However, it should be noted that qualitatively the same phenomenon exists in photosystem I (Sêtif et al., 1984), the only difference being that the balance is much more in favor of forward electron transfer.

Highly reducing conditions largely prevent the low-temperature charge separation by chemically prereducing the FeS acceptors. Yet, a complete chemical reduction of center F_A was never possible (about 15% of center F_A cannot be reduced), which indicates that this center is somewhat more negative than centers F_A/F_B in photosystem I.

Thus, concerning the midpoint potentials of F_A and F_B, *H. chlorum* seems to take an intermediate position between green sulfur bacteria, where the FeS centers can hardly be chemically reduced, and photosystem I, where both FeS centers are completely reducible by dithionite at high pH.

An E_m of −410 mV for the reduction of a terminal acceptor has been published (Smit et al., 1987). However, in this redox titration, the redox mediators methyl viologen and benzyl viologen were present. In our hands, addition of these chemicals under reducing conditions abolishes long-lived P^+ formation (microseconds to milliseconds) and induces the narrow triplet spectrum. A comparable effect can also be observed in PS I, PS II, and green sulfur bacteria and has been ascribed to the double reduction of the quinone acceptor (Sêtif & Bottin, 1989; Van Mieghem et al., 1989; Nitschke et al., 1990). Thus, we assume that the value of −440 mV is erroneously high due to additional effects caused by some of the redox mediators.

According to our results, center F_A is considerably more negative than center F_B. From the observation that in the presence of excess dithionite at high pH only 85% of center F_A is reduced, we estimate an E_m of about −600 mV. Once center F_B is chemically prereduced, center F_A could be expected to function as an alternative electron acceptor, thereby maintaining long-lived P_{798}^+ formation at room temperature. This is in contradiction to the reported E_m of −510 mV for this effect (Prince et al., 1985). Thus, either F_A cannot take over this role once center F_B is reduced, or the argument of an artifactually high value due to the use of some mediators applies also to this value. Indeed, methyl viologen and benzyl viologen were also used in this study. Thus, a precise knowledge of the E_m values of the acceptors has to await redox titrations taking into account possible complications associated with A_1 double reduction.

It should be mentioned that according to our data only 20% of the total content of FeS centers in the membrane are terminal acceptors of the reaction center. The so-called light-induced FeS center reported by Brok et al. (1986), which was calculated as a difference of a sample frozen under illumination and one frozen in darkness, has g values different from those of centers F_A and F_B reported here; however, it does not necessarily represent one of the terminal acceptors. At room temperature, the rather negative E_m of the terminal acceptors could well result in nonspecific reduction of other FeS centers in the membrane. Such effects could explain the different g factors reported by Brok et al. (1986) compared to our data.

Fischer (1990) reported the spectrum of an iron–sulfur center which could only be observed *under* illumination. The spectral changes reversed when the light was switched off. As it is very difficult to maintain a stable sample temperature during illumination, we consider it likely that this signal might be due to heating artifacts. The shifted g value of the spectrum under illumination as compared to the dark spectrum could be due to differing relaxation properties of the various FeS centers present in the dark sample. In fact, the composite spectra arising from the many FeS centers which can be chemically reduced in our membrane samples are significantly different at 15 and 4 K, pointing toward such an effect.

As already suggested by Trost and Blankenship (1989), the absence of chemically reducible or photoinducible FeS centers in their isolated reaction center from *H. chlorum* probably results from the loss during purification of a protein subunit carrying the FeS centers F_A and F_B.

The similarities of the terminal FeS acceptors and their photochemistry at low temperature in *H. chlorum* to those observed in green sulfur bacteria (Nitschke et al., 1990) led us to search for a center analogous to center F_X, which is present in photosystem I and green sulfur bacteria. No signal attributable to center F_X could be detected. This might indicate that center F_X does not exist in *H. chlorum*. However, in view of the high similarity concerning the remaining primary and terminal acceptors (see below), this seems rather unlikely to us. The broad and weak signal of center F_X could easily escape detection, even if it was only slightly broader than observed in the other systems. Alternatively, it might be possible that the efficiency to reduce center F_X, even at the conditions of photoaccumulation at 200 K, is too low to trap detectable amounts of this center. According to recent results in PS I, center F_X may not be a physiological electron acceptor (Brettel, 1989), in which case its midpoint potential could be unrelated to those of the components of the electron acceptor chain and could therefore vary considerably between different organisms.

