Site-directed mutations at D1-His198 and D1-Thr179 of photosystem II in *Synechocystis* sp. PCC 6803: deciphering the spectral properties of the PSII reaction centre

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Site-directed mutations were constructed in photosystem II of *Synechocystis* sp. PCC6803 in which the axial ligand, D1-His198, of special pair chlorophyll P_{D1} was replaced with Gln and where D1-Thr179, which overlies monomeric chlorophyll Chl_{D1}, was replaced with His. The D1-His198Gln mutation produces a 3 nm displacement to the blue of the bleaching minimum in the Soret and in the Q_y region of the (P⁺Q_A⁻-PQ_A) absorbance difference spectrum. To a first approximation, the bleaching can be assigned to the low-energy exciton transition of the special pair chlorophylls P_{D1}/P_{D2}. The D1-Thr179His mutation produces a 2 nm displacement to the red of the bleaching minimum in the Q_y region of the (³P-¹P) absorbance difference spectrum. Analysis of the flash-induced (P⁺Q_A⁻-PQ_A) and (³P-¹P) absorbance difference spectra of both mutants compared with wild-type at 80 K indicate that the cation of the oxidized donor P⁺ is predominantly localized on the chlorophyll P_{D1} of the special pair and that the reaction centre triplet state, produced upon charge recombination from ³[P⁺Pheo⁻], when the primary quinone electron acceptor Q_A is doubly reduced, is primarily localized on Chl_{D1}.

Keywords: photosystem II; P680; P_{D1}; Chl_{D1}; site-directed mutagenesis; reaction-centre triplet

1. INTRODUCTION

X-ray crystallography has recently provided a great deal of structural information on the content and arrangement of the cofactors of photosystem II (PSII; Kamiya & Shen 2003; Ferreira *et al.* 2004; Loll *et al.* 2005). According to the most recent structural analysis (Loll *et al.* 2005), the core complex of PSII of *Thermosynechococcus elongatus* comprises 20 polypeptide subunits and some 77 cofactors which include 35 Chl *a*, two Pheo *a*, 11 β-carotenes, one b-type cytochrome (cyt_b559), one c-type cytochrome (cyt_c550), two plastoquinones, a non-haem iron and the Mn₄Ca cluster responsible for water oxidation. Of these, six Chl *a*, the two Pheo *a*, two β-carotene, the two plastoquinones, cyt_b559 and the Mn₄Ca cluster are located in the reaction centre. Of these, with the exception of the cyt_b559 and one ligand to the Mn₄Ca cluster, all are coordinated by D1 and D2, two

homologous polypeptides that form a complex with C2 symmetry. All of the light-driven primary and secondary electron transfer reactions of the photosystem occur within the reaction centre.

Although, Chl *a* and Pheo *a* have replaced Bchl and Bpheo, and plastoquinone has replaced ubiquinone, the cofactors of the PSII reaction centre are arranged almost identically to those of the purple bacterial reaction centres. There are two branches of cofactors (A and B) in each of these reaction centres, though only the A-branch appears to be active for light-driven electron transport under normal physiological conditions. Absorption of a photon in reaction centre preparations results in the formation within 6–11 ps of P⁺Pheo_{D1}⁻ (Groot *et al.* 2005; Holzwarth *et al.* 2006) and in 200–500 ps of the charge-separated state (P⁺Q_A⁻; Nuijs *et al.* 1986; Schatz *et al.* 1988; Trissl & Leibl 1989; Bernarding *et al.* 1994). Charge separation is orthogonal to the plane of the photosynthetic membrane. Moving across the membrane from the luminal towards the stromal side of PSII, there is a pair of excitonically coupled chlorophylls P_{D1} and P_{D2}, followed on each branch by a monomeric chlorophyll Chl_{D1} (A-branch) and Chl_{D2} (B-branch), followed by Pheo_{D1} (A-branch)

