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Both chlorophylls *a* and *d* are essential for the photochemistry in photosystem II of the cyanobacteria, *Acaryochloris marina*

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This paper is dedicated to the memory of Professor Michael (Mike) C. W. Evans

Abstract

We have measured the flash-induced absorbance difference spectrum attributed to the formation of the secondary radical pair, P^+Q^-, between 270 nm and 1000 nm at 77 K in photosystem II of the chlorophyll *d* containing cyanobacterium, *Acaryochloris marina*. Despite the high level of chlorophyll *d* present, the flash-induced absorption difference spectrum of an approximately 2 ms decay component shows a number of features which are typical of the difference spectrum seen in oxygenic photosynthetic organisms containing no chlorophyll *d*. The spectral shape in the near-UV indicates that a plastoquinone is the secondary acceptor molecule (Q_A). The strong C-550 change at 543 nm confirms previous reports that pheophytin *a* is the primary electron acceptor. The bleach at 435 nm and increase in absorption at 820 nm indicates that the positive charge is stabilized on a chlorophyll *a* molecule. In addition a strong electrochromic band shift, centred at 723 nm, has been observed. It is assigned to a shift of the Qy band of the neighbouring accessory chlorophyll *d*, Chl_{D1}. It seems highly likely that it accepts excitation energy from the chlorophyll *d* containing antenna. We therefore propose that primary charge separation is initiated from this chlorophyll *d* molecule and functions as the primary electron donor. Despite its lower excited state energy (0.1 V less), as compared to chlorophyll *a*, this chlorophyll *d* molecule is capable of driving the plastoquinone oxidoreductase activity of photosystem II. However, chlorophyll *a* is used to stabilize the positive charge and ultimately to drive water oxidation.

Keywords: Photosystem II; Chlorophyll *d*; Water oxidation; Charge separation; *Acaryochloris marina*

1. Introduction

In the unusual phototrophic cyanobacterium, *Acaryochloris marina*, most of the chlorophyll (Chl) is of the *d*-type [1]. Recently other strains have been found, some associated with a red alga [2] and others free-living in a saline lake [3], but only *A. marina* MBIC-11017 has been biochemically characterised in any detail [4–6]. Chl *d* has a formyl group on ring 1 of the porphyrin head group, in place of the vinyl group in Chl *a*, and this shifts the Qy absorption maximum ~35 nm more to the red as compared to Chl *a*. *A. marina* thus appears to exploit the far red-rich light that penetrates the extreme shade of the environment where it lives [7]. The shade is intensified because it lives underneath a marine colonial ascidian which has a thick layer of the prochlorophyte, *Prochloron didemnii*, living symbiotically within the tunic. This absorbs most of the visible light [7] before it can reach *A. marina*. The other strains also live in a shade environment (see Ref. [8]).

The red shift in absorbance of Chl *d* relative to Chl *a* results in the electronic energy gap of the excited state being 0.1 V lower as compared to Chl *a* and it has been questioned, ever since the organism was first discovered, whether this is

Abbreviations: Chl, chlorophyll; FeCy, ferricyanide; Pheo, pheophytin; PS, photosystem; Q_A, primary plastoquinone acceptor in PS II

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sufficient to drive water splitting in photosystem II (PS II) (e.g. Ref. [9]). Analysis of the cell pigment content shows that there is always some Chl *a* present in both of the *A. marina* strains analysed so far [1,3,5,10], but only at a very low level, $<5\%$ of the total Chl. The fact that some Chl *a*, the main chlorophyll found in oxygenic photosynthetic organisms, is always present in *A. marina*, has thus been suggested to indicate that Chl *d* alone cannot drive water splitting and that it is essential that there is some Chl *a* in the reaction centre for the operation of PS II [5,9]. For this reason various models have been proposed in which Chl *a* is involved in the primary electron transfer steps of PS II (see Refs. [11,12]). *A. marina* has also been shown to have a low level of pheophytin (Pheo) *a* present (and perhaps surprisingly no Pheo *d*). It was suggested therefore that the PS II reaction centre structure is similar to PS II in Chl *a* organisms, with Pheo *a* serving as the primary electron acceptor and Chl *a* as the primary donor [10]. It was also assumed, by analogy to the known X-ray structure of PS II reaction centres [13–15], that there is a pair of Pheo *a* molecules in PS II of *A. marina*, only one of which is redox active and that, as in Chl *a* oxygenic organisms, two molecules of Pheo *a* can be used as a measure of the PS II reaction centre stoichiometry. Recently the electrochromic band of the Qx absorption of Pheo *a*, known as the C550 change, which is induced by the negative charge on Q_A, has clearly been demonstrated in whole cells of *A. marina* [16]. This indicated that Pheo *a* is indeed the primary electron acceptor for PS II in this organism.

