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Electron transport and triplet formation in membrane fragments of the green sulfur bacterium *Prosthecochloris aestuarii*

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Abstract

Under conditions where normal electron transport is blocked, at high pH in the presence of dithionite, three triplets could be observed upon flash illumination of membrane fragments of the green sulfur bacterium *Prosthecochloris aestuarii*. The first triplet decayed in 7.5 μs and is assigned to carotenoid. The second triplet decays in 67 μs and is assigned to bacteriochlorophyll (BChl) *a* of the Fenna-Matthews-Olson (FMO) complex, since a triplet with the same spectrum and lifetime was also formed in the isolated FMO complex. The third triplet decayed in 165 μs and is assigned to BChl *a* of the reaction center core complex, based on its main bleaching at 837 nm. There is insufficient evidence to decide whether this triplet is located on the primary electron donor P840 or on a long-wavelength antenna BChl *a* of the core. A multiple-flash experiment indicated the presence of two photo-oxidizable hemes per reaction center (RC), both having a difference spectrum centered around 553 nm. The oxidation time was 25 μs for both cytochromes. However, a 75-μs delay was observed for the oxidation of the second heme, indicating that another process must take place before this reaction can occur. This result, together with the observed low efficiency of oxidation of the second heme, suggests the presence of a four-heme cytochrome (as observed in some other species of green sulfur bacteria), with only one heme in direct contact with the RC, rather than a model with two cytochromes symmetrically attached to the RC, as proposed by others. The observed delay can then be explained by a relatively slow heme-to-heme electron transfer. The cytochrome oxidation time of both hemes increased with viscosity, suggesting that some molecular motion is involved in the oxidation process.

Keywords: Cytochrome; Bacteriochlorophyll *a*; Triplet; Electron transfer; (Green sulfur bacterium); (*Prosthecochloris aestuarii*)

1. Introduction

In some respects, less is known about the reaction center processes in green sulfur bacteria than of related, much more recently discovered, heliobacteria. Although much progress has been made describing both the donor and the acceptor chain of electron transport [1], a generally accepted electron transfer model has not yet been established. In this publication an effort is made to elucidate some properties of the donor side of the electron transfer chain.

Already in 1971 [2] it was proposed that a cytochrome molecule functions as a first electron donor to P840, the BChl *a* dimer forming the primary donor of the reaction center in green sulfur bacteria. However, there is still no agreement as to the exact

Abbreviations: BChl, bacteriochlorophyll; Cyt, cytochrome; FMO complex, Fenna-Matthews-Olson complex; FWHM, full width at half maximum; P840, primary electron donor; RC, reaction center.

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nature and function of this cytochrome. Some reports suggest a four-heme cytochrome model [1,3–6], as in certain species of purple bacteria [6], whereas others indicate the presence of a mono-heme cytochrome *c*-551 of a new type [7–11]. Also the reported oxidation time of the cytochromes by $P840^+$ in the literature varies substantially [9,12–15]. A major problem analyzing cytochrome oxidation is that the time-scale on which this process occurs coincides with that of triplet decay. Therefore, in order to study cytochrome oxidation, one should be able to identify the signals due to the triplets present in green sulfur bacteria. Although several reports dealt with triplet formation, a complete characterisation was still absent.

This paper describes a study of flash-induced absorbance changes in isolated membranes of *Prosthecochloris aestuarii*. These membranes contain the reaction center core complex. Attached to the membrane is the so-called FMO complex [16], but the membranes are largely devoid of the chlorosomes, the main antenna components of green sulfur bacteria. In the Q_Y region, the absorption spectrum between 790 and 830 nm is dominated by the BChl *a* of the FMO complex, whereas transitions at longer wavelengths are due to the reaction center core complex [17]. We first performed a spectral and kinetic characterisation of the three triplets observed in isolated membranes of the green sulfur bacterium *Prosthecochloris aestuarii*. Then, knowing the contributions of triplet state formation to the signals due to electron transfer, the cytochrome oxidation process was studied, resulting in a model for the donor side of the electron transport chain.

The results were reported at the XIIth Intern. Congress of Biophysics, Amsterdam, 1996 (Abstract Nr. P-E1-04).

