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Electric field effects on red chlorophylls, β-carotenes and P700 in cyanobacterial Photosystem I complexes

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Abstract

We have probed the absorption changes due to an externally applied electric field (Stark effect) of Photosystem I (PSI) core complexes from the cyanobacteria *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* and *Spirulina platensis*. The results reveal that the so-called C719 chlorophylls in *S. elongatus* and *S. platensis* are characterized by very large polarizability differences between the ground and electronically excited states (with $\mathrm{Tr}(\Delta\alpha)$ values up to about 1000 $\mathrm{Å}^3\ \mathrm{f}^{-2}$) and by moderately high change in permanent dipole moments (with average $\Delta\mu$ values between 2 and 3 D f^{-1}). The C740 chlorophylls in *S. platensis* and, in particular, the C708 chlorophylls in all three species give rise to smaller Stark shifts, which are, however, still significantly larger than those found before for monomeric chlorophyll. The results confirm the hypothesis that these states originate from strongly coupled chlorophyll *a* molecules. The absorption and Stark spectra of the β-carotene molecules are almost identical in all complexes and suggest similar or slightly higher values for $\mathrm{Tr}(\Delta\alpha)$ and $\Delta\mu$ than for those of β-carotene in solution. Oxidation of P700 did not significantly change the Stark response of the carotenes and the red antenna states C719 and C740, but revealed in all PSI complexes changes around 700–705 and 690–693 nm, which we attribute to the change in permanent dipole moments of reduced P700 and the chlorophylls responsible for the strong absorption band at 690 nm with oxidized P700, respectively.

Keywords: *Synechocystis* PCC 6803; *Synechococcus elongatus*; *Spirulina platensis*; Stark spectroscopy; Red chlorophyll; Photosystem I; Exciton interaction

1. Introduction

The role of Photosystem I (PSI) in photosynthesis is to catalyze electron transport from reduced plastocyanin or cytochrome c_6 to ferredoxin. This process involves the capture of (sun-)light, the equilibration and funnelling of the excited state energy towards the photochemically active pigment called P700, the release of an electron by P700 and the subsequent transfer of electrons by the electron transfer chains.

The crystal structure of the PSI core complex from the cyanobacterium *Synechococcus elongatus* has been resolved at 2.5 Å resolution [1]. In this structure, 31 transmembrane α-helices were resolved in each monomeric unit as well as nine surface α-helices. The cofactors of the electron transfer chain are organized along a pseudo-C2 symmetry axis, much like the purple bacterial reaction center. The two major core proteins (PsaA and PsaB) form a heterodimer and bind the primary donor (P700) and four other chlorophylls (Chls) of the reaction center, as well as the majority of the so-called bulk or core-antenna Chl *a* molecules. In *S. elongatus*, each monomeric PSI core complex binds 96 Chl *a* molecules, 22 β-carotenes (of which 17 are in the all-*trans* form and 5 contain one or two *cis* bonds), two phylloquinones and three [4Fe–4S] clusters [2].

The 2.5 Å structure has revealed new details on the organization of the cofactors of the electron transfer chain [1,2]. The distance between the Mg^{2+} ions of the two chlorophylls of P700 is only 6.3 Å, shorter than the distance between the corresponding bacteriochlorophyll molecules in

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the purple bacterial reaction center. The electronic interaction, however, is weaker because of a less favorable overlap between the π electron systems of the two chlorophylls [3]. The primary acceptor A_0 probably absorbs maximally around 690 nm [4].

A remarkable aspect of PSI is the absorption of light at wavelengths longer than that of the primary electron donor. The number, size and absorption maxima of these long-wavelength Chls are species dependent [5,6]. There have been several proposals for the function of these so-called 'red' Chls. They could focus the excitation energy near P700 and thus help to guide the excitations to P700 [7], but this proposal is now less likely because increasing numbers of red pigments have been shown to slow down the trapping of the excitation energy [6]. More likely, possibilities are that they help to increase the cross-section for absorption of light in 'shady' conditions [8,9], and/or are involved in photoprotection [10].

The differences of contents and energies of red pigments among PSI complexes from various species can most easily be observed by their low-temperature emission spectra. The 4 K emission spectrum of PSI from the cyanobacterium *Synechocystis* PCC 6803 shows a maximum near 720 nm [11,12], which was suggested to arise from three to four Chls peaking at about 708 nm [6] or from two pools of two Chls peaking near 708 and 714 nm [12]. The 4 K emission of PSI from *S. elongatus* peaks near 735 nm [13], which was suggested to arise from about four Chls peaking near 719 nm and about four others near 708 nm [13,14]. Also in this complex, a new red state was proposed, peaking at 715 nm [15]. Trimeric PSI complexes from *Spirulina platensis* show a 4 K fluorescence maximum at 760 nm, which originates from an absorption band peaking near 740 nm [6,16,17]. This fluorescence was shown to be strongly quenched by oxidized P700, while in monomeric complexes, this extremely long-wavelength chlorophyll is completely missing [17]. In these complexes, the long-wavelength chlorophylls are very similar to those in *S. elongatus* [5].

A common feature of the long-wavelength emission of all PSI particles is that the emitting species are characterized by a substantial inhomogeneous broadening, of the order of 200–400 cm^{-1} [11–13,18,19] and furthermore by a relatively large Stokes' shift (160–200 cm^{-1} for the red chlorophylls in *Synechocystis* PCC 6803 [11,12]). An even larger Stokes' shift of $\sim$ 420 cm^{-1} has been proposed for the red-most pigments in the plant LHCI complex [20]. The similarities of all these features with those of the special pair of the bacterial reaction center has led to the idea that the chlorophylls responsible for the long-wavelength emission in PSI are strongly excitonically coupled [11,19,21,22] and have significant charge transfer character [12].

A powerful tool to further investigate the electronic nature of the pigments in the core complex of PSI is given by Stark spectroscopy, which monitors the effect of a strong electric field on the spectroscopic properties of the chromophores and allows to probe the strength of the excitonic coupling between pigments and the influence of the protein matrix on this coupling [23]. The interpretation of the Stark spectra relies mainly on two molecular parameters that can be derived from the comparison of the absorption spectrum and the Stark absorbance difference spectrum. The first is given by the change of the permanent dipole moment upon optical excitation ($\Delta\mu$), which results in a Stark spectrum with a line shape resembling the second derivative of the absorption spectrum. The second is given by the difference in polarizability $Tr(\Delta\alpha)$ between the ground- and excited state and gives rise to a Stark spectrum resembling the first derivative of the absorption spectrum.

Monomeric Chl *a* was reported to have a $\Delta\mu$ value of about 0.9–1.0 D f^{-1} and a $Tr(\Delta\alpha)$ value of 1.5–4 $Å^3$ f^{-2}, the variation being dependent on the number of axial ligands [24]. For a Chl *a* dimer, both the $\Delta\mu$ and the $Tr(\Delta\alpha)$ values were shown to be considerably larger ($\Delta\mu \cong 5$ D f^{-1} and $Tr(\Delta\alpha) \cong 90$ $Å^3$ f^{-2}) [24]. In the case of B850 of the bacterial light-harvesting antenna LH2 [25] and the special pair of the purple bacterial reaction center [26], similarly high values were found for $\Delta\mu$ (3–7 D f^{-1}), but $Tr\Delta\alpha$ turned out to be even higher (500–1500 $Å^3$ f^{-2}), which can only be explained by a mixing of charge transfer (CT) states into the excited (excitonic) state by the external electric field [27,28]. High $\Delta\mu$ values can arise from the internal electric field generated by the protein, which transforms the difference polarizability into a difference dipole moment when a Stark experiment is performed [26]. On the other hand, very small $\Delta\mu$ values are expected when the pigments have strongly contrasting orientations, even in case of considerable orbital overlap, as has been shown for chlorosomes [29]. In the CP43 complex of Photosystem II, the Stark spectrum is dominated by excitonic couplings and the $\Delta\mu$ value was even smaller than that of monomeric Chl [30]. For other complexes, such as the plant light-harvesting complex II (LHCII) and the water-soluble bacteriochlorophyll *a* antenna complex (FMO), the Stark parameters did not differ very much from those of free Chl *a* molecules [31–33], which was taken to imply that in these complexes excitonic interactions are not very significant.

