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Spectroscopic characterization of PS I core complexes from thermophilic *Synechococcus* sp.

Identical reoxidation kinetics of A_1^- before and after removal of the iron-sulfur-clusters F_A and F_B

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Abstract

Monomeric and trimeric PS I complexes missing the three stromal subunits E,C and D (termed PS I core complexes) were prepared from the thermophilic cyanobacterium *Synechococcus* sp. by incubation with urea. The subunits E,C and D are sequentially removed. In the monomeric PS I the subunit C is removed with a half life of approx. 5 min. This is about eight times faster than in the trimeric PS I complex. In parallel with the removal of the $F_{A/B}$ containing subunit C the reduction kinetics of $P700^+$ changed from a half life of about 25 ms to about 750 μs. The partner of $P700^+$ in the 750 μs charge recombination was identified to be F_X by the difference spectrum of this phase. There are some minor differences in the spectra of trimeric and monomeric PS I core complexes. At 77K the forward electron transfer from A_1^- to F_X is blocked in the major fraction of the PS I core complexes and $P700^+A_1^-$ recombines with a half life of about 220 μs. In the remaining fraction $P700^+F_X^-$ is formed and decays with a half life of approx. 10 ms at 77 K. The kinetics of the forward electron transfer from A_1^- to the iron-sulfur-clusters was measured in the native PS I and the corresponding core complexes. The reoxidation kinetics of A_1^- are identical in both cases ($t_{1/2}$ = 180 ns). We conclude that F_X is an obligatory intermediate in the normal forward electron transfer.

Key words: Photosystem I; Core protein; Iron sulfur center; Electron transfer; Absorption difference spectroscopy

1. Introduction

Photosystem I (PS I) is a membrane protein complex, consisting of at least 11 subunits, that mediates the light-driven electron transfer from reduced plastocyanin or cytochrome c_6 to ferredoxin (for review, see [1,2]). The PS I complex of *Synechococcus* contains two large subunits (A, B of about 83 kDa) and five small membrane-intrinsic polypeptides (I, J, K, L and M of 3–15 kDa). Three of the four extrinsic subunits (C, D and E (8–16 kDa)) are located on the stromal side, whereas F (15 kDa) is located on the lumenal side of the membrane. The subunits are denoted according to the respective genes psa A to psa M [3]. The A/B-heterodimer binds about 100 antenna chlorophyll *a* molecules and the following redox centers: P700 (presumably a chlorophyll *a* dimer), A_o (a monomeric chlorophyll *a*), A_1 (vitamin K_1, a phylloquinone) and F_X (a 4Fe-4S iron-sulfur-cluster). The terminal electron acceptors F_A and F_B (two further 4Fe-4S iron-sulfur-clusters) are both coordinated by subunit C. The PS I complex can be isolated from the membrane in a monomeric and trimeric form [4,5]. The trimer is proposed to be the native structure in intact membranes [6].

After excitation of PS I by light the primary radical pair $P700^+A_o^-$ is formed. Subsequent charge stabilisation is achieved by electron transfer from A_o^- to the secondary acceptor A_1 (vitamin K_1). For the reoxidation of A_1^- a half life of 200 ns was found in PS I complexes from *Synechococcus* [7], whereas in PS I from spinach A_1^- decays biphasically with $t_{1/2} \simeq 25$ ns and 150 ns and relative amplitudes depending on the preparation [8]. It is widely accepted that the electron from A_1^- is transferred to F_X.

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Abbreviations: BV, benzylviologen; CAPS, (3-(Cyclohexylamino)-1-propane sulfonic acid; Chl, chlorophyll *a*; d, optical path for the measuring light; DPIP, 2,6-dichlorophenolindophenol; DTT, dithiothreitol; FeS, iron-sulfur-cluster; MES, 2-(*N*-morpholino)-ethane sulfonic acid; PS, photosystem; PS I core complex, PS I without the extrinsic subunits C, D and E; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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However, there is little direct evidence for this assumption. Since the iron-sulfur-clusters can hardly be distinguished by optical spectroscopy, the state $P700^+F_X^-$ could not yet been monitored as an intermediate in normal forward electron transfer. Photoreduction of F_X has been observed by EPR at low temperatures, but the results more likely indicate that F_x may represent an acceptor on a pathway parallel to $F_{A/B}$ (for review see [9]). The observation, that the 200 ns forward electron transfer from A_1^- to an iron-sulfur-center is blocked in *Synechococcus* after chemical pre-reduction of both F_A and F_B, may even indicate that F_X is not involved in normal forward electron transfer [10]. Remarkably, in PS I from spinach the 150 ns phase disappears, whereas the 25 ns phase is still observed, when both F_A and F_B are pre-reduced [8]. On the other hand, in PS I core preparations lacking the $F_{A/B}$ containing subunit C, the state $P700^+ F_X^-$ is formed with high quantum yield decaying by charge recombination with a half life of about 1.2 ms [8]. This result does not exclude that F_X is located on a side path of electron transfer and functions as an electron acceptor only after depletion of $F_{A/B}$. Furthermore the removal of the extrinsic polypeptides C, D and E may effect the electron transfer in the A/B heterodimer.

