Comparison of Primary Charge Separation in the Photosystem II Reaction Center Complex Isolated from Wild-type and D1-130 Mutants of the Cyanobacterium Synechocystis PCC 6803*

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We compare primary charge separation in a photosystem II reaction center preparation isolated from a wild-type (WT) control strain of the cyanobacterium Synechocystis sp. PCC 6803 and from two site-directed mutants of Synechocystis in which residue 130 of the D1 polypeptide has been changed from a glutamate to either a leucine residue (mutant D1-Gln130Leu) or a glutamate residue (mutant D1-Gln130Glu). The D1-130 residue is thought to be close to the pheophytin electron acceptor. We show that, when P680 is photoselectively excited, the primary radical pair state sequences, or a leucine residue (mutant D1-Gln130Leu). The D1-130 residue is thought to be close to the pheophytin electron acceptor. We show that, when P680 is photoselectively excited, the primary radical pair state is formed with a time constant of 20–30 ps in the WT and both mutants; this time constant is very similar to that observed in Pisum sativum (a higher plant). We also show that a change in the residue at position D1-130 causes a shift in the peak of the pheophytin Q\textsubscript{b} band. Nanosecond and picosecond transient absorption measurements indicate that the quantum yield of radical pair formation (\(\phi_{RP}\)), associated with the 20–30-ps component, is affected by the identity of the D1-130 residue. We find that, for the isolated photosystem II reaction center particle, \(\phi_{RP}\) higher plant > \(\phi_{RP}\) D1-Gln130Glu mutant > \(\phi_{RP}\) WT > \(\phi_{RP}\) D1-Gln130Leu mutant. Furthermore, the spectroscopic and quantum yield differences we observe between the WT Synechocystis and higher plant photosystem II, seem to be reversed by mutating the D1-130 ligand so that it is the same as in higher plants. This result is consistent with the previously observed natural regulation of quantum yield in Synechococcus PS II by particular changes in the D1 polypeptide amino acid sequence (Clark, A. K., Hurry, V. M., Gustafsson, P., and Oquist, G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11985–11989).

Photosystem II (PS II)

Photovoltaic properties of the primary electron donor of PS II; Ph, pheophytin; maltoside, n-dodecyl \(\beta\)-d-maltoside; Q, quinone; WT, wild-type; \(\phi_{RP}\), quantum yield of radical pair formation.

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1 The abbreviations used are: PS II, photosystem II; P680, primary electron donor of PS II; Ph, pheophytin; maltoside, n-dodecyl \(\beta\)-d-maltoside; Q, quinone; WT, wild-type; \(\phi_{RP}\), quantum yield of radical pair formation.

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Synecocystis PCC 6803 has been applied to the study of secondary electron transfer reactions in PS II (20). More recently, this technique has also been applied to the identification of these residues important to the function and assembly of the oxygen-evolving complex (22–24). However, to date, there have been no reports of the application of site-directed mutagenesis to the study of primary photochemistry in PS II. Although studies of primary photochemistry in PS II have been performed, these have been limited to reaction centers isolated from higher plants (see above) and, more recently, from the green alga C. reinhardtii (Ref. 25 and see Ref. 26 for isolation procedure of PS II reaction centers from Chlamydomonas). Higher plants, however, are not readily amenable to genetic engineering. It is therefore highly desirable to study the primary photochemistry of PS II reaction centers isolated from organisms that are easy to manipulate genetically, in order to determine whether they are suitable model systems for the higher plant PS II.

Comparison of the primary structures of the Synechocystis D1 and D2 polypeptides with those of the higher plant, Pisum sativum (27) indicates several significant differences in primary structure between the two organisms. Of particular relevance to this paper is the difference in the residue at position 130 on the D1 polypeptide. In Synechocystis, the D1–130 residue is a glutamine, while in P. sativum, and all other higher plants studied, it is a glutamate; this residue is thought to lie close to the putative binding site of the pheophytin electron acceptor (28). This particular residue is analogous to residue 104 on the L subunit in Rhodobacter capsulatus. In a study of these bacterial reaction centers, it was found that, converting the L–104 residue from a glutamate to a glutamine or a leucine resulted in a blue shift (of different amounts) in the absorption maximum of the pheophytin Qy band and also in slightly slower primary electron transfer than in the wild type (29).

