Biochimica et Biophysica Acta, 973 (1989) 212–219
Elsevier

BBA 42919

Charge separation and trapping efficiency in membranes of *Heliobacterium chlorum* at low temperature

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(Received 5 May 1988)

Key words: Electron transfer; Energy transfer; Reaction center; Low temperature; Triplet formation; (*H. chlorum*)

Photooxidation of the primary electron donor, P-798, and the transfer of excitation energy to the reaction center in membranes of *Heliobacterium chlorum* were studied at low temperature. The difference spectrum of P-798 photooxidation at 5 K is dominated by a bleaching at 793 nm. P-798$^+$ was rereduced after a flash with a time constant of 2.3 ms, presumably by a back reaction with a reduced electron acceptor. Analysis of the kinetics at various wavelengths showed the contribution of two other components to the absorbance changes at 5 K. The first one could be attributed to the photooxidation, within 10 μs, of bound cytochrome *c*-553 in about 3% of the reaction centers; the second one to the generation of the triplet states of P-798 and bacteriochlorophyll (BChl) *g* with (average) lifetimes of 350 ± 50 μs. Measurements of the relative efficiency of energy transfer from the antenna to the reaction center indicated that this transfer is still quite efficient at 5 K and essentially equal for the three BChl *g* species, BChl *g* 778, BChl *g* 793 and BChl *g* 808. The function of the latter pigment thus may be to focus the excitation energy on pigments close to the reaction center, similar to what has been proposed for long-wave components in the antenna of purple bacteria. However, in *H. chlorum* this process would appear to imply 'uphill' transfer to P-798. Possible explanations to avoid this difficulty are discussed.

Introduction

The heliobacteria, *Heliobacterium chlorum* [1] and the more recently discovered *Heliobacillus mobilis* [2], are characterized by the presence of bacteriochlorophyll *g* [3]. The structure of BChl *g* resembles that of BChl *b* [3,4]. The Q_y absorption band of BChl *g* in organic solution is at 763 nm [3]; in vivo it absorbs around 790 nm. At least three spectrally distinct BChl *g* species can be distinguished in *H. chlorum*, which, according to their Q_y absorption maxima at 4 K, were named BChl *g* 778, BChl *g* 793 and BChl *g* 808 [5]. From the fluorescence emission and excitation spectra it was concluded [5] that efficient energy transfer occurs from the BChl *g* species absorbing at shorter wavelength to BChl *g* 808 at low temperature.

The primary electron donor of *H. chlorum*, P-798 [6–9], is probably a dimer of BChl *g* [7,10]. Upon excitation P-798 transfers in less than 35 ps an electron to a primary acceptor which absorbs near 670 nm and is probably a BChl *c*-like pigment [8]. Transfer to a secondary acceptor, the identity of which is unknown, occurs with a time constant of about 500 ps. P-798$^+$ is rereduced by cytochrome *c*-553, but this reaction is rather slow [7,9] and was observed to occur in only a fraction of the reaction centers [9].

The present communication extends these measurements to low temperatures. Light-induced difference spectra and kinetics at 5 K will be presented. It will be shown that efficient photooxidation of P-798 can be observed at 5 K, while excitation with flashes of different wavelengths indicated that energy transfer from BChl *g* 808 to the reaction center occurred with high efficiency, in spite of the fact that the absorption band of this pigment is situated at significantly longer wavelengths than the Q_y band of P-798. A small amount of cytochrome *c*-553 oxidation was also observed at low temperature, while a rapid kinetic component could be ascribed to the formation of reaction center and antena triplets.

Materials and Methods

Heliobacterium chlorum was grown anaerobically in medium 112 of the American Culture Collection [1],

Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; P-798, primary electron donor.

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containing 2.5 mM ascorbate. Membrane fragments were prepared as described earlier [9], and stored at −20 °C until use. Before the measurements the samples were diluted in a medium containing 66% v/v glycerol (to prevent crystallization), 10 mM Tris, 10 mM ascorbate and 2 mM dithiotreitol at pH = 8.0 in the presence of glucose, glucose oxidase and catalase to maintain anaerobiosis.

Flash-induced absorption changes were measured with a single-beam spectrophotometer. The measuring light, obtained from a 250 W tungsten-halogen or a 900 W Xe lamp, passed through a monochromator, the sample, suitable optical filters to diminish effects of stray actinic light, and a second monochromator. Actinic light was provided by a Xe flash tube (13 μs halfwidth) equipped with suitable absorption and interference filters or by a Q-switched, frequency-doubled Nd-YAG laser (15 ns halfwidth, 532 nm). The intensity of the exciting light was measured with a calibrated photodiode.