Recently the structure of PS I was determined at 6 Å resolution [14,15]. In this structure the positions of the three iron sulfur clusters could be unambiguously identified, indicating that F_X has not only an important structural function, but might be directly involved in electron transfer because of the distances between the electron carriers. Therefore it is of great importance to investigate experimentally whether F_X is an intermediate in the electron transfer from A_1^- to $F_{A/B}$. In order to get information which can be directly compared to the crystal structure, in the present work core complexes were prepared from the same PS I material that was used for crystallization.

In this work we compare the kinetics of the reoxidation of A_1^- (i.e. the rate of forward electron transfer) in native PS I complexes and in PS I core complexes both isolated from Synechecoccus. In PS I core complexes lacking the iron-sulfur-clusters F_A and F_B it is clear that the electron is transferred to F_X. The identical kinetics found in the presence and absence of $F_{A/B}$ give direct evidence that F_X is an obligatory intermediate in forward electron transfer.

2. Materials and Methods

2.1. Samples and biochemical methods

PS I trimers from *Synechococcus* sp. were isolated according to the method of Witt et al. [15]. PS I monomers were obtained from trimers by a new, very mild procedure as described in [16]. Both PS I complexes contain 80–100 Chl/P700.

PS I core complexes were prepared from monomers and trimers by incubation with urea according to a method described in [11]. PS I complexes suspended at a chlorophyll concentration of approx. 1.7 mM in buffer A (20 mM MES, 0.02% β-DM (w/v), 25 mM $MgSO_4$, pH 6.8) were added to a concentrated urea solution (9 M urea, 10 mM CAPS, pH 10.0) at 37°C. The final urea concentration was 6.8 M. The incubation time, that has been varied from 5 min to 14 h, is indicated in the figure legends. The reaction mixture (500–1,000 μl) was applied on a gel filtration column (Sephadex G 50 fine from Pharmacia, equilibrated with buffer A) to separate the urea and the small subunits from the remaining core complex. All experiments were performed with freshly prepared core complexes stored at 4° C.

HPLC runs were performed on a Knauer apparatus (pump 64 with programmer 50) combined with gel filtration column TSK 4000 SW and pre-column from Beckman. The columns were equilibrated with buffer A at a flow rate of 0.8 ml · min^{-1}.

SDS-PAGE were carried out on an automated PhastSystem apparatus, using high density gels, both from Pharmacia. Prior to SDS gel electrophoresis the samples were desalted on Sephadex G-50 centrifugation columns [17], which were equilibrated with a buffer containing 20 mM MES, pH 6.4, 5 mM $MgSO_4$ and 0.02% β-DM. The samples were incubated with Laemmli sample buffer containing 2.5% (w/v) SDS and 0.2% (w/v) DTT for 45 min at 56°C. The gels were run at 15°C and protein was visualised by silver staining. The SDS-gels were scanned using a Quick Scan Densitometer from DESAGA, Heidelberg.

2.2. Spectroscopic methods

The chlorophyll concentration was determined in buffer A using an extinction coefficient of 74,000 $M^{-1} \cdot cm^{-1}$ at 679 nm. The Chl/P700 ratio for the PS I complexes was calculated from the flash-induced absorption change at 703 nm due to the photooxidation of P700, using a differential molar extinction coefficient of 64,000 $M^{-1} \cdot cm^{-1}$.