Triplet States. In line with previously published results (Fischer, 1990), we observe two distinct triplet spectra. Only z peaks show up in the spectra of relatively intact material (i.e., cells and isolated membranes), whereas after treatment with detergents the x troughs become visible as well. The y signals are obscured by the radical signal. This situation is reminiscent of what has been found in photosystem I (Rutherford, unpublished results), where the x and y peaks are less intense in more intact material.

Fischer (1990) attributed the triplet having the wider splitting ($|D| = 242 \times 10^{-4}$ cm^{-1}) to the reaction center triplet ^3P. We are led to challenge this hypothesis on the basis of four lines of evidence:

(a) The wider triplet is randomly oriented, whereas the narrow triplet is carried by a BChl molecule which is oriented perpendicular to the membrane plane. This orientation is expected for $^3P_{798}$ since the same orientation has been reported in purple bacteria (Hales & Gupta, 1979; Tiede & Dutton, 1981), green sulfur bacteria (Nitschke et al., 1990), and photosystem I (Rutherford & Sêtif, 1990).

(b) Only the narrow triplet is influenced by the presence or absence of reduced electron acceptors in the reaction center.

(c) The triplet minus singlet bleaching seen in the optical experiment under conditions where the narrow triplet can be detected by EPR is maximal at wavelengths close to those of the P_{798}^+/P_{798} bleaching. The bleaching maximum of the antenna triplet, by contrast, is at considerably longer wavelengths.

(d) In some samples, the wider triplet can be induced even when P_{798} is oxidized.

No in vitro data on the zero-field splitting parameters for BChl g have been reported. However, taking the $|D|$ and $|E|$ values determined for BPheo g (Michalski et al., 1987) and using the empirical rule that the zero-field splitting parameter $|D|$ for (bacterio)chlorophylls is higher by a factor of 1.15–1.2 than that of (bacterio)pheophytins, then a $|D|$ of (250–260) $\times 10^{-4}$ cm^{-1} for BChl g in vitro seems likely. This is reasonably close to the value of 242×10^{-4} cm^{-1} found for the wider triplet and is in line with our interpretation that this triplet is located on a BChl g monomer in the antenna. The slightly smaller $|D|$ value for the reaction center triplet would indicate that P_{798} is a BChl g dimer. Thus, *H. chlorum* would again be intermediate between green sulfur bacteria, where a considerable narrowing of the reaction center triplet with respect to the in vitro data is observed (Swarthoff et al., 1981b; Nitschke et al., 1990), and photosystem I, where the values for the reaction center triplet are almost indistinguishable from that of the isolated Chl a (Frank et al., 1979; Rutherford & Mullet, 1981).

Comparison of the Electron Acceptor Chain in H. chlorum to That in Photosystem I and Green Sulfur Bacteria. Two distinct FeS centers (F_A and F_B) acting as terminal acceptors are found in *H. chlorum*. F_B has a less negative E_m than F_A and is predominantly photoreduced at 4 K. F_A seems to be more reducing than the FeS centers in photosystem I, but is still largely reducible by dithionite at high pH. Thus, both F_A and F_B are considerably less reducing than their counterparts in green sulfur bacteria, where neither of them can be reduced chemically (Nitschke et al., 1990). According to their EPR spectra and low-temperature photochemistry, both centers are similar to their respective counterparts in PS I and green sulfur bacteria.

As has been shown by Nuijs et al. (1985a), the primary electron acceptor in *H. chlorum* is a pigment absorbing at 670 nm, most probably a BChl species. This transiently flash reduced acceptor is reoxidized by forward electron transfer to a subsequent acceptor with a time constant of 800 ps at room temperature (van Kan et al., 1989).

In green sulfur bacteria, a similar bleaching at 670 nm is observed which reverses with 600–700 ps (Nuijs et al., 1985b; Shuvalov et al., 1986a). In photosystem I, the primary electron acceptor is a Chl molecule called A_0, which is reoxidized by the quinone-type acceptor A_1 within 20 ps (Shuvalov et al., 1986b). In view of the high homology of the acceptor chains in green sulfur bacteria and photosystem I, we designated the 670-nm pigment in *Chlorobium* as A_0 (Nitschke et al., 1990), and we propose the same for *H. chlorum*.