Results

Kinetics and spectrum of P-798 oxidation and rereduction

The kinetics of flash-induced absorbance changes at 5 K at various wavelengths are shown in Fig. 1. It can be seen that the kinetics at these wavelengths showed a very simple pattern and consisted of a change of absorbance which was too rapid to be resolved by the apparatus, followed by a mono-exponential decay with a time constant (1/e) of 2.3 ms. At most wavelengths the 2.3 ms decay component was the only or the dominant decay observed, but at some wavelengths other, faster components were observed which will be discussed below.

The amplitude of the 2.3 ms kinetic component, plotted as a function of wavelength, is shown in Fig. 2. The spectrum is dominated by a strong bleaching at 793 nm and a positive band at 782 nm. Additional, smaller changes are seen around 670 and 576 nm, whereas in the blue region the spectrum shows a number of positive bands above and negative bands below 430 nm. Similar features have been observed in the room-temperature difference spectrum of P-798 oxidation [6–9], indicating that the spectrum represents the difference spectrum (oxidized minus reduced) of P-798 together with a, presumably minor [9], contribution caused by an unidentified electron acceptor. The positive and negative bands at 670 and 666 nm, respectively, may be due to a change in the band shape of the primary electron acceptor which is thought to absorb in this region [8].

Upon closer inspection the low-temperature spectrum shows some interesting differences as compared to that measured at room temperature. First of all the minimum of the main bleaching shifts from 799 to 793 nm upon cooling. In view of what has been observed in other material, a blue shift of the absorption band of P-798 upon cooling is unlikely. The shape of the difference spectrum in the region 760–800 nm suggests that, like at room temperature [8], the spectrum is composed of a bleaching of the Q_y band of P-798, and a concomitant blue shift of neighboring BChl *g* molecules. In this context it is of interest to note that the difference spectrum ascribed to the formation of the triplet of P-798 [9] has a minimum at 793 nm when measured at room temperature. Since the triplet difference spectrum, unlike that of $P\text{-}798^+$, is not affected by electrochromic effects on neighboring pigment molecules, this would suggest that P-798 in fact absorbs maximally at a wavelength close to 793 nm at all temperatures and that the apparent red shift to 799 nm in the oxidized-minus-reduced difference spectrum at 300 K is caused by the blue shift of neighboring BChl *g* molecules. Due to the sharpening of the absorption bands upon cooling the effect of such a blue shift on the apparent position of the bleaching band would be diminished upon cooling.

A second difference as compared to the room-temperature spectrum concerns the Q_x region of BChl *g* (560–600 nm). At room temperature negative bands at 595 and 570 nm are observed [7,9], and from comparison with the triplet spectrum it was concluded that the spectrum might be composed of a bleaching of the Q_x band of P-798 at 575 nm and a band shift of neighbor-

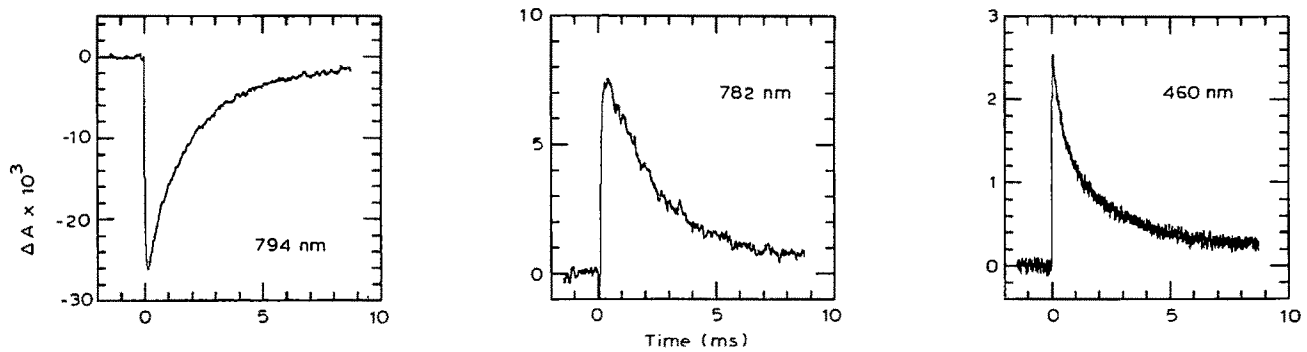

Fig. 1. Kinetics of absorbance changes induced at 5 K by a 15 ns, 532 nm saturating laser flash in membranes of *H. chlorum*. Repetition rate, 2.5 Hz. The absorbance of the sample was 0.5 at 788 nm at room temperature.