Carotenoids usually give rise to very strong Stark effects, because their long polyene chains allow high polarizability values [34]. Some carotenoids, however, are mostly characterized by a high change in permanent dipole moment in the excited state [35], which is generally ascribed to a static field originating from charged groups in the surrounding protein matrix. This field induces a difference dipole, which then interacts with the applied field. Although β-carotene is not much different from the carotenoids reported by Gottfried et al. [35], it does not respond as strongly to variations of polarity in different solvents [36,37].

With regard to PSI, only a few Stark studies have been reported so far. Stark spectroscopy studies have been presented of PSI reaction centers from spinach, which have been treated with strong detergent [38] or organic solvents [39], but these preparations lack at least some of the antenna

chlorophylls and red chlorophylls and are therefore only relevant for the Stark properties of the reaction center components. These studies revealed that the Stark spectrum of P700 resembles the second derivative of the absorption spectrum and that P700 has a large change in permanent dipole moment ($\Delta\mu \cong 5$ D f^{-1}), in line with its attribution to an exciton-coupled dimer. With oxidized P700, a Stark feature around 687 nm with a second-derivative line shape was seen and attributed to the chlorophyll of P700 that remains neutral after photooxidation.

Recently, Stark holeburning studies have been presented of PSI complexes from *Synechocystis* PCC 6803 and some mutants [12] and from *S. elongatus* [15,40]. The Stark holeburning technique, however, is mainly sensitive to permanent dipole effects, although it more clearly distinguishes between the various electronic states [12,15].

Here we present the results of electric field modulated absorption spectroscopy studies on PSI complexes obtained from the cyanobacteria *Synechocystis* PCC 6803, *S. elongatus* and *S. platensis*. The results reveal strong electric field responses for the pigments involved in the various red states of these PSI complexes, which we attribute to strong polarizability effects rather than to a difference dipole moment. The results thus confirm their special properties, characteristic for strongly coupled dimers or larger aggregates of chlorophyll *a*. We show that the Stark response of the β-carotene molecules in the various complexes is relatively strong and that oxidation of P700 leads to characteristic Stark changes in the Q_y absorption region of the chlorophylls, but not in the main absorption region of the β-carotenes.

2. Materials and methods

Trimeric PSI complexes from *Synechocystis* sp. PCC 6803 were isolated as described by Kruip et al. [41], trimeric PSI complexes from *S. elongatus* were isolated as described by Fromme and Witt [42], and monomeric and trimeric PSI complexes from *S. platensis* were isolated as described by Kruip et al. [43]. The PSI complexes and thylakoid membranes from *Synechocystis* sp. PCC 6803 and *S. elongatus* were dissolved in a buffer containing 20 mM Bis–Tris, 20 mM $MgCl_2$, 10 mM $CaCl_2$, 0.04% *n*-dodecyl-β-maltoside and 55% (w/v) glycerol at pH 6.5, while for the PSI complexes from *S. platensis* the Bis-Tris in the buffer was replaced by 10 mM Tris–HCl at pH 7.8. For the preparation of samples with reduced P700, 5 mM ascorbate and 3 μM phenazine methosulfate were added to the buffer and the samples were slowly frozen to 77 K in the dark for 30 min. For the preparation of samples with oxidized P700, the addition of ascorbate and phenazine methosulfate was omitted, and the complexes were subjected to continuous white light illumination during 10 min, after which they were slowly cooled to 77 K in the light for 20 min. We note that it is not exactly known to which extent P700 was reduced or oxidized during our measurements under both preparation protocols. The absorbance difference spectra between the corresponding samples with oxidized and reduced P700 resembled those recorded before at 5 K [44], but it is hard to quantify the changes observed in these spectra. Nevertheless, subtraction of the Stark spectra recorded with 'reduced' and 'oxidized' P700 should reveal the Stark features of P700 oxidation.

Stark and absorbance spectra were recorded simultaneously on a homebuild setup [29,45] at a spectral resolution of 1 nm and at 77 K. The applied electric fields were 2.3×10^5 V cm^{-1} and the angle between the electric field and the probing light beam was set at magic angle.

For randomly oriented and fixed molecules, the Stark line shape can be described by a sum of the zeroth, first and second derivatives of the ground-state absorption spectrum [27,46,47] and is given by Eq. (1):

$$\Delta A(\nu) = (fF_{\mathrm{ext}})^2 \left\{ A_\chi A(\nu) + \frac{B_\chi}{15hc}\nu\frac{\mathrm{d}}{\mathrm{d}\nu}\left[\frac{A(\nu)}{\nu}\right] + \frac{C_\chi}{30h^2c^2}\nu\frac{\mathrm{d}^2}{\mathrm{d}\nu^2}\left[\frac{A(\nu)}{\nu}\right]\right\} \quad (1)$$

In Eq. (1), ν is the energy in wave numbers, h is Planck's constant, c is the speed of light, F_{ext} is the externally applied field and f is the local field correction factor that relates the applied electric field to the electric field at the site of the molecule. A_χ, B_χ and C_χ are weight factors depending on the experimental angle χ between the electric vector of the linearly polarized probe light and the direction of the applied field. If we neglect the electric field effect on the optical transition moment [26], A_χ becomes zero and B_χ and C_χ are described by Eqs. (2) and (3), respectively:

$$B_\chi = \frac{1}{2}\mathrm{Tr}(\Delta\alpha)\left[5 + (3\cos^2\chi - 1)\left(3\frac{\boldsymbol{p}\cdot\Delta\alpha\cdot\boldsymbol{p}}{Tr(\Delta\alpha)} - 1\right)\right] \quad (2)$$

$$C_\chi = |\Delta\mu|^2[5 + (3\cos^2\chi - 1)(3\cos^2\zeta - 1)] \quad (3)$$

in which $\boldsymbol{p}$ is a unit vector in the direction of the transition dipole moment and ζ is the angle between $\boldsymbol{p}$ and $\Delta\mu$. When χ is at magic angle, all angle dependencies vanish. The second derivative contribution scales with the size of the difference in permanent dipole moment $\Delta\mu$ between the excited- and ground state of the molecule. The first derivative contribution scales with the size of $\mathrm{Tr}(\Delta\alpha)$, which is a measure for the difference polarizability between the excited- and ground state $\Delta\alpha$. The zeroth derivative is a measure for the field dependent changes of the oscillator strength of the optical transition.

The Stark and absorbance spectra were fitted simultaneously using a nonlinear least squares fitting program as described in Ref. [29]. The absorption spectra were fitted with a number of Gaussian functions, while the Stark spectra were fitted with the first and second derivatives of these functions (Gaussian fit). Alternatively, the absorption of the red chlorophylls can also be assumed to be caused by

a single homogeneously broadened transition, which was then fitted with a polynomial function (spline) and the Stark spectrum with the zeroth, first and second derivative of this function (spline fit). The method used to extract Stark parameters is stated in the text and figure legends. Because the local field correction factor f is hard to estimate, all values for $\Delta\mu$ and $\mathrm{Tr}(\Delta\alpha)$ are represented in terms of D f^{-1} and $Å^3$ f^{-2}, respectively (1 D = 3.34×10^{-30} C m, 1 $Å^3$ = 1.113×10^{-40} C m^2 V^{-1}).

