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Spectroscopic studies of the chlorophyll *d* containing photosystem I from the cyanobacterium, *Acaryochloris marina*

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ABSTRACT

Absorbance difference spectroscopy and redox titrations have been applied to investigate the properties of photosystem I from the chlorophyll *d* containing cyanobacterium *Acaryochloris marina*. At room temperature, the ($P740^+ - P740$) and ($F^-_{A/B} - F_{A/B}$) absorbance difference spectra were recorded in the range between 300 and 1000 nm while at cryogenic temperatures, ($P740^+A_1^- - P740A_1$) and ($^3P740 - P740$) absorbance difference spectra have been measured. Spectroscopic and kinetic evidence is presented that the cofactors involved in the electron transfer from the reduced secondary electron acceptor, phylloquinone (A_1^-), to the terminal electron acceptor and their structural arrangement are virtually identical to those of chlorophyll *a* containing photosystem I. The oxidation potential of the primary electron donor P740 of photosystem I has been reinvestigated. We find a midpoint potential of 450±10 mV in photosystem I-enriched membrane fractions as well as in thylakoids which is very similar to that found for P700 in chlorophyll *a* dominated organisms. In addition, the extinction difference coefficient for the oxidation of the primary donor has been determined and a value of 45,000±4000 M^{-1} cm^{-1} at 740 nm was obtained. Based on this value the ratio of P740 to chlorophyll is calculated to be 1:~200 chlorophyll *d* in thylakoid membranes. The consequences of our findings for the energetics in photosystem I of *A. marina* are discussed as well as the pigment stoichiometry and spectral characteristics of P740.

1. Introduction

Two large membrane-embedded pigment-protein complexes, photosystem I (PS I) and photosystem II (PS II), catalyze the primary processes of oxygenic photosynthesis in cyanobacteria, algae and plants. The light-induced electron transfer reactions start, in almost all organisms, from the lowest excited singlet state of chlorophyll *a* (Chl *a*). The cyanobacterium *Acaryochloris marina* (*A. marina*) which was discovered in 1996 represents a remarkable exception. Its pigment composition is dominated by Chl *d*. However, ≤5% of the total Chl is Chl *a* [1–3]. Chl *d* is different from Chl *a* in only one regard: it contains a formyl group at the C-3 position on ring A instead of the vinyl group found in Chl *a*. Due to this substitution the Q_y absorption maximum is shifted approximately 30 nm more to the red corresponding to an approximately 0.1 V lower electronic energy gap between ground and lowest excited singlet state as compared to Chl *a*. However, because of the consistent but low level presence of Chl *a* it has been speculated that Chl *a* is likely to be involved as a cofactor in the electron transfer in both PS II and PS I [4] and it was questioned, whether the lower photon energy, due to the red-shifted Chl *d* absorption, is sufficient to drive water splitting in PS II. A study of Schlodder et al. [5], using absorption difference spectroscopy gave direct evidence that a Chl *a* molecule plays an important role in PS II. The bleaching band at 435 nm and the spectrum of the absorbance increase in the NIR associated with the formation of the secondary radical pair $P^+Q_A^-$ showed unambiguously that the cation is located on a Chl *a* rather than a Chl *d* molecule. This Chl *a* molecule has been assigned to be the P_{D1} cofactor in the special pair [5] (nomenclature of Zouni et al. [6] is used for naming cofactors in PS II). The ($P^+Q_A^- - PQ_A$) difference spectrum in the Soret region measured by time-resolved flash absorption spectroscopy was recently confirmed by Itoh et al. [7]. Nevertheless the authors suggest that a Chl *d* molecule becomes photo-oxidized and forms the radical cation P^+ in accordance with a proposal of Tomo et al. [8]. This suggestion however can explain neither the observed bleaching at 435 nm nor the lack of the characteristic bleaching at 455 nm associated with the oxidation of Chl *d*. It is also inconsistent with the spectrum of the absorbance increase in the NIR attributed to the formation of the radical cation P^+ [5].

Abbreviations: A_0, intermediary chlorophyll acceptor in PS I; A_1, primary quinone acceptor in PS I; β-DM, *n*-dodecyl-β-maltoside; Chl, chlorophyll; Cyt, cytochrome; FeS, iron–sulphur-cluster; F_X, F_A, F_B, three [4Fe–4S] clusters in PS I; FeCy, ferricyanide; MES, 2-(*N*-morpholino)ethanesulfonic acid; MV, methyl viologen; Pheo, pheophytin; PMS, phenazine methosulphate; PS I (II), photosystem I (II); PhQ, phylloquinone; RT, room temperature; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride; T−S, triplet-minus-singlet

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It was shown earlier that in PS I of *A. marina*, Chl *d* rather than Chl *a* seems to function as the primary donor [9]. The spectrum of the flash-induced absorbance changes due to photo-oxidation of the primary donor exhibits strong bleaching bands around 455 nm and around 740 nm, which led to the designation as P740 [9]. In organisms containing only Chl *a*, bleaching bands around 435 nm and 700 nm are observed due to the photo-oxidation of the primary electron donor P700. In this case the primary electron donor is a dimer comprised of one Chl *a* and one Chl *a*′ (the 13^2 epimer of Chl *a*) as first suggested from HPLC analysis [10]. This suggestion has since been substantiated by the determination of the three dimensional structure of PS I [11]. ENDOR [12] and FTIR studies [13,14] suggest that P740 is also a dimer of two Chl *d* molecules being structurally similar to P700. Recent analysis of the pigment content indicates that P740 is most probably also a heterodimer composed of Chl *d* and Chl *d*′ [15].

As purified PS I complexes from *A. marina* contain about one Chl *a* per P740 [9], the question arises of where this is located. Based on studies with femtosecond optical spectroscopy [16] it has been suggested that this Chl *a* molecule acts as the primary electron acceptor A_0 in PS I of *A. marina*. Indeed, the rise and decay of absorbance changes around 680 nm with apparent time constants of about 7 and 50 ps were tentatively assigned to the reduction and re-oxidation of a Chl *a* molecule functioning as A_0 [7,16].

Although the pigment composition of PS I from *A. marina* containing predominantly Chl *d* and α-carotene is very different as compared to PS I from organisms containing Chl *a* and β-carotene, the amino acid sequences of the two large subunits, PsaA and PsaB, are remarkably similar (e.g. 75.2% and 77.4% identity, respectively with *T. elongatus* [17]). The strong sequence homology of the PsaA and PsaB proteins between *A. marina* and other cyanobacteria suggests that the 3-dimensional structure of PS I from *A. marina* is likely to be very similar to that of PS I from the cyanobacterium *T. elongatus* [11]. Furthermore nearly all binding sites of chlorophyll molecules in the antenna as well as for the cofactor molecules are conserved except for one remarkable exception: the methionine ligand of eC-B3 (MetB668 in *T. elongatus* [11]) is replaced by a leucine [17]. It is assumed that one or both of the two chlorophylls denoted as eC-A3 and eC-B3 [11] serve as the primary electron acceptor, A_0. Based on the replacement of the methionine ligand of eC-B3 it has been proposed that in PS I of *A. marina* the electron transfer from P740 to the iron–sulphur clusters occurs uni-directionally via the A-branch [18].