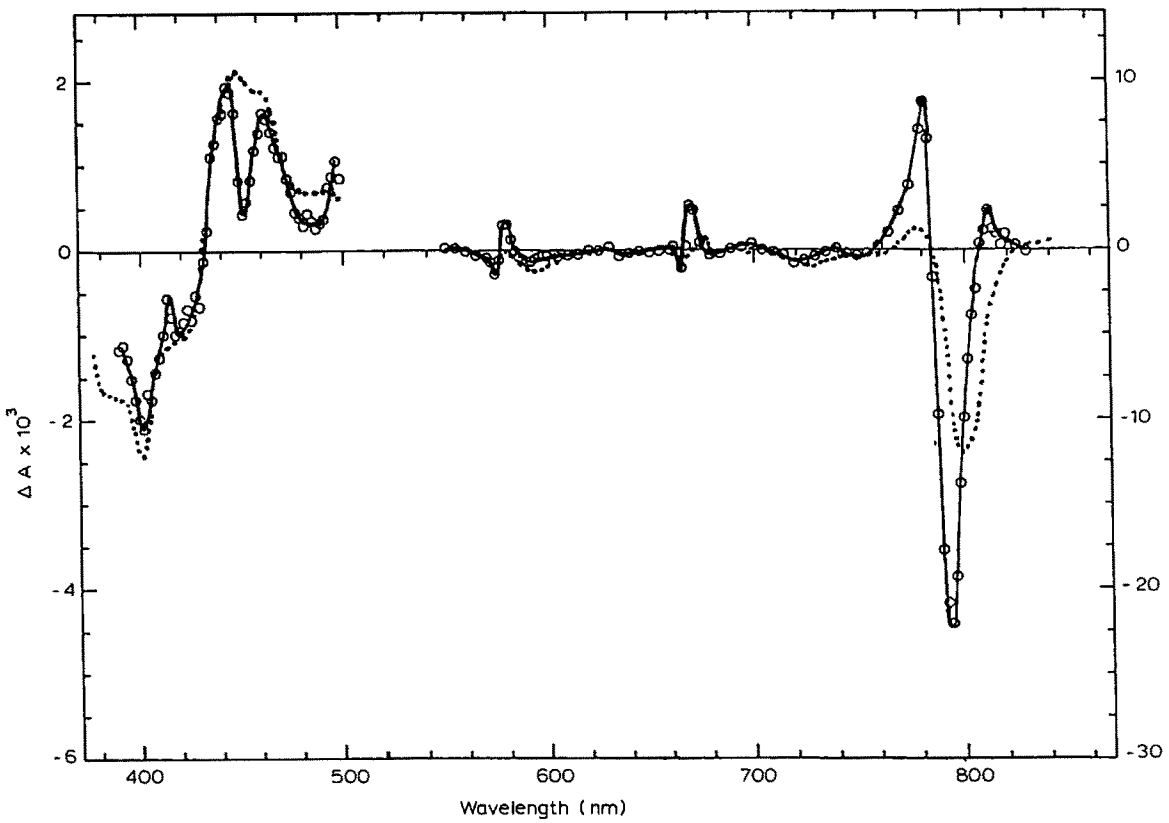

Fig. 2. Absorbance-difference spectrum of the 2.3 ms decay component. Conditions as for Fig. 1. The spectrum was obtained by plotting the amplitude of the absorbance changes at 2 ms after a flash, subtracting the 'irreversible' component (see text) and extrapolating the absorbance change to $t = 0$. The left-hand scale applies to the region below 500 nm, the right-hand scale to the region above 550 nm. The dotted line gives the difference spectrum induced by a flash given at room temperature (redrawn from Ref. 9).