In this paper we report the isolation of a PS II reaction center preparation from Synechocystis, using a different version of the isolation procedure described in Ref. 30. Our main aim has been to produce a Synechocystis PS II particle that is active and stable and that contains sufficiently low levels of chlorophyll to allow photoelective excitation of P680. Photoelective excitation of P680 has been used in the transient absorption experiments to avoid those slow energy transfer steps that can confound the interpretation of the kinetic data (10). We present data comparing the primary photochemical properties of the PS II reaction center preparation isolated from a wild-type control strain of Synechocystis and from two different site-directed mutants of Synechocystis, one in which the D1–130 residue has been changed from a glutamine to a glutamate (mutant D1-Gln130Glui), and a second in which the D1–130 residue has been changed to a leucine (mutant D1-Gln130Leu). This is the first report of such studies on PS II reaction center particles that have been subjected to protein engineering.

MATERIALS AND METHODS

Cyanobacterial Strains—The control strain of Synechocystis referred to as wild-type (WT) in this study is the TCC31 strain (23), a deletion mutant of Synechocystis 6803 in which there is only one copy of the psbA gene, psbA3, rather than three, as in the glucose-tolerant strain originally isolated by Williams (31). We have used the Synechocystis WT plus the D1-Gln130Glui and D1-Gln130Leu preparations to study the primary photochemistry of PS II (using the procedure given below) from the original glucose-tolerant strain of Synechocystis, and we have found this to be identical biochemically and spectroscopically to that isolated from the WT TCC31 strain (data not shown).

The D1-Gln130Glui mutant and the D1-Gln130Leu mutant were created using methods described in Ref. 23. The mutations were verified by sequencing the psbA3 gene after amplification by the polymerase chain reaction (23).

Sample Preparation—A PS II reaction center preparation was isolated as follows from both WT and mutant strains of Synechocystis. TCC31 cells were grown (20-liter cultures) and harvested as described in Ref. 30. All subsequent steps were then carried out in the dark. The concentrated TCC31 cells were resuspended in 20 mM Mes-NaOH (pH 6.35) and 25% (w/v) glycerol to give a final volume of approximately 200 ml with 90–100 mg of chlorophyll. The cells were then frozen in liquid N2 and stored at −80°C.