The electron transfer reactions in PS I of organisms containing mainly Chl *a* have been extensively studied for many years and a detailed knowledge has been established. Upon illumination P700 is excited either directly or via energy transfer from the antenna molecules. During the subsequent initial charge separation an electron is transferred from P700* to a chlorophyll *a* molecule A_0 within a lifetime of about 1 ps. To stabilize charge separation the electron is transferred further to the phylloquinone acceptor A_1 and from there on to the terminal acceptors; three iron–sulphur clusters F_X, F_A and F_B. The arrangement of these cofactors [11] is mainly defined by the protein environment namely the PsaA and PsaB subunits of PS I.

Despite the great interest that PS I of *A. marina* has attracted since its discovery, the precise nature of its electron transfer cofactors, especially on the acceptor side, has not yet been fully clarified. Recent studies showed that the distance between the secondary electron acceptor and the primary electron donor is very similar to that of PS I, containing no Chl *d* [19], where the secondary donor A_1 is a phylloquinone molecule. There are indications that A_1 is also a phylloquinone in *A. marina* [9] but spectroscopic evidence is still missing. The terminal acceptors in *A. marina* are believed to be iron–sulphur clusters as in Chl *a* binding PS I [9,19]. Indeed, the recent EPR experiments suggested that at least the F_A and F_B cofactors are structurally identical to those in organisms containing only Chl *a* [18]. On the presence and nature of a F_X cofactor little is known.

In the present work we have used transient absorbance difference spectroscopy to characterize PS I of *A. marina* at room temperature and at cryogenic temperatures in order to obtain spectroscopic fingerprints of the cofactors involved in the electron transfer reactions. At room temperature, the ($P740^+ - P740$) absorbance difference spectrum has been measured between 300 nm and 1000 nm. The molar extinction difference coefficient for the bleaching at 740 nm upon photo-oxidation of P740 has been determined to be 45,000±4000 M^{-1} cm^{-1}. The difference spectrum for the reduction of the terminal acceptor resembles closely those observed upon reduction of the terminal iron–sulphur clusters, $F_{A/B}$, in Chl *a* containing PS I.

As the oxidation potential of Chl *d* in organic solvents was recently found to be more positive than that of Chl *a* [20] we have re-measured the redox potential of P740. We find it not to be as low as originally proposed [9]. The consequences of our findings are discussed in terms of the energetics in PS I of *A. marina*. Our measurements have also allowed us to define the pigment stoichiometry of *A. marina* PS I and the spectral characteristics of its primary electron donor, P740.

2. Materials and methods

2.1. A. marina samples

Cells were grown in artificial sea water plus iron (4 mg/L) at 6–10 μE/ (m^2s) (otherwise as described in Ref. [21]) and thylakoid membranes were isolated essentially as before [5,22]. Sucrose density gradient fractionation of the detergent solubilised (1% *n*-dodecyl-β-D-maltoside for 45 min at 4 °C) *A. marina* thylakoid membranes was carried out as described previously [21]. This procedure normally yields three main bands with a high Chl concentration [5,9] and several much fainter, heavier bands [21]. The top band (F1) contains mainly the intrinsic antenna complexes, known as Pcb proteins, and carotenoid, in the second band (F2) both PS I and PS II have been found (probably monomeric complexes). The third band (F3) contains predominantly PS I trimers and to some extent PS II [5,21]. F3, which is enriched in PS I, was collected, concentrated and stored at −80 °C.

Chlorophyll *d* concentrations were determined using an extinction coefficient of 69,000 M^{-1} cm^{-1} at 697 nm in methanol. This value is based on the published extinction coefficient for Chl *d* in diethyl ether [23] and the ratio between the maximum absorbance of Chl *d* in the Q_Y region determined in diethyl ether and methanol. We find a ratio of 0.7 in excellent agreement with Ritchie [24].

2.2. Transient absorption spectroscopy

Time resolved absorption difference spectroscopy is applied to monitor the changes in absorption upon light-induced formation of transient functional states (charge separated states or ^{3}P740) within the reaction centre. Because of the difference, only the pigment that changed its electronic state and pigments which are coupled to this pigment contribute to the spectra. Transient absorption difference spectra (e.g. $\Delta A(P740^+ - P740)$) were measured as described previously [25] using a laboratory-built flash spectrophotometer. In brief, the measuring light of a 250-watt tungsten halogen lamp (Osram) passed through a monochromator placed between light source and sample and a combination of interference filters (Schott) and coloured glasses (Schott) in front of the photomultiplier (EMI 9668BQ). The spectral resolution was 3 nm and the optical path length was 1 cm. The signals were digitized and averaged by a transient recorder (Tektronix TDS 540). A similar set-up was used for measurements in the wavelength region between 700 and 1000 nm except that the wavelength of the measuring light was selected by interference filters (Schott) placed before the sample and in front of the silicon photodiode detector (OSD 100-5T, Centronic) that was coupled to an amplifier (TEK AM 502,

Tektronix). The signals were again digitized and averaged by a transient recorder (Tektronix TDS 320).

The samples were excited by flashes of about 15 μs duration from a Xe flash lamp filtered by coloured glasses (Schott). The flash intensity was adjusted to be nearly saturating. The time resolution was determined by the duration of the flash. For low temperature measurements, the cuvette was placed in a variable temperature liquid nitrogen bath cryostat (Oxford DN1704) or an Oxford liquid helium flow cryostat (CF1204).

The time courses of the absorption changes were fitted to a (multi) exponential decay using an algorithm that minimizes the sum of the unweighted least squares. The amplitudes of each individual exponential component and the initial amplitude (=sum of amplitudes) are then depicted as a function of wavelength to obtain absorbance difference spectra.

Transient absorption difference spectra (e.g. $\Delta A(P740^+ - P740)$) were measured at room temperature using thylakoid membranes and the PS I-enriched fraction from a sucrose density gradient (band F3). The concentrated samples were thawed and diluted to a final chlorophyll concentration of 10–15 μM in 20 mM Tricine (pH 7.5), 25 mM $MgCl_2$, 100 mM KCl and 0.02% β-DM. The reaction mixture also contained: 5 mM ascorbate and about 10 μM phenazine methosulfate (PMS) (in this case reduced PMS functions as artificial electron donor and oxidized PMS as acceptor) or 1–5 mM ascorbate, 200 μM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) and 50 μM methyl viologen (MV) especially for measurements between 300 nm and 450 nm.