3. Results and discussion

3.1. Stark spectra of PSI from Synechocystis sp. PCC 6803

The 77 K absorption and Stark spectra of trimeric PSI complexes from *Synechocystis* PCC 6803 with reduced and oxidized P700 are shown in Fig. 1. The absorption spectrum in the Chl *a* Q_y region (Fig. 1A) is characterized by a peak near 678 and shoulders at 670 and 705 nm, in agreement with earlier reports (see, e.g., Ref. [48]). The 670 and 678 nm bands represent the two main pools of bulk antenna pigments, while the 705 nm shoulder represents the pool(s) of red pigments [6,12]. More to the blue, the β-carotenes absorb with maxima near 500 and 466 nm and a shoulder at 528 nm.

The 77 K Stark spectrum of these complexes with reduced P700 is shown in Fig. 1B. A first inspection indicates that the Stark response of the carotenes is quite pronounced (the magnitude of the Stark response should be compared with the magnitude of the absorption band from which it originates) and shows peaks with maxima at 521 and 480 nm, a minimum at 498 nm and a shoulder at 540 nm. In the Q_y-region, the Stark spectrum shows a broad positive band centered at 716 nm, a positive peak at 695 nm and negative peaks at 701, 687, 675 and 666 nm. In diethyl ether treated PSI particles, reduced P700 was reported to give a second-derivative-like feature with a strongly negative band at 701 nm and weak positive contributions near 688 and 715 nm [39]. Because the spectrum in Fig. 1B also shows a small negative band at 701 nm, it is likely that part of the spectrum in Fig. 1B originates from P700.

The Stark spectrum with oxidized P700 is shown in Fig. 1C. This spectrum is rather similar to that with reduced P700 at all wavelengths shorter than about 670 nm, but is significantly different at longer wavelengths. The differences between these spectra will be discussed in more detail in Section 3.6 together with the corresponding Stark spectra of the other investigated PSI core complexes.

3.2. Stark spectra of PSI from S. elongatus

Fig. 2 shows the simultaneously recorded absorption and Stark spectra of the trimeric PSI core complex with reduced and oxidized P700 from *S. elongatus* at 77 K. The absorption spectrum (Fig. 2A) shows maxima in the Q_y region at

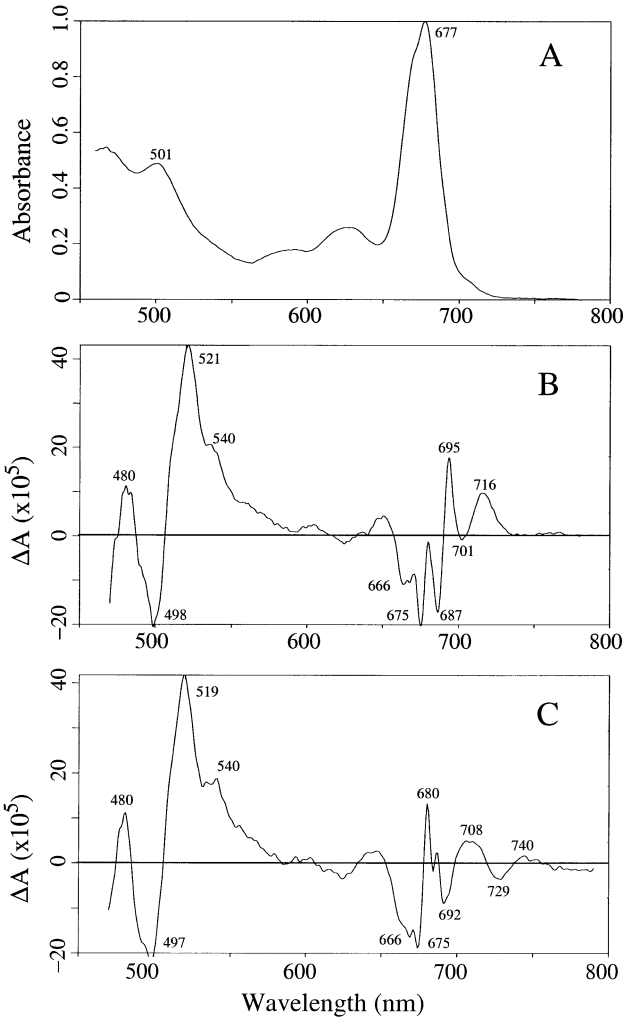

Fig. 1. Absorption (A) and Stark spectra at 77 K with reduced (B) and oxidized (C) P700 of trimeric PSI core complexes from *Synechocystis* sp. PCC 6803. All spectra were normalized to OD = 1 at 680 nm. The Stark spectra were recorded at magic angle and with electric field strength $V_{rms} = 2.3 \times 10^5$ V cm^{-1}.

679 with a shoulder at 670 and at 707 nm. The red-most pool at 707 nm has a slight shoulder at 715 nm. The 4 K absorption spectrum has been shown to have fine structure with pronounced bands at 708 and 719 nm [13–15], but otherwise, these spectra are very comparable.

The Stark spectrum with reduced P700 is shown in Fig. 2B. In the red-most part, it shows a positive peak at 725 nm and a negative peak at 705 nm with a zero crossing at 715 nm. We will show below (Section 3.6) that only a small part of the negative peak at 705 nm is caused by P700. Further to the blue, positive peaks are found at 692 and 681 nm and negative peaks at 678 and 664 nm. Comparison with the corresponding spectrum of *Synechocystis* PCC 6803 in the Q_y absorption region of the chlorophylls (Fig. 1B) reveals that differences manifest themselves especially above 690 nm, though the magnitude of the field effect is similar. Especially the Stark effect of the red-most bands is very different from what we found for *Synechocystis* PCC 6803.

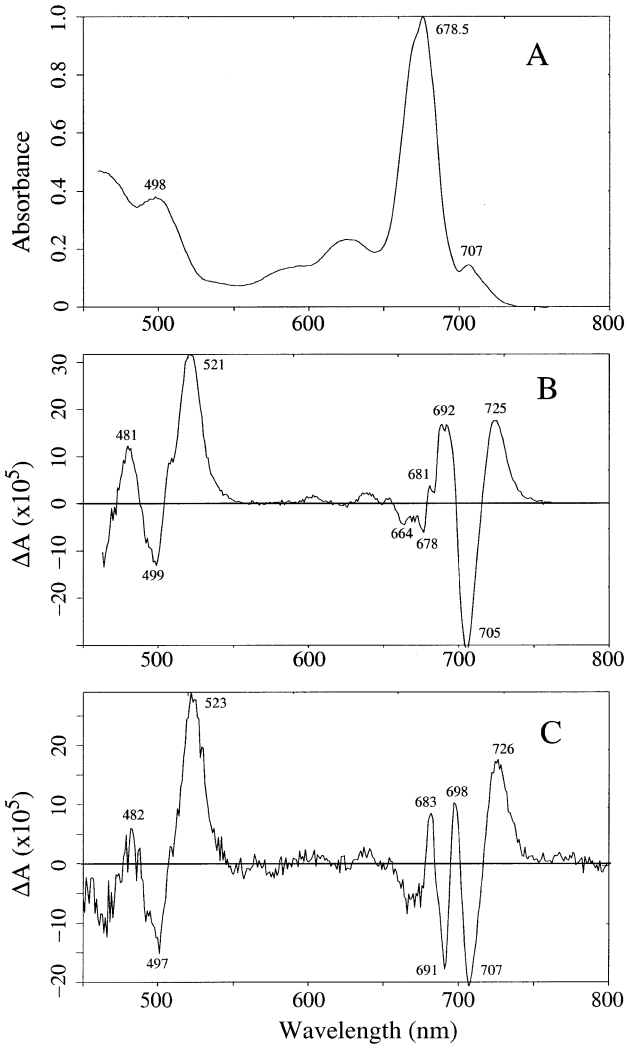

Fig. 2. Absorption (A) and Stark spectra at 77 K with reduced (B) and oxidized (C) P700 of trimeric PSI core complexes from *S. elongatus*, recorded as in Fig. 1.