The unanswered question, however, is whether the small amount of Chl *a* found in *A. marina* cells is actually associated with PS II. It was suggested, in various models proposed for the reaction centre, that there are 4 Chl *a* per centre [9–11]. However more recently it has been shown that the proportion of Chl *a* to Chl *d* decreases with growth light intensity and the lowest ratio seen is two Chl *a* per two Pheo *a*. This gives an upper limit of two Chl *a* molecules per reaction centre [12]. It was suggested that these are the two central chlorophylls constituting the special pair [12], named in the recent crystal structures P_{D1} and P_{D2} [14,15]. There is no direct evidence that the Chl *a* is actually in the PS II reaction centre and no unambiguous spectroscopic data has been presented so far for the involvement of Chl *a* in the primary photochemistry of PS II.

In PS I of *A. marina* the primary electron donor special pair has been demonstrated to be either a homodimer of the 13^2 epimer Chl *d*′ or a heterodimer (Chl *d*/*d*′) [11]. It is named P740 because it absorbs around 740 nm [17], whereas P700, the primary donor of PS I from oxygenic photosynthetic organisms containing no Chl *d*, absorbs at 700 nm. In contrast the actual pigment composition of the PS II reaction centre is still unclear. No pure PS II core preparations have been fully characterised nor have the spectroscopic properties of the secondary radical pair ($P^+Q_A^-$) been measured.

In this paper we have used a PS II-enriched preparation from *A. marina* and transient absorption difference spectroscopy to measure the $P^+Q_A^-/PQ_A$ absorbance difference spectrum at low temperature in the UV/VIS and the near IR regions. Charge recombination of $P^+Q_A^-$ occurs at 77 K with a half-life of about 2 ms. The spectrum of this decay component clearly indicates that the cation is located on Chl *a* rather than Chl *d* and that the negative charge is on a quinone molecule. We discuss the implication of this finding for primary radical pair formation and stabilization of the positive charge in PS II of *A. marina* and also the significance for the energetics of water splitting in oxygenic organisms in general.

2. Materials and methods

2.1. Acaryochloris marina samples

Cells were grown in artificial sea water plus iron (4 mg/L) at 6–10 μE (m^2 s) (otherwise as described in Ref. [18]) and thylakoid membranes were isolated essentially as before [19]. Sucrose density gradient fractionation of the detergent solubilised (1% n-dodecyl-β-D-maltoside for 45 min at 4 °C) *A. marina* thylakoid membranes were carried out as described [18]. This procedure normally yields three main bands with a high Chl concentration [17] and several much fainter, heavier bands [18]. The top band, (F1) has been identified as mainly containing the intrinsic antenna complexes, known as Pcb proteins, and carotenoid, the second (F2) has both PS I and PS II (probably monomeric complexes) and F3 has a mixture of PS II dimers and PS I trimers [18]. The much less abundant heavier bands were expected to be highly enriched in PS II as the heavy band (F5) has been shown by single particle analysis to contain supercomplexes of PS II double dimers plus Pcb proteins [18]. This is only the case when *A. marina* is grown in the presence of iron (as were the cells used in this study). Only if cells are grown under iron-stress conditions do the heavier bands contain supercomplexes of PS I trimers and Pcb proteins [20]. In order to increase the yield of the PS II-enriched fraction we ran sucrose gradients with very high levels of chlorophyll. Though this procedure did not yield clearly separated fractions, we harvested the lowest region of the gradient, which should contain the PS II supercomplexes, and will refer to it as the 'heavy fraction' (HF).

Transient absorption difference spectra of $P740^+$/P740 [17,21] were measured at room temperature using thylakoids, the third band (F3) and the heavy fraction (HF) in the presence of phenazine methosulphate and ascorbate, in order to determine the PS I content of the different preparations (Chl *d* per PS I). The following ratios were obtained for the PS I content: 1:1.4:0.5 (thylakoids: F3:HF). We determined the PS II content from low temperature absorption difference spectra attributed to the formation and decay of the secondary radical pair in PS II (see Results). The following ratios were obtained for the relative PS II content: 1:0.5:3 (thylakoids:F3:HF). The heavy fraction, which was enriched in PS II was collected, concentrated and stored at −80 °C.