2. Materials and methods

Prosthecochloris aestuarii was grown in a mixed culture as described by Holt et al. [18]. Membrane fragments were prepared essentially as described by Swarthoff and Amesz [19], and stored in the presence of 10 mM ascorbate and 50 mM Tris at pH 8.3 at 77 K until used. The isolated FMO complex was prepared according to Ref. [20].

Flash-induced absorption difference kinetics and spectra were measured with a single-beam spectrophotometer, similar to the one described in Refs. [21] and [22]. The samples were contained in a 1-cm cuvette at an absorbance of about 0.75 at 810 nm. Excitation flashes were provided by a Q-switched frequency doubled Nd-YAG laser (15 ns FWHM, 532 nm, 90 mJ per pulse), or a Nd-YAG laser pumped, tunable optical parametric oscillator (OPO) used at 810 and 845 nm (10 ns FWHM, 5–10 mJ per pulse), both having a maximal repetition rate of 10 Hz. Double pulse experiments were performed utilising a second frequency doubled Nd-YAG laser (10 ns FWHM, 532 nm, 90 mJ per pulse), producing a second flash at an adjustable time after the first one. Measuring light was provided by a 250 W tungsten-iodine lamp, passing a monochromator, the sample (perpendicular to the excitation beam) and a second monochromator. Suitable optical filters were used to prevent detection of higher order transmitted light and to reduce flash and fluorescence artifacts. The transmitted light was detected by a photomultiplier tube (EMI 9658 R or Hamamatsu R5600U-01). Signals were amplified using a 100-MHz or a 1-MHz bandwidth differential amplifier (with an adjustable low-pass filter) and recorded on a LeCroy 9310 300 MHz oscilloscope. All results were obtained by averaging 10–1000 flashes.

3. Results

3.1. Triplet formation

Various authors have observed the formation of BChl *a* and carotenoid triplets in FMO and core complexes of green sulfur bacteria [12,13,23–25], but so far no systematic study of their room temperature spectra and lifetimes has been reported. For this reason, and also in order to obtain information needed to distinguish their contribution from those caused by electron transfer to be described below, we studied the light-induced absorption difference spectra and kinetics of membranes of *P. aestuarii* under conditions where no interference by signals due to electron transfer in the μs time region were to be expected. These experiments were done at high pH in the presence of dithionite. Under these conditions, most of the secondary electron acceptors are reduced or

photo-accumulated in the reduced form, resulting in a rapid reversal of the charge separation [1].

Fig. 1 shows the kinetics of the absorbance changes observed upon excitation by a flash at 532 nm. At most wavelengths multi-exponential decays were observed, but at the wavelengths shown in the figure the decays could be fitted with or were dominated by a single exponential. Corresponding time constants were 7.5 μs (Fig. 1A), 67 μs (Fig. 1B) and 165 μs (Fig. 1C), respectively. At other wavelengths the decay could be fitted by a combination of two or three of these exponentials, and the difference spectra of the three components thus obtained are shown in Figs. 2 and 3.

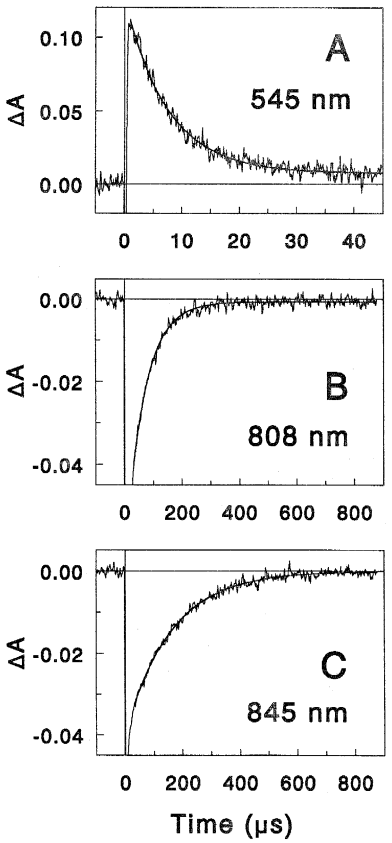

Fig. 1. Kinetics of absorbance changes at 545 (A), 808 (B) and 845 nm (C) upon excitation of isolated membranes of *P. aestuarii* with a series of 15-ns 532-nm flashes, given at 10 Hz. The smooth lines represent mono-exponential fits of the decays with time constants of 7.5, 67 and 165 μs, respectively. The measurements were done at pH 11.0 in the presence of 150 mM Caps buffer and 10 mM dithionite. The signals are attributed to triplets of carotenoid (A), BChl *a* in the FMO complex (B) and BChl *a* in the reaction center core complex (C), respectively.