The difference between the molar extinction coefficients of $P740^+$ and P740 at 740 nm was calculated from the flash-induced absorption change of TMPD according to Hiyama and Ke [26,27]. TMPD is oxidized by the flash-induced $P740^+$. Measurements were performed at pH 7.5 in the presence of 200 μM TMPD.

For the measurement of $(P740^+A_1^- - P740A_1)$ absorbance difference spectra at low temperature, the PS I samples were diluted with a buffer containing 20 mM Tricine (pH 7.5) or 20 mM MES/NaOH (pH 6.5), 20 mM $CaCl_2$, 20 mM KCl, 0.02% β-DM, 5 mM ascorbate, 2 μM PMS and with glycerol as cryoprotectant to give a final glycerol concentration of about 65% (v/v). The final Chl *d* concentration was 10–15 μM. For comparison, PS I complexes were suspended in a buffer containing 100 mM CAPS (pH 10), 25 mM $MgCl_2$, 0.02% β-DM, 65% (v/v) glycerol and 10 mM sodium dithionite. Subsequently, the sample was frozen in the dark. The absorbance difference spectra measured under both conditions were virtually identical.

To study the triplet state of P740, flash-induced T–S spectra have been recorded at 5 K. PS I complexes were diluted to about 10 μM Chl *d* in 100 mM CAPS (pH 10), 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.02% β-DM. Glycerol was added to a final concentration of 65% (v/v). 10 mM dithionite plus 7 μM PMS were added to this solution under argon. The samples were then illuminated at 260 K for 3 min with a 250-watt focused tungsten lamp filtered by water and additional heat-absorbing filters. This procedure leads to the pre-reduction of the secondary electron acceptor A_1 whereby further electron transfer to A_1 is blocked. The primary radical pair $P740^+A_0^-$ formed under these conditions recombines with a high yield to the triplet state of P740.

2.3. Redox titration

To determine the oxidation midpoint potential of P740, the flash-induced absorbance change at 826 nm, associated with oxidation of P740, was measured as a function of the redox potential. PS I complexes or thylakoid membranes were diluted to about 20 μM Chl in 20 mM Tricine (pH 7.5), 100 mM KCl, 25 mM $MgCl_2$, 0.02% β-DM, and the redox potentials were adjusted by adding ferricyanide and ferrocyanide. After each experiment, the potential was measured using a combination Pt/Ag/AgCl electrode (Schott PT5900A) which was calibrated against the redox potential of a saturated solution of quinhydrone as a function of pH. A pH-meter (Knick PHM82) was used to read out the redox potential. All redox potentials are given relative to the standard hydrogen electrode (normal hydrogen electrode).

3. Results

3.1. Absorbance difference spectroscopy at room temperature

Absorbance difference spectroscopy was used to probe the charge separation between the primary donor P740 and the terminal iron–sulphur clusters [19] in a PS I-enriched sucrose gradient fraction (F3) as well as in thylakoid membranes isolated from *A. marina*. The measurements were performed on a millisecond timescale in the presence of artificial electron donors and acceptors (see Materials and methods). The time course of the flash-induced absorbance changes has been fitted by a fast and a slow exponential component. The initial amplitude obtained by the fit represents the absorbance changes coupled to the formation of $P740^+F_{A/B}^-$ because preceding electron transfer steps are too fast to be resolved. Fig. 1 (triangles) shows the $(P740^+F_{A/B}^- - P740F_{A/B})$ difference spectrum between 300 nm and 600 nm. The main bleaching band in the Soret region is located at about 455 nm as reported earlier [9,28]. This corresponds to a red shift of around 22 nm compared to Chl *a* containing PS I of higher plants and cyanobacteria, containing no Chl *d* and gives clear evidence that the cation is located on a Chl *d* molecule. The absorbance changes are suppressed in the presence of ferricyanide which inhibits the photochemistry of PS I by chemical oxidation of P740 (data not shown). These control measurements gave clear evidence that we are observing pure PS I spectra. Indeed electron transfer reactions in PS II are most likely to be too fast to be resolved on the millisecond timescale.

The amplitude of the fast component with a half-life between 0.5 ms and 5 ms can be attributed to the oxidation of the reduced terminal iron–sulphur cluster. [29,30] The oxidation of $F_{A/B}^-$ occurs to a small extent by charge recombination with $P740^+$ (approx. 14% under our conditions based on measurements at 740 nm where the iron–sulphur cluster does not contribute to the absorbance changes) and predominantly by electron donation to an external electron acceptor (e.g. oxidized PMS or MV). Therefore the half-life of this reaction

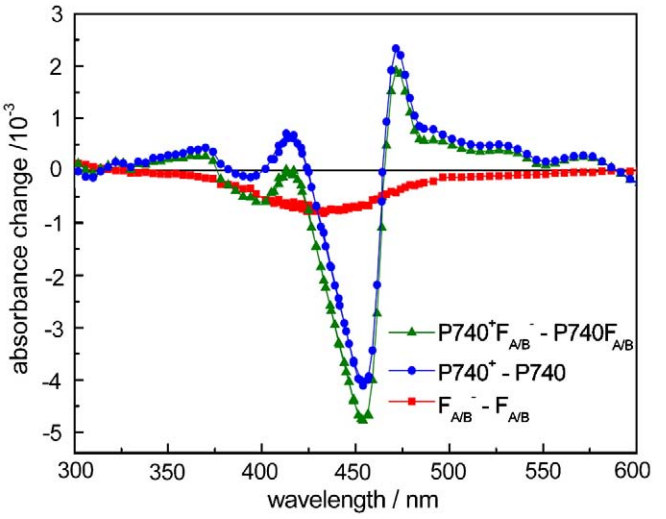

Fig. 1. Flash-induced absorbance difference spectra of PS I complexes from *A. marina* for the oxidation of P740 (circles), for the reduction of the terminal iron–sulphur cluster (squares) and the formation of $P740^+F_{A/B}^-$ (triangles) measured in the presence of 5 mM Na-ascorbate and 7 μM PMS. Samples were excited with saturating flashes of about 15 μs in duration from a Xenon flash lamp (for experimental details, see Materials and methods).