2.2. Transient absorption spectroscopy

For the measurement of absorbance difference spectra, concentrated samples of HF and the other preparations were diluted with a buffer containing 20 mM Mes/NaOH (pH 6.5), 20 mM $CaCl_2$, 20 mM KCl, 0.02% n-dodecyl-β-D-maltoside 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. 1 mM potassium ferricyanide (FeCy) was added to block photochemistry in PS I. For low temperature measurements, the cuvette was placed in a variable temperature liquid nitrogen bath cryostat (Oxford DN1704). Measurements of flash-induced absorbance changes in the micro- to millisecond time range were performed with the apparatus described previously [22]. The spectral resolution was 3 nm. The optical path length was 0.4 cm or 1 cm. The sample was excited with flashes from a Xe-flash lamp (pulse duration 10 μs) whose emission was filtered by a coloured glass filter.

3. Results

Flash-induced absorbance changes at 77 K were measured in a PS II-enriched preparation isolated from *A. marina* (the heavy fraction, HF, see Materials and methods). FeCy was added to pre-oxidize P740 and cytochrome *b-559*. Thereby, we prevented

any absorbance changes due to photochemistry in PS I and the accumulation of Q_A^--Cyt b-559+, which is irreversibly formed with a low quantum yield at low temperature [22]. The time-course of the absorbance changes observed under these conditions is close to a mono-exponential decay. Fitting the decay with a single exponential plus a constant yielded a half-life of 2 (±0.3) ms which resembles that found in oxygenic photosynthetic organisms containing no Chl *d* [22,23]. Based on the kinetics and spectrum (see below) the flash-induced absorbance changes are attributed to the formation and subsequent decay of the secondary radical pair in PS II, $P^+Q_A^-$. It should be noted that measurements on a shorter time scale revealed an additional faster decay component with a half-life of about 200 μs which can be attributed to the formation and decay of a Chl *d* triplet state [Schlodder et al., unpublished results].

In order to obtain the absorbance difference spectrum associated with the formation of the secondary radical pair in PS II from *A. marina* the amplitude of the flash-induced absorbance changes was measured as a function of the wavelength. Fig. 1 shows the amplitude of the 2 ms component (see above) for the red region between 630 nm and 770 nm at 77 K and 260 K. The 77 K spectrum is dominated by the negative peak around 727 nm and the positive peak around 719 nm. The areas enclosed by the positive and negative peak are equal indicating an electrochromic blue shift of the Qy band of a neighbouring chlorophyll *d* molecule within the reaction centre. The shift to the blue is induced by the electric field of P^+ and Q_A^-. In addition, the spectrum reveals two negative bleaching bands; the stronger one is centred at 698 nm and the smaller one at 672 nm. A smaller band shift centred at 693 nm has also been resolved at 77 K. Upon repeated measurements at the same wavelength a decrease of the signal amplitude was observed and the difference spectra had to be corrected for the signal loss. This decrease can be explained by an electron transfer from secondary donors, e.g. carotenoid, to

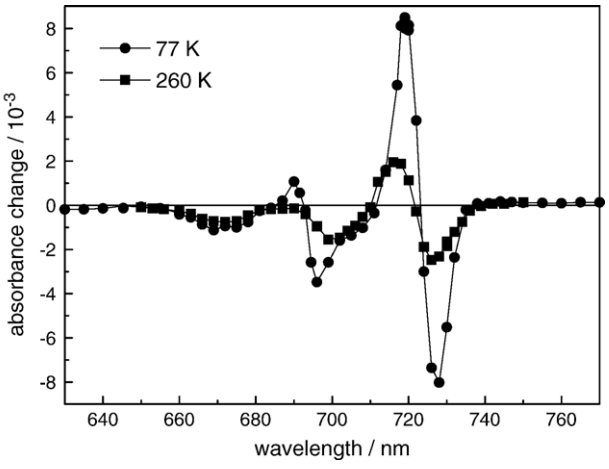

Fig. 1. Spectrum of the flash induced absorbance changes in the wavelength region between 630 nm and 770 nm measured in a PS II-enriched sample (HF) from *A. marina* in the presence of 1 mM FeCy at 260 K (squares) and 77 K (circles) attributed to the formation of the secondary radical pair, P^+Q^-. The circles represent the amplitude of the flash induced absorption changes at 77 K decaying with a half-life of ~2 ms. The squares represent the amplitude of the flash induced absorption changes at 260 K decaying with a half-life of ~1 ms.

