Fluorescence of the various red antenna states in photosystem I complexes from cyanobacteria is affected differently by the redox state of P700

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Abstract
Photosystem I of cyanobacteria contains different spectral pools of chlorophylls called red or long-wavelength chlorophylls that absorb at longer wavelengths than the primary electron donor P700. We measured the fluorescence spectra at the ensemble and the single-molecule level at low temperatures in the presence of oxidized and reduced P700. In accordance with the literature, it was observed that the fluorescence is quenched by P700+. However, the efficiency of the fluorescence quenching by oxidized P700+ was found to be extremely different for the various red states in PS I from different cyanobacteria. The emission of the longest-wavelength absorbing antenna state in PS I trimers from Thermosynechococcus elongatus (absorption maximum at 5 K: ≅719 nm; emission maximum at 5 K: ≅740 nm) was found to be strongly quenched by P700+ similar to the reddest state in PS I trimers from Arthrospira platensis emitting at 760 nm at 5 K. The fluorescence of these red states is diminished by more than a factor of 10 in the presence of oxidized P700. For the first time, the emission of the reddest states in A. platensis and T. elongatus has been monitored using single-molecule fluorescence techniques.

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1. Introduction
Photosystem I (PS I) is a pigment-protein complex located in the thylakoid membranes of cyanobacteria, algae and plants that mediates light-induced electron transfer from plastocyanin or cytochrome C6 on the lumenal side to ferredoxin on the stromal side (for a review see Refs. [1,2] and references therein). PS I of higher plants and algae (named PS I-200) consists of the PS I core complex and the peripheral light-harvesting complex LHC I. Cyanobacteria lacking LHC I contain only the PS I core. The PS I core complex coordinates all the peripheral antenna pigments and the following redox cofactors involved in the electron-transfer reactions: the primary electron donor P700 (a heterodimer of Chl a (eC-B1; FpA) and a’ (eC-A1; FpA)) located on the luminal side (nomenclature of Ref. [8] is used for naming cofactors). Two branches of cofactors related by pseudo-C2 symmetry connect P700 and the first [4Fe-4S] iron sulfur cluster Fx. Each branch is composed of two Chls (Acc-A (eC-B2) and Aα-A (eC-A3) in the A-branch and Acc-B (eC-A2) and Aα-B (eC-B3) in the B-branch) and one phyloquinone A1 (Qx-A and Qx-B, respectively). The terminal electron acceptors FxA and FxB (two [4Fe-4S] iron–sulfur-clusters) are both coordinated by subunit PsAc, one of the three extrinsic subunits located on the stromal side.

After absorption of light by an antenna pigment, the excitation energy is efficiently trapped via charge separation in the reaction center leading to P700+A0 . Charge stabilization is achieved by subsequent electron transfer to the secondary acceptor A1, then further to Fx and finally to the terminal electron acceptors FxA and FxB. An interesting aspect of the electron transfer in PS I is the reported heterogeneity at low temperature [9,10]. In one fraction of the PS I complexes, an irreversible charge separation due to the stable formation of P700+Fx and P700+Fx takes place, whereas in the

A high resolution (2.5 Å) X-ray structure is available for trimeric PS I core complexes from the cyanobacterium Thermosynechococcus elongatus [7], whereas for plant PS I a crystallographic model at 3.3 Å resolution has been reported [8]. The two large subunits, PsAA and PsAB, each consisting of 11 transmembrane helices, coordinate most of the antenna pigments and the following redox cofactors involved in the electron-transfer reactions: the primary electron donor P700 (a heterodimer of Chl a (eC-B1; FpA) and a’ (eC-A1; FpA)) located on the luminal side (nomenclature of Ref. [8] is used for naming cofactors). Two branches of cofactors related by pseudo-C2 symmetry connect P700 and the first [4Fe-4S] iron sulfur cluster Fx. Each branch is composed of two Chls (Acc-A (eC-B2) and Aα-A (eC-A3) in the A-branch and Acc-B (eC-A2) and Aα-B (eC-B3) in the B-branch) and one phyloquinone A1 (Qx-A and Qx-B, respectively). The terminal electron acceptors FxA and FxB (two [4Fe-4S] iron–sulfur-clusters) are both coordinated by subunit PsAc, one of the three extrinsic subunits located on the stromal side.

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other fraction forward electron transfer to the terminal iron–sulfur-clusters is completely blocked at cryogenic temperatures [10]. In this fraction the charge separation is reversible at low temperature and can be attributed to the formation and decay of P700\(^+\)A\(_{1}\) and P700\(^+\)F\(_{x}\), respectively. Charge recombination of P700\(^+\)A\(_{1}\) occurs almost independent of temperature with \(t_{1/2} \sim 170\ \mu s\) [10]. The charge recombination between F\(_{x}\) and P700\(^+\) takes place in the ms time range at low temperature.