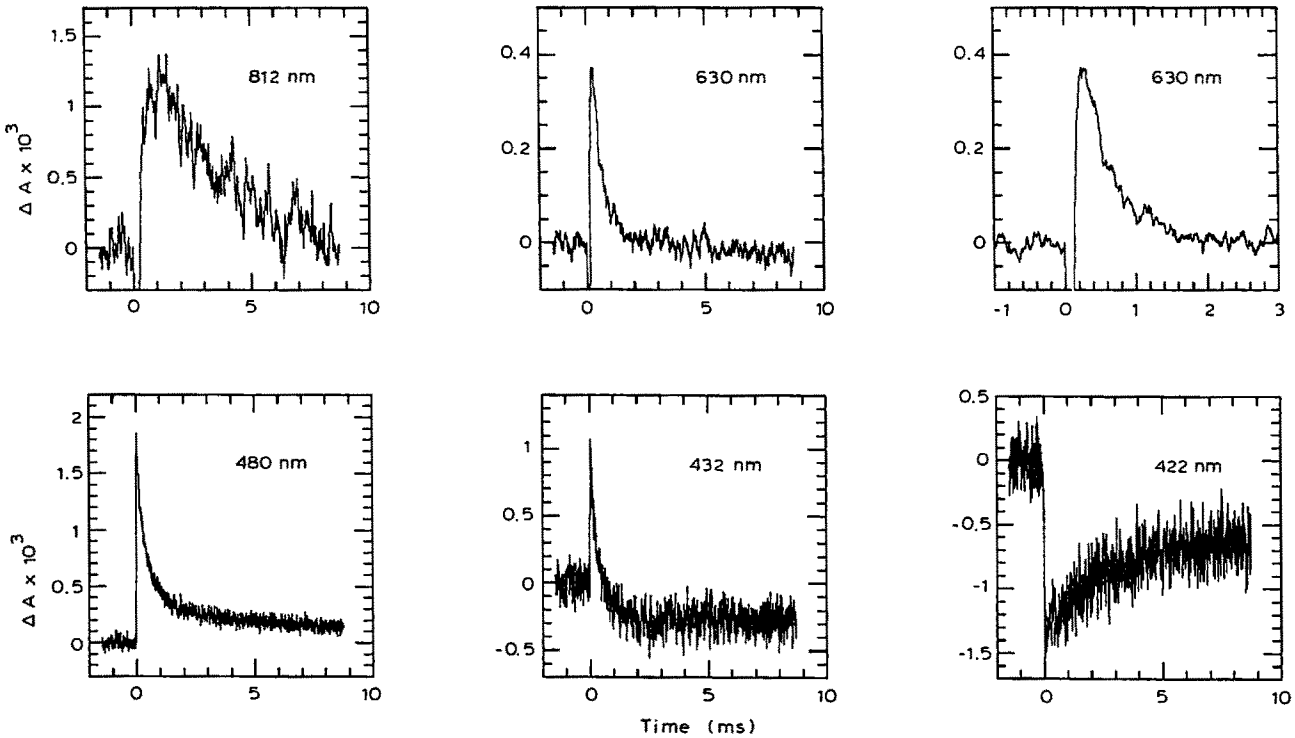

Fig. 3. Kinetics of the flash-induced absorbance changes at wavelengths selected to show the 'irreversible' and 350 μs decay components (see text). The negative spikes at 630 and 812 nm are artifacts due to the laser flash. Conditions as for Fig. 1.

ing BChl *g* molecules absorbing at longer wavelengths. A similar reasoning might apply to the low-temperature spectrum where the negative and positive bands at 576–581 nm suggest a change in the shape of a BChl *g* band, but in this case the net bleaching is very weak. It thus appears that at low temperature the total oscillator strength in the Q_x region decreases only little upon P-798 oxidation, in contrast to what is observed in reaction centers of purple bacteria [11]. Apart from the small negative band at 720 nm, which is also seen in the room temperature spectrum [9] and which may reflect bleaching of a vibrational band of P-798 and from the feature near 670 nm, discussed above, the spectrum of $P\text{-}798^+$ almost perfectly matches that of P-798 in the region 600–760 nm, and the same applies to the region above 815 nm. This indicates that both species show very little absorption in these spectral regions.

A third difference between the difference spectra at room temperature and at 5 K concerns the amplitude of the main bleaching, which was about 2 times higher that that at 300 K. This effect can be partially explained by the sharpened P-798 band at 5 K, but when the absorption difference spectra at 5 K and at room temperature were integrated from 760 to 820 nm, the absorbance changes at 5 K were still significantly larger than those at 300 K. This would suggest an increase in oscillator strength of the Q_y band of P-798 upon cooling, but the mechanism for such an effect is not clear, unless one assumes that the positive charge on $P\text{-}798^+$ is shared by more molecules at low than at room temperature (see also Ref. 10).

In the blue region the main difference is a better resolution of the positive bands at 440–445 and 460–465 nm. The presence of the first band indicates that, as at room temperature, photooxidation of P-798 was accompanied by the reduction of a secondary electron acceptor, which was called X_2 [9]. At room temperature X_2^- shows a back reaction with $P\text{-}798^+$ with time constants of approx. 6 and 30 ms [9]. Our data indicate that this back reaction is considerably faster at liquid helium temperature. Similar increases in the rate of back reaction upon cooling have been observed in reaction centers and chromatophores of purple bacteria [12,13]. Approx. 6% of $P\text{-}798^+$ was rereduced at much longer time scale. The difference spectrum of this 'irreversible' component was very similar to that of Fig. 2, except in the blue region (see below).

Cytochrome oxidation and triplet formation

As mentioned above, at some wavelengths components other than the 2.3 ms decay were clearly observed in the kinetics of the absorbance changes. Examples are given in Fig. 3. Analysis of these kinetics at selected wavelengths showed that they could be deconvoluted in three components: (1) the 2.3 ms decay component due to the back reaction of $P\text{-}798^+$; (2) a component which decayed with a time constant of 350 ± 50 μs; and (3) a component which did not reverse at the time-scale of our experiments. All three components were formed within the effective time resolution of the apparatus, which varied between 10 μs in the blue and 60 μs in the red and near-infrared (due to the laser artifact which occurred in this region).