P^+ that occurs with low quantum yield in competition with the recombination of $P^+Q_A^-$ (not shown) [22].

At the higher temperature (260 K) the absorption changes due to electrochromic band shifts decreased in size but the amplitudes of the bleached bands were essentially temperature independent. The dominant half-life of the secondary radical pair decay, however, decreased from about 2 ms at 77 K to about 1 ms at 260 K. In oxygenic photosynthetic organisms containing no Chl *d* the recombination of $P^+Q_A^-$ has been reported to be multiphasic with half-lifetimes of 170 μs, 800 μs and 6 ms at room temperature [24]. Different decay phases were not resolved in this work, but this is probably because of the lower signal-to-noise ratio of the *A. marina* preparations.

We have also seen very similar low temperature absorption difference spectra in the F3 fraction, thylakoid membranes and even in cells, in the presence of FeCy, although the signal size decreased relative to the chlorophyll concentration, as expected for less PS II-enriched material. Based on the size of the absorbance changes the following ratios were obtained for the PS II content of the different samples: 1:1:0.5:3 (cells: thylakoids:F3:HF). The fact that the same spectrum can be seen in this range of preparations and in cells supports the conclusion that this difference spectrum is due to the formation of the PS II secondary radical pair, $P^+Q_A^-/PQ_A$. A similar room temperature absorption difference spectrum has been reported by Itoh and coworkers measured in thylakoids of *A. marina* [25]. In solubilized PS II complexes, however, they found only one bleaching band, centred at 725 nm. The absorption difference spectrum they observed in solubilised PS II samples resembles that of the faster decay component we found. The difference spectrum of our fast decay, which has a half-life of about 200 μs, showed a negative peak at 727 nm with a full width at half maximum of about 9 nm (not shown).

The $P^+Q_A^-/PQ_A$ difference spectrum in the Qy region, shown in Fig. 1, looks rather different from those observed in PS II of higher plants or cyanobacteria (see e.g. Refs. [22,26]) because of the presence of Chl *d*. It does not allow a straightforward and definite conclusion as to whether the cation is located on a Chl *a* or Chl *d* molecule or whether Chl *d* is used in the special pair as proposed by Itoh and coworkers [25].

We therefore measured the $P^+Q_A^-/PQ_A$ absorbance difference spectrum in PS II from *A. marina* from 270 nm to 610 nm at 77 K using the HF preparation to achieve a good signal-to-noise ratio (Fig. 2). Surprisingly, the spectrum strongly resembles that of $P680^+Q_A^-/P680Q_A$ normally seen in this wavelength region in Chl *a* organisms (see e.g. Refs. [22,24]). The C550 feature, due to the electrochromic shift of the Qx band of Pheo *a* by the negatively charged Q_A, is well resolved and is located at exactly the same wavelength position (543 nm) as in PS II core complexes from *Thermosynechococcus elongatus*. The main bleaching in the Soret region caused by the oxidation of P is at about 435 nm which is the same position as observed with PS II core complexes from *T. elongatus*, within the accuracy of the monochromator [22,24]. This result is evidence that the positive charge is located on a Chl *a* molecule and not Chl *d*. The oxidation of Chl *d* gives rise to bleaching of a band at about 455 nm in the Soret region, as observed in PS I complexes from

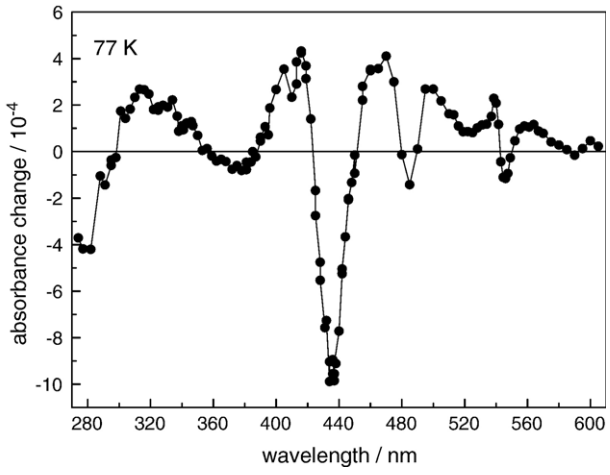

Fig. 2. Spectrum of the flash-induced absorbance changes at 77 K decaying with a half-life of ~2 ms in the wavelength region between 270 nm and 610 nm measured in PS II HF from *A. marina* in the presence of 1 mM FeCy. The absorption difference spectrum is attributed to the formation of the secondary radical pair, P^+Q^-.