This points towards a different origin of the red absorbing transitions for the two species. We note that many pigments are involved in these systems and that variations in the orientations and/or interaction strengths of a small fraction of these pigments can in principle give rise to large variations in the Stark effect.

Below 550 nm, the carotenes show an absorption maximum at 498 nm (Fig. 2A) and a strong Stark effect with a positive features at 521 and 480 nm and a negative feature at 498 nm (Fig. 2B). These features are essentially identical to those in *Synechocystis* PCC 6803 (Fig. 1B), except that the shoulder at 540 nm is not present in *S. elongatus*.

3.3. Stark spectra of PSI from S. platensis

Fig. 3 shows the simultaneously recorded absorption and Stark spectra of the monomeric PSI core complex with reduced and oxidized P700 from *S. platensis* at 77 K. The absorption spectrum (Fig. 3A) is very similar to that of *S. elongatus* (Fig. 2A), though the amplitude of the C719 band seems to be smaller. A comparison between the 6 K spectra of both complexes has been reported before by Gobets et al. [6] and Gobets and van Grondelle [5].

The Stark spectrum (Fig. 3B) is very similar to the corresponding spectrum of PSI from *Synechococcus* (Fig. 2B). The most prominent Stark effect is again found for the red absorbing species, with a positive peak at 724 nm and a negative peak at 707 nm. The relative amplitudes of these bands, however, are smaller than those of *Synechococcus*, which correlates with the lower content of C719 in *Spirulina*. Further towards the blue, positive peaks are found at 690 and 683 nm and negative peaks at 677 and 668 nm. The largest difference with the Stark spectrum of PSI from *S. elongatus* is the larger negative amplitude of the 677 nm band. Also, the carotenoids show similar Stark shifts with corresponding magnitudes, though all bands are red-shifted by 2–4 nm compared to those in PSI from *Synechococcus* and *Synechocystis*.

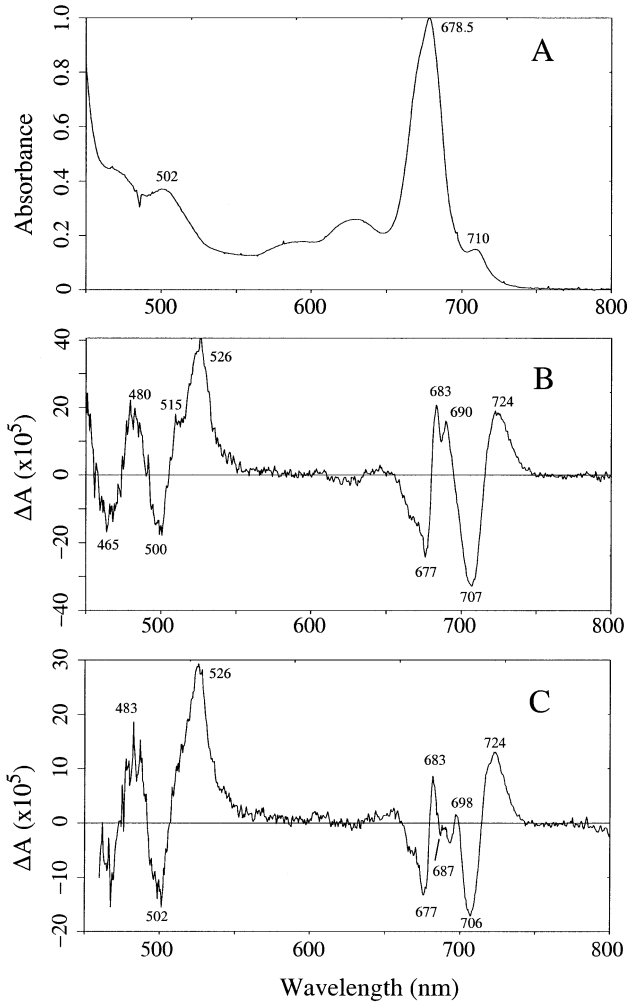

Fig. 3. Absorption (A) and Stark spectra at 77 K with reduced (B) and oxidized (C) P700 of monomeric PSI core complexes from *S. platensis*, recorded as in Fig. 1.

The highly similar Stark fingerprints indicate that the monomeric PSI complexes from *S. platensis* and the trimeric PSI complexes from *S. elongatus* are essentially of the same type. Also, their excited state dynamics are very similar [6]. It is therefore tempting to postulate that the chlorophylls of both systems have a very similar organization and electronic interactions with the molecular environment.

Trimeric PSI complexes from *S. platensis* exhibit the largest red shifted-absorption band of all PSI core complexes. In Fig. 4, the 77 K absorption (A) and Stark spectra (B,C) are shown. The absorption spectrum shows a separate absorption band at 739 nm, commonly denoted as C740. All other features in the absorption spectrum, including the 710 nm band, seem identical to those of the monomeric PSI complexes from *S. platensis* (Fig. 3, see also Ref. [5]). The difference spectrum between the 77 K absorption spectra of the trimeric and monomeric PSI complexes suggests that the C740 absorption in the trimers did arise at the expense of

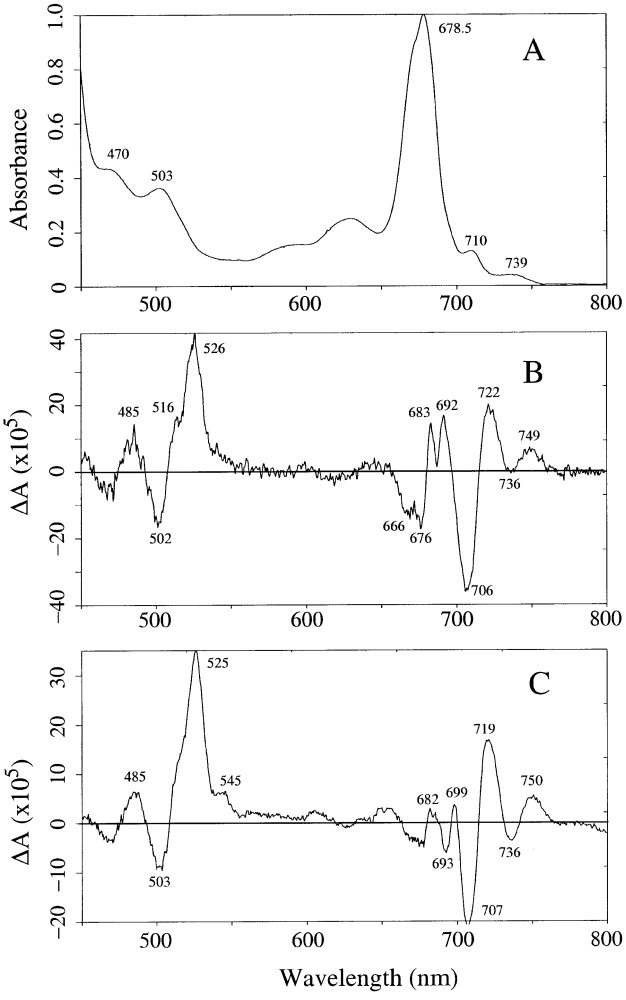

Fig. 4. Absorption (A) and Stark spectra at 77 K with reduced (B) and oxidized (C) P700 of trimeric PSI core complexes from *S. platensis*, recorded as in Fig. 1.