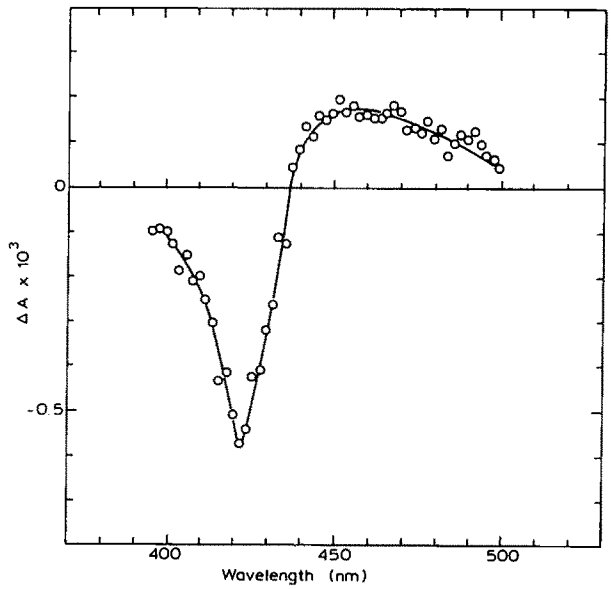

Fig. 4. Absorbance-difference spectrum of cytochrome *c*-553 oxidation, obtained by plotting the 'irreversible' component at 8.7 ms after a flash after correction for the contribution due to $P\text{-}798^+$.

As mentioned already, the irreversible component was largely due to P-798. When an appropriate correction was made by subtracting the normalized difference spectrum of Fig. 2, the resulting difference spectrum (Fig. 4), showed a minimum at 422 nm. At room temperature the difference spectrum of cytochrome *c*-553 photooxidation shows a minimum at 418–420 nm [8,9]. This indicates that in addition to P-798 a small amount of cytochrome *c*-553 was oxidized at low temperature, which was rereduced by a slow back reaction between flashes. As judged from the amplitude of the negative band at 422 nm, the amount of cytochrome *c* oxidized in a flash was quite small, and corresponded to roughly one molecule per 30–40 reaction centers. The amount of cytochrome oxidized was independent of the dark time between flashes in the range of 0.4–10 s.

The 350 μs component could be most easily seen at wavelengths where absorbance changes due to P-798 oxidation were very small, e.g., 630 or 432 nm (see Fig. 3). Fig. 5 shows the corresponding absorption difference spectrum. The spectrum was obtained by plotting the absorbance difference measured at a short time after the flash after correction for the contribution caused by the photooxidation of P-798 and cytochrome *c*-553. In the visible region, the spectrum shows a positive band at 430 nm and negative bands at 400, 576 and 665 nm. The latter two are superimposed on a broad positive