To isolate thylakoid membranes, the TCC31 cells were initially washed, after thawing, with an equal volume of “break” buffer (20 mM Mes-NaOH (pH 6.35), 5 mM CaCl2, 5 mM MgCl2, 25% (w/v) glycerol, 1 mM e-aminocaproic acid, and 1 mM benzamidine) and pelleted by centrifugation at 4°C. (α-amino caproic acid is a protease inhibitor). The membrane washed cells was resuspended in break buffer and then broken immediately (without any incubation period) in a prechilled 360-ml beadbeater chamber (Biospec Products), as described by Kirillovsk et al. (32). After breaking the cells and removing the cellular material and residual beads (32), the thylakoid membranes in the supernatant were pelleted by centrifugation at 45,000 rpm for 45 min, at 4°C. The pellet was resuspended in a buffer containing 50 mM Mes-NaOH (pH 6.5), 20 mM sodium pyrophosphate, 1 mM e-aminocaproic acid, and 1 mM benzamidine, and the membranes were pelleted again. This additional wash step helped to remove the phycobiliproteins from the membranes. The pelleted thylakoid membranes were resuspended in break buffer to a final volume of approximately 90 ml, and homogenized with approximately 5–8 passes at 4°C. The membranes were frozen in liquid N2 at −80°C. A typical way to carry out this procedure was to prechill a 360-ml beaker at −80°C and then add 90 ml of the membrane suspension, with gentle stirring to homogenize the suspension. After breakage, the membranes were pelleted by centrifugation at 45,000 rpm for 45 min. After breaking the second wash, was resuspended in buffer A only, Vmax optical density units and no longer changed very much. The supernatant and any soft pellet were discarded. For the third wash, the hard pellet, from the second wash, was resuspended in buffer A only, to give a final chlorophyll concentration of approximately 0.14 mg/ml. The membranes were centrifuged at 14,000 rpm for 40 min in a Beckman J A14 rotor (~30,000 g) at 4°C. The supernatant and any soft pellet of chlorophyll containing membranes, typically 60–75 mg of chlorophyll, were subjected to three further washing steps, at very low chlorophyll concentrations, to remove any residual phycobiliproteins. For the first two washes, the membranes were diluted to a concentration of approximately 0.1 mg/ml chlorophyll with 50 mM Mes-NaOH (pH 6.5), 1 mM e-aminocaproic acid, 1 mM benzamidine (buffer A), containing 20 mM sodium pyrophosphate, and centrifuged at 14,000 rpm for 40 min in a Beckman J A14 rotor (~30,000 g) at 4°C. The supernatant and any soft pellet were discarded. For the third wash, the hard pellet of chlorophyll-containing membranes was resuspended in as little buffer A as possible, and a chlorophyll concentration determination was carried out according to the method of Arnon (33). The solution was then diluted with a 30% (w/v) stock of Triton X-100 and more buffer A, if necessary, to give final concentrations of 0.45 mg/ml chlorophyll and 4.5 mM (w/v) Triton X-100. This material was then homogenized, with 12 passes, at room temperature and left to incubate in the dark, on ice and stirring gently, for 2 h. It was then centrifuged at 45,000 rpm for 45 min in a Kontron TFF 65.38 rotor (~145,000 g) at 4°C. The pellet of unsolubilized material was discarded, and the supernatant containing the solubilized material was further diluted to three anion exchange chromatography columns for the separation of PS I and PS II and the subsequent purification of PS II. The supernatant of solubilized material was applied to a column (16 x 200 mm) of Fractogel TSK DEAE-650 (S) (Merck-BDH) maintained at 4°C and equilibrated with buffer A containing 0.2% (w/v) Triton X-100 (Triton buffer). The column length was approximately 150 mm. The sample was loaded at 2.0–2.5 ml min−1 and then washed overnight with 550–650 ml of Triton buffer containing 50 mM NaCl, at a flow rate of approximately 0.5 ml min−1. At this concentration of 50 mM NaCl, some of the PS I contained within the sample was eluted from the column. Following this overnight wash, the absorbance at 450 nm of the eluant was reduced to a level where the absorbance was approximately 0.25–0.35 optical density units and no longer changed very much. The column was then washed with 200–250 ml of Triton buffer containing 75 mM NaCl, at a flow rate of approximately 2.5–2.8 ml min−1. Again, the absorbance at 450 nm of the eluant was monitored, and washing was continued until the absorbance was approximately 0.20–0.30 optical density units and no longer changed very much. A NaCl concentration of 175 mM NaCl was then used to wash the column. The column was then washed with 200–250 ml of Triton buffer containing 75 mM NaCl, at a flow rate of approximately 2.5–2.8 ml min−1. Again, the absorbance at 450 nm of the eluant was monitored, and washing was continued until the absorbance was approximately 0.20–0.30 optical density units and no longer changed very much. A NaCl concentration of 175 mM NaCl was then used to wash the column.
be less than or equal to 1.1. Fractions satisfying these criteria were pooled, the absorption spectrum of the pooled material was recorded, and the chlorophyll a to cytochrome b$_{559}$ ratio estimated. The pooled fractions after the first column typically contained 16–20 chlorophyll a/cytochrome b$_{559}$ and a total of 240–270 $\mu$g of chlorophyll. It should be noted that, when determining the chlorophyll a to cytochrome b$_{559}$ ratio of the pooled fractions after this and the second column, care was taken not to overestimate the oxidized minus reduced cytochrome b$_{559}$ change due to a contribution to the signal by cytochrome c$_{553}$. This contaminating cytochrome c$_{553}$ was usually removed completely after the second column. Its loss could be followed clearly by monitoring the absorption spectrum of the eluant; cytochrome c$_{553}$ has a large absorption in the Soret region (data not shown).