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and Pheo_{D2} (B-branch), followed by Q_A (A-branch) and Q_B (B-branch). In the bacterial reaction centres, primary electron transfer is initiated by excitation energy localized at the P_A/P_B chlorophyll special pair (homologue to P_{D1}/P_{D2}) from which an electron is transferred to B_A (homologue to Chl_{D1}) and then to Pheo_A (homologue to Pheo_{D1}) and then to Q_A and ultimately to Q_B. The strong electronic interaction between P_A and P_B (500–1000 cm⁻¹; Woodbury & Allen 1995) produces a dominant low-energy exciton band in the Q_Y region, which constitutes the lowest excited singlet state of the reaction centre and which consequently concentrates the excitation energy at the site of primary electron transfer. In PSII, the greater physical separation of P_{D1} and P_{D2}, the smaller dipole strength of the Q_Y transition of Chl *a* and slight differences in macrocycle orientation produce a substantially weaker electronic interaction of approximately 150 cm⁻¹ between the two (Raszewski *et al.* (2005); see also Diner & Rappaport (2002) for a review of earlier literature), such that these chlorophylls do not represent the lowest energy sink for the excitation energy. Even though the monomeric Chl_{D1} has the lowest site energy (Diner *et al.* 2001; Diner & Rappaport 2002; Raszewski *et al.* 2005), the chlorophylls of the PSII reaction centre show overall much less spectral differentiation from each other in the Q_Y region than is observed in the bacterial reaction centres. At liquid helium temperatures, however, the excitation energy is likely to be highly localized on Chl_{D1}, making it probable that charge separation, which still occurs at this temperature, will be initiated by Chl_{D1}^{*} (Diner *et al.* 2001; Diner & Rappaport 2002; Raszewski *et al.* 2005), rather than from the excited [P_{D1}/P_{D2}]^{*} special pair. A consequence of the weak spectral differentiation is that the excitation energy is much more distributed over the reaction centre pigments at physiological temperatures, including Chl_{D1}, from which charge separation could still be initiated (Tetenkin *et al.* 1989; Rutherford & Nitschke 1996; Dekker & Van Grondelle 2000; Prokhorenko & Holzwarth 2000; Barter *et al.* 2003). Recent reports from two groups (Groot *et al.* 2005; Holzwarth *et al.* 2006) place the reduction of Pheo_{D1}, even at ambient temperature, occurring prior to the oxidation of P_{D1}/P_{D2}, implying that the cation of the primary radical pair is localized at another site, most probably Chl_{D1}.

In order to better describe the localization of the excitation energy and of the electron transfer steps in PSII, we have been using site-directed mutagenesis to enhance the spectral differentiation of the pigments of PSII. Mutations that replace the axial ligand or that perturb a hydrogen bond to the C₉=O ring V keto group of a particular chlorophyll modify the absorbance and vibrational spectra and occasionally the redox properties of that chlorophyll. The modified spectra provide spectroscopic labels on individual chlorophylls, allowing one to examine, to a first approximation, the role of that chlorophyll in energy and electron/hole localization.

2. MATERIAL AND METHODS

(a) Mutant construction

The glucose-tolerant strain of the cyanobacterium *Synechocystis* sp. PCC 6803 (Williams 1988) was used for the construction

of the site-directed mutants described in this paper. All strains were grown on BG-11 medium as described by Williams (1988) and Metz *et al.* (1989). Five millimolar glucose was included in the medium to allow the propagation of mutants inactive in PSII. Mutations at codons 179 and 198 of the *psbA3* gene encoding the D1 polypeptide were introduced into the TD41 *psbA* triple deletion strain of *Synechocystis* sp. PCC 6803 according to Nixon *et al.* (1992).

(b) Isolation of PSII complexes

PSII core complexes were isolated according to the combined procedures of Rögner *et al.* (1990) and Tang & Diner (1994) in that order and stored at -80°C until use.

(c) Transient absorbance spectroscopy

Flash-induced absorbance difference spectra of (P⁺Q_A⁻-PQ_A) and (³P-¹P) were recorded at low temperature as previously described (Hillmann *et al.* 1995) using a laboratory-built flash spectrometer. The (³P-¹P) absorbance difference spectra were monitored in the presence of doubly reduced Q_A. Q_A was doubly reduced by the addition of 10 mM Na₂S₂O₄ and preillumination at room temperature under anaerobic conditions. Under these conditions, the lifetime of ³P680 is similar to that of the chlorophyll *a* triplet state in solvents ($\tau \approx 1.4$ ms (70%)/7 ms (30%) at 5 K and $\tau \approx 1.4$ ms at $T > 80$ K). The samples were excited with saturating flashes of approximately 15 μ s in duration from a Xe flash lamp filtered by coloured glass (model CS4-96 from Corning).