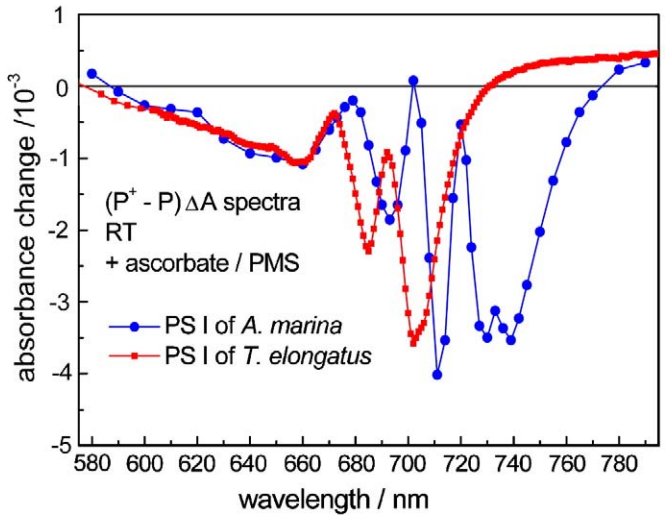

Fig. 2. Flash-induced absorbance difference spectra of PS I complexes from *A. marina* (circles) for the oxidation of P740 in the Q_Y region measured in the presence of 5 mM Na-ascorbate and 7 μM PMS. For comparison the ($P700^+$–P700) absorbance difference spectrum of PS I complexes from *T. elongatus* is shown (squares). It should be noted that the negative band at 703 nm is normalized to the bleaching at 740 nm in the ($P740^+$–P740) absorbance difference spectrum.

depends on the type and concentration of the added electron acceptor. The remaining $P740^+$ (≈86%) is reduced by the added artificial donor (either reduced PMS or TMPD) giving rise to the slow recovery phase. In the presence of ascorbate and PMS, the half-life of this slow phase due to $P740^+$ reduction was confirmed to be proportional to the concentration of the added artificial donor (reduced PMS) with a slope of 6×10^6 M^{-1} s^{-1} at pH 7.5 which corresponds to the second order rate constant of this reaction (not shown). The amplitude of the slow component as a function of wavelength represents the difference spectrum for the oxidation of P740. In order to account for 100% of the PS I complexes the amplitudes were multiplied by the factor of 1.16. Fig. 1 (circles) shows the ($P740^+$–P740) spectrum. It exhibits the major bleaching at 455 nm, positive bands at 472 nm and 415 nm and a broad absorption increase in the range of 480 nm to 588 nm. These features are similar to the ($P700^+$–P700) spectrum except for the red-shift of about 22 nm, due to the fact that in PS I of *A. marina* a Chl *d* is oxidized instead of a Chl *a*. Oxidation of P740 causes only small absorbance changes in the range between 300 nm and 400 nm, particularly between 375 nm and 400 nm the absorbance changes due to oxidation of P740 are nearly zero or slightly negative. The difference spectrum for the reduction of the terminal iron–sulphur cluster ($F^-_{A/B}$–$F_{A/B}$) (see Fig. 1, squares) was obtained by subtracting the ($P740^+$–P740) spectrum from the ($P740^+F^-_{A/B}$–$P740F_{A/B}$) spectrum. The broad bleaching band centred at 432 nm agrees well with the ($F^-_{A/B}$–$F_{A/B}$) spectrum reported for PS I of higher plants and cyanobacteria, containing no Chl *d* [30,31]. This result supports strongly the conclusion based on EPR data [19] that the terminal acceptors of PS I from *A. marina* are identical to those of Chl *a* containing PS I.

Fig. 2 shows the room temperature ($P740^+$–P740) absorbance difference spectrum of PS I complexes from *A. marina* in the Q_Y region (Fig. 2, circles). It resembles closely that reported by Hu et al. [9] and Mi et al. [28]. The absorbance difference spectrum exhibits a very broad bleaching band which probably results from the overlap of two bands centred at 740 nm and 730 nm. The zero crossing in the red region is at about 774 nm. There is also a sharp bleaching band at 711 nm and bleaching bands at 693 nm and 660 nm. The zero crossing on the short wavelength side is at 588 nm, which is about 14 nm more to the red than in PS I of higher plants and cyanobacteria, containing no Chl *d*. For comparison the ($P700^+$–P700) absorbance difference spectrum of PS I complexes from *T. elongatus* is shown in Fig. 2 (squares). It should be noted that the negative band at 703 nm is normalized to the bleaching at 740 nm in the ($P740^+$–P740) absorbance difference spectrum.

The difference between the molar extinction coefficients of $P740^+$ and P740 at 740 nm was determined by measuring the flash-induced absorption change of TMPD as described by Hiyama and Ke [26]. The reduction of $P740^+$ after the actinic flash is well coupled with the oxidation of TMPD. TMPD oxidation was measured at the isosbestic point of P740 oxidation, i.e. 588 nm (see Figs. 1 and 2). An extinction coefficient of 10,200 M^{-1} cm^{-1} has been determined for oxidized TMPD at this wavelength at pH 7.5. The size of the absorption changes at 740 nm and 588 nm were measured for the same sample. From the ratio of the amplitudes of these absorption changes the difference extinction coefficient for the formation of $P740^+$ was calculated to be 45,000±4000 M^{-1} cm^{-1} for the bleaching at 740 nm. This value is significantly lower than that reported earlier [9]. Using this molar extinction difference coefficient the Chl *d*/P740 ratio has been calculated from the maximum flash-induced absorbance decrease in the Q_y region due to photoxidation of P740 measured in F3 preparations and thylakoids. The following ratios of Chl *d* per P740 were obtained: 150±10 for F3 preparations and 200±10 for thylakoids (data not shown).

Fig. 3 shows the room temperature ($P740^+$–P740) absorbance difference spectrum of PS I complexes from *A. marina* in the NIR region (circles) using the same measuring conditions as described above. Above 780 nm the absorbance difference spectrum reflects the absorbance spectrum of the radical cation ($P740^+$), because the neutral P740 does not absorb in this wavelength region. As noted previously [5] the radical cation of the primary electron donor $P740^+$ absorbs approximately 40 nm more into the near infrared relative to the Chl *a* radical cation ($P700^+$) from PS I of higher plants and cyanobacteria, containing no Chl *d*, which is consistent with the 40 nm red shift of the most long-wave Q_Y bleaching band due to the oxidation of P740 relative to P700.

It should be noted that the magnitudes of the absorbance changes in the Soret, Q_Y and NIR region shown in Figs. 1–3 can be compared directly. We find that with the same sample the absorbance change in the NIR around 840 nm is about five times smaller than that at 740 nm. From this result it follows that the extinction coefficient for the maximum absorbance of the radical cation $P740^+$ in the near infrared is ~9000 M^{-1} cm^{-1} in good agreement with the expected value for a Chl cation. The extinction coefficient of $P700^+$ is somewhat smaller

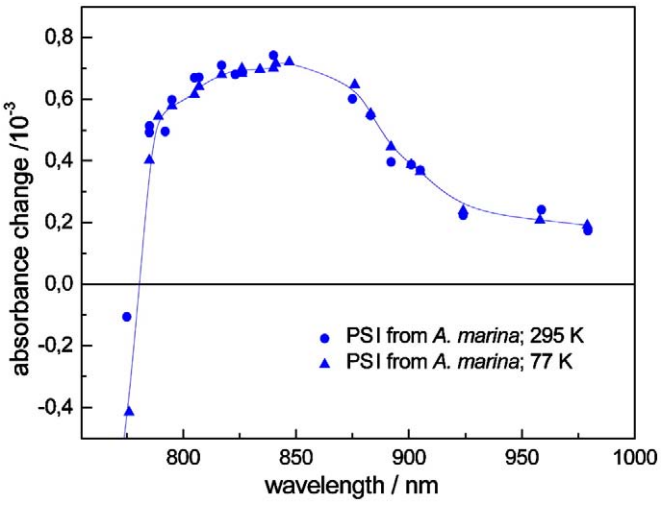

Fig. 3. Flash-induced absorbance difference spectra of PS I complexes from *A. marina* for the oxidation of P740 in the NIR measured in the presence of 5 mM Na-ascorbate and 7 μM PMS at room temperature (circles). For comparison the ($P740^+$–P740) difference spectrum at 77 K is depicted (triangles). For details see text.