All of the pooled material from the first column was diluted 4-fold with Triton buffer and applied to a second column (10 × 98 mm), packed, and equilibrated with Triton buffer, as described for the first column. The packed column length was approximately 5 cm. The sample was then washed with 60–80 ml of Triton buffer containing 75 mM NaCl, at a flow rate of 0.6 ml min$^{-1}$, in repeated cycles of washing with 20 ml and stopping the flow for 5 min. The purpose of this wash was to remove chlorophyll and any residual cytochrome. The eluted material after the second column typically contained 9–10 chlorophyll a/cytochrome b$_{559}$ and a total of 90–120 $\mu$g of chlorophyll. This material was frozen in liquid N$_2$ and stored at −80°C overnight.

A third column was prepared as for the second column. All of the PS II particles from the second column were slowly thawed on ice and then diluted 4-fold with Triton buffer. They were applied to a third column and then washed, exchanged from Triton X-100 to n-dodecyl β-o-maltoside (maltoside) by washing with approximately 15 ml of buffer A containing 2 mM maltoside (maltoside buffer) and containing 75 mM NaCl. PS II particles with reduced chlorophyll content were then eluted in a single salt step with maltoside buffer containing 200 mM NaCl and at a flow rate of approximately 0.6 ml min$^{-1}$. The eluted material after the second column typically contained 7–8 chlorophyll a/cytochrome b$_{559}$ and a total of 40–50 $\mu$g of chlorophyll. This material was frozen in liquid N$_2$ and stored at −80°C. It was subsequently thawed slowly on ice for determination of pigment and cofactor composition and spectroscopic characterization.

Higher plant PS II reaction center particles were isolated from P. sativum as described previously (35).

Determination of Pigment and Cofactor Composition of Sample—Chlorophyll a and phaeophytin a ratios were measured by phaeophytinization of chlorophyll by a 60:40 mixture of HCl and H$_2$O$_2$. Samples containing 3 $\mu$g of chlorophyll were dissolved in 0.2 ml of acetone. The absorption spectrum from 500–580 nm was recorded to resolve clearly the 536 nm phaeophytin peak. 10 ml H$_2$O$_2$ was then added to the sample to phaeophytinize all the chlorophyll a present and the spectrum recorded again. Comparison of the peaks at 536 nm, before and after the addition of H$_2$O$_2$, gives the chlorophyll a to phaeophytin a ratio. It is assumed that there are 2.0 phaeophytin a molecules/PS II reaction center particle.

Quantitative analyses of the individual pigments were carried out by high performance liquid chromatography (HPLC). The samples were protected from light at all stages of the procedure. The pigments were extracted from the proteins dissolved in buffer by addition of 9 volumes of 100% HPLC grade cold acetone (0.5 ml of acetone in 0.1-ml sample). After stirring and centrifugation for 5 min at 10,000 rpm in a bench centrifuge, at 4°C, the green supernatant was filtered through a 0.2-$\mu$m fluoropolymer filter (ACROBUCO LC13). 20-$\mu$l aliquots (equivalent to 200–300 $\mu$g of chlorophyll) were injected in the chromatograph for analysis on a reverse phase HPLC column (Spherisorb S5ODS1; 5 mm, 250 × 4.6 cm inner diameter). An isocratic mobile phase of methanol/ethyl acetate (68:32, v/v) with a flow rate of 1.0 ml min$^{-1}$ was used for separation. The peaks were detected with a UV-visible Kontron spectrophotometer at 450 nm for β-carotene and xanthophylls, at 663 nm for chlorophyll a and phaeophytin a, at 645 nm for chlorophyll b, and at 225 nm for plastocyanine-9. The extinction coefficients for chlorophyll a, phaeophytin a, and β-carotene were calculated from the absorption spectra in a pigments in a methanol/ethyl acetate (68:32 v/v) mixture, i.e., the isomeric mobile phase used in the HPLC analysis, and by comparison with the known extinction coefficients of these pigments in methanol and 80% acetone (36). The extinction coefficients calculated were, in units of $\text{m}^{-1}\text{cm}^{-1}$, as follows: 86.9 for chlorophyll a at 663 nm; 49.3 for phaeophytin a at 663 nm; 135.0 for β-carotene at 450 nm. These values are only slightly different to those in Ref. 36. The pigment stoichiometry given in Table I, as determined by HPLC, also assumes that there are 2.0 phaeophytin aPS II reaction center particle.