A. marina by Hu et al. [17] and others [21,25]. Below 400 nm the spectrum exhibits the characteristic features due to Q_A (plastoquinone) reduction [22]. The only significant differences between the $P^+Q_A^-/PQ_A$ absorbance difference spectra in the 270 nm to 610 nm region measured with PS II from *A. marina* (see Fig. 2) and PS II from oxygenic photosynthetic organisms containing no Chl *d* (see e.g. Ref. [22]) are observed between 460 nm and 500 nm where the spectrum shown in Fig. 2 exhibits a rather pronounced negative peak at about 485 nm and two positive peaks at 465 nm and 497 nm. These features might be caused by electrochromic band shifts of Chl *d* present in the PS II reaction centre.

To confirm the conclusion that the cation radical in PS II of *A. marina* is stabilized on Chl *a* and not Chl *d,* we then measured the absorption spectrum of the radical cation in the near infrared. In Fig. 3A the $P^+Q_A^-$ spectrum in PS II of *A. marina* is compared with that from a *T. elongatus* PS II core preparation, measured at 77 K in the presence of FeCy. The absorption of the radical cation (P^+) clearly peaks around 820 nm in both cases and the spectra are strikingly similar above 750 nm. The large negative band of the *A. marina* spectrum below 750 nm is due to the electrochromic shift shown in Fig. 1. For comparison Fig. 3B shows the absorption spectra of the radical cation (P^+) in PS I from the two organisms, measured in the presence of phenazine methosulphate and ascorbate. Here the broad absorption peak from the *A. marina* radical cation ($P740^+$) absorbs approx. 40 nm more into the near infra red relative to the Chl *a* radical cation ($P700^+$) from *T. elongatus*, which is consistent with the 40 nm red shift of the Qy bleach [17] of P740 relative to P700.

4. Discussion

To understand the photochemistry in PS II of the predominantly chlorophyll *d* containing cyanobacterium, *A. marina,* it is of crucial importance to identify the cofactors involved in electron transfer from water to plastoquinone. The X-ray structure of PS II core complexes from the cyanobacterium *T. elongatus* is available down to 3.0 Å resolution [15]. The strong sequence homology of the D1 and D2 proteins between *A. marina* and other cyanobacteria suggests that the 3-dimensional structure of the reaction centres is very similar. In fact all the known pigment and other cofactor binding sites are conserved [27]. The most interesting question is which cofactors are exchanged in order to adapt the reaction centre to the Chl *d* containing antenna system which powers the transfer of electrons from water to plastoquinone.

In the present work, we have measured the complete spectrum of flash-induced absorbance changes in PS II-enriched samples at low temperature. The spectrum obtained, when PS I absorption changes are prevented by the presence of the oxidant FeCy, can be attributed to the formation and subsequent charge recombination of the secondary radical pair, $P^+Q_A^-/PQ_A$, in PS II. In the Soret and the near infrared region the difference