The function of the antenna is to harvest solar energy and to transfer this energy to the reaction center, where the excitation energy is converted into a charge separated state. One striking feature of the light-harvesting antenna of PS I is the presence of long-wavelength (also called red or low-energy) Chls (LWC) that absorb at energies lower than that of the primary electron donor P700 (for reviews see Refs. [11,12]). Higher plants contain their most-red Chls in the light-harvesting complex LH\(_{1}\), which absorb around 716 nm and give rise to the emission around 735 nm at low temperature, whereas long-wavelength Chls in the PS I core of plants absorb around 705 nm and emit at 719 nm. In the green algae Chlamydomonas reinhardtii absorption and emission of the reddest state are at ~701 nm and ~712 nm, respectively [13]. The occurrence of LWC in the core antenna, which absorb even further into the red, is unique to cyanobacteria. The content and the spectral characteristics of long-wavelength Chl \(a\) antenna states are species dependent. PS I trimers contain usually more red Chls than monomers (Ref. [12] and references therein). The 5 K absorption and linear dichroism spectra of PS I trimers of \(T\.\ elongatus\) exhibit Chl \(a\) antenna states absorbing at 708 (C708) and 719 nm (C719) [14,15]. Hole-burning experiments indicate a third spectral form named C715 [16]. PS I trimers of Synchocystis sp. PCC 6803 contain red Chls peaking at 708 and 714 nm [17]. The most-red Chl \(a\) antenna state absorbing at 740 nm (C740) and emitting at 760 nm (F760) at low temperature is present in PS I trimers of \(A\.\ platensis\) [18,19].

The function of the red Chls in photosynthesis may be different depending on their location in the antenna system and therefore on the distance between the red Chls and P700. At physiological temperatures, the quantum efficiency of photochemistry is virtually not affected by the long-wavelength Chls. Thermal energy of the surrounding enables efficient uphill energy transfer to bulk Chls and then to P700. Therefore the quantum yield of P700 oxidation is independent of the wavelength of the excitation, even at wavelengths of up to 760 nm [14]. At lower temperatures, however, the red Chls act as traps for excitations, thereby decreasing the quantum efficiency of charge separation and increasing the fluorescence yield [14,19]. It has been suggested that the extension of the spectral range for light harvesting to longer wavelengths increases the efficiency of the antenna system for the utilization of far-red light for photochemistry. This might be the result of the adaptation of the cyanobacteria to low light conditions [20,21]. Red Chls may also be involved in the protection of PS I complex against excess excitation light energy [12,22 and Refs. therein].

Remarkably, the fluorescence yield of PS I was found to be very similar in PS I with P700 in the reduced and oxidized state at room temperature [12,23] indicating that P700\(^+\) quenches the fluorescence as efficiently as P700. P700\(^+\) exhibits a broad, flat absorption band that extends into the far red beyond 800 nm [14,24] and provides spectral overlap with the emission bands of all antenna pigments, so that excitation energy transfer from the excited long-wavelength chlorophylls to P700\(^+\) can occur by the inductive resonance mechanism (Förster transfer). The subsequent fast radiationless decay of P700\(^+\) probably constitutes the quenching mechanism [23,25].

Quenching of fluorescence from long-wavelength Chls by P700\(^+\) has also been observed at cryogenic temperatures. The intensity of the fluorescence at 760 nm (F760) in PS I trimers of \(A\.\ platensis\) is highly dependent on the redox state of P700 at 77 K [19,27,28]. The maximum F760 level was observed for PS I trimers if P700 was reduced [19]. The 760 nm fluorescence is diminished by more than a factor of 10 in the presence of oxidized P700 at cryogenic temperatures. In accordance with the difference in the low temperature yield of 760 nm fluorescence in PS I trimers with P700 and P700\(^-\), respectively, the lifetime of the fluorescence decay at 760 nm was found to be significantly shorter in PS I trimers with P700 oxidized than that in PS I trimers with P700 reduced [28]. The Förster overlap integral between F760 and the absorption spectrum of P700\(^-\) is sufficiently large (about ~1/3 of the overlap between two isoenergetic chlorophylls with a Stokes shift of 130 cm\(^{-1}\)) to explain the quenching by direct resonance energy transfer from the LWC to P700\(^+\) [19]. From the decay rate of the fluorescence at 760 nm a distance between C740 and P700 of ~27 Å has been estimated [19]. A decrease of the fluorescence from red Chl \(a\) antenna states by P700\(^+\) formation was observed also for PS I trimers of \(T\.\ elongatus\) at low temperature [23], but it is still an open question whether fluorescence of different red states is quenched with the same efficiency or not. At cryogenic temperatures it can be assumed that quenching by P700\(^+\) is based on a direct energy transfer from the excited red states to P700\(^-\) since uphill energy transfer from the low-energy Chls via bulk Chls to P700\(^+\) is highly unlikely. In this case, the quenching efficiency will depend on (a) the distance between the individual red states and P700 and (b) the mutual orientation of the transition dipole moment of the low-energy exciton transition of the LWC aggregate with respect to that of P700\(^+\) [29].

Because the long-wavelength chlorophylls dominate the emission spectra of PS I and because they have pronounced effects on energy transfer and trapping, the purpose of this paper is to measure fluorescence of PS I complexes from various cyanobacteria with oxidized and reduced P700. Thereby, we want to clarify whether the emission of the various red antenna states is affected differently by the redox state of P700. The positions of the most-red chlorophylls with respect to P700 are estimated and consequences of our results on single-molecule spectroscopy of PS I complexes are discussed.