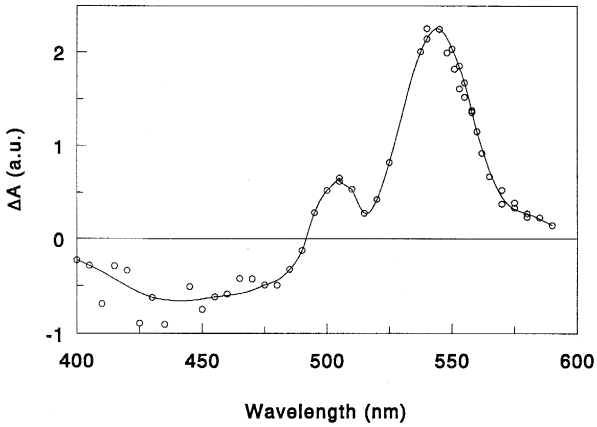

Fig. 2. Difference spectrum of the 7.5 μs decay component, ascribed to a carotenoid triplet. Conditions as for Fig. 1 (a.u., arbitrary units).

The difference spectrum of the 7.5 μs component (Fig. 2) showed absorption increases at 505 and 545 nm, and a broad bleaching at 440 nm, thus featuring the characteristics of a carotenoid triplet [12,26]. This component was absent at wavelengths above 600 nm. Apart from the newly resolved band at 505 nm, the spectrum is similar to that of an 8–10 μs component observed by Smit and Amesz [12]. The carotenoid triplet signal was obtained upon excitation at 532 nm as well as at 810 and 845 nm, respectively, indicating that it is, at least partially, generated by energy transfer from BChl *a* in the core complex, as proposed by Kingma et al. [26]. A similar signal, showing a comparable difference spectrum and triplet decay time, but with lower amplitude, was also observed upon 532 nm excitation of isolated chlorosomes (not shown).

The difference spectrum of the 67 μs component is shown in Fig. 3A,B. The spectrum is obviously due to BChl *a*. A signal with the same difference spectrum and a single lifetime of 67 μs at all wavelengths was obtained upon flash excitation of the isolated FMO complex (Fig. 3C,D). Thus we conclude that the 67 μs triplet in isolated membranes must be likewise due to a triplet located on the BChl *a* of the FMO complex. The spectra differ considerably from that obtained with isolated FMO at 4 K [25]. The bandshift near 813 nm is missing at room temperature, and in addition to a bleaching of the low energy band (823 nm) there is also a broad bleaching centered at 808 nm, close to the absorption maximum of

the complex. A bleaching in the Q_X region is located at 605 nm, whereas there are broad absorption increases in the regions 400–580 and 620–720 nm.

Fig. 3E,F shows the difference spectrum of the 165 μs component. This spectrum is clearly different from that of Fig. 3A,B and shows negative bands at 410 and 837 nm, together with broad absorption increases around 500 and 700 nm. The position of the bleaching band in the Q_y region shows that it must be located on the reaction center core complex. We therefore ascribe it to P840 or antenna BChl *a* of the reaction center core complex. A similar bleaching in the Q_Y region was observed in an FMO-reaction center core complex obtained by solubilization of membranes of *P. aestuarii* [23]. BChl *a* triplet signals earlier observed in membranes of *P. aestuarii* were probably due to a combination of the FMO and core triplets as indicated by their difference spectra and lifetimes [12,13].

3.2. Electron transfer

Electron transfer was studied in the presence of ascorbate, at pH 8.3. Fig. 4A shows the kinetics of flash-induced absorbance changes at 830 nm. The reversible part of the signal could be fitted with decay components of 25 μs and 165 μs, with relative amplitudes of 1:0.3. In addition there was an irreversible component accounting for about 10% of the total absorbance change.