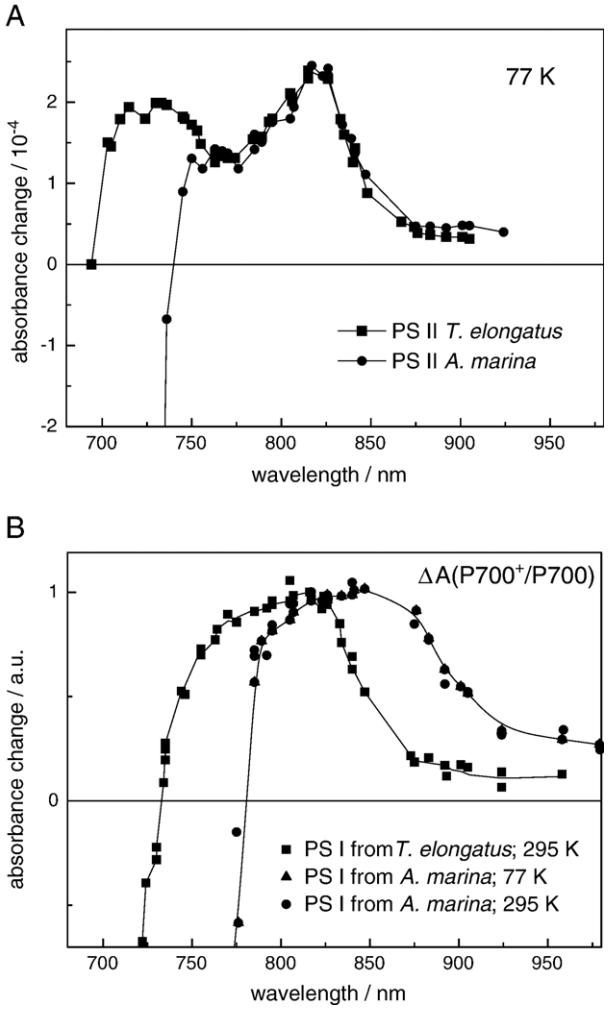

Fig. 3. Absorption difference spectra of PS I and PS II from *A. marina* and *T. elongatus* measured in the NIR region. (A) Difference spectrum of P^+Q^-/PQ in PS II of *T. elongatus* (squares) and *A. marina* (circles) at 77 K. (B) Difference spectrum of P^+/P in PS I of *T. elongatus* (squares) measured at 295 nm and *A. marina* (circles and triangles) at 295 K and 77 K respectively, in the presence of 5 mM ascorbate and 10 μM phenazine methosulphate.

spectrum (Figs. 2 and 3A) exhibits a number of spectral features characteristic of $P^+Q_A^-/PQ_A$ formation in PS II of higher plants and cyanobacteria: the bleaching around 270 nm, the absorbance increase around 320 nm and the C-550 band shift in the green wavelength region indicate the reduction of the plastoquinone acceptor, Q_A. The C-550 band shift confirms earlier reports that Pheo *a* is a component of the active electron transfer branch in the reaction centre, i.e. it is the primary electron acceptor in PS II of *A. marina* [16,19]. The narrow bleaching around 435 nm [22,24] and the spectrum in the near-infrared, with the typical maximum around 820 nm [28], shows clearly that the species oxidised is a Chl *a* molecule. In PS II from oxygenic photosynthetic organisms containing no Chl *d* the cation has been shown to be stabilized mainly on P_{D1}, the chlorophyll adjacent to Tyr_Z [26,29]. Assuming that this is also the case in *A. marina* we conclude that this cofactor (P_{D1}) is Chl *a* and not Chl *d*.

Thus far the spectroscopic data are consistent with a pigment organisation which is the same as in the PS II reaction centre of oxygenic photosynthetic organisms containing no Chl *d*, i.e. P_{D1} is a Chl *a* molecule, $Pheo_{D1}$ is a pheophytin *a* and Q_A is a plastoquinone. The $P^+Q_A^-/PQ_A$ absorbance difference spectrum in the red (Fig. 1) is, however, very different from that seen for PS II complexes from *T. elongatus* or *Synechocystis* 6803 [26,30]. The strong electrochromic band shift, centred at 723 nm, which is observed in the $P^+Q_A^-/PQ_A$ difference spectrum (see Fig. 1), can be attributed to an electrochromic effect of P_{D1}^+ and Q_A^- on the neighbouring accessory Chl_{D1}. In PS II of higher plants and cyanobacteria, containing no Chl *d*, a strong band shift has been observed in the low temperature $P^+Q_A^-/PQ_A$ difference spectrum. In this case the blue shift is centred at 681 nm and can be attributed to an electrochromic effect of P_{D1}^+ and Q_A^- on the neighbouring accessory Chl *a* molecule, Chl_{D1} [26]. The Qy band of the Chl being blue shifted in PS II of *A. marina* at 77 K is around 727 nm (see Fig. 1). This long wavelength absorption maximum indicates that it is a Chl *d* molecule. Taking into account an upper limit of two Chl *a* molecules per reaction centre [12], it is reasonable to assume that the accessory Chl on the inactive branch Chl_{D2} is also a Chl *d* molecule.

The above conclusions, that the cation is stabilized on a Chl *a* molecule and that the accessory Chl of the active branch of the RC of PS II (Chl_{D1}) is a Chl *d*, are not affected by the residual PS I content or a knowledge of the pigment stoichiometry of the sample since the corresponding bands of the difference absorbance spectra can clearly be assigned to PS II (see above) and are also observable in thylakoids and even in whole cells of *A. marina* (not shown). Additionally, other artefacts can be excluded because the absorption changes, decaying with about 2 ms, are susceptible to the oxidation state of Q_A. If Q_A is reduced before freezing, either chemically or by preillumination in the presence of DCMU, the 2 ms signals are not observed.