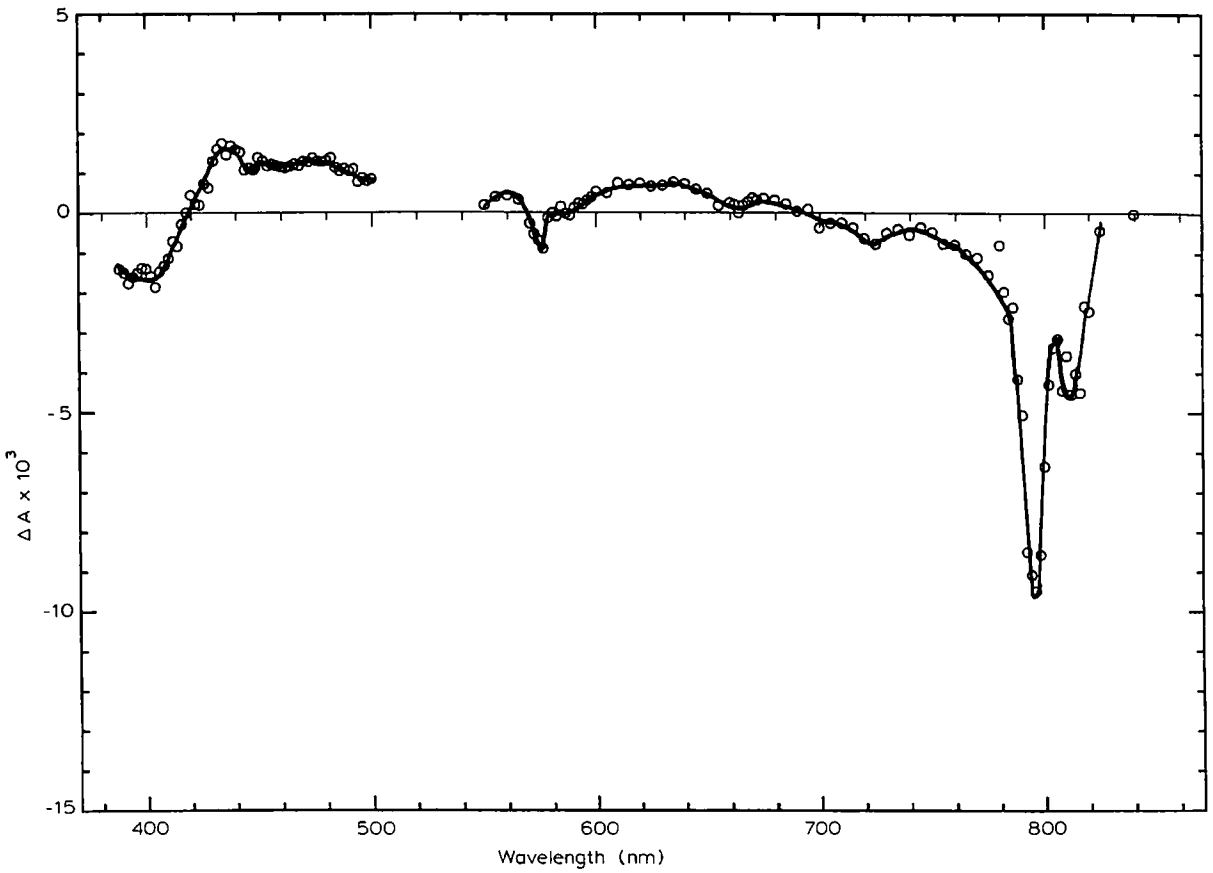

Fig. 5. Absorbance-difference spectrum was of the 350 μs component. The spectrum obtained by plotting the absorbance change at 75 μs (for the region 390–500 nm) or 300 μs after a flash, and subtracting the 'irreversible' and 2.3 ms components. The resulting signals were extrapolated to $t = 0$.

signal. In the near-infrared region negative bands are seen at 725, 795 and 812 nm. It should be noted that there is some uncertainty in the amplitude of the 795 nm band, because in this region the difference spectrum is sensitive to errors in the correction for the relatively large signal due to P-798 oxidation. However, the absence of a positive signal at 780 nm indicates that a contribution by P-798$^+$, if any, to the spectrum of Fig. 5 must be rather small. The negative band at 812 nm is clearly not due to an artifact, as can also be seen from an inspection of the kinetics at this wavelength (Fig. 3), since P-798 oxidation causes only small absorbance changes above about 800 nm.

Except for the band at 812 nm, the difference spectrum is similar to that obtained under reducing conditions at room temperature [9]. This indicates that it can be mainly attributed to the formation of the triplet of P-798. In addition, some triplets appear to be generated on a BChl *g* species absorbing at longer wavelengths, perhaps the long-wave antenna component BChl *g* 808.

Energy transfer to the reaction center

The presence of a long-wave antenna pigment (BChl *g* 808) raises questions about the pathway of energy transfer to the reaction center, especially since the fluorescence emission spectrum [5] and the absorption difference spectrum in the picosecond region [14] indicate that excitations are rapidly transferred to BChl *g* 808 at low temperature. Two pathways of energy transfer to the reaction center may be envisaged. In the first one, energy is transferred directly from BChl *g* 793 to P-798. This implies that the rate of energy transfer to

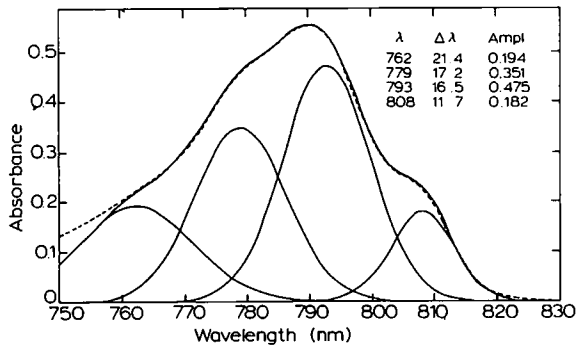

Fig. 6. Gaussian deconvolution of the 5 K absorption spectrum of *H. chlorum* membranes. The dashed line indicates the measured absorption spectrum. Band centers, half-widths and amplitudes of the four components are given in the figure.

TABLE I

Characteristics of the three filter combinations used and relative absorbances by the three main BChl g species

Absorption by the component at 762 nm was not taken into consideration. The absorbance by the sample (column 3) was calculated for each filter combination by convolution of the transmittance spectrum of the filter with the absorbance spectrum of the sample.