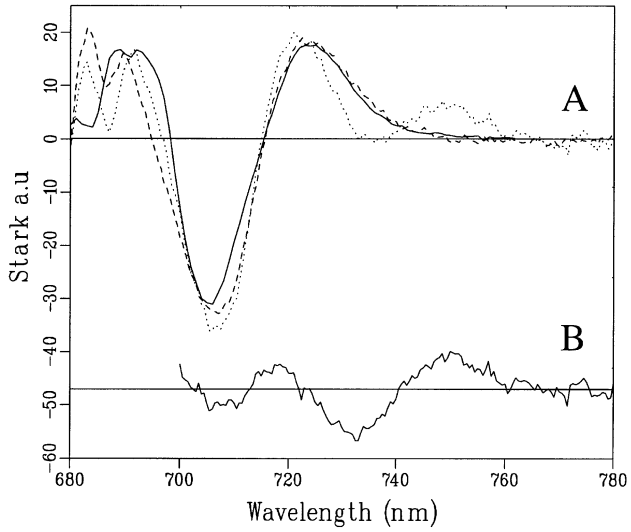

Fig. 5. (A) Comparison of the normalised Stark spectra (from Figs. 1, 3 and 4), respectively) with reduced P700 of PSI complexes from *S. elongatus* (full line) and of monomeric (dashed line) and trimeric (dotted line) PSI complexes from *S. platensis*. (B) Difference Stark spectrum (direct subtraction of spectra as depicted in A) of the trimeric and monomeric PSI complexes from *S. platensis*.

part of the absorption around 710 nm (not shown). It is not known, however, if the diminishing of the 710 nm absorption in the trimers directly results from the formation of C740, or that the 710 nm absorption is highly sensitive to photobleaching in the trimers [19].

Also the Stark spectrum with reduced P700 (Fig. 4B) is similar to those of the PSI monomers from *Spirulina* (Fig. 3B) and the PSI trimers from *S. elongatus* (Fig. 2B), except for the extra feature around 749 nm. Fig. 5 shows that this extra feature has the appearance of an S-shaped signal with a positive band at 749 nm, a negative band near 730 nm and a zero-crossing at 740 nm, very close to the absorption maximum of C740. This shape resembles that of the first derivative of C740, suggesting that differences in polarizability between ground and excited state dominate the Stark properties of C740. A more quantitative analysis is presented below.

3.4. Modelling absorption and Stark spectra of the red chlorophylls

For the analysis of the Stark response of the chlorophylls, we note that every single electronic transition may have a different response to the applied electric field and that the measured Stark spectrum is a sum of the field effects on all these transitions. Each chlorophyll should therefore be modelled separately, which is in practice impossible given the large number of chlorophylls in the PSI complex. We focus our attention, therefore, on the red chlorophylls absorbing at wavelengths longer than 700 nm, because only a few different electronic transitions contribute to the absorption in this region. However, even in this region,

several very broad bands overlap. For instance, in the 2.5 Å structure of the PSI complex from *S. elongatus* four groups of two or three chlorophylls each were assigned as candidates for the long-wavelength pigments [1,2]. Since each dimer or trimer will give rise to its own homogeneously and inhomogeneously broadened absorption band, it is obvious

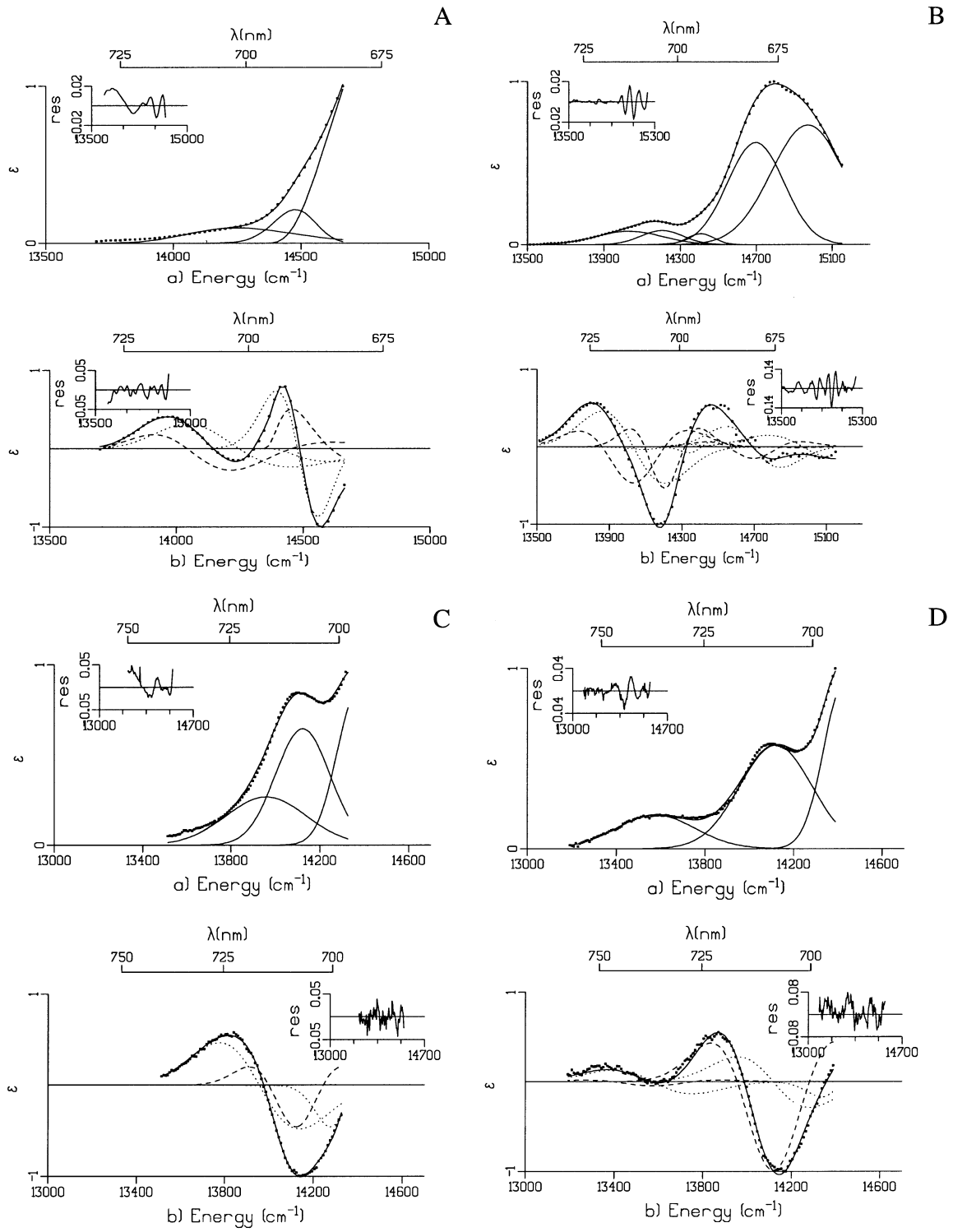

Fig. 6. Simultaneous Gaussian fit of the red parts of the 77 K absorption (top spectrum) and Stark (lower spectrum) spectra of PSI complexes with reduced P700 of (A) *Synechocystis* sp. PCC 6803, (B) *S. elongatus*, (C) PSI monomers from *S. platensis* and (D) PSI trimers from *S. platensis*. The absorption spectrum (closed circles, upper panels) was fitted with three independent Gaussian functions (solid lines), while the Stark spectrum (closed circles, lower panels) was fitted with a combination of the first derivatives (dotted lines) and second derivatives (dashed lines) of these Gaussians. The sums of the Gaussians are represented by solid lines. The insets show the residuals of the fits. Absorption/Stark spectra are normalised to unity at the maximum/minimum.

that a unique fit will be very hard to obtain, even in the red part of the spectrum, also because it is not precisely known if the various bands have Gaussian line shapes or not.