(~7500 M^{-1} cm^{-1} around 830 nm) than that of $P740^+$ which might be due to a different absorption in the NIR of the Chl *a* cation and the Chl *d* cation, respectively.

3.2. Charge recombination kinetics

Charge recombination between $P740^+$ and the terminal iron–sulphur clusters $F_{A/B}$ measured in the F3 fraction occurs with a half-life of approximately 60 ms which is similar to that determined for Chl *a* binding PS I complexes (see for example Ref. [29]). Upon incubation with 6.8 M urea from 1 min up to 180 min the slow decay of the flash-induced absorbance changes is progressively replaced by a faster decay phase with a half-life of about 2 ms (not shown). As described earlier [25,32,33] urea treatment removes the stromal PsaC subunit that coordinates the terminal iron–sulphur clusters F_A and F_B. Under these conditions, the recovery of the oxidized primary donor takes place by charge recombination with the reduced iron–sulphur cluster F_X that is located on the PsaA/PsaB heterodimer. It should be mentioned that the 2 ms decay is somewhat slower than the approximately 1 ms half-life, which has been reported for PS I complexes from cyanobacteria lacking the PsaC subunit [25,32,33]. When F_A and F_B were pre-reduced in F3 by dithionite at pH 10, the flash-induced absorbance changes decayed predominantly with a half-life of about 190 μs (not shown). In accordance with the literature [34,35], we assign this phase to the charge recombination between $P740^+$ and A_1^- (see also below).

3.3. Oxidation midpoint potential of P740

To determine the oxidation potential (E_m) of $P740^+$/P740, the flash-induced absorbance change at 826 nm, associated with the oxidation of P740, was measured as a function of the potential adjusted by adding varying amounts of ferricyanide and ferrocyanide. The dependence of the amplitude of the absorbance change could be satisfactorily fitted using the one-electron Nernst equation (see Fig. 4, solid line). Titrations have been performed with solubilised PS I complexes (F3 band) and also with thylakoid membranes. Within the error limits, the midpoint potential was identical for all samples with a value of 450±5 mV. This is about 10 mV more positive than the oxidation potential of P700 in PS I from *T. elongatus* which we determined under the same conditions. The value of 440 mV measured for the midpoint potential of P700 is in good agreement with previous studies [27]. Taking into account the uncertainty of the calibration of the electrode we estimate an error of ±10 mV for the E_m absolute values. It should be noted that the value of 450 mV is significantly higher than the value of 335 mV reported by Hu et al. [9]. In that work the titration was performed at 740 nm which might have the problem that P740 was to some extent oxidized by the measuring light. The higher midpoint potential of P740 has been reported previously in preliminary publications by us [36] as well as by Benjamin et al. [37].

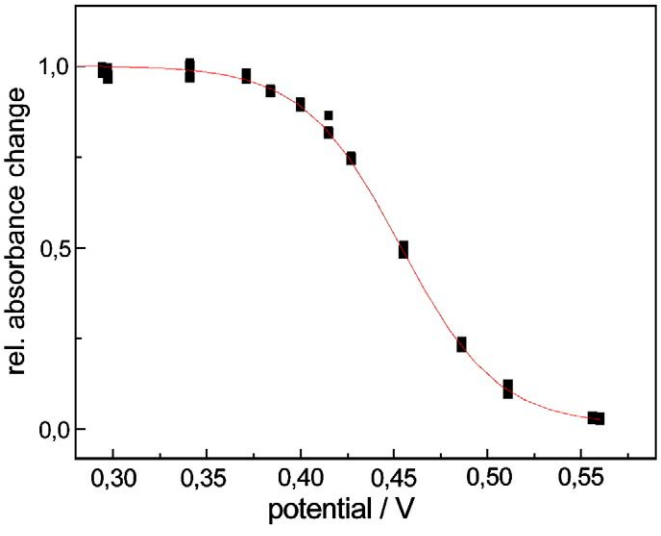

Fig. 4. Redox titration of the primary donor (P740) of *A. marina* PS I. The initial extent of the absorption change at 826 nm after a flash-induced excitation was plotted against the redox potential of the medium. The midpoint redox potential value was obtained to be +455 mV by fitting with the one-electron Nernst's curve (solid line).

3.4. Absorbance difference spectroscopy at cryogenic temperatures

As seen in Chl *a* containing PS I of higher plants and cyanobacteria, containing no Chl *d*, we find that electron transfer in PS I of *A. marina* is heterogeneous at low temperature [34]. At 77 K, an irreversible charge separation due to the stable formation of $P740^+F_A^-$ and $P740^+F_B^-$ takes place in one fraction of the PS I complexes (~20%), whereas in the largest fraction (~70%) forward electron transfer is restricted to the formation and decay of the secondary radical pair $P740^+A_1^-$. These conclusions are derived from measurements of the initial amplitude of the flash-induced absorption changes at 826 nm as a function of the flash number at 77 K as described earlier [34]. Charge recombination of $P740^+A_1^-$ occurs at 77 K with $t_{1/2}$ ~150 μs. This half-life is virtually identical to that observed in PS I of higher plants and cyanobacteria, containing no Chl *d* [34].

Fig. 5A shows the ($P740^+A_1^- - P740A_1$) absorbance difference spectrum in the Q_Y region measured at 5 K. The amplitude of the ~150 μs decay phase is depicted as a function of the wavelength. The ($P740^+A_1^- - P740A_1$) absorbance difference spectrum measured at 77 K (not shown) resembles closely that measured at 5 K (Fig. 5A). The low temperature spectrum exhibits the most long-wavelength bleaching band, which we attribute to the disappearance of the low energy exciton band upon oxidation of P740, at about 744 nm plus an even larger negative band at 730 nm. These two bands are clearly separated in contrast to the RT ($P740^+ - P740$) spectrum (see Fig. 2) which is probably due to the narrowing of absorption bands with decreasing temperature. In addition, negative bands are observed at 711 nm, 687 nm and 664 nm. A broad positive band appears at 700 nm, smaller positive bands are observed at 719 nm and 674 nm. The shape of the ($P740^+A_1^- - P740A_1$) absorbance difference spectrum indicates contributions from electrochromic band shifts induced by the positive charge localized on oxidized P740 and the negative charge located on A_1.