Thus, the primary electron-transfer steps in *H. chlorum* and green sulfur bacteria seem to be similar.

Extending the analogy to PS I, we suggest that A_0 in Heliobacteria is the electron acceptor involved in the radical pair

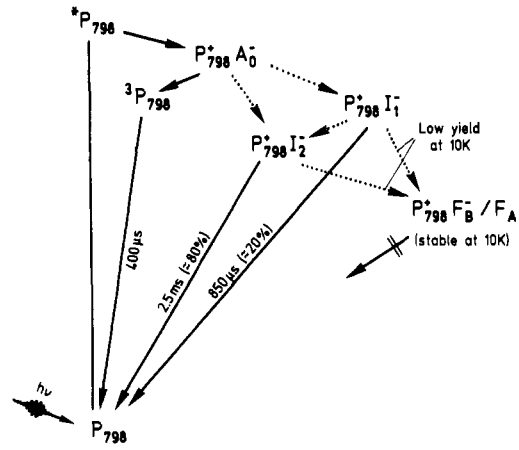


FIGURE 8: Schematic representation of the electron-transfer steps in the reaction center of *H. chlorum* as determined in this work. I_1 and I_2 represent two possibly different electron acceptors (as detailed in the text) with I_2 being less negative in redox potential than I_1. The dotted arrows indicate that an exact picture of the sequence of electron-transfer events cannot be deduced from our data. Thus, it is possible that electron transfer from A_0 proceeds in parallel toward I_1 and I_2 or in a consecutive manner via I_1 toward I_2. The same considerations apply to the forward transport from I_1/I_2 toward F_B/F_A.

recombination that generates the triplet.

In addition, the spectrum of the transiently reduced acceptor A_0 around 670 nm (Nuijs et al., 1985a) is clearly different from the absorption changes observed in this spectral region under the conditions where the millisecond recombination phases are seen (Smit et al., 1989). This shows that the species recombining with P_{798}^+ in the millisecond range (I_1 and I_2, see Figure 8) are different from A_0, providing evidence for the presence of at least one additional acceptor acting between A_0 and F_A/F_B. The differential influence of the redox potential on the extents of the two phases seen in this time range, 850 µs and 2.5 ms, suggests the presence of two different acceptors. By analogy with photosystem I, two likely candidates for these intermediate acceptors exist: the FeS center F_X and the quinone acceptor A_1.

Except for the analogy, there is no evidence for the existence of F_X in *H. chlorum*; however, our result that methyl viologen plus dithionite is capable of producing conditions where F_A and F_B are essentially oxidized but still a large triplet signal can be induced, is in favor of the existence of a quinone acceptor.[2] In PS I and green sulfur bacteria, it has been observed that the same treatment induces changes which can be explained by the double reduction of a quinone molecule acting as the electron acceptor A_1 (Sêtif & Bottin, 1989; Nitschke et al., 1990).

According to our results and to the interpretation of previously published data in light of these results, a model of the electron-transfer reactions in the photosynthetic reaction center of *H. chlorum* can be drawn (Figure 8). A model with more specific assignments of the acceptors I_1 and I_2 will have to await detailed spectrophotometric studies of these components.

[2] A quinone as an electron acceptor in *H. chlorum* has already been suggested on the basis of a high *g*-value radical signal seen in EPR when samples were frozen under illumination (Brok et al., 1986). A similar signal has previously been taken as evidence for the identification of the acceptor A_1 as a quinone in photosystem I (Gast et al., 1983; Mansfield & Evans, 1985) and green sulfur bacteria (Nitschke et al., 1987). However, the attribution of this so-called "A_1" EPR signal to the actual electron acceptor A_1 has since then become rather doubtful [Ziegler et al., 1987; Barry et al., 1988; see also Hauska (1988)]. The signal reported by Brok et al. (1986) in *H. chlorum* is seen under exactly the conditions where the formation of the probably artifactual "A_1" EPR signal would be expected. Thus, the evidence in favor of a quinone acceptor brought about by the high *g*-factor EPR signal is probably questionable.

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