Filter transmitting wavelength (nm)	Halfwidth (nm)	Absorbance by sample (%)	Relative absorbances (%) by		
			BChl *g* 778	BChl *g* 793	BChl *g* 808
774	12	54.8	75.1	23.6	1.3
800	16	49.4	16.1	67.2	16.7
810	14	34.1	10.6	44.3	45.1

the reaction center should be quite rapid in order to compete effectively with energy transfer to BChl *g* 808 at low temperature. Another possibility, which is more in line with the proposed role of the long-wave absorbing antenna (bacterio)chlorophylls in purple bacteria and in Photosystem II of green plants [15–17], would be that energy transfer to the reaction center occurs via BChl *g* 808. However, at low temperature the efficiency of this transfer would be expected to be strongly reduced, since little thermal energy is available to overcome the energy difference between P-798 and BChl *g* 808 at 5 K. It was therefore of interest to measure the efficiencies of charge separation upon excitation of the different spectral forms of BChl *g* at low temperature.

To perform such a study adequately quantitative information about the relative contributions of the BChl *g* species to the 5 K absorption spectrum of membranes of *H. chlorum* was needed. Fig. 6 shows an analysis into Gaussian-shaped bands of this spectrum for the region 750–820 nm. At least four bands located at 762, 779, 793 and 808 nm are needed to obtain a good fit. The positions agree well with the maxima observed in the second derivative spectrum [5]. The rather broad band at 762 nm may contain contributions by partially overlapping vibrational bands of the three main BChl *g* transitions.

The extent of charge separation upon selective excitation in the three main BChl *g* bands was monitored by measuring the absorbance changes at 460 nm induced by xenon flashes filtered by suitable combinations of Balzers B-40 interference and absorption filters. The characteristics of the filter combinations used are given in Table I. It can be seen that for the first two filters, with maximum transmittances at 774 and 800 nm, the major fraction of the excitation energy was absorbed by BChl *g* 778 and BChl *g* 793, respectively. When the excitation was provided through the filter transmitting maximally at 810 nm, the energy was equally shared by BChl *g* 808 and BChl *g* 793, due to the higher amplitude and larger width of the absorption band of the latter pigment relative to that of BChl *g* 808. Each filter combination had a negligible transmittance beyond 980 nm.

The relative efficiencies of the three filter combinations in bringing about oxidation of P-798 are shown in Fig. 7. The intensity of the actinic light was varied by means of neutral density filters (Schott NG11). The extent of the signal at 460 nm, plotted as a function of

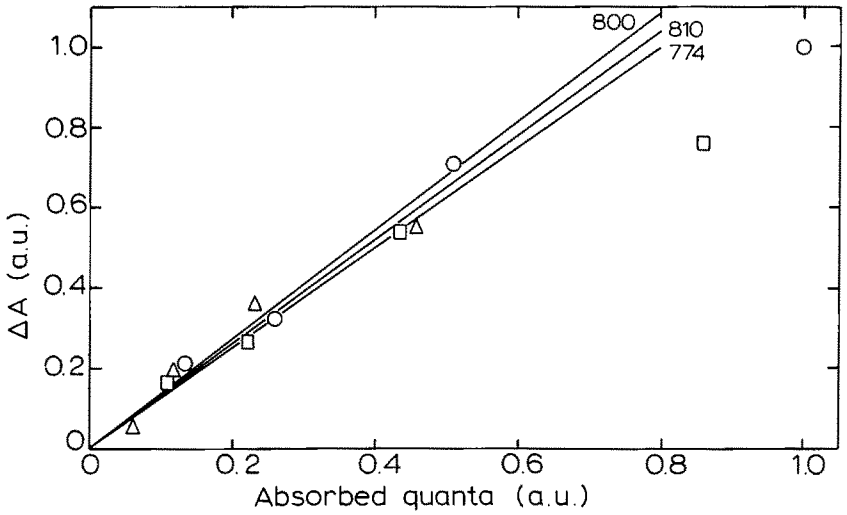

Fig. 7. Integrated absorbance changes at 460 nm as a function of the number of absorbed quanta for three different wavelengths of excitation (□, 774 nm; ○, 800 nm; and △, 810 nm). A.U., arbitrary units.

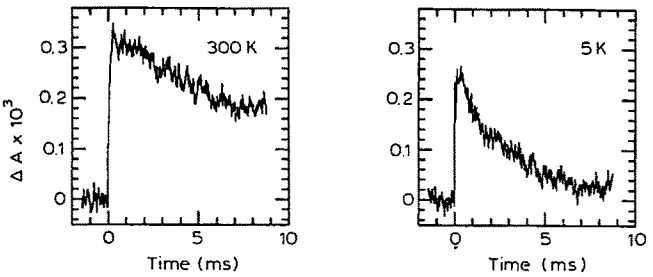

Fig. 8. Kinetics of the absorbance changes at 460 nm excited through the 810 nm filter in the presence of a NG 11 neutral grey filter and measured at 300 K and at 5 K.

the absorbed intensity, is plotted in arbitrary units, because the absorbance increases at this wavelength were integrated with time from 0.35 to 3.35 ms after the actinic flash to enhance the accuracy of the measurement.