Fig. 5 shows the absorption difference spectra of the two decay components. The spectrum of the 25 μs component is characterized by bleachings at 830 and 842 nm and a smaller one near 790 nm. This spectrum closely resembles the difference spectra obtained by Swarthoff and Amesz [19] upon continuous illumination of membranes of *P. aestuarii* (Fig. 5, solid line) and may be ascribed to $P840^+$. The spectrum of the 165 μs component resembles that of

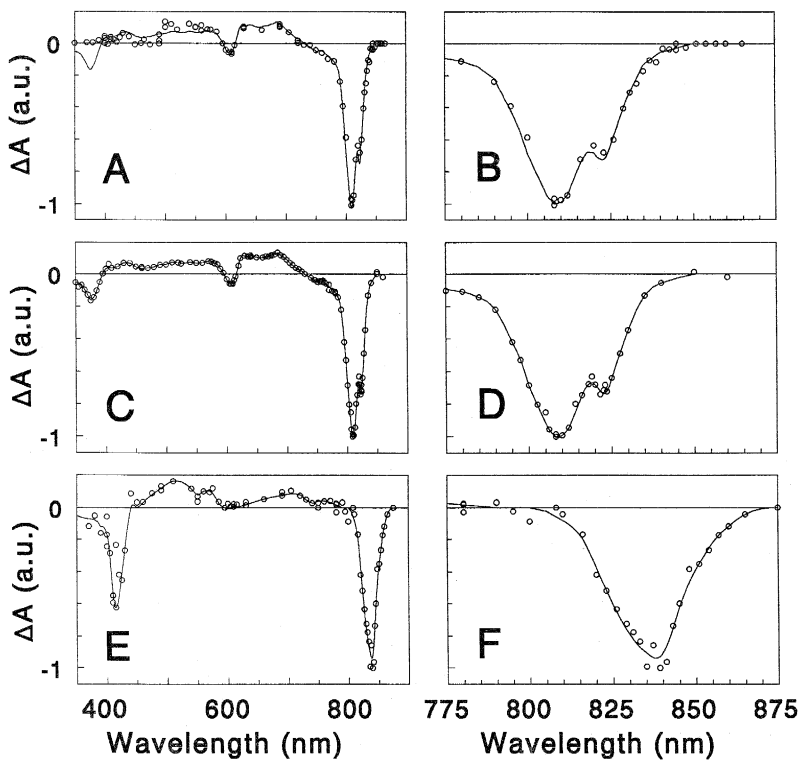

Fig. 3. Difference spectra of the 67 μs decay component, measured in isolated membranes (A and B) and in the isolated FMO complex (C and D). For comparison the FMO spectrum is also plotted in A and B (solid line). E and F: difference spectra of the 165 μs decay component, ascribed to reaction center core BChl *a*. Conditions as for Fig. 1.

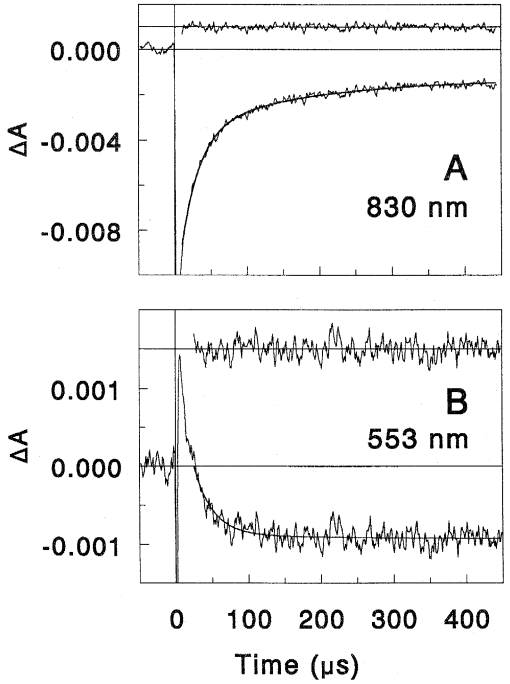

Fig. 4. Kinetics of absorbance changes at 830 (A) and at 553 nm (B) upon excitation with a 532-nm flash. The measurements were done at pH 8.3 in the presence of 50 mM Tris and 100 mM ascorbate. Dark time between the flashes: 10 s. The decays were fitted (smooth lines) with exponential time constants of 25 and 165 μs, with relative amplitudes of 1:0.3 (A) and 1:0.05 (B), respectively, in addition to an irreversible component. The residuals of the fits are given at the top of the figures.

the reaction center core triplet (Fig. 5, broken line). The spectrum of the irreversible component (not shown) was similar to that of the 25 μs component, indicating that this was due to a fraction of $P840^+$ that did not react with an electron donor.