The spectrum of the absorption changes in the range from 350 to 500 nm and from 650 to 740 nm was measured with a flash photometer that is characterized by a time resolution of about 50 μs. The measuring light of a 250 W tungsten halogen lamp (Osram) passed through a monochromator with 7 nm bandwidth placed between light source and sample and a combination of interference filters and colored glasses in front of the photomultiplier (EMI 9668BQ). The signals were digitized and averaged by a transient recorder (Biomation 4500 from Gould). The samples were excited by a saturating Xe-flash of about 15 μs duration filtered by colored glasses.

For measurements of absorption changes at 384 and 434 nm in the ns time range a Xe-flash lamp was used as measuring light source [18] and the sample was excited by laser flashes at 532 nm (pulse duration ≅ 3 ns; repetition rate 1 Hz).

Absorption changes at 820 nm were measured as described previously [19]. For measurements in the nanosecond time range, the detection system had an electrical bandwidth of 500 Hz - 100 MHz. In the μs- and ms-range, the measuring light was monitored by a photodiode (SGD 444 from EG&G) loaded with 1 kΩ.

All measurements were performed at room temperature in a medium containing 20 mM Tricine pH 8.2, 25 mM $MgSO_4$, 0,02% β-DM, 5 mM Na-ascorbate and artificial electron donors and acceptors as given in the figure legends. In experiments at 77 K, glycerol was added to the sample to a final concentration of about 60% (v/v) before freezing in a liquid nitrogen cryostat (Model DN 1704 from Oxford Instruments).

The time course of the absorption changes was fitted to a (multi)exponential decay using an algorithm that minimizes the sum of the unweighted least squares.

3. Results and discussion

3.1. Biochemical characterization of the core complexes

Both, PS I monomers and PS I trimers were incubated with 6.8 M urea in order to remove the stromal subunits. After different incubation times the urea and the removed subunits were separated from the remaining PS I complexes by gel filtration as described in materials and methods. The protein composition of the PS I complexes was analyzed by SDS-gelectrophoresis. Fig. 1 shows a silver stained SDS gel in the range from 2 to 96 kDa of the trimeric PS I after different incubation times. The

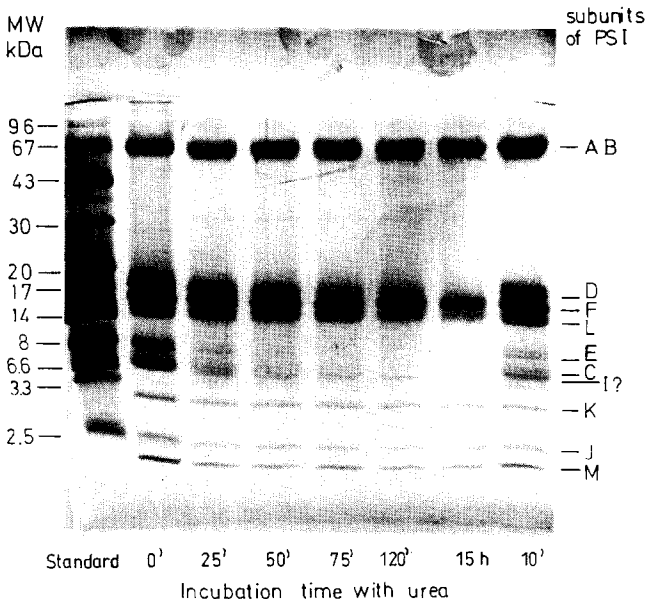

Fig. 1. SDS-PAGE of trimeric PS I complexes prepared from the thermophilic cyanobacterium *Synechococcus* sp. after different incubation times with 6.8 M urea at 37°C. Lane 1: molecular mass marker, lane 2: untreated PS I complex, lane 3 to 8: PS I complexes incubated for 10 min (lane 8), 25 min (3), 50 min (4), 75 min (5), 120 min (6) and 15 hours (7); on the right hand side the subunits are assigned according to Mühlenhoff et al. [3].