As the pigment composition of the PS II reaction centres from *A. marina* is heterogeneous (i.e. contains Chl *a*, Chl *d* and Pheo *a*) it is obvious that multimer models to describe the spectral properties can be excluded (see Ref. [31]). These models assume that the transition energies of the pigments are nearly equal, which is certainly not the case for Chl *a* and Chl *d*. Additionally, taking into account the smaller distance between the special pair chlorophylls as compared to the distance between the other cofactors (seen in the recent structural studies; [14,15]), P_{D1} and P_{D2} may be regarded as an excitonically coupled dimer. Upon oxidation of P, the excitonic interaction is lost, resulting in two non-interacting Chls, one in the oxidized state (P_{D1}) and one in the singlet ground state. Thereby, both excitonic absorption bands of P are bleached and the two bleaching bands, observed below 700 nm, may be explained in this simple model as follows. The main bleaching band around 698 nm is attributed to the bleaching of the lower Qy exciton band upon oxidation of P and the smaller bleaching band around 672 nm would correspond to the bleaching of the upper Qy exciton band. From the separation in energy of the two bands the excitonic interaction energy can be calculated to be about 270 cm^{-1}. From the ratio between the dipole strengths of the two bands the angle between the transition dipoles of the two unperturbed Qy transitions can be estimated to be about 110°. Similar values can be derived from the PS II structures for the special pair [14,15] using the point dipole approximation and the assumption that P is constituted of two Chl *a* molecules with approximately the same transition energy. The exciton interaction is weak compared with the 550–950 cm^{-1} interaction energy of the strongly coupled special pair in bacterial reaction centres [32,33] and the intermediate value of 420 cm^{-1} calculated within the point dipole approximation for P700 [34]. It should be noted that there is no direct spectroscopic evidence for the assumption that P_{D2} is also a Chl *a* molecule. We cannot exclude that the special pair is a heterodimer composed of one Chl *a* molecule (P_{D1}) and one Chl *d* molecule (P_{D2}).

Based on the assignments discussed above, the accessory Chl *d* molecules have the lowest transition energy and function as a long-wavelength trap for the excitation energy within the reaction centre. The transition energy of Chl_{D1} corresponds to a wavelength of ~727 nm (see above), i.e. the accessory Chl *d* of the active branch, Chl_{D1}, is significantly red shifted compared to the Qy absorption maximum of HF (708 nm at 77 K) or that of thylakoids from *A. marina* (710 nm at 77 K). Therefore the singlet excitation is presumably exclusively trapped on Chl_{D1}, in particular, at low temperature. The localization of singlet excitation on Chl_{D1} (727 nm) over P (698 nm) would be favoured much more strongly than in 'normal' PS II. The Boltzmann factor $\exp(-\Delta E/kT)$, where ΔE is the difference between the excitation energies of Chl_{D1} and P, is about 15, even at room temperature, whereas at 5 K it would be a factor of 10^{67} smaller. Therefore, as in the mechanism proposed for 'normal' PS II (see Ref. [26]), we assume that Chl_{D1} (i.e. Chl *d*) accepts excitation energy from the antenna and, at least at low temperature, the excited state, Chl_{D1}^*, initiates primary charge separation. The cation, formed by the primary charge separation in PS II, is then stabilized primarily on P_{D1}, a Chl *a* molecule. Based on a photon echo study a similar mechanism was suggested for primary charge separation in D1–D2–cyt-b559 reaction centres from spinach [35]. It should be noted that the P^+ cation may be localized to a minor extent on P_{D2} as proposed earlier [26,36–38]. Light-induced electron transfer in PS II has

been observed at 77 K (see above), and even at 1.7 K Q_A reduction and cytochrome *b-559* oxidation has been demonstrated by Razeghifard et al. [16]. Therefore, it seems reasonable to assume that the transition energy of Chl_{D2} is higher than that of Chl_{D1}.