In Fig. 6 and Table 1, we present a simultaneous fit of the red-most parts of the absorption and Stark spectra of the four investigated PSI complexes. All parameters were free, including those of the first and second derivatives of all bands. In *Synechocystis*, there was no need to assume more than one long-wavelength transition, while in *Synechococcus* and *Spirulina* PSI monomers one extra red band appeared necessary. In *Spirulina* PSI trimers, a reasonable fit was obtained with one extra band absorbing at 737 nm, but a description of two extra bands at about 740 and 719 nm is also possible (not shown). We note that in all cases, each band can in principle consist of more than one optical transition, which means that the Stark parameters found for this band can in principle be the combination of more than one transition.

The results of the fits suggest that in all cases, the shape of the Stark spectrum of the red-most band has equally first-derivative and second-derivative character. This means that the Stark spectra of these bands are characterised by a large polarizability difference Tr($\Delta\alpha$) besides a moderate change in dipole moment. For C719, the values for Tr($\Delta\alpha$) are in the range of 500–1000 $Å^3\ f^{-2}$ (Table 1), very similar to those observed before for the B850 bacteriochlorophylls of the bacterial light-harvesting antenna LH2 [25] and the special pair of the purple bacterial reaction center [26]. In these complexes, the high values were explained by a mixing of charge transfer (CT) states into the excited (excitonic) state [28]. These results are also in line with the conclusions from Stark holeburning on C719 from *Synechococcus* [15,40]. For C740, the values for Tr($\Delta\alpha$) are perhaps lower than those for C719 (Table 1), in agreement with the data in Fig. 6, where the red-most band (C740) shows a smaller first-derivative feature than the second band (C719). The Tr($\Delta\alpha$) values of C708 are much lower than those of C719 (Table 1), though these values in *Synechococcus* and *Spirulina* are hard to evaluate because of the considerable overlap with C719. The values for $\Delta\mu$ of the red chlorophylls in PSI were found to be somewhat smaller than those found for B850 and the purple bacterial special pair (3–7 D f^{-1} [25,26]). We note that precise values for $\Delta\mu$ are hard to evaluate, because of the relatively small second-derivative contribution to the spectra. Our values for $\Delta\mu$ are roughly consistent with the more precisely determined values of 2.3–2.4 D f^{-1} obtained for the red edge of the PSI spectra from *Synechococcus* and *Synechocystis* by Stark holeburning [15].

In another approach, to describe the simultaneously recorded absorption and Stark spectra, we treated the long-wavelength absorption bands as one band, described the absorption above 700 nm by a polynomial function, and fitted the Stark spectrum by a combination of first and second derivatives. The advantage of this method is that no a priori assumptions on the line shape of the transitions have to be made. The disadvantage is that not only the red chlorophylls will be included in the analysis, but also the red-absorbing parts of reduced or oxidized P700 and even the red-most absorbing part of the bulk antenna pigments. The results for the PSI core complexes with reduced P700 are shown in Fig. 7 and Table 2, except for those on *Synechocystis*, because in this case the red chlorophylls only give rise to a shoulder in the absorption spectrum and a reasonable description by this method is not possible. The results for *Synechococcus* and *Spirulina* point to lower values for Tr($\Delta\alpha$) and higher values for $\Delta\mu$ than in the fits with the separate transitions in Fig. 6 and Table 1, but confirm the large values of these parameters for the red chlorophylls, and are consistent with the idea that the red transitions in PSI are caused by two or more strongly coupled chlorophylls [11] with significant charge-transfer character [12]. The higher $\Delta\mu$ values in this type of analysis may be explained by an overestimation of the second derivative contribution in this type of modelling. These $\Delta\mu$ values are very similar to those reported before from a similar method of analysis of Stark spectra (CP1 and P700 enriched particles: $\Delta\mu \cong 5$ D f^{-1} [38,39]). In all complexes, the Stark parameters are slightly larger with oxidized P700 than with reduced P700, suggesting an increase of the charge-transfer character upon oxidation of P700. We note that in the Stark holeburning studies from Small et al. the $\Delta\mu$ values around 700 nm were considerably lower ($\Delta\mu \cong 0.6$ D f^{-1} [12,15]) and similar to those of monomeric Chl *a*. It is possible, though, that P700 was not monitored under their experimental conditions. Earlier persistent pho-

Table 1
Stark parameters calculated from Gaussian band analysis of two pools of red chlorophylls in four different PSI complexes with reduced P700, isolated either as monomers (mon) or as trimers (trim)

Species	Aggr. state	C708 pool			C719/C740 pool		
		λ_{max} (nm)	$\Delta\mu$ (D f^{-1})	Tr($\Delta\alpha$) ($Å^3\ f^{-2}$)	λ_{max} (nm)	$\Delta\mu$ (D f^{-1})	Tr($\Delta\alpha$) ($Å^3\ f^{-2}$)
Synechocystis	trim	708	0.4	275			
Synechococcus	trim	706	2.4	0	714	3.6	600
Spirulina	mon	708	2.2	0	717	0	1200
Spirulina	trim	708	4.2	265	737	1.7	415

λ_{max} represents the wavelength maximum of the Gaussian used for the fit of the absorption spectrum. The errors for the values of the change in permanent dipole moment $\Delta\mu$ and the polarizability Tr($\Delta\alpha$) are at least 15%.