Kinetics of cyt *c*-553 oxidation were measured at 553 nm (Fig. 4B). After an initial positive absorbance change, which we ascribe to formation of carotenoid and BChl *a* triplets (see Section 2) and of $P840^+$, the absorbance change reversed to a negative value. No accurate fit of the decay of the carotenoid triplet was possible, but the decay remaining after 25 μs (i.e., when the decay of the carotenoid triplet is completed) could be fitted with a decay component of 25 ± 5 μs, together with a minor one of 165 μs. When extrapolated to $t = 0$, the relative amplitudes of these components were 1:0.05, respectively. The latter component may be ascribed to the decay of the reaction center core triplet, which has a positive absorbance

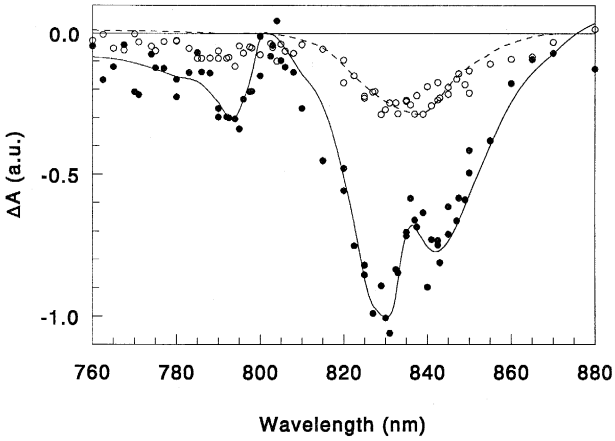

Fig. 5. Absorbance difference spectra of the 25 and 165 μs components in the Q_Y region. Conditions as for Fig. 4. The solid line is the difference spectrum for P840 oxidation obtained upon continuous illumination [19]; the broken line is that of reaction center core BChl *a* triplet, taken from Fig. 3F.

change at 553 nm (Fig. 3). The 25 μs component can be ascribed to oxidation of cyt *c*-553 with a small contribution due to re-reduction of $P840^+$. This time constant is smaller than earlier reported for the same material [13], which is perhaps due to an improved time resolution. The time constant is in good agreement with that of $P840^+$ re-reduction as measured in the Q_Y region (see above), confirming that cytochrome *c*-553 acts as first electron donor to $P840^+$

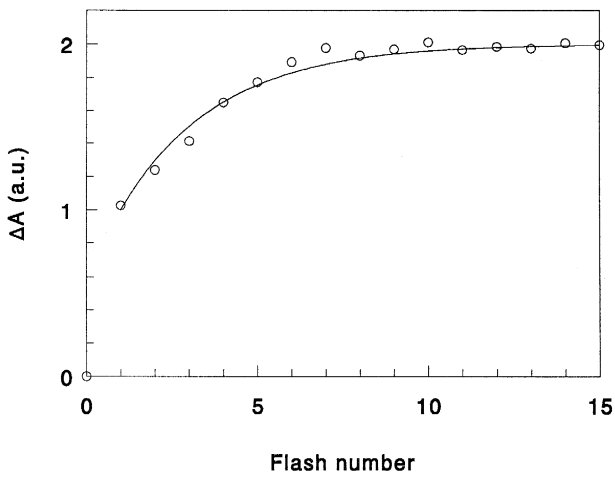

Fig. 6. The extent of cytochrome oxidation, measured at 553 nm, upon a series of 15 saturating 532-nm flashes, separated by 100 ms, with 30 s dark time between the series. Conditions as for Fig. 4, except ascorbate concentration 2.5 mM.