The arrangement of the cofactors deduced from the data corresponds to a pigment stoichiometry of 2 Pheo *a* ($Pheo_{D1}$ and $Pheo_{D2}$):2 Chl *d* (Chl_{D1} and Chl_{D2}): 2 Chl *a* (P_{D1} and P_{D2}). This would be in agreement with the pigment analysis of whole cells [12] giving an upper limit of two Chl *a* per two Pheo *a* if we assume that all other chlorophylls bound to PS II from *A. marina* are of the *d*-type i.e. also $Chlz_{D1}$ and $Chlz_{D2}$ must be replaced by Chl *d* molecules. Indeed, our spectroscopic data give strong evidence for the model of Mimuro and coworkers for the pigment arrangement [11,12] based on their pigment analysis. The proposed pigment composition is however in contradiction to two recent papers [19,39]. In the former the following stoichiometry for a 'crude PS II reaction centre' preparation has been determined: 20 Chl *d*:2 Pheo *a*:0.5 Chl *a*:1 cytochrome *b559*. Even though we cannot be certain that the assignment of the pigments located on the inactive cofactor branch is correct, the data presented here show that at least 1 Chl *a* per reaction centre must be present. Swingley et al. [39] also found Chl *a* to be low in an analysis of high iron grown *A. marina* cell pigment content; 1.4 Chl *a* per 2 Pheo *a.* which might indicate that the special pair is indeed a heterodimer composed of one Chl *a* molecule (P_{D1}) and one Chl *d* molecule (P_{D2}).

The data presented in this work give clear evidence that the positive charge is stabilized on at least one chlorophyll *a* molecule when the 'stable' radical pair, $P^+Q_A^-$, has been formed. Based on the proposed assignment that the accessory chlorophyll Chl_{D1} is a Chl *d* molecule we suggest that Chl *d** initiates the primary charge separation in PS II of *A. marina* and hence Chl *d* must be 'tuned' by the protein to a high oxidising potential. The oxidation potential of Chl *d* in acetonitrile has been recently reported to be somewhat more positive than that of Chl *a* (+0.88 V vs. +0.81 V) [M. Kobayashi et al., see this issue of BBA Bioenergetics]. The stabilization of the positive charge on Chl *a* is intriguing. It was proposed to be the primary electron donor in PS II because Chl *d* has a lower excitation energy than Chl *a*, and it has been suggested that the energy gap of the excited state of Chl *d* (1.7 eV) is insufficient to drive water oxidation. However, as we have shown here, the Chl *d* suggested to be in the Chl_{D1} site is presumably the most oxidising species in the reaction centre, because it must be able to oxidize P. If primary charge separation occurs from Chl *d* this means that there is little need for uphill energy transfer (as energy transfer is only occurring between Chl *d* molecules). Indeed we would not observe efficient $P^+Q_A^-$ formation at low temperature if this was not the case (this work and ref. [16]). Uphill energy transfer has been reported [9] but a different explanation for the delayed fluorescence data was argued by Nieuwenburg et al. [40]. In a more recent and extended study by Petrášek et al. [41] no long-lived recombination fluorescence with an emission spectrum typical for Chl *a* was observed.

It has been speculated that a minor amount of Chl *a* is always present in *A. marina* because Chl *a* is necessary for the operation of PS II. This was suggested because PS II has to achieve a very high oxidising potential in order to extract electrons from water (see [42]) and the ubiquity of Chl *a* in oxygenic photosynthetic reaction centres seems to back up this theory. However, as the whole reaction sequence in PS II leading to water oxidation starts with the excitation of a Chl *d* in the antenna, the lower excitation energy for Chl *d* must be sufficient to drive charge separation and water oxidation in *A. marina*. The Chl *d* from which primary charge separation occurs must have a very high oxidising potential so it is likely that the donor side has very similar redox properties to the 'normal' PS II reaction centre. Indeed Razeghifard et al. [16] concluded this is the case as Tyr_Z oxidation was unperturbed. It is probable that the result of having Chl *d* in the reaction centre means that the redox potential of the excited Chl_{D1} (Chl *d**) is less reducing and so the stability of the primary radical pair may be affected. To clarify this point, measurements of the lifetime of the primary radical pair are in preparation.

Our findings raise the new question why Chl *a* is used to stabilize the positive charge in PS II from *A. marina*? This is unknown but it is possible that adaptation to a Chl *d* antenna in order to exploit the light above 700 nm of the *A. marina* habitat might be a relatively recent adaptation to life in an environment depleted of visible light by Chl *a*-containing organisms [8]. Thus the Chl *a* reaction centre of 'normal' oxygenic organisms has probably been adapted minimally in order to use Chl *d* for the ejection of an electron, to avoid the necessity of too great uphill energy transfer and yet to use a Chl *a*/Pheo *a* redox couple to stabilize the charge separated state.

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