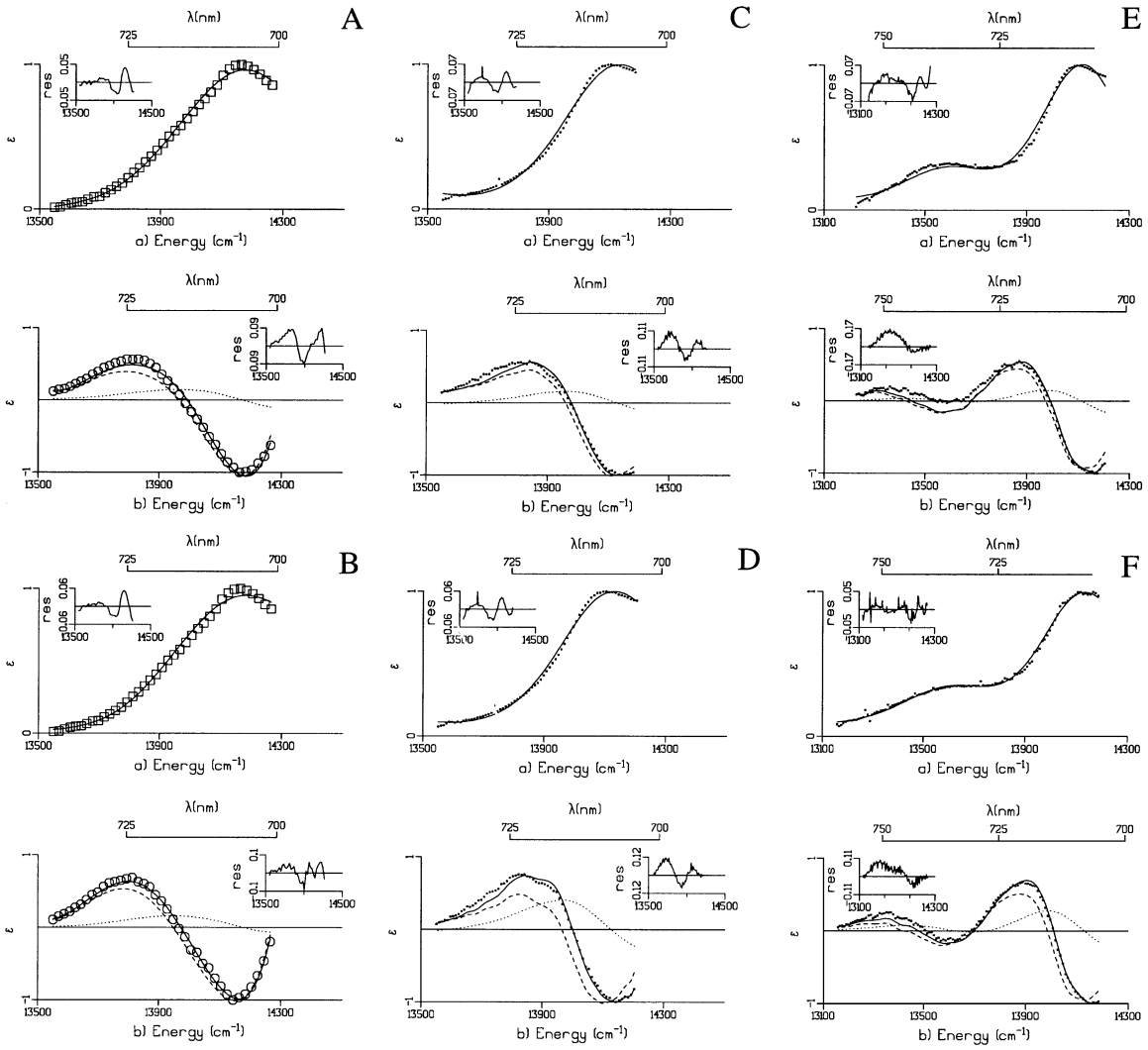

Fig. 7. Simultaneous spline fits of the red parts of the 77 K absorption and Stark spectra of trimeric PSI complexes from *S. elongatus* (A,B), monomeric PSI complexes from *S. platensis* (C,D) and trimeric PSI complexes from *S. platensis* (E,F). The spectra were recorded with reduced P700 (A,C,E) or oxidized P700 (B,D,F). The absorption spectra (circles) were fitted with a polynomial function, while the first derivatives (dotted lines) and second derivatives (dashed lines) of this function were used to fit the Stark spectrum. The (sums of the) functions are represented by solid lines. The insets show the residuals of the fits.

tochemical holeburning studies have revealed that P700 is a strongly coupled dimer [49], which should give rise to relatively large permanent change in dipole moment.

3.5. Modelling absorption and Stark spectra of the β-carotenes

All species show similar Stark effects of the β-carotene molecules. This implies a very similar organization and structure of the carotenoids in the PSI complexes from all three different organisms. A rough estimate of the Stark values for the red-most band of the carotenes using a polynomial fit gives values of $\Delta\mu = 6-10$ D f^{-1} and $\mathrm{Tr}(\Delta\alpha) = 1000-1900$ Å^3 f^{-2} (not shown). These values are in the upper limit of those reported recently for β-carotene in different solutions [37] and are considerably larger than measured for the β-carotenes in CP47 (De Weerd et al., in preparation). The high change in dipole moment values of β-carotene in PSI could arise from polarizability elements converted by the internal electric field (generated by the surrounding protein) into a permanent dipole moment when

Table 2
Stark parameters calculated from polynomial fit analysis of the red parts of the 77 K absorption and Stark spectra of three different PSI complexes, isolated either as monomers (mon) or as trimers (trim)

Species	Aggr. state	Redox state	λ_{max} (nm)	Δμ (D f^{-1})	Tr(Δα) (Å^3 f^{-2})
Synechococcus	trim	reduced	705	5.0	150
		oxidized	705	6.8	250
Spirulina	mon	reduced	707	3.0	120
		oxidized	707	3.4	185
Spirulina	trim	reduced	707	3.9	155
		oxidized	707	3.9	180

λ_{max} represents the wavelength of the maximal value of the polynomial function. The errors for the values of the change in permanent dipole moment $\Delta\mu$ and the polarizability Tr(Δα) are at least 15%.

probed by an external electric field [35]. Also the mixture of *cis* and *trans* β carotenes in PSI [1] could increase the dipolar contribution in the Stark spectra because of possible differences in polarizability [37].

3.6. Stark spectra of PSI with oxidized P700

We also monitored the effect of continuous white light illumination during freezing to 77 K on the Stark properties of the various PSI complexes. This type of illumination leads to the accumulation of oxidized P700 and a reduced iron–sulfur center [14]. The absorbance difference spectra brought about by this type of illumination indeed confirmed the oxidation of P700 (not shown), though the extent of the oxidation remains uncertain. The oxidized Stark spectra for the different species are shown in the panels C of Figs. 1–4. The results reveal large differences in the Q_y absorption region of the chlorophylls upon P700 oxidation (discussed in detail below), but very small differences in the β-carotene absorption region. This result is remarkable, because the absorption spectra of carotenoids are usually very sensitive to internal electric fields (see, e.g., Ref. [35]). The most straightforward explanation for these results is that most of the β-carotene molecules of PSI are located far away from the positive and negative charges on P700 and the iron–sulfur centers, respectively. The 2.5 Å crystal structure of PSI from *S. elongatus* [1,2] indeed shows that most carotenes are located far away from the central reaction center components. The similarity of the carotenoid Stark features of the PSI complexes of *Synechocystis* PCC 6803 and *S. platensis* then suggests that also in these complexes most carotenes will be located far away from the central electron transfer components.

All species show pronounced differences in the Chl Q_y region between the Stark spectra with different P700 redox state. At first sight, the strongest oxidation feature occurs in PSI from *Synechococcus* (Fig. 2C), where with oxidized P700, but not with reduced P700, a strong negative band is seen at 691 nm. The other investigated PSI complexes also show negative features at about 691–693 nm upon oxidation of P700 (Figs. 1C, 3C and 4C), which thus seems to be a general characteristic of P700 oxidation. We would like to stress that the differences found between the Stark spectra of both redox states shows the necessity of strict control of the redox state when investigating the electronic nature of PSI.

Oxidation of P700 also induced some changes in the Stark spectra of the red states of all species. However, the magnitude and peak position of the positive peak at ~ 725 nm in *Synechococcus* and *Spirulina* monomers, attributed to the red part of the first derivative line shape of C719, remained unchanged, indicating a significant distance between C719 and P700. Also, the Stark feature of C740 did not change very much upon oxidation of P700 (Fig. 4B,C), in line with the estimated long distance of 42 Å between C740 and P700 [10]. For C708, the relatively small Stark signal and the considerable overlap with the Stark spectrum of P700 (see below) prevent conclusions on a possible dependence of its Stark line shape on the redox state of P700.

For PSI from *Synechocystis* with oxidized P700, Stark shifts can be observed as far as 750 nm (Fig. 1C). In Fig. 8, we show that these shifts resemble to some extent those in the PSI trimers from *Spirulina*, though the peak position is less far to the red in *Synechocystis* and the amplitude is smaller. Nevertheless, the presence of these shifts suggest that also PSI from *Synechocystis* may contain a far red absorbing pigment under certain conditions.