2. Materials and methods

2.1. Preparation of PS I complexes

Monomeric and trimeric PS I core complexes from \(A\.\ platensis\) have been prepared as described before [19]. PS I trimers and monomers from \(T\.\ elongatus\) have been prepared as described in [30,31].

2.2. Measurement of steady-state fluorescence spectra

Fluorescence spectra were recorded in a FluorMax 2 (Jobin Yvon) photon counting spectrophotometer. The spectra were corrected for the spectral sensitivity based on the measurement of a calibrated light source.

For the measurements, PS I complexes were diluted to a final Chl concentration of about 5 \(\mu M\) with buffer (either buffer A, containing 20 mM tricine, pH 7.5, 25 mM MgCl\(_2\) and 0.02% (w/w) \(\beta\)-DM, or buffer B, containing 100 mM AMPSO or CAPS, pH 9.0–9.5, 20 mM CaCl\(_2\), 10 mM MgCl\(_2\), and 0.02% (w/w) \(\beta\)-DM) and glycerol (final concentration about 65% (v/v)), to obtain a transparent glass at low temperatures.

Measurements were carried out under three different redox conditions: (a) Samples with P700 in the oxidized state were prepared by the addition of 1 mM ferricyanide to oxidize P700 chemically or by freezing a sample without addition of redox mediators under illumination. In this case P700 is irreversibly oxidized by the light. The two ways of oxidizing P700 gave virtually the same results. (b) Samples with P700 in the reduced state were prepared by using buffer B (pH 9–9.5) and addition of 20 mM dithionite. In the presence of the strong reductant at basic pH values
(pH ~9) the terminal iron–sulfur clusters F₆ and F₇ are chemically pre-reduced. The efficiency of pre-reduction can be enhanced by additional illumination during freezing. In the presence of pre-reduced terminal iron–sulfur cluster, light-induced electron transfer is limited to the formation and decay of P700⁺A₇ or P700⁺F₆, respectively. The charge recombination of P700⁺A₇ occurs with a half-life of about 150 μs, whereas P700⁺F₆ decays in the ms time range [10]. Since the rate of charge separation induced by the excitation light in the spectrophotometer is small compared to the decay rate of the charge separated states, P700 remains in the neutral (reduced) state during fluorescence measurements. (c) Samples frozen in the dark with 5 mM ascorbate and 5 μM PMS contain all redox cofactors initially in the neutral ground state. However, the charge-separated states P700⁺F₆ and P700⁺F₇ are irreversibly formed in a fraction of the PS I complexes by the excitation light when recording the spectra, whereas in the other fraction forward electron transfer to the terminal iron–sulfur-clusters is completely blocked at cryogenic temperatures [9,10]. In the latter fraction the charge separation is reversible at low temperature and P700 is maintained in the reduced state. Therefore the sample contains a mixture of reduced and oxidized P700 during fluorescence measurements.

For low temperature measurements, the cuvette was placed in a variable temperature liquid nitrogen bath cryostat (Oxford DN1704) or an Oxford liquid helium flow cryostat (CF1260) and a home-built cryostat holder was used in the spectrophotometer. Due to small variations in the position of the samples the reproducibility of the peak amplitudes for identical samples was about ± 10%.

2.3. Single-molecule fluorescence spectroscopy

Purified PS I trimers were at first diluted with buffer A or B and glycerol to a final Chl concentration of about 20 μM. The concentration of detergent in the buffer was increased to 4 mM or 0.2% (w/w). This concentration was found to be adequate to avoid completely PS I aggregation. In further steps this PS I containing solution was diluted to a PS I trimer concentration of about 3 μM. Additionally, 20 mM dithionite was added to the sample diluted in buffer B to achieve pre-reduction of the terminal iron–sulfur-clusters. The yield of pre-reduction was increased by additional illumination during sample preparation and mounting. Less than 1 μl was placed between two cover slips assuring spatial separation of individual PS I trimers. To observe fluorescence emission of single molecules, a home-built confocal microscope was used. The experimental setup was described recently in Ref. [32]. The sample and the microscope objective were immersed in superfluid He, with a sample temperature of 1.4 K. The excitation power measured directly behind the beam-scanning module was varied between 0.05 μW and 100 μW, and the excitation wavelength was 680 nm for all experiments.

3. Results and discussion

3.1. Photosystem I from A. platensis

Fig. 1 shows 77 K steady-state fluorescence spectra of isolated PS I trimers from A. platensis (former name Spirulina platensis) under different redox conditions (see Materials and methods). The excitation wavelength was 500 nm. Virtually identical fluorescence spectra were recorded at an excitation wavelength of 430 nm. The spectra are in agreement with those reported in the literature [26,27]. In the presence of reduced P700 (solid line) the emission maximum is observed at 760 nm. The emitter is most likely the LWC pool C740. A much weaker peak is located at 726 nm, which might be ascribed to the C708 pool. A minor fluorescence band is observed at about 690 nm which originates presumably from bulk chlorophylls.