3. RESULTS AND DISCUSSION

According to the 3 Å (Loll *et al.* 2005) and 3.5 Å (Ferreira *et al.* 2004) X-ray structures of PSII, D1-H198 and D2-H197 are the axial ligands, respectively, of the special pair chlorophylls P_{D1} and P_{D2}. Site-directed mutations constructed at D1-His198 and D2-His197 were introduced and their effects on the (P⁺Q_A⁻-PQ_A) difference spectrum were examined for both sets of mutants in the Soret at ambient temperature using flash detection optical spectroscopy (Diner *et al.* 2001). Flash-induced absorbance changes in the Q_Y region at 80 K, attributed to the formation and the decay of P680⁺Q_A⁻, were measured in the presence of ferricyanide to pre-oxidize cytochrome-*b*559, thereby preventing the accumulation of Q_A⁻cyt*b*559⁺, which is irreversibly formed with low quantum yield at low temperature (Hillmann & Schlodder 1995). The recombination of P680⁺Q_A⁻ takes place within a few milliseconds. A satisfactory fit of the decay in wild-type (WT) PSII is obtained using two exponentials with half-lives of 1.8 ms (47%) and 6.3 ms (53%) plus a constant (1%). These difference spectra are dominated by P⁺-P with only a small contribution from Q_A⁻-Q_A. Mutations introduced at D1-198 produce displacements of the main bleaching bands to the blue of the (P⁺Q_A⁻-PQ_A) difference spectrum in both spectral regions. One of the most marked of these mutations is D1-His198Gln, which produces only a very small change in the P⁺/P reduction potential (Diner *et al.* 2001, 2004). The (P⁺Q_A⁻-PQ_A) difference spectrum of the WT measured at 80 K shows a large bleaching centred at 672.5 nm and a band shift to the blue with maxima and minima at 679 and 683 nm, respectively, and an inflection at 682 nm (figure 1a).

Figure 1. Flash-induced ($P680^+Q_A^- - P680Q_A$) absorbance difference spectra of PSII core complexes from the wild-type (WT) and the (a) D1-His198Gln mutant and from the wild-type (WT) and the (b) D1-Thr179His mutant measured at 80 K. The spectra were normalized to -1 at the bleaching minimum to allow better comparison. The PSII core complexes were diluted in a glycerol/buffer mixture (pH 6.5; 65 : 35; v : v). The OD at the maximum in the Q_Y region was approximately 1 cm^{-1} . 3 mM ferricyanide was added to oxidize cyt $b559$. The absorbance change at the bleaching minimum was approximately -3×10^{-2} for the wild-type and the mutant core complexes. Flash-induced ($^3P-^1P$) absorbance difference spectra of PSII complexes from the wild-type and the (c) D1-His198Gln mutant and from the wild-type and the (d) D1-Thr179His mutant measured at 80 K. The PSII core complexes were suspended in 100 mM MES, pH 6.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 0.02% β -dodecylmaltoside, and 65% glycerol. The OD at the maximum in the Q_Y was approximately 1 cm^{-1} . Ten millimolar dithionite was added and the samples were preilluminated for approximately 1 min to doubly reduce Q_A . The spectra were normalized to the bleaching minimum which corresponds to $\Delta A \approx -0.006$ for wild-type and the D1-His198Gln mutant and approximately -0.0023 for the D1-Thr179His mutant.

The replacement of the D1-His198 by Gln produces a 3 nm displacement to the blue of the band bleaching with no change in the localization of the band shift. The band bleaching at 672.5 nm is assigned as a first approximation to the bleaching of the absorbance band of P680 due to its oxidation or, expressed more properly, to the disappearance of the low-energy exciton band of the special pair as in the bacterial reaction centres or in PSI. We would like to make it clear that the position of the bleaching band at 672.5 nm does not reflect the transition energy (site energy) of P_{D1} alone but corresponds to the transition from the groundstate to an exciton state which is dominated by a contribution from P_{D1} and P_{D2} . That the spectral shift of the bleached band has indeed contributions from the P_{D1} chlorophyll was shown by measurement of the $(Y_Z^+ - Y_Z)$ difference spectrum (Diner et al. 2001) which, owing to retention within the reaction centre of the phenolic proton which dissociates upon oxidation, produces an electrochromic shift of the nearby P_{D1} . The $(P^+ - P)$ difference spectrum measured in the Soret also produces a 3 nm displacement to the blue (Diner et al. 2001). The equivalent site-directed

mutation introduced at the P_{D2} axial ligand D2-197 (His to Gln) produces practically no change in the $(P^+ - P)$ difference spectrum in the Soret region. These experiments are interpreted as signalling the localization of the P^+ cation primarily on P_{D1} (Diner et al. 2001). The band shift to the blue in the $(P^+ - P)$ difference spectrum is attributed to an electrochromic shift of the nearby Chl_{D1} , induced by the positive charge on P_{D1} and a negative charge on Q_A . The band shift of Chl_{D1} is unaffected by the D1-His198Gln site-directed mutation.