[2]. The relative amplitudes of the 25 μs decay component at 553 and 830 nm were 1:3.4, in reasonable agreement with the ratio of the estimated differential extinction coefficients for cyt *c* ($\epsilon_{551\text{-}540\ nm} = 20$ mM^{-1} cm^{-1} [27]) and P840 ($\epsilon_{830\ nm} = 90$ mM^{-1} cm^{-1} [28]) of 1:4.5. The ratio of the amplitudes of the core triplet at 553 and 830 nm (1:20) corresponds with the relative amplitude ratio in the spectrum of Fig. 3E.

Thus, the above experiments indicate that one heme is photo-oxidized per 'active' reaction center upon a single saturating flash. So far, the experiments were done with a dark time of 10 s between flashes, sufficient to obtain complete re-reduction of the cytochrome. Experiments were also done with multiple flashes spaced at 100 ms. Fig. 6 gives the result of such an experiment, indicating the presence of two photo-oxidizable cytochromes per active reaction center, since the saturated absorption difference is twice that after the first flash. The oxidation of the second cytochrome was much less efficient, and 6–7 flashes were needed to obtain complete oxidation. Comparable results, also indicating the presence of two cytochromes per RC, were obtained for various preparations of other green sulfur bacteria [14,29,30]. Using continuous illumination, Swarthoff et al. [13] found three cytochromes per reaction center in membranes of *P. aestuarii*, of which two were slowly oxidizable.

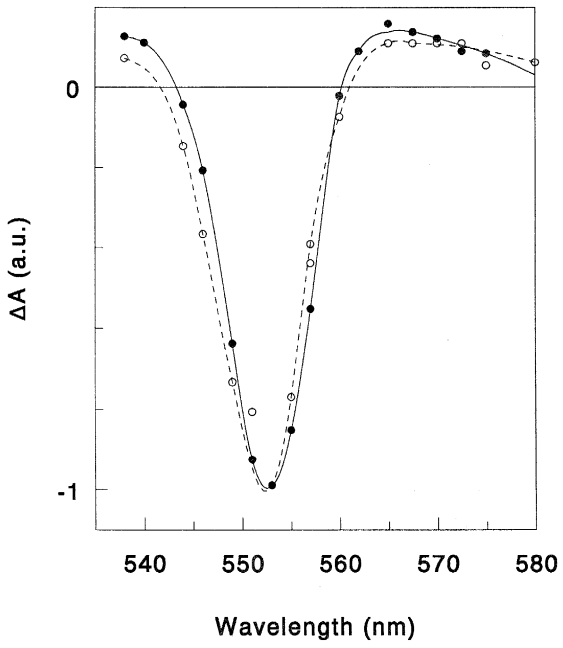

Fig. 7. Normalized absorbance difference spectra of the oxidation of the first (open circles) and the second heme, measured after the first flash and after a subsequent series of 8 flashes.

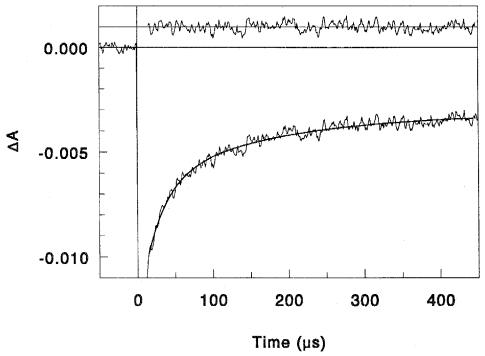

Fig. 8. Kinetics at 830 nm after the second of two flashes, spaced at 100 ms. The smooth line gives a fit with time constants of 25 and 165 μs, with relative amplitudes 1:1, together with an irreversible component.