In the presence of oxidized P700, the emission of the C740 pool is almost completely quenched and appears only as a shoulder of the 726 nm band (dashed line). The amplitude of the 726 nm peak is only slightly lower. The data confirm that oxidized P700 quenches effectively the emission of the C740 pool, whereas the emission of the C708 pool is not affected significantly [26,27].

The dipole strength of the 740 nm band in the 5 K absorption spectrum of PS I trimers corresponds to that of approximately three monomeric Chl molecules [19]. It is known that C740 arises upon trimerization of isolated monomeric PS I complexes [33] indicating that at least three Chls of each monomer are red-shifted due to structural reorganisations and/or to altered interactions induced by the trimerization. Therefore, a location of C740 close to the trimerization domain has been proposed [19,22,33]. To explain the quenching by direct resonance energy transfer from C740 to P700⁺ the rate of energy transfer must be higher by at least a factor of 10 compared to the decay rate in the absence of energy transfer. This consideration leads to a time for energy transfer of approximately 100 ps. In PS I with oxidized P700 the decay kinetics of F760 has been reported to be dominated by a 107 ps component, whereas in samples with P700 reduced nanosecond decay components were observed predominantly [28]. Taking into account that the observed 107 ps lifetime originates from two competing processes - the intrinsic ns fluorescence decay as observed in centers with reduced P700 and direct quenching by P700⁺ - a lifetime of about 115 ps results for the latter process. From this value a distance between C740 and P700 of ~28 Å has been estimated using Förster theory [19]. This distance is in reasonable agreement with the assumption that the involved Chls are located near the monomer–monomer interfaces [7,33].

PS I monomers from A. platensis contain only the LWC form with an absorption maximum around 708 nm. Accordingly, the 77 K fluorescence spectrum in the presence of reduced P700 (see Fig. 2, solid line) exhibits the main emission band at 727 nm and a minor fluorescence band at about 690 nm. The shoulder at longer wavelengths can be assigned to a vibronic sideband corresponding to the mirror image of the Q₅(0→1) absorption band of the LWC C708. With P700 in the oxidized form, the fluorescence intensity on the long-wavelength side of the main emission band is diminished (see Fig. 2, dashed line). Thereby, the maximum is shifted by 1 to 2 nm to the blue and the peak amplitude is slightly lower. The difference between both spectra (reduced – minus – oxidized) is given by the dotted line. The difference spectrum has its maximum at about 731 nm.
reported substantial inhomogeneous broadening [12 and Refs. therein]. Thus chlorophylls [7,34]. It should be noted that more than one aggregate is strongly coupled aggregates consisting of 2 to 3 complexes at 295 K and 77 K. The low temperature absorption spectra of trimeric PS I complexes from T. elongatus: the fluorescence intensity on the long-wavelength side of the main emission band is decreased (see Fig. 4, dashed line). Thereby the maximum is shifted by 2 nm to the long-wavelength side of the main emission band. This means that at least two different strongly coupled aggregates contribute to the C719 pool: (a) one red antenna state that is present in both monomeric and trimeric complexes and (b) another one that emerges as a result of the trimerization [31]. The results presented below demonstrate that the fluorescence yield of the latter red state depends in a similar way on the redox state of P700 as shown above for the C740 pool in A. platensis.

The situation is very different in PS I trimers from T. elongatus: the fluorescence intensity at low temperature was found to be strongly affected by the redox state of P700. Fig. 5 shows 77 K fluorescence spectra of PS I trimers from T. elongatus in the presence of reduced P700 (solid line) and oxidized P700 (dashed line). In the presence of oxidized P700 (dashed line) the fluorescence spectrum resembles that of PS I monomers form this organism (see Fig. 4). If P700 is reduced, the fluorescence emission maximum is shifted ~5 nm to the red and the total fluorescence intensity has increased by a factor of two. The reduced – minus – oxidized difference spectrum (dotted

maximum at 715 nm at low temperature has been suggested on the basis of hole-burning experiments [16]. This LWC form cannot be resolved as a separate band in the 5 K absorption spectrum due to strong overlap with the C719 absorption band. Whereas monomeric PS I complexes of T. elongatus contain about the same number of red Chls absorbing around 708 nm, the monomers have a lower number (2 instead of 4) of C719 chlorophylls. This means that at least two different strongly coupled aggregates contribute to the C719 pool: (a) one red antenna state that is present in both monomeric and trimeric complexes and (b) another one that emerges as a result of the trimerization [31]. The results presented below demonstrate that the fluorescence yield of the latter red state depends in a similar way on the redox state of P700 as shown above for the C740 pool in A. platensis.

Fig. 4 shows 77 K steady-state fluorescence spectra of isolated PS I monomers from T. elongatus under different redox conditions (see Materials and methods). The main emission band in the presence of reduced P700 (see Fig. 4, solid line) is located around 730 nm. As in monomeric PS I from A. platensis, there is a minor fluorescence band at about 690 nm and a shoulder at longer wavelengths that can be assigned to the mirror image of the Qx(0 → 1) absorption band of the LWC C708. In comparison to monomeric PS I from A. platensis, the position of the main band is shifted 3 nm to longer wavelengths. This is due to contributions from LWC C715 and/or C719 to the emission spectrum. These LWC are present in addition in PS I monomers from T. elongatus.