subunit composition of the untreated PS I complex (lane 2) corresponds to that identified by Mühlenhoff et al. [3]. All subunits are resolved and labelled on the right hand side of Fig. 1. The attribution of the band below subunit C to subunit I is under investigation. This subunit is possibly encoded by the gene *Psa* I but up to now this has not been verified by N-terminal sequencing of the protein [3]. It is obvious (see Fig. 1, lanes 2 to 8) that the subunits E, C and D are removed from the PS I complex with increasing incubation time. All other subunits, including the stromal subunit F and all small subunits (K, J and M) remain on the core complexes even after very long incubation. The SDS-gel (lane 2 to 8) was scanned with a densitometer and the area under each peak (IS) has been determined. In each lane the areas were normalized to the peak area related to subunit F (IF). Only the peak areas assigned to the subunits C, D and E decrease with increasing incubation time. A plot of ln (IS/IF) vs. incubation time gives a straight line for each of the subunits C, D and E, which is expected for a first order reaction (data not shown). The half lives for the removal of C, D and E were calculated from the slope of the straight lines. Subunit E is removed first, with a half life of about 25 min, followed by subunit C with a half life of about 40 min. The removal of subunit D is significantly slower with a half life time of approx. 200 min. This subsequent removal of the subunits is in good agreement with proposals for the subunit arrangement by biochemical data [20]. After that, subunit E is located on top of the stromal hump, which was found in the electron density map at 6 Å resolution [14]. Using monomeric PS I complexes the subunits are removed in the same order: first E, after that C and finally D. However the kinetics of the removal is significantly faster. Subunit C has for example a half life of only 5 min (data not shown).

The trimeric and monomeric organization of PS I was not influenced by the incubation with urea. Fig. 2 compares the elution profiles on a TSK 4000SW gel filtration column of untreated monomeric and trimeric PS I complexes (controls) to that of the corresponding core complexes obtained by urea treatment. In both cases practically the same elution volumes (i.e. size) are observed after depletion of the extrinsic subunits C,D and E. This demonstrates for the first time that trimeric PS I complexes from a thermophilic cyanobacterium do not disintegrate into monomers upon urea incubation [21].While this work was in progress Kruip et al. [22] reported the existence of monomeric and trimeric PS I core complexes (named P700-F_X complexes) from the mesophilic cyanobacterium *Synechocystis* PCC 6803.

3.2. Electron transfer in PS I core complexes

Flash-induced absorption changes at 820 nm have been measured in order to analyse the effect of removal of the extrinsic subunits C, D and E on the reduction kinetics of $P700^+$. Fig. 3 shows the time course of the absorption changes on two time scales for monomeric PS I before and after incubation with urea for 25 min. In the untreated sample (Fig. 3, control) a stable charge separation is observed. The absorption increase caused by the photooxidation of P700 decays with a half life of about 25 ms (not shown). Under the measuring conditions (no artificial electron acceptor and low concentration of external electron donors (see figure legend)) $P700^+$ is for

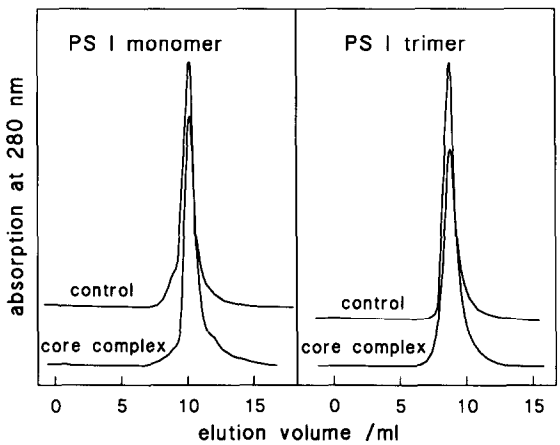

Fig. 2. Elution profiles on a TSK 4000 SW gel filtration column of PS I complexes. Left: Chromatograms of PS I monomers: untreated (control) and after incubation with 6.8 M urea for 15 min (core complex); right: Chromatogram of PS I trimers: untreated (control) and after incubation with urea for 55 min (core complex). Flow rate was 0.8 ml/min.

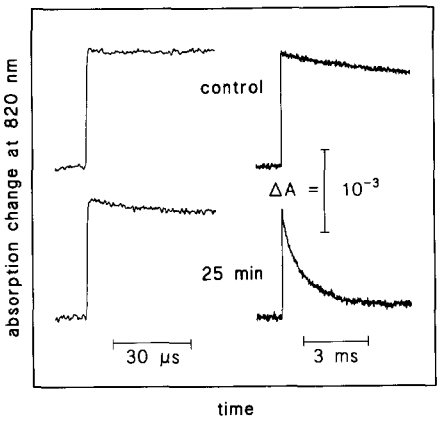

Fig. 3. Flash-induced absorption changes at 820 nm in the μs- (left) and ms-time range (right) attributed to the photooxidation and re-reduction of P700 in monomeric PS I complexes from *Synechococcus* sp.: untreated (control) and after 25 min incubation in concentrated urea solution. Excitation by 3 ns laser flashes at 532 nm, d = 5 cm, 3.2 μM Chl, pH 8.2, 75 μM DPIP.