Fig. 7 shows the normalized difference spectra of the absorbance changes of the first and the second cytochrome, measured as the absorbance difference induced by the first and by the following 8 flashes of a series of saturating flashes, respectively. Both spectra show a negative band at 553 nm. Kinetics at 830 nm measured after a set of two flashes are shown in Fig. 8. The decay could again be fitted with time

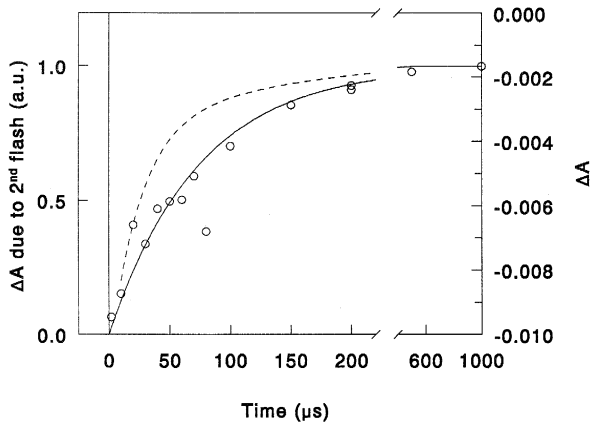

Fig. 9. The absorption decrease at 553 nm due to cytochrome oxidation induced by a second saturating flash, as a function of the time between the first and the second flashes (circles) and the corresponding fit (solid line). Broken line (right hand scale): re-reduction of $P840^+$ by the first heme (from Fig. 4A).

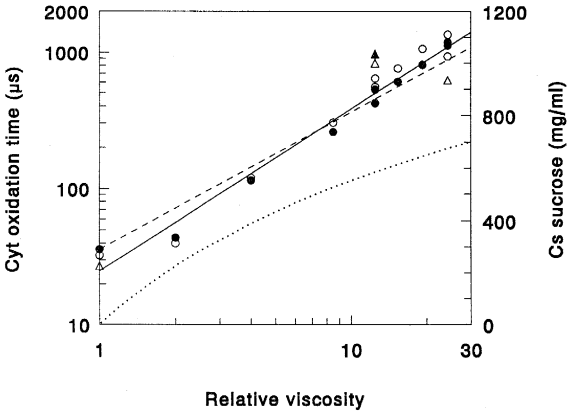

Fig. 10. Time constant for the oxidation of the first (circles) and the second (triangles) heme as a function of viscosity, measured at 553 nm (filled symbols) and at 830 nm (re-reduction of $P840^+$, open symbols). Conditions as for Fig. 4. Measurements were done at 20°C. The oxidation times of the first and the second cytochrome were measured after a first and a second 532-nm flash spaced at 100 ms. The solid line represent a best fit, the dashed line gives a fit for a diffusion-limited process. The dotted line gives the relation between viscosity and sucrose concentration [34].

constants of 25 and 165 μs, together with an irreversible component. This indicates that the rate of oxidation of a second cyt *c* is the same as of the first one. The amplitude of the 25 μs decay component had decreased considerably as compared to Fig. 4A, as was to be expected.

In the above experiments, the flashes were given at 100 ms intervals, i.e., after re-reduction of essentially all $P840^+$. Fig. 9 shows an experiment in which the time between the first and second flashes was varied, and the amount of cytochrome oxidized by the second flash was measured as a function of the time between the flashes. The time constant for this effect was 75 μs. Comparison with the time constant for $P840^+$ re-reduction (25 μs) shows that oxidation of the second cytochrome is not solely determined by the availability of reduced P840. Apparently, some other process must take place before $P840^+$ is able to react with the second cytochrome. The results will be discussed in terms of a serial scheme for cytochrome oxidation in the next section.

The filled circles in Fig. 10 show the time constant for cytochrome oxidation measured at 553 nm at different viscosities, achieved by applying increasing sucrose concentrations. As can be seen the cytochrome oxidation time increased with viscosity. Approximately the same results were obtained by measuring the rate of $P840^+$ re-reduction at 830 nm (open circles). The broken line, representing a fit for a diffusion-limited process, with its reaction rate being inversely proportional to the viscosity [31], gives a reasonable fit of the results (solid line). Approximately the same time constants were found for the oxidation of the second cytochrome (Fig. 10, triangles). The viscosity dependence of the cytochrome oxidation time suggests that the cytochromes are not tightly bound to the RC, but more or less flexibly attached to the membrane. An abrupt change in the oxidation rate, as was reported with some cryoprotectants for cytochrome oxidation by the purple bacterium *Rubrivivax gelatinosus* [32] was not observed.