With P700 in the oxidized form, the fluorescence intensity on the long-wavelength side of the main emission band is decreased (see Fig. 4, dashed line). Thereby the maximum is shifted by 2 nm to the blue and the peak amplitude is somewhat lower. The difference between the two spectra (reduced – minus – oxidized) is given by the dotted line. The difference spectrum has its maximum at about 734 nm. Similar to monomeric PS I from A. platensis the total fluorescence intensity is only slightly affected by the redox state of P700 except that of this red-shifted emitter, which might be assigned to an aggregate from the C715 or C719 pool.

The situation in monomers from A. platensis is quite different. The dissolved crystals of trimeric PS I complexes from T. elongatus at 295 K and 77 K. The spectra presented in Fig. 3 do not confirm the result of Riley et al. [35], but are virtually identical to our previously published spectra [14,34] and the estimated number of red pigments is also about the same. The reason for this discrepancy is not clear. A third LWC form with an absorption

Fig. 2. 77 K fluorescence spectra of PS I monomers from A. platensis with P700 in the reduced (solid line) and oxidized (dashed line) state. The excitation wavelength was 500 nm. The dotted line represents the difference between both spectra.

3.2. Photosystem I from T. elongatus

The low temperature absorption spectra of trimeric PS I complexes of T. elongatus show long-wavelength absorption bands at about 708 nm and 719 nm with oscillator strengths corresponding to approximately five and four Chls, respectively [14,34]. More recently, it has been reported that PS I samples prepared by dissolving crystals of trimeric PS I complexes exhibit a much stronger absorption in the red antenna region [35]. To verify this result, we also measured the absorbance spectrum of freshly dissolved crystals of trimeric PS I complexes from T. elongatus at 295 K and 77 K. The spectra presented in Fig. 3 do not confirm the result of Riley et al. [35], but are virtually identical to our previously published spectra [14,34] and the estimated number of red pigments is also about the same. The reason for this discrepancy is not clear. A third LWC form with an absorption

Fig. 3. Absorbance spectra of a sample with freshly dissolved crystals of trimeric PS I complexes at 295 K and 77 K.

Fig. 4. 77 K fluorescence spectra of PS I monomers from T. elongatus with P700 in the reduced (solid line) and oxidized (dashed line) state. The excitation wavelength was 430 nm. The dotted line represents the difference between both spectra.
The luminal side close to the trimerization domain (see Fig. 6 for the excitonically coupled chlorophylls A31–A32–B7 are located on the phylls) is probably the best candidate for this red state. The wavelength range between 715 nm and 730 nm [15]. The energy of the transition dipole of the low-energy exciton state parallel to the membrane plane matching the reported linear dichroism of trimeric PS I at 5 K shows a decay component with a lifetime of a few nanoseconds in rough agreement with spectroscopic studies. In the case of reduced P700, time-resolved fluorescence measurements reveal a decay component with a lifetime of a few nanoseconds [23,29]. Its decay-associated spectrum peaks at 745 nm at 5 K [22,28]. The lifetime decreases to a few 100 ps in the presence of P700+ [29, Meyer, J., Schmitt, F.J. and Schlodder, E., unpublished results] due to the efficient quenching of the emission from this red state by oxidized P700. The similarities between this red state and C740 in trimeric PS I from A. platensis indicates the same structural organization or even a structural identity of the reddest antenna state in both organisms. The reason, why the absorption of the aggregate in A. platensis is shifted about 20 nm further to the red, remains to be clarified. The chlorophyll trimer B31–B32–B33 has often been proposed as another candidate for the C719 LWC. B31, B32 and B33 are among the most strongly coupled chlorophylls [36] and were assigned to C719 on the basis of modeling of time-resolved fluorescence data [34]. Apparently, the trimer cannot be assigned to the reddest state because of the large distance to P700 (about 50 Å). This would not be consistent with the efficient quenching of the emission by oxidized P700. It is however possible that this Chl trimer is identical with the C719 aggregate that exists in both monomeric and trimeric PS I complexes.

Interestingly, the fluorescence intensity of monomeric PS I complexes (see Figs. 2 and 4) is only slightly affected by the redox state of P700. This means that the emission of nearly all LWC is not quenched strongly by oxidized P700 except one LWC aggregate emitting around 732 nm (see Figs. 2 and 4). This indicates a large distance of nearly all LWC to P700 and to a location at the periphery of the PS I complex. Among others: B37–B38, A38–A39, B18–B19, A16–A17–A25 (see Fig. 6) are potential candidates which meet the necessary conditions: (a) strong coupling between the chlorophylls of the aggregate (see Table 3 in Ref. 33), (b) orientation of the transition dipole of the low-energy exciton state parallel to the membrane plane [15] and (c) large distance to P700 [7]. These aggregates might be responsible for C708. For B24–B25 and A26–A27, a short energy-transfer time to P700+ can be expected. These dimers might be involved in C715 with an emission around 734 nm that was found to be quenched by P700+ (see Fig. 4).