Fig. 5B shows the 77 K ($P740^+A_1^- - P740A_1$) absorbance difference spectrum between 300 and 550 nm. The main bleaching band around 455 nm is primarily due to the oxidation of P740 (see Fig. 1, circles). In addition, there are some electrochromic band shifts. The absorbance increase in the wavelength region between 350 nm and 400 nm can be attributed mainly to the reduction of A_1 because the oxidation of P740 causes only very weak absorbance changes in this spectral range (see Discussion of the ($P740^+ - P740$) difference spectrum (Fig. 1, circles) above). In PS I complexes containing only Chl *a* the absorbance increase in the 350–400 nm range is also observed and has been assigned to the reduction of phylloquinone (called PhQ_A and PhQ_B in Ref. [11]) [25,31,38]. The kinetics of charge recombination between $P740^+$ and A_1^-, measured in PS I of *A. marina*, are also very similar to those between $P700^+$ and A_1^-, measured in Chl *a* containing PS I [34,38]. Therefore, our spectroscopic and kinetic data are consistent with phylloquinone functioning as the secondary electron acceptor in PS I of *A. marina* as well as in Chl *a* containing PS I of plants and cyanobacteria.

The 77 K ($P740^+A_1^- - P740A_1$) absorbance difference spectrum of PS I from *A. marina* in the NIR is shown in Fig. 3 (triangles) for comparison with the RT spectrum. For measurements of ($P740^+A_1^- - P740A_1$) absorbance difference spectra the samples contained 5 mM ascorbate and about 2 μM PMS and were frozen in the dark. Under these conditions P740 is fully reduced and all electron acceptors are

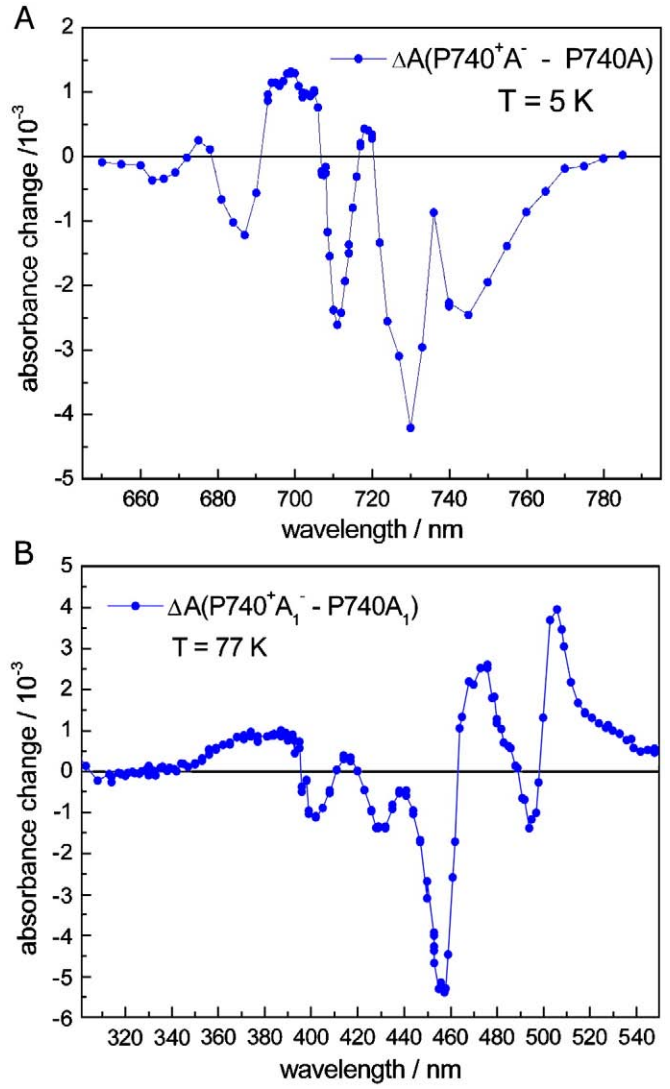

Fig. 5. ($P740^+A_1^-$ – $P740A_1$) absorbance difference spectrum of PS I complexes of *A. marina* measured by flash-induced absorbance changes at 5 K in the Q_Y region (A) and in the wavelength region between 300 nm and 550 nm (B).

oxidized. Reversible absorbance changes with a half-life of 150 μs are attributed to $P740^+A_1^-$ recombination in PS I, as described above. Under these conditions, flash-induced formation and decay of $P^+Q_A^-$ occur in PS II. $P^+Q_A^-$ decays at low temperature with a half-life of about 2 ms [5] which is more than a factor of 10 slower than the decay of the secondary radical pair $P740^+A_1^-$ in PS I. Therefore, contributions of PS II to the flash-induced absorbance changes can be separated kinetically. As an additional control the ($P740^+A_1^-$ – $P740A_1$) absorbance difference spectrum has also been measured in a sample which was frozen in the dark in the presence of dithionite. Under these conditions, the plastoquinone acceptor in PS II is chemically pre-reduced. The observed ($P740^+A_1^-$ – $P740A_1$) absorbance difference spectrum was virtually identical (data not shown).

To measure the triplet-minus-singlet absorbance difference spectrum of P740 we used the approach common for PS I from higher plants and cyanobacteria, containing no Chl *d*. Measurements are performed with PS I under reducing conditions in which the secondary acceptor A_1 is in the reduced state. Pre-reduction is typically achieved by illumination of the sample at about 270 K in the presence of dithionite at high pH before freezing. With pre-reduced A_1 electron transfer to A_1 is blocked and the primary radical pair, $P^+A_0^-$ recombines to the triplet state of P with high yield. We applied this treatment to PS I from *A. marina* and observed at 5 K the flash-induced absorbance difference spectrum shown in Fig. 6 (circles). For comparison the (^{3}P700 – P700) absorbance difference spectrum of PS I complexes from *T. elongatus* (taken from Ref. [27]) is shown (squares). It should be noted that the negative band at 700 nm is normalized to the bleaching at 740 nm in the (^{3}P740 – P740) absorbance difference spectrum. The flash-induced absorbance difference spectrum observed in PS I from *A. marina* (circles) exhibits an extremely broad main bleaching around 738 nm. The zero crossing in the red region is at about 776 nm. On the short wavelength side of the main band, a spectral feature is observed that can be interpreted as a band shift centred at 712 nm due to altered excitonic interactions after triplet formation comparable to the feature located at 685 nm in PS I from *T. elongatus* (see Discussion and Ref. [27]). A smaller bleaching band is observed at around 688 nm. The comparison shows that the flash-induced absorbance difference spectrum observed in PS I from *A. marina* (circles) exhibits the same fingerprint-like features as observed in the triplet-minus-singlet spectrum of P700 from PS I of higher plants and cyanobacteria, containing no Chl *d*, which supports strongly the assignment of the flash-induced absorbance changes in PS I of *A. marina* observed in the presence of pre-reduced A_1 to the formation and decay of ^{3}P740. At 5 K, the decay kinetics of ^{3}P740 can be described by two components with half-lives of 170 μs (≈70%) and 1.4 ms (≈30%). The non-monoexponential decay of the triplet state at very low temperatures is expected because interconversion between the three spin sublevels becomes slow [39]. Above 50 K, the decay could be fitted with a single exponential with $t_{1/2}$≈200 μs. The decay of ^{3}P740 at low temperatures is significantly faster than the reported kinetics of the decay of ^{3}P700 ($t_{1/2}$≈0.7 ms (65%) and 7 ms (35%)) [40]. In order to check if the difference between the triplet decay kinetics originates from the difference between Chl *d* and Chl *a*, we measured the formation and decay of the triplet state of Chl *d* in detergent micelles i.e. under similar conditions as described earlier for Chl *a* [41]. The flash-induced absorbance changes yielded the characteristic triplet-minus-singlet difference spectrum (not shown). It resembles closely the spectrum reported by Di Valentin et al. [42]. The observed decay was found to be a factor of 5 faster than that of Chl *a*, indicating that the intersystem crossing rate of Chl *d* is indeed significantly faster than that of Chl *a*. These results show that at low temperatures the decay kinetics of