Fig. 9 shows the 77 K reduced-minus-oxidized Stark difference spectra of the four investigated PSI complexes. All complexes show some differences, but also some common features, such as a negative band at 700–705 nm and a positive band at around 690–693 nm (the negative band at 691 nm of PSI with oxidized P700 mentioned above, but with opposite sign because the reduced-minus-oxidized spectrum is monitored). For the interpretation of these features, we first refer to the Stark difference spectra of diethyl ether treated PSI particles with either reduced or oxidized P700 [39]. These particles lack most antenna chlorophylls, but retain P700 and therefore allow to observe the Stark features of P700 without much disturbance of (red) antenna chlorophylls. The Stark spectrum with reduced P700 was shown to have a second-derivative-like appearance with a strong negative signal at 701 nm and small positive signals near 715 and 687 nm [39]. We monitored the reduced-minus-oxidized spectrum in Fig. 9, so the signs in the spectra in Fig. 9 and in Krawczyk and Ikegami [39] should have the same sign. The Stark difference spectra of all cyanobacterial PSI complexes show indeed a negative

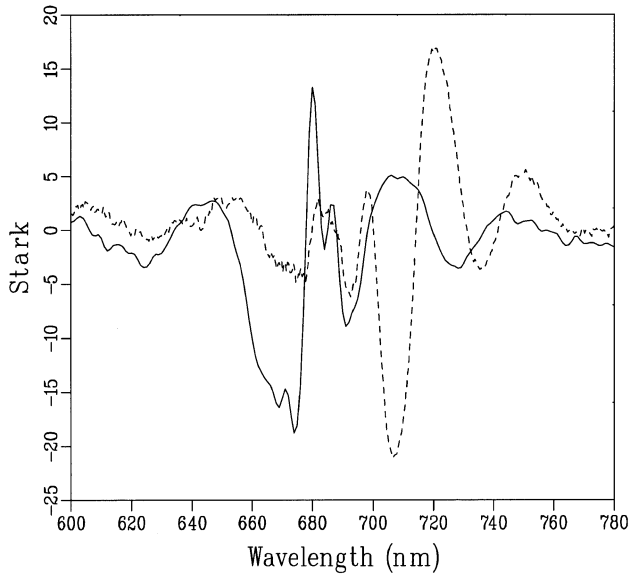

Fig. 8. Comparison of the 77 K Stark spectra of trimeric PSI complexes with oxidized P700 from *Synechocystis* sp. PCC 6803 (solid line) and *S. platensis* (dashed line). Absorption/Stark spectra are normalised to unity at the maximum/minimum.

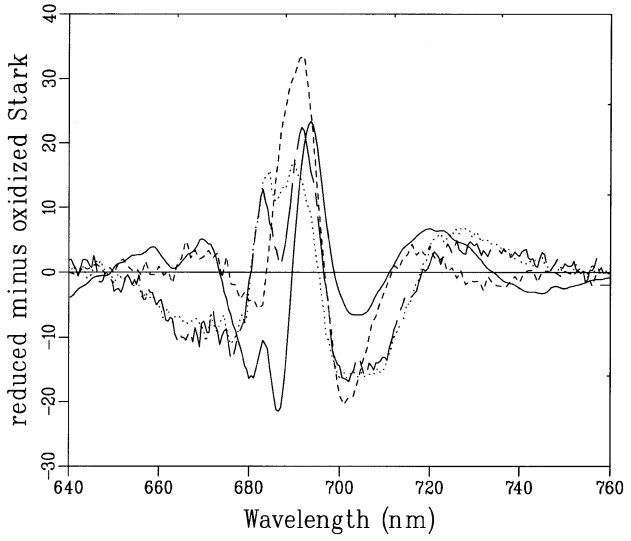

Fig. 9. Reduced-minus-oxidized Stark difference spectra at 77 K of trimeric PSI complexes from *Synechocystis* sp. PCC 6803 (full line), trimeric PSI complexes from *S. elongatus* (dashed line), monomeric PSI complexes from *S. platensis* (dotted line) and trimeric PSI complexes from *S. platensis* (chain-dashed line).

band at 700–710 nm and a positive feature at about 720 nm (Fig. 9), which suggests that the Stark features of reduced P700 are visible in all our spectra and are similar to those observed before in diethyl ether treated PSI. The size of the change in dipole moment of reduced P700 is roughly comparable to the earlier estimate of $\Delta\mu = 5$ D f^{-1} [38,39], but because of the overlap with the strong signals of the red chlorophylls and the uncertainty of the extent of the P700 oxidation, a more precise value cannot be given.

The Stark spectrum of diethyl ether treated PSI particles with oxidized P700 shows a distinct second-derivative feature dominated by a negative band at 687 nm, which was attributed to the absorption of the neutral chlorophyll molecule of $P700^+$ [39]. In Fig. 9, this feature should appear as a narrow positive band at 687 nm, because the reduced-minus-oxidized spectrum is recorded in this figure. However, there is no positive band at 687 nm in the Stark spectra of the cyanobacterial PSI complexes, though there is a very pronounced positive band at 690–693 nm. For an explanation of these results, we first note that a very pronounced positive 690 nm band has also been observed in the low-temperature oxidized-minus-reduced absorbance-difference spectrum of P700 in PSI from *S. elongatus* [44]. This band does not occur in the 5 K triplet-minus-singlet absorbance difference spectrum of P700 [50,51], which suggests that it must at least in part be due to electrochromic bandshifts induced by the positive charge on $P700^+$. We also note that the six reaction center chlorophylls are at relatively short distances from each other, that excitonic interactions between these chlorophylls are likely, just like in Photosystem II [52], and that new excitonic interactions can be induced if P700 is oxidized. In particular, the neutral chlorophyll of $P700^+$ can give rise to new excitonic interactions if it absorbs at about the same wavelength as other reaction center chlorophylls. Indeed, both the neutral chlorophyll of $P700^+$ and A_0 have been suggested to peak at 690 nm [4,39]. Whatever the origin, if the sharp positive band at 690–693 nm in the reduced-minus-oxidized Stark spectrum originates from the 690 nm absorption band in the oxidized-minus-reduced absorbance-difference spectrum of P700, its Stark line shape must resemble its second derivative and its Stark features are therefore dominated by the difference permanent dipole moment of this transition.

4. Conclusions

The results in this paper indicate that the red chlorophylls, β-carotenes and primary electron donor P700 in all investigated cyanobacterial PSI complexes give rise to very considerable electric field induced absorption changes. The magnitude of the Stark effect is of the order C719 > C740>C708, and the Stark effect of all red chlorophylls is clearly dominated by the changes in polarizability. For C719 and C740, the Stark shifts do not vary significantly upon changing the redox state of P700, and for C719 the magnitude of $Tr(\Delta\alpha)$ is almost as high as has been reported before for the special pair of the purple bacterial reaction center [26] and for the bacteriochlorophylls in the purple bacterial antenna complexes LH1 and LH2 [25,45]. In all these cases, the huge values of $Tr(\Delta\alpha)$ have been explained by mixing of intradimer charge-transfer states into the lower exciton states of these molecules. Based on Stark holeburning experiments, Small et al. [12,15,40] also concluded that the red chlorophylls in PSI are strongly coupled and have considerable charge-transfer character. The β-carotene molecules show Stark responses that are equal in magnitude or slightly larger than those observed for β-carotene in solution [37] and considerably larger than those found for the CP47 antenna complex of Photosystem II (de Weerd et al., unpublished observation). The Stark response of P700 oxidation can most easily be explained by the concept that in the reduced state the $\Delta\mu$ originates primarily from the pair of chlorophyll molecules that make up P700, while in the oxidized state, the $\Delta\mu$ originates from one or more chlorophyll molecules that give rise to the absorption band peaking at 690 nm. Also in diethyl ether-treated PSI, the Stark response of P700 is dominated by $\Delta\mu$ [39]. This situation is strongly reminiscent of that observed in the heterodimer mutant of the purple bacterial reaction center [53].

Apart from the details on the electric field responses of the various chromophores, the Stark spectra provide highly specific fingerprints of the pigment organizations in the various PSI complexes, from which we conclude that the overall pigment organization is similar in PSI complexes from *S. elongatus* and *S. platensis* and is at least in some aspects different in *Synechocystis* PCC 6803.

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