the most part rereduced by charge recombination with $F_{A/B}^-$. Upon incubation with urea the 25 ms phase was gradually replaced by a faster decay ($t_{1/2}$ = 750 μs ± 200 μs) in monomeric as well as trimeric PS I complexes. The error limits include variations observed with different preparations of PS I core complexes. It should be mentioned that the 750 μs decay is significantly faster than the 1.2 ms half life, which has been reported for PS I core preparations from mesophilic cyanobacteria [11]. The half life depends only slightly on the pH in the range from pH 5 to pH 9 (data not shown).

In PS I monomers the amplitude of the 25 ms phase decreases by 50% in approx. 5 min. After 25 min of urea treatment (see Fig. 3, lower trace) the 750 μs phase accounts for 80–90% of the decay. Correspondingly, the amplitude of the slow component ($t_{1/2}$ 25 ms) has dropped to a minimum level of about 10%. Additionally, a small proportion of a phase with $t_{1/2} \simeq 10$ μs appears upon longer incubation times (10% after 25 min) (see Fig. 3).

Using PS I trimers remarkably longer incubation times are required in order to induce the 750 μs decay phase. The half maximal effect is observed after about 40 min incubation with urea. This half life agrees well with the half life for the depletion of subunit C determined by SDS PAGE (see above). This gives clear evidence that the increase of the amplitude of the 750 μs phase is correlated with the removal of the $F_{A/B}$ containing subunit C. Therefore the 750 μs phase is attributed to the charge recombination of $P700^+$ with an acceptor located on the A/B-heterodimer.

After longer incubation times there is still about 10% of a slow phase with a half life of approx. 150 ms (see Fig. 3). A small proportion of this phase remains even if the subunit C cannot be detected any more on the SDS gels. Therefore we conclude, that this phase is not due to an incomplete removal of the $F_{A/B}$ containing subunit C. We assume that in these PS I centers the electron is transferred from F_X^- to a not identified acceptor (possibly oxygen) and $P700^+$ is reduced by DPIP.

Upon very long incubation times a rapid phase of about 10 μs increases in amplitude. In this core complexes most probably the iron sulfur center F_X has been lost. We attribute this phase to the charge recombination of A_1^- with $P700^+$[23].

The difference spectrum of the 750 μs phase has been measured in the range from 350 to 500 nm, in order to identify the partner of $P700^+$ in the charge recombination. In Fig. 4 left the amplitude of the 750 μs phase measured in trimer PS I core complexes is depicted as a function of the wavelength (△). The circles (control) represent the initial amplitude of the flash-induced absorption changes in the untreated PS I trimer. Since the time resolution was limited to approx. 50 μs, the initial amplitude reflects the absorption changes due to the formation of $P700^+F_{AB}^-$. The corresponding difference spectra of the PS I monomers are shown in Fig. 4 right. Each spectrum is normalised to the maximum of the bleaching band that is characterized by a measured differential molar extinction coefficient of about 50,000 $M^{-1} \cdot cm^{-1}$.

The control spectrum obtained with PS I trimers (Fig. 4 left, circles) agrees well with $P700F_{A,B}/P700^+F_{A,B}^-$ difference spectra reported for the same material [7] and for PS I from spinach [24]. A comparison with the control