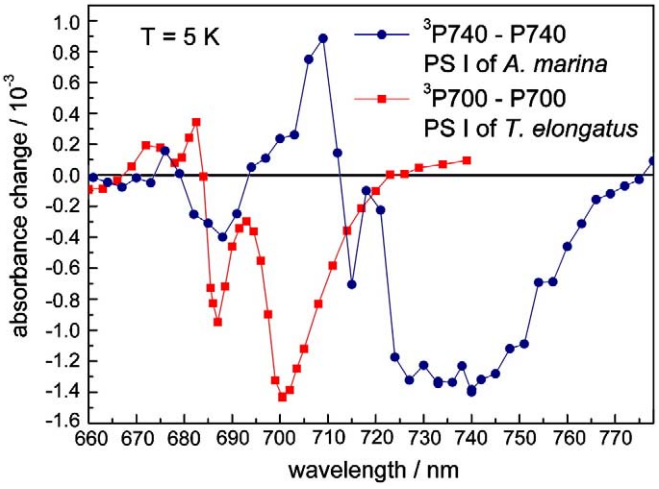

Fig. 6. Triplet-minus-singlet spectrum of P740 from *A. marina* measured by flash-induced absorbance changes attributed to ^{3}P740 formation at 5 K. For details see text. For comparison the (^{3}P700 – P700) absorbance difference spectrum of PS I complexes from *T. elongatus* (taken from Ref. [27]) is shown (squares). It should be noted that the negative band at 700 nm is normalized to the bleaching at 740 nm in the (^{3}P740 – P740) absorbance difference spectrum.

^{3}P740 in vivo are almost identical to those of ^{3}Chl *d* in vitro, as are the slower decay kinetics of ^{3}P700 in PS I from Chl *a* binding organisms essentially the same as those of ^{3}Chl *a* in vitro.

4. Discussion

We have applied transient absorbance difference spectroscopy and redox titrations to investigate the Chl *d* binding PS I of the cyanobacterium *A. marina*.

4.1. Acceptor side of PS I of A. marina

Spectroscopic and kinetic evidence is presented that the cofactors involved in the electron transfer from the reduced secondary electron acceptor A_1^- to the terminal electron acceptor and their structural arrangement are virtually identical to those of Chl *a* containing PS I. The absorbance difference spectrum for the reduction of the terminal acceptor (see Fig. 1, squares) agrees well with those reported for PS I complexes from spinach [30,43] and cyanobacteria [31]. This indicates that the terminal acceptors are highly likely to be identical to the [4Fe–4S] cluster F_A and F_B present in PS I complexes containing no Chl *d*. Further evidence has been reported recently in the literature to support this conclusion: (i) The EPR difference spectrum observed upon illumination at 12 K is characteristic of reduced [4Fe–4S] clusters and the features are reminiscent of those attributed to F_A and F_B present in Chl *a* containing PS I [19]. (ii) The kinetics of charge recombination between $P740^+$ and the reduced terminal acceptor are similar to those between $P700^+$ and $F_{A/B}^-$ (see Refs. [9,29] and Results section). (iii) The amino acid sequence of the PsaC subunit from *A. marina* and Chl *a* containing organisms is highly conserved. (e.g. 98.8% identity with *T. elongatus*) [17].

An indication for the presence of F_X comes from our observation that in urea treated PS I lacking F_A and F_B the oxidized primary donor $P740^+$ is reduced with a half-life of about 2 ms which may be assigned by analogy to Chl *a* binding PS I [25,32] to charge recombination between P^+ and F_X^-.

The low temperature ($P740^+A_1^- - P740A_1$) absorbance difference spectrum (see Fig. 5B) exhibits an absorbance increase in the wavelength region between 350 nm and 400 nm, a feature also observed in PS I complexes containing no Chl *d* and assigned to the reduction of phylloquinone [25,31,38].

The kinetics of charge recombination between $P740^+$ and A_1^- measured in PS I of *A. marina* are also very similar to those between $P700^+$ and A_1^- measured in Chl *a* binding PS I [34,38]. This spectroscopic and kinetic data yields strong evidence that phylloquinone also functions as the secondary electron acceptor in PS I of *A. marina*. Recently Santabarbara et al. [19] determined the distance between the electronic spins of $P740^+$ and A_1^- in *A. marina* as 25.23±0.05 Å by the analysis of the electron spin echo envelope modulation which is remarkably close to the distance between $P700^+$ and PhQ^- in Chl *a* organisms [44]. Assuming the midpoint potential of the phylloquinone is not altered in PS I of *A. marina*, the standard free energy change ΔG^0 for charge recombination between P^+ and A_1^- should be identical in Chl *a* and Chl *d* containing PS I, as the oxidation potentials of P740 and P700 were also found to be about the same (see results section).

Regarding the similarity of the cofactors involved and the protein surrounding them, it seems reasonable to assume that the reorganization energy is virtually identical in Chl *a* and Chl *d* containing PS I. Since the electron transfer rate is mainly depending on the following parameters: distance between the redox cofactors, driving force (ΔG^0) and reorganization energy λ [45], our observation of identical rates for $P740^+A_1^-$ and $P700^+A_1^-$ charge recombination is in agreement with established electron transfer theory.