Cytochrome b$_{559}$ was measured by determining the concentration of cytochrome b$_{559}$ from the reduced (dithionite) minus oxidized (ferriyanide) absorption difference spectrum obtained for the sample resuspended in buffer A; this difference spectrum peaked at 559 nm for the Synechocystis PS II preparations. An extinction coefficient of 23.4 $\text{m}^{-1}\text{cm}^{-1}$ (37) was used for the concentration of chlorophyll a at 559 nm. The concentration of the sample was calculated from the reaction center concentration, assuming a chlorophyll a to phaeophytin a ratio of 7.9±2.0 (taken from the HPLC data, Table I). The reaction center concentration was calculated from the absorbance at 663 nm for the 800 acetone extracted reaction center particle and using an extinction coefficient calculated by assuming 7.9 chlorophyll a and 2.0 phaeophytin a/reaction center (from the HPLC data) and applying Lichtenthaler’s (36) extinction coefficients for these pigments in 80% acetone. On this basis, the extinction coefficient for an acetone-extracted reaction center particle containing 7.9 chlorophyll a and 2.0 phaeophytin a is 780.4 $\text{m}^{-1}\text{cm}^{-1}$. It should be noted that, for these samples, the absorbance at the Q$_y$ absorption peak for the aqueous suspension was found to be the same as the absorbance at 663 nm for the acetone-extracted sample.

Spectroscopic Characterization of Sample—Steady state absorption measurements were recorded using a Shimadzu MPS2000 or a PerkinElmer 554 spectrophotometer.

Femtosecond transient absorption measurements were carried out as described previously (5, 10). The time resolution of the spectrometer was approximately 250 fs for the experiments reported here. The excitation pulses were centered at 694 nm for selective excitation of P680 (10). Transient absorption data were collected using a multichannel detector at 150 time delays between 0 and 60 ps and were globally analyzed as described in Ref. 10. The experiments were repeated several times on different samples in order to assess the reproducibility of the data and to establish the precision of the results. Precision in the determination of the measured lifetimes is quoted as ± one standard error. Data were collected with the polarization of the probe beam rotated by 54.7° relative to the pump. This “magic angle” configuration avoids any contributions to the data from depolarization processes. The Synechocystis PS II reaction center particles used in these experiments were those that were eluted directly from the third chromatographic column, without further dilution apart from the addition of the oxygen trap, 5 mm glucose, 0.1 mg ml$^{-1}$ glucose oxidase, and 0.5 mg ml$^{-1}$ catalase (16), and contained typically 30–35 $\mu$g of chlorophyll (in a volume of approximately 0.75 ml). The samples were rotated in a circular cuvette at 60 Hz and maintained at 10°C.

Femtosecond transient absorption measurements were carried out as described previously (18), with the following modifications. Output pulses from a dye laser, pumped by a nitrogen laser, were used as the excitation source; the pulses were 500 ps in duration, at a repetition...
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Fig. 1. Steady state absorption spectrum of the WT Synechocystis PS II reaction center particle compared with that for the higher plant, P. sativum, PS II reaction center. Samples were resuspended in buffer A, containing 2 mM maltoside and 200 mM NaCl.

RESULTS

Fig. 1 shows the room temperature steady state absorption spectrum for the control WT Synechocystis PS II reaction center preparation compared with that for the higher plant, P. sativum, PS II reaction center. The room temperature steady state absorption spectrum for the PS II preparation isolated from the D1-Gln130Glu and D1-Gln130Leu mutants is similar to that for WT Synechocystis (data not shown). The Q band of the WT Synechocystis PS II reaction center particle peaks at 675.0–675.3 nm, which is slightly to the blue of the Q band maximum of the higher plant PS II reaction center, which peaks at 675.5–676.0 nm. In the Soret region, the short wavelength peak at approximately 417 nm predominates, as it does in higher plant PS II reaction centers; however, differences can be seen at approximately 434 nm. Another notable difference is the magnitude of the absorbance in the carotenoid region at approximately 483–489 nm; it is nearly a factor of 2 lower in the WT Synechocystis preparation than in the