The strong influence of the reddest antenna state on the emission spectrum of trimeric PS I from T. elongatus is illustrated in Fig. 7. It shows fluorescence spectra measured under different redox conditions at 5 K and normalized to the respective peak maximum. With P700 in the oxidized state, the fluorescence spectrum (dashed curve) peaks at 732 nm. In the presence of reduced P700, the emission maximum at 5 K is red-shifted to 741 nm (solid curve). This red shift is due to the fact that under these conditions, the reddest antenna state contributes strongly to the fluorescence, since its emission is not quenched in the presence of reduced P700. In addition, the contribution of the long-wavelength chlorophylls, which emit at 734 nm, is larger (see Fig. 4). In the literature [12 and Refs. therein] it has been concluded that the fluorescence intensity of monomeric PS I complexes is only slightly affected by the redox state of P700. This means that the emission of nearly all LWC is not quenched strongly by oxidized P700 except one LWC aggregate emitting around 732 nm (see Figs. 2 and 4). This indicates a large distance of nearly all LWC to P700 and to a location at the periphery of the PS I complex. Among others: B37–B38, A38–A39, B18–B19, A16–A17–A25 (see Fig. 6) are potential candidates which meet the necessary conditions: (a) strong coupling between the chlorophylls of the aggregate (see Table 3 in Ref. 33), (b) orientation of the transition dipole of the low-energy exciton state parallel to the membrane plane [15] and (c) large distance to P700 [7]. These aggregates might be responsible for C708. For B24–B25 and A26–A27, a short energy-transfer time to P700+ can be expected. These dimers might be involved in C715 with an emission around 734 nm that was found to be quenched by P700+ (see Fig. 4).

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the fluorescence maximum of trimeric PS I from *T. elongatus* is reported to be located at 732 nm indicating that most of the low temperature fluorescence studies have been performed using PS I with P700 in the oxidized state.

For samples with a mixture of reduced and oxidized P700, the fluorescence spectrum peaks at 738 nm (see Fig. 7, dotted line). These samples containing ascorbate and PMS (which keeps both P700 and the acceptors in the neutral state) have been frozen in the dark. Under these conditions P700 is initially completely reduced. Illumination of such a sample at low temperature will lead to the irreversibly formed radical pair P700\(^+\)A\(_1\) in a fraction (~50% at 5 K [10]) of the photosystems, whereas in the other fraction charge recombination maintains P700 in the reduced state (see Materials and methods). Thus, it is possible to prepare samples containing a mixture of reduced and oxidized P700 due to light-induced oxidation of P700 prereduced by ascorbate at 77 K.

### 3.3. Single-molecule spectroscopy (SMS)

The first single-molecule spectra of PS I at cryogenic temperature have been reported by Jelezko et al. [37]. SMS offers the possibility to obtain more detailed information about the spectral characteristics of the antenna states. SMS enables the observation of the emission of single photosystems [32]. As a consequence, spectral features that are characteristic of single molecules can be revealed. At low temperatures, these are a narrow zero-phonon line (ZPL) and a broad phonon wing. The ZPL belongs to an electronic transition between the lowest-lying energy levels in the ground and excited state. The phonon wing on the low-energy side of the ZPL is related to transitions from the ground vibrational state of the excited electronic state to higher-lying vibrational levels of the electronic ground-state due to the interaction of the chromophore with its surrounding (electron–phonon coupling). SMS experiments using PS I complexes from cyanobacteria show sharp emission lines at 705–715 nm assigned to ZPLs from C708 and a broad emission band centered around 725–730 nm [32,35]. It has been proposed that the longer-wavelength antenna states are characterized by increased electron–phonon coupling and strong spectral diffusion. The rates of the spectral diffusion process are much higher than the actual acquisition rate and the wavelength range covered by these lines is in the range of more than 10 nm [32,37].

To detect the weak fluorescence of single molecules (photosystems) high excitation laser intensities of about 100 \(\mu\)W at 680 nm are often used, yielding an excitation intensity in the focus on the order of 6·10\(^{2}\) \(\text{W/cm}^2\) [32]. This corresponds to a photon flux of about 6.8·10\(^{20}\) photons/(cm\(^2\)-s). Because of the very large photon flux P700 is kept in the oxidized state. At low temperature, the highest rate for the recovery of the ground state of P700 is achieved in centers with prereduced iron–sulfur cluster F\(_{A}\) and F\(_{B}\). Under these conditions the radical pair P700\(^+\)A\(_1\) is formed which decays by charge recombination of P700\(^+\)A\(_1\) with a half-life of 170 \(\mu\)s [10] corresponding to a rate \(k_d = 4.1 \cdot 10^3\) s\(^{-1}\). The lifetime of the primary radical pair P700\(^+\)A\(_0\) would be shorter (~50 ns) but it decays dominantly in the triplet state P700, which decays by intersystem crossing to the singlet ground state with a significantly longer half-life of about 1 ms at low temperature. The rate of formation of P700\(^-\)A\(_1\)\(^-\) is \(k_{cs} = \sigma \cdot \Phi \cdot I\), where \(\sigma\) is the absorption cross section at 680 nm (~2·10\(^{-14}\) cm\(^2\)), \(\Phi\) is the quantum yield of charge separation at low temperature (~50% [10]) and I is the photon flux. The fraction of centers in the ground state P700A\(_1\) is then given by:

\[
P700A_1 = \frac{k_d}{k_d + \frac{\sigma \cdot \Phi \cdot I}{k_d} + \frac{1}{\tau}}
\]