4.2. Energetics of electron transfer

The oxidation midpoint potential (E_m) of $P740^+$/P740 has been measured in solubilised PS I complexes (F3 band) and also with thylakoid membranes to be 450±5 mV (see Fig. 4). It appears that the previous measurement of the redox potential of P740 by Hu et al. [9], who reported 335 mV, may be incorrect and this is possibly due to the fact that they measured the absorption changes at 740 nm where the measuring beam may have had an actinic effect. It should be noted that the oxidation potential in acetonitrile and dimethylformamide of Chl *d* (0.88 V and 0.92 V, respectively) is somewhat higher than for Chl *a* in the same solvents (0.81 V and 0.86 V, respectively) [20]. Our value for P740, which is, indeed, very similar to that found for P700 in chlorophyll *a* dominated organisms, is in agreement with recent results of Benjamin et al. [37] based on their measurements of intersystem electron transfer equilibria and a reassessment of the redox potential of P740. The intersystem electron transfer equilibria determined in their work are also consistent with the difference in potential of cytochrome *f* and P740 based on the new value of the midpoint potential of P740.

The consequence of an oxidation potential of 450 mV is that the lowest excited singlet state of P740 is about 90 mV lower than that of P700. Nevertheless the reduction potential of P740* ($E_m \approx -1.23$ V) should be sufficient to reduce Chl *a* ($E_m \approx -1.12$ V in acetonitrile) as well as Chl *d* ($E_m \approx -0.91$ V in acetonitrile) [20]. If the redox potential of the phylloquinone A_1 in *A. marina* is assumed to be the same as in Chl *a* containing PS I the driving force for electron transfer from the excited primary donor to the secondary electron acceptor is smaller by 90 mV. If A_0 is a Chl *a* molecule as previously suggested [7,16] and the accessory chlorophylls are Chl *d*, the reduction of A_0 by the reduced accessory Chl *d* would be uphill assuming a sequential electron transfer and reduction potentials similar to those in acetonitrile. However, this problem may be solved within the PS I of *A. marina* by fine tuning the redox potentials of the cofactors by appropriate protein-cofactor interactions.

4.3. Pigment stoichiometry

In this work, the extinction difference coefficient for the oxidation of the primary donor has been determined and a value of 45,000±4000 M^{-1} cm^{-1} at 740 nm was obtained which is significantly lower than that reported by Hu et al. [9]. The reason for the discrepancy is unclear. Our value is in agreement with the ratio of absorbance changes at 740 compared to those around 840 nm (approx. 5:1) resulting in an extinction coefficient at 840 nm of about 9000 M^{-1} cm^{-1} for the Chl *d* cation. Based on our value of 45,000 M^{-1} cm^{-1} the ratio of P740 to chlorophyll is thus calculated to be 1:~200 Chl *d* in thylakoid membranes and 1:~150 Chl *d* in samples from band F3 of the sucrose density gradient (see Materials and methods). The Chl *d*:P740 ratio of about 200 in thylakoid membranes is consistent with the following simple consideration. Assuming PS I:PS II ≈ 1 [15,46,47], Chl *d*:PS I core ≈ 100 as in Chl *a* binding PS I [11,48], Chl *d*:PS II ≈ 40 [49,50] and 4 Pcb proteins with approximately 15 chlorophylls each per PS II [21] one obtains around 200 chlorophyll molecules per P740. The ratio of 150 Chl *d* per P740 in samples from the band F3 indicates that this fraction also contains PS II, which has been proven by transient absorbance difference spectroscopy, and some Pcb proteins (Telfer et al. unpublished).

4.4. Spectral properties of the reaction centre of PS I

The light-induced bleaching band around 455 nm and the spectrum of the absorbance increase in the NIR upon oxidation of P740 (see Figs. 1, 3 and 5B) show unambiguously that the cation is located on a Chl *d* molecule. ENDOR studies of $P740^+$ indicate that the

cation is mainly localized on one of the two Chls *d* constituting P740 [12]. They suggest that the molecular arrangement of the special pair of P740 is very similar to that of P700 where the latter is comprised of one Chl *a* and one Chl *a′* (the 13^2 epimer of Chl *a*) [10,11]. ENDOR spectra of $P700^+$ show that more than 85% of the spin density is located on P_B [51], the Chl *a* molecule of P700 coordinated by PsaB (named eC-B1 in Ref. [11]). Therefore it seems likely that P740 is comprised of one Chl *d* and one Chl *d′* (the 13^2 epimer of Chl *d*) [15] with similar electronic properties to its Chl *a* counterpart.

In the Q_Y region, a broad bleaching band around 740 nm is observed upon both oxidation of P740 (see Figs. 2 and 5A) and formation of the triplet state ^{3}P740 (see Fig. 6). The absorbance decrease around 740 nm most probably corresponds to the transition from the ground state to the lowest excited exciton state of the reaction centre Chls. If one of the special pair Chls of P740 is oxidized or in the triplet state the transition dipole strength of this Chl is very small and the excitonic coupling is restricted to the remaining 5 Chls expected to be bound in the reaction centre core. This leads to the disappearance of the low energy exciton band of all six reaction centre Chls. The complex shape of the (^{3}P740 −P740) and ($P740^+$−P740) absorbance difference spectra (see Figs. 2, 5A and 6) gives evidence for coupling between all reaction centre Chls and hence for the delocalization of excited states. In the case of a coupled dimer the appearance of just two negative bands is expected at the position of the lower and upper exciton transition energy of the dimer and one positive band due to the absorbance of one of the special pair Chls which is not carrying the positive charge or the triplet state, respectively. Because of the delocalization of the excited singlet states the whole spectrum of the excitonically coupled Chls is changed upon converting one pigment into a triplet or an oxidized state. Difference spectra associated with the formation of charge separated states, e.g. $P740^+A_1^-$, may additionally contain electrochromic (Stark) shifts of absorbance bands of pigments located in the vicinity of the cofactors carrying a positive or negative charge.

A comparison between the (^{3}P−P) and (P^+−P) absorbance difference spectra of P700 and P740 (see e.g. Fig. 2 as an example of the latter) shows clearly that the difference spectra of P740 do indeed exhibit more bands over an extended wavelength range than those of P700. This could be an indication that PS I of *A. marina* contains one Chl *a* molecule as proposed by Kumazaki et al. [16]. Interestingly the differences in the spectral region between 570 and 670 nm upon oxidation of the primary electron donor are almost identical for the two different species *T. elongatus* and *A. marina*. Theoretical calculations of the flash-induced absorbance difference spectrum of ($P740^+$ −P740) are in preparation in collaboration with T. Renger in order to enable the assignment of the observed bands. An exciton Hamiltonian determined previously for Chl *a* containing photosystem I complexes [52] will be modified to take into account the occupancy of binding sites by chlorophyll *d* instead of chlorophyll *a*.

5. Note added in proof

Since this paper was submitted, Tomo et al. (J. Biol. Chem, [2008] 283, 18198–18209), using purified PS I particles isolated from *A. marina*, have confirmed our reassessment of the redox potential of P740 as being essentially the same as that of P700 in Chl *a* containing organisms.

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