To investigate the effect of a high sucrose concentration on the structure of the core-FMO complex, we monitored the triplet decay times of the core BChl *a*, FMO and carotenoid triplets. Even at the highest sucrose concentration the decay times of the FMO and of the carotenoid triplet remained unchanged, and the decay time of the core BChl *a* triplet decreased by only 15% (data not shown). Therefore we may conclude that the high sucrose concentrations did not affect the structure of FMO or core.

4. Discussion

Three different triplets are observed upon excitation of membranes of *P. aestuarii*, which can be distinguished by their difference spectra and decay times. The fastest decay is shown by a carotenoid triplet, in agreement with earlier measurements [12]. Two BChl *a* triplets, with decay times of 67 and 165 μs, are located on the FMO and on the reaction center core complex, respectively. The FMO triplet was earlier characterized at cryogenic temperatures [25], but as far as we know not yet at room temperature. The other BChl *a* triplet, with a main bleaching at 837 nm is located on the reaction center core complex; however, there is insufficient evidence to decide whether it is located on P840, as originally proposed by Swarthoff et al. [13], or on a long-wavelength antenna BChl *a* of the core.

Although it is generally accepted that a *c*-type

cytochrome acts as electron donor to P840 in green sulfur bacteria, the identity of this cytochrome is still a matter of contention. On one hand, there is evidence that in *Chlorobium phaeovibrioides* and *C. limicola* a four-heme cytochrome *c*, cyt *c*-553, is associated with the reaction center [4–6,9], and this 32 kDa cytochrome was recently purified and characterized [3]. On the other hand, the presence of a mono-heme cytochrome of a 'new' type of 18 kDa, cyt *c*-551, was reported in reaction center preparations from *C. vibrioforme* [10] and a similar cytochrome may be present in other species of green sulfur bacteria [7–9,11]. No evidence is available about the type of cytochrome(s) in *P. aestuarii*, but the light-induced difference spectrum, with minimum at 553 nm (Fig. 7 and Refs. [14] and [19]) clearly suggest the presence of cytochrome *c*-553.

The kinetics of cytochrome photo-oxidation as well as those of $P840^+$ re-reduction indicate that cytochrome *c*-553 is oxidized in a direct reaction with $P840^+$, with a time constant of 25 μs. The minor 165 μs component in the kinetics can be ascribed to the core BChl *a* triplet mentioned above. It may be noted that our observations indicate that the cytochrome is missing or inoperative in some reaction centers, most likely due to the isolation procedure. No evidence was found for a slow phase for cytochrome oxidation, as reported earlier for various preparations [9,10,13–15]. Various time constants for cyt *c* oxidation have been reported for different species and preparations of green sulfur bacteria ([9,10,12–15]; see also Ref. [1]), and it has been suggested that the actual cytochrome oxidation time in intact cells would be faster than in cellular preparations [1], as was also observed in heliobacteria [33]. The influence of viscosity on the cytochrome oxidation time suggests that the cytochrome is more or less flexibly attached to the membrane, since apparently some molecular motion is needed for the cytochrome in order to react with $P840^+$.

The second cytochrome (or heme) is oxidized with the same time constant as the first one, and the effect of viscosity is the same in both cases. This either suggests a symmetrical arrangement with two identical hemes [29,30] or a sequential scheme, in which the same heme is oxidized twice after re-reduction by the second one. However, our observation, and those of others [14,29,30], that several flashes are needed to obtain complete oxidation of the second heme, is more easily explained by a serial scheme, where only one heme is directly oxidized by $P840^+$, and cytochrome oxidation by the second and subsequent flashes only occurs after this heme has been re-reduced by a second one of approximately the same oxidation reduction potential. This implies, however, that there must be a bottleneck at the acceptor chain, presumably at one of the iron-sulfur centers [1], causing a re-reduction of $P840^+$ by a back-reaction when the heme is not yet available for oxidation. The time constant of 75 μs observed in the experiment of Fig. 9 could then reflect the rate of reaction between the two hemes. This again would favor the four-heme cytochrome model. A third, slowly oxidizable cytochrome as reported by Swarthoff et al. [13] using continuous illumination, could then be a third heme in the four-heme cytochrome, not detectable upon flash illumination.

Acknowledgements

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