At a photon flux of 6.8·10\(^{20}\) photons/(cm\(^2\)-s) it follows that this fraction is 6.0·10\(^{-4}\). That means P700 is completely in the oxidized state under these experimental conditions and fluorescence of the reddest long-wavelength chlorophylls would escape detection because their emission is largely quenched by P700\(^+\). A reduction of the excitation power by a factor of about 1000 would be necessary to have at least about 40% of P700 in the neutral state.

Fig. 8 shows single-molecule fluorescence emission spectra of one trimeric PS I complex from *A. platensis* at 1.4 K. The laser intensity was varied during the experiment between 100 \(\mu\)W and 0.05 \(\mu\)W, corresponding to an excitation intensity in the focus in the order of

![Fig. 7. 5 K fluorescence spectra of PS I trimers from *T. elongatus* with P700 in the reduced (solid line) and oxidized (dashed line) state. The spectra are normalized to their respective peak maximum. The excitation wavelength was 500 nm. The dashed-dotted line shows the fluorescence spectrum of a trimeric PS I sample that was frozen in the presence of 5 mM ascorbate and 5 \(\mu\)M PMS in the dark. These samples contain a mixture of reduced and oxidized P700 during fluorescence measurements (see Materials and methods).](image-url)

![Fig. 8. Single-molecule fluorescence spectra of one trimeric PS I complex from *A. platensis*. 20 mM dithionite was added to the sample diluted in a buffer B (pH 9–9.5). The pre-illuminated sample has been frozen to 1.4 K; excitation wavelength was 680 nm. The excitation power was varied between 10 \(\mu\)W (top trace) and 0.05 \(\mu\)W (bottom trace). The accumulation time was chosen in each case so that a sufficient signal/noise ratio was achieved (5 s–10 min). Original data (solid curve), data smoothed by a moving average filter (dashed curve).](image-url)
6 · 10^1 W/cm² to 0.3 W/cm². The accumulation time was chosen in each case so that a sufficient signal-to-noise ratio was achieved.

20 mM dithionite was added to the sample diluted in buffer B (pH 9–9.5). The sample has been frozen under illumination to 1.4 K. This treatment leads to chemical pre-reduction of the terminal iron-sulfur clusters so that light-induced electron transfer is restricted to the formation and decay of the secondary radical pair P700⁺⁻A₁. At an excitation power of 100 μW (not shown), 10 μW and even 1 μW the emission of the C740 pool is almost completely quenched and appears only as a shoulder of the broad emission band around 727 nm band (see Fig. 8). If the excitation power is further decreased to 0.1 μW and 0.05 μW, the emission of C740 becomes visible as a separate broad band. ZPLs are observed only in the region around 715 nm. Strong electron–phonon coupling and ZPL dynamics [32,37] are most likely the reasons that emission bands at 727 nm and 760 nm are broad and unstructured. Spectral jumps of ZPLs on a time scale much shorter than the acquisition time would lead to an increase of the line-width of ZPLs at first and then after prolonged averaging to the complete loss. The single-molecule fluorescence spectra of one trimeric PS I complex from A. platensis monitored with an excitation power ≥ 1 μW resemble the bulk emission spectrum in the presence of oxidized P700 (see Fig. 1, dashed line) except for the ZPLs. Below 1 μW the amplitude of the F760 band is growing. At 0.05 μW the F760/F726 ratio is about one. This indicates that a fraction of P700 is in the reduced state in accordance with our estimation above. If P700 is completely reduced, the F760/F726 ratio is about ten (see Fig. 1, solid line). A further reduction of the excitation power was, however, not possible for experimental reasons and because of the significantly decreasing signal-to-noise ratio.

Single-molecule fluorescence spectra in dependence of the excitation power were also recorded for trimeric PS I complexes from T. elongatus (see Fig. 9). For an excitation power of ≥ 10 μW the maximum of the unstructured broad emission band is located at 732 nm which is typical for PS I with oxidized P700 (see Fig. 5, dashed line). If the excitation power is reduced to 0.1 μW, the maximum shifts to the red up to 739 nm. Bulk emission spectra of a sample with a mixture of reduced and oxidized P700 show at 5 K the maximum at this wavelength (see Fig. 7 dotted line). The result suggests that a mixture of reduced and oxidized P700 is present at an excitation power of 0.1 μW. Consistently the emission of the reddest antenna state starts to contribute to the fluorescence spectrum leading to the observed red shift of the maximum from 732 to 739 nm.

The correlation between the content of oxidized P700 and the extent of fluorescence quenching contradicts the photochemical quenching mechanism that was proposed recently [39] in order to explain that the fluorescence yield in PS I with reduced and oxidized P700 is almost the same at room temperature. In this hypothesis, it was assumed that the primary electron donor is rather the accessory chlorophyll than P700. If so, photochemistry can occur regardless of whether P700 is reduced or oxidized. If the initial charge separation forming Acc⁻⁻A₀ in the presence of P700⁺ is followed by a fast radiationless charge recombination, the quenching of the excited state would be the same but no stable photoproduc would be detectable. As a consequence of this mechanism, the fluorescence quenching would be the same regardless of the redox state of P700. At low temperature, however, PS I with oxidized P700 (also named closed PS I) quenches the fluorescence significantly better than PS I with reduced P700 (also named open PS I) (see Figs. 2 and 5). Especially fluorescence from the reddest long-wavelength Chls is strongly quenched by P700⁺ at cryogenic temperatures. Since uphill energy transfer is unlikely at low temperatures, the most likely explanation for the observed quenching is that described in the Introduction. Excitation energy is transferred from the excited long-wavelength chlorophylls to P700⁺ by the inductive resonance mechanism ( Förster transfer) followed by the radiationless decay of (P700⁺⁻)⁺ [23,25].

The absorption of the oxidized donor P700⁺ resembles most likely that of Chl a in organic solvents [40,41], which exhibits a broad and almost constant absorption between 500 nm and 850 nm. The broad absorption of the cation P700⁺ provides about the same spectral overlap (about 1/3 of the overlap between two isoen energetic chlorophylls with a Stokes shift of 130 cm⁻¹) with the emission bands of all the core-antenna pigments, so that P700⁺ can effectively act as an excitation energy acceptor for bulk and long-wavelength Chls. The excitation in the antenna is removed by the decay of (P700⁺⁻)⁺ which is expected to be extremely fast. Therefore, the distance between P700⁺ and the LWC and the mutual orientation of the transition dipoles determine the rate and efficiency of the quenching. At low temperature, the LWC act as traps for the excitation. Our data indicate that most of the LWC are localized at the periphery of the PS I complex and only the reddest antenna states seems to be close enough for an efficient quenching.

At room temperature, the described quenching mechanism should also be effective. The excited state is, however, more extensively distributed between all antenna chlorophylls, because thermal energy is sufficient to enable uphill energy transfer from LWC to bulk chlorophylls. During their migration through the antenna all excitations will come close enough to reaction center to be quenched efficiently by P700⁺. Therefore, the quenching by P700⁺ is sufficient to explain the fast fluorescence decay within about 30 ps in closed PS I.

To explain the surprisingly small difference between the fluorescence quenching efficiency of P700⁺ (photochemical quenching via charge separation) and P700⁺ at room temperature, various possibilities have been considered in the literature (see e.g. [23]). If, however, there would be additional photochemical quenching in the presence of P700⁺ via charge separation starting from the excited accessory Chls similar to the photochemical quenching in the presence of P700, the fluorescence decay in closed PS I (P700 oxidized) should be significantly faster than that in open PS I (P700 reduced). The faster decay would result from two efficient quenching mechanisms which would be effective in the case of oxidized P700⁺. Therefore, additional photochemical quenching in the presence of P700⁺ seems to be unlikely.

Our data do not allow conclusions regarding the mechanism of the primary charge separation in open PS I (P700 reduced), but we would like to note that photochemistry starts most likely from the lowest excitonic state of the reaction center. Especially at low temperatures, this must be the case, because only the lowest excited state of the reaction center chlorophylls state is populated according to Boltzmann. Therefore, the proposal in which the initial charge separation starts from the excited accessory Chl in one or both branches,
Acc-A and/or Acc-B [42], are based on the assumption that the accessory Chls contribute significantly to the lowest excitonic state, that drives the initial charge separation. The problem with this assumption is that steady-state and transient polarized absorption spectroscopy of PS I yields evidence that the lowest excitonic state of the reaction center is dominated by P700. This is in agreement with the exciton calculations [43,34]. On the other hand, a certain contribution of the accessory chlorophylls to the lowest excitonic state cannot be ruled out. Additionally, the situation might be different at room temperature, because higher exciton states will be populated thermally.

4. Conclusions

This work demonstrates for the first time the emission of the reddest antenna state for single PS I complexes. Assignments of LWC aggregates to emission bands in previously published work must be re-made since the emission of the reddest antenna state escaped detection due the strong quenching by P700<sup>−</sup>. The data presented in this work indicate a simple rule. The emission band of the LWC is red-shifted by about 20 nm relative to the Q<sub>y</sub> absorption band. The efficient fluorescence quenching, which is observed for the reddest antenna states, may represent a protective mechanism with physiological relevance [12,23]. Excess energy in the antenna may be dissipated because of the quenching by P700<sup>−</sup>, which is present under high light conditions. Thereby, the formation of triplet states in the antenna and subsequently the formation of harmful singlet oxygen are avoided.

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