When electron transfer to the first PSII quinone acceptor, Q_A , is blocked, the primary radical pair, $P680^+Pheo^-$, decays with high yield at low temperature, after singlet-triplet mixing in the radical pair, by recombination to the lowest excited triplet state of P680, 3P680 (Schlodder et al. 1998). The P680 triplet state has been monitored in the presence of singly reduced Q_A by transient absorbance spectroscopy (van Mieghem et al. 1995; Schlodder et al. 1998) and time-resolved electron paramagnetic resonance (EPR) (Feikema et al. 2005). Under these conditions, the triplet decay is two or three orders of magnitude faster

than when Q_A is doubly reduced. Double reduction of Q_A can be achieved by illumination of PSII core complexes in the presence of dithionite (van Mieghem *et al.* 1995). Under these conditions, 3P680 can be detected by its characteristic spin-polarized EPR spectrum (Rutherford *et al.* 1981). High triplet yields have also been observed at low temperature in D1/D2/cytb559 reaction centre preparations that lack Q_A (Durrant *et al.* 1990; Kwa *et al.* 1994). Orientation studies by van Mieghem *et al.* (1991) indicated that the triplet state was localized on a chlorophyll, the macrocycle ring plane of which was oriented at 30° with respect to the membrane plane. This orientation is consistent with the localization of the triplet state on either Chl_{D1} or Chl_{D2} but could not distinguish between the two. Fourier transform infrared spectroscopy (FTIR) measurements of the triplet also indicated that the triplet state was localized at $T < 80$ K on a chlorophyll other than that on which the P^+ cation was localized (Breton *et al.* 1997; Noguchi *et al.* 1998, 2001; Sarcina *et al.* 1998). This conclusion was based on differences in the position of the stretch vibrational mode of the $C_9=O$ keto carbonyl arising from differences in hydrogen bond strength. Noguchi *et al.* (2001) attributed the triplet localization to Chl_{D1} at $T < 80$ K but could not in principle distinguish Chl_{D1} from Chl_{D2} . The triplet is expected to be localized on the chlorophyll with the lowest triplet energy, and not necessarily on the one on which it was initially formed. A comparison of the (^3P-P) absorbance difference spectra in the WT and D1-His198Gln strains of *Synechocystis* 6803 at 80 K (Figure 1*b*) and at 5 K (not shown) indicated practically no effect of the mutation, consistent with the localization of the triplet state on a chlorophyll other than P_{D1} and probably Chl_{D1} considering that the triplet bleaching at 80 K, centred at approximately 682 nm, matches the inflection of the band shift observed in the ($P^+Q_A^- - PQ_A$) difference spectrum (figure 1*a*; Diner *et al.* 2001).

In order to pin down the assignment of the band shifted pigment in the ($P^+Q_A^- - PQ_A$) difference spectrum and the band bleaching in ($^3P - ^1P$) spectrum to Chl_{D1} , site-directed mutations were constructed at D1-Thr179. According to the X-ray crystal structures, this threonine overlies the Mg^{2+} of the Chl_{D1} (figure 2; Ferreira *et al.* 2004; Loll *et al.* 2005). However, the distance between the -OH group of this residue and the Mg^{2+} is too great (4.8 Å; Loll *et al.* 2005) for this residue to be a direct ligand to Chl_{D1} . Instead, it is probable that a water molecule, hydrogen bonded to D1-Thr179, is the axial ligand. Figure 1*c* shows that when D1-Thr179 is replaced by His, there is a marked displacement of approximately 2 nm to the red relative to WT of the band shift observed in the ($P^+Q_A^- - PQ_A$) difference spectrum. In addition, the amplitudes of the positive and negative peaks become more equal. There is, however, no significant change in the location of the bleached component of the difference spectrum. These observations are the inverse of what was observed in the case of the D1-His198Gln mutation (figure 1*a*). Measurement of the ($^3P - ^1P$) difference spectrum in the D1-Thr179His mutant cores at 80 K, generated in the presence of Q_AH_2 , shows a displacement to the red of approximately 2 nm relative to WT

Figure 2. Environment of P_{D1} , P_{D2} and Chl_{D1} showing the location of D1-Thr179. Adapted from Ferreira *et al.* (2004).