From Fig. 7 it can be concluded that, except for high flash intensities where some saturation seemed to occur, the amplitudes of the absorbance increase at 460 nm were proportional to the excitation density. By means of a linear regression technique the relationship between the excitation density and the signal at 460 nm was calculated for the region where this proportionality seemed to hold. The slopes of the thus obtained lines were very similar: 1.25, 1.36 and 1.30 relative units for excitation at 774, 800 and 810 nm, respectively. Since light transmitted by the 810 nm filter is about equally absorbed by BChl *g* 793 and BChl *g* 808, we conclude that no significant loss of the photochemical efficiency occurs upon excitation in the latter pigment. This would imply that the efficiencies of transfer of excitation energy to the reaction center are approximately equal for BChl *g* 778, BChl *g* 793 and BChl *g* 808.

Fig. 8 compares the absorbance changes at 460 nm induced by a weak, non saturating flash at 810 nm at 300 K and 5 K. The result indicates that the efficiency of charge separation does not decrease strongly upon cooling to liquid helium temperature. Thus we conclude that energy transfer from the antenna to the reaction center at 5 K (and by inference also at room temperature) occurs via the long-wave pigment BChl *g* 808. Energy transfer from short-wave antenna BChls *g* to BChl *g* 808 and from BChl *g* 808 to the reaction centers occurs with high efficiency, even at 5 K.

Discussion

The results presented in this paper indicate that illumination of membranes of *H. chlorum* at liquid helium temperature serves to bring about a charge separation in the reaction center with considerable efficiency. In addition to photooxidation of P-798, which is responsible for most of the changes of absorption observed in the visible and near-infrared region, smaller changes are observed due to photooxidation of cytochrome *c*-553 and to the formation of triplet states of BChl *g*.

Our experiments indicate that a small fraction (roughly 3%) of the reaction centers contain a bound cytochrome *c*-553 which is oxidized by P-798$^+$ in less than 10 μs at 5 K and slowly rereduced by a back reaction in the dark. The amount of rapidly oxidized cytochrome is too small and its oxidation too fast to be resolved in the kinetics of P-798$^+$. The relatively low rate of cytochrome *c*-553 oxidation observed at room temperature [7,9] would then be due to the large dissociation constant of the reaction center-cytochrome *c* complex and may be diffusion-controlled as in, e.g. *Rhodospirillum rubrum* [18,19]. A small, rapid contribution by bound cytochrome *c*-553 would not be observed in the room-temperature kinetics.

The difference spectrum of Fig. 5 appears to be composed of the triplet-minus-singlet spectrum of P-798 and a contribution by a BChl *g* species absorbing at longer wavelengths. Both triplets appear to have roughly the same rate of decay, with a weighted average of 350 ± 50 μs, as shown by the kinetics at 630 and 432 nm (Fig. 3), where absorbance changes due to P-798$^+$ are absent. The long-wave BChl *g* component may conceivably be BChl *g* 808. At present we have no evidence whether this triplet originates from the reaction center or is generated in the antenna, e.g., by intersystem crossing.

There is evidence [15,16] that the long-wave absorbing BChl *a* component which is present in small amounts in the antenna of purple bacteria facilitates energy transfer from the antenna to the reaction center. Our measurements indicate that BChl *g* 808 serves the same purpose in *H. chlorum*, and that energy transfer from the antenna to the reaction center proceeds via BChl *g* 808. The efficiency of this process appears to be high, even at low temperature.

However, such a pathway may involve 'uphill' energy transfer to the reaction center. Although the exact position of its Q_y absorption maximum might be somewhat uncertain, it seems clear that the excited state of P-798 has a significantly higher energy than of BChl *g* 808. This raises a serious problem at low temperatures, because of the lack of overlap between the absorption band of the primary donor and the fluorescence emission band of BChl *g* 808. At 5 K, the latter band is located at 819 nm [5] and has an extremely small overlap with the proposed absorption band of P-798 with a maximum near 793 nm. According to the Förster model for energy transfer [20], this poor overlap can only be compensated for by a very short distance between BChl *g* 808 and P-798. To overcome this problem one could postulate the existence of a (weak) low energy band of the reaction center which, due to its red-shifted absorption maximum, would have a large Förster overlap with the emission of BChl *g* 808, but which is not

observed in the absorbance-difference spectrum of P-798. In this connection it is of interest to refer to the recent model of Fischer and Scherer [21] for the early charge separation in purple bacteria, which implies a charge-transfer transition involving the so-called accessory BChl and bacteriopheophytin. This step is followed by a rapid transfer of an electron from the primary electron donor P to the accessory BChl, yielding the state P^+BPh^-. One might speculate that an analogous mechanism applies to *H. chlorum*, but further evidence will be needed before such speculation would become profitable.

Acknowledgement

The investigation was supported by the Netherlands Foundation for Biophysics and for Chemical Research (SON) financed by the Netherlands Organization for Scientific Research (NWO).

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