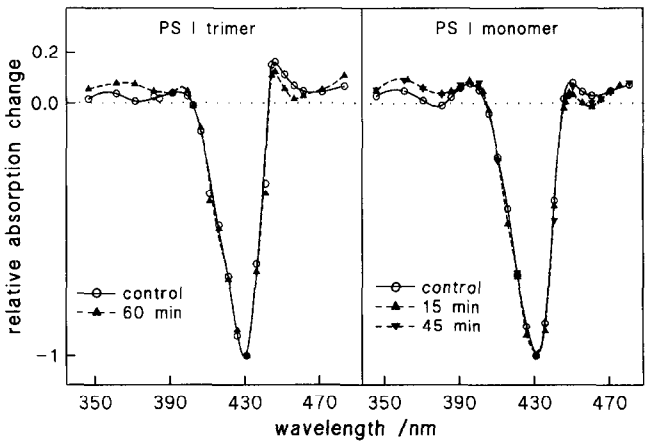

Fig. 4. Absorption difference spectra measured in trimeric (left) and monomeric (right) PS I compexes. The circles (○) represent the initial amplitude of the flash-induced ΔA, measured in untreated PS I complexes with a time resolution of about 50 μs. These spectra (control) are attributed to the formation of $P700^+F_{A/B}^-$. The triangles (△, △) represent the amplitude of the pre-dominant decay phase $t_{1/2}$ = 750 200μs. The results of the fit were $t_{1/2}$ = 850 μs (△, left), 600 μs (△, right) and 900 μs (△, right) measured in PS I core complexes prepared by the incubation with urea for the indicated times. This phase is attributed to the charge recombination of $P700^+$ F_X^-. Each spectrum is normalized to the maximum of the bleaching band. Control samples contain 500 μM DPIP and 10 μM BV, samples of core complexes contain only 50 μM DPIP; [Chl] · d = (90 20) μM · mm for all samples.

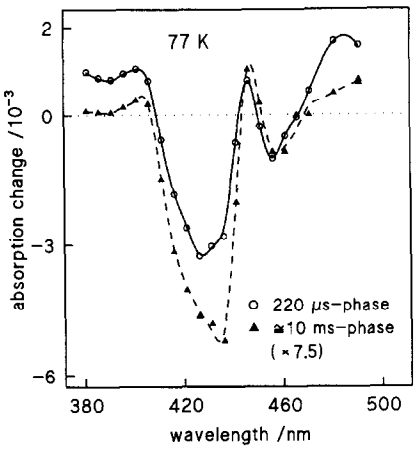

Fig. 5. Absorption difference spectra of the 220 μs- (○, solid line) and of the approx. 10 ms decay phase (△, dashed line, expanded by a factor of 7.5) measured in monomeric PS I core complexes at 77 K. 44 μM Chl, d = 2.4 mm, 62% (v/v) glycerol, pH 6.8.

spectrum of monomeric PS I (Fig. 4 right, circles) shows slight differences. Especially the absorption increase around 445 nm is less pronounced in the monomeric sample.

In both the monomeric and trimeric PS I the spectrum of the 750 μs phase (Fig. 4 left, △ and 4 right, △, △) resembles closely the corresponding control spectrum, except for a slightly larger absorption increase between 350 and 390 nm and for smaller absorption changes from 445 to 470 nm. The similarity between the spectrum of the 750 μs phase and the $P700F_{A/B}/P700^+F_{A/B}^-$ difference spectrum (control) indicates that the partner of $P700^+$ in the 750 μs charge recombination is most likely also a FeS-cluster. In accordance with previous reports concerning PS I core complexes from mesophilic cyanobacteria and spinach [9–11] we conclude that in PS I complexes depleted of F_A and F_B a charge separation between P700 and F_X takes place after excitation. Slightly different contributions of $F_{A/B}^-/F_{A/B}$ and F_X^-/F_X might be the reason for the minor deviations between the control spectra and the spectra of the 750 μs phase in Fig. 4.

Flash-induced absorption changes at 77K have also been studied using monomeric PS I core complexes. The reduction kinetics of $P700^+$ followed at 825 nm can be described by a dominant phase with a half life of about 220 μs and a minor component (10–15%) with $t_{1/2} \simeq 10$ ms. The spectrum of the longer-lived component (see Fig. 5, dashed line) resembles closely the difference spectra depicted in Fig. 4 except that the band shift around 450 nm is larger at 77 K. We attribute this phase to the charge recombination between $P700^+$ and F_X^- in a small proportion of the PS I centers. The decrease of the half life from 750 μs at room temperature to about 10 ms at 77K can be explained by an activation energy for the charge recombination of $P700^+$ and F_X^-[25]. The spectrum of the 220 μs component (see Fig. 5, solid line) is significantly different. The difference spectrum of this component is very similar to that of $P700^+A_1^-/P700A_1$ [10,26]. Compared to the spectra shown in Fig. 4 the absorption increase in the range from 380 nm to 400 nm is larger due to the contribution of A_1^-/A_1 and the bleaching around 430 nm is diminished, since the absorption decrease related to the reduction of an iron-sulfur- center is missing. We conclude that in the major proportion of the PS I core complexes the forward electron from A_1^- to F_X is blocked at 77K and, instead, $P700^+A_1^-$ decays by charge recombination with a half life of about 220 μs. This heterogeneity at low temperature has been also observed in the untreated PS I complexes from *Synechococcus*. At least three fractions of PS I centers can be distinguished which exhibit flash induced charge separations up to $F_{A/B}$ (≃ 30%), F_X (≃ 20%) and A_1 (≃ 50%) (not shown). This effect of temperature has not been reported for the core comlexes prepared with lithium dodecyl sulfate from spinach [25]

3.3. Reoxidation kinetics of A_1^-

Concerning the question whether F_X participates in normal forward electron transfer in native PS I complexes, it is of particular interest to compare the kinetics of the forward electron transfer from A_1^- to the succeeding acceptor in untreated PS I and PS I core complexes. Fig. 6 shows flash-induced absorption changes on a nanosecond time scale at 385 nm and 435 nm measured in monomeric PS I and the corresponding core complexes lacking F_A and F_B. The formation and the decay of A_1^- are best monitored around 384 nm, since absorption changes due to the formation of $P700^+$ or FeS^- are rather small at this wavelength (see Fig. 4). The kinetics of the reoxidation of A_1^- followed at 384 nm (see Fig. 6) is virtually the same for PS I and the corresponding core complexes. In both samples the initial (at t ≃ 5 ns) absorption change due to the formation of $P700^+A_1^-$ is fol-

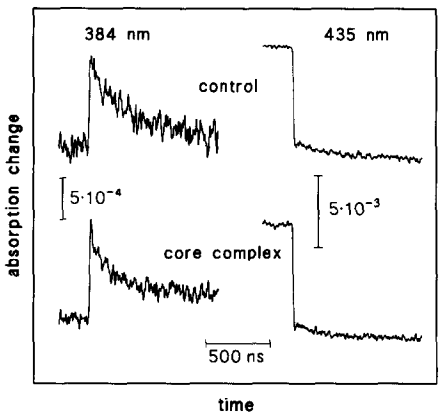

Fig. 6. Flash induced absorption changes at 384 nm (left) and 435 nm (right) in the ns time range measured in monomeric PS I complexes: untreated (control) and after 15 min of urea incubation (core complex). Both samples contain 25 μM DPIP, d = 1.2 mm, pH 8.2. The Chl-concentration was 99 μM (control) and 82 μM (core complex).

lowed by a kinetic phase with a half life of about 180 ns. This phase has been attributed to the electron transfer from A_1^- to an iron-sulfur-center but it was not possible to clarify which of the iron-sulfur-centers accepts the electron, since the spectral properties of the FeS-centers are nearly identical [7]. For quantitative analysis of the kinetics, the relative size of the 180 ns component in both samples has been determined by fitting the data with one exponential plus a constant. We obtained at 384 nm: 83% in the control and 76% in the PS I core complex and at 435 nm: 18% in the control and 15% in the PS I core complexes lacking $F_{A/B}$. The slightly smaller relative size of the 180 ns phase in the PS I core complex is probably related to the observation that a small proportion of a 10 μs component appears upon incubation with urea (see above). This phase has been attributed to the charge recombination of $P700^+A_1^-$ in core complexes that have lost F_X [23]. The quantitative analysis confirms that the kinetics of forward electron transfer is the same in intact PS I, containing the terminal iron sulfur centers F_A and F_B and in the core complexes, where only electron transfer to F_X is possible. Since the kinetics of the electron transfer is independent of the presence and absence of $F_{A/B}$, these results show unambiguously that F_X is an obligatory intermediate in the normal forward electron transfer. The observation that the reoxidation ofC A_1^- is blocked, if both F_A and F_B are chemically pre-reduced [10], might be explained by an electrostatic effect of the negative charges located on the terminal iron-sulfur-centers. Furthermore it can be concluded that the structure of the A/B heterodimer, especially the distances between the electron carriers, is not altered by the removal of the extrinsic subunits C, D and E, because there is no change in the electron transfer kinetics up to F_X in the core complexes compared to the native PS I.

Our result fits well to the proposed arrangement of the electron carriers based on the three-dimensional structure of PS I at 6 Å resolution [12]. From the distances between A_1, F_X and $F_{A/B}$ an unidirectional electron transfer with F_X as unambiguous intermediate has been predicted.

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