(figure 1*d*), whereas the D1-His198Gln mutation (Figure 1*b*) produced no change. The minimum of the ($^3P - ^1P$) difference spectrum measured at 80 K in the mutant is located at 684.5 nm. The location of the band shift inflection point in the ($P^+Q_A^- - PQ_A$) difference spectra and the minima of the ($^3P - ^1P$) difference spectrum all measured at 80 K are at about the same wavelength and track each other in the wild-type and in the two mutants. It must therefore be the same pigment molecule that produces the electrochromic shift in response to the charge on P^+ (primarily on P_{D1}) and on which the reaction centre triplet state is localized at ≤ 80 K. As the mutation at D1-Thr179 produces the same displacement of both spectra, it must be the monomeric Chl_{D1} that detects the charge on P_{D1}^+ and on which the triplet is localized.

The loss of histidine as the axial ligand in the D1-His198Gln and the replacement of Thr with His in the case of the D1-Thr179His produce 2–3 nm displacements of P_{D1} to the blue and of Chl_{D1} to the red, respectively. These observations are consistent with the electronic polarizability of the π -system of the imidazole moiety of histidine which stabilizes the excited state of nearby chromophores by dispersive interactions, shifting the absorbance spectrum of the pigment to the red whenever it is present. We cannot be sure that in the case of the D1-Thr179His mutant His has replaced a water molecule as a ligand to Chl_{D1} . In fact, substitution of Thr with His using the program Swisspdbviewer and the *T. elongatus* crystal structure (Ferreira *et al.* 2004; Loll *et al.* 2005) suggests that His may still be too distant to be a direct ligand. However, even if a water molecule remains the immediate ligand, it is probably hydrogen bonded to the imidazole group of histidine. The polarizable His residue is still close enough to Chl_{D1} to exert a stabilizing effect on the excited state.

The ($^3\text{P}-^1\text{P}$) difference spectra were recorded on the millisecond time scale. The clear localization of the triplet energy on Chl_{D1} indicates that at 1 ms there is no triplet energy in the CP43 and CP47 antenna complexes. Presumably, any triplet generated in CP43 and CP47 disappears on a much more rapid time scale ($\ll 1$ ms), quenched by antenna carotenoids.

Having introduced spectroscopic tags that permit one to distinguish between the reaction centre core pigments that are probably involved in primary and secondary charge separation, we are now attempting to track the pathways for electron transfer involved in primary and secondary radical pair formation.

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Discussion

G. Brudvig, Yale University, Yale, USA. When measuring the equilibrium-binding constant for Mn²⁺ during the first step of photooxidation, it is important to determine the site occupancy before the flash, otherwise there will be a second-order Mn²⁺ oxidation reaction from oxidation of Mn²⁺ that diffuses into the OEC site during the lifetime of the charge separation. This could be a factor for different values for *K_m* determined by different types of experiments.

B. Diner. The value that you are asking for is a *K_d*, a dissociation constant. We have been careful to specify that what we are measuring is a *K_m*, which combines the binding and oxidation of Mn²⁺ following a light flash in a second-order mechanism, and not a *K_d*. There is a small amount of prebound Mn²⁺ (typically 20–30%), the oxidation of which appears to be first order. It is, however, very difficult to measure the *K_d*, both because the fraction of centres with prebound Mn²⁺ is small in wild-type (smaller still or non-existent in some D1-Asp170 mutants, see for example Diner & Nixon (1992)) and because the *K_d* is so low (<< 1 μM) where adventitious Mn²⁺ is difficult to exclude. As long as one is clear about what one is measuring (*K_m* versus *K_d*) and the concentration of the core complexes is submicromolar, it is possible to demonstrate a clear-cut dependence of the *K_m* on site-directed mutations constructed at the oxygen-evolving complex (OEC). The binding and oxidation, represented by the *K_m*, is consistent with what occurs physiologically in the first step of assembly of the OEC.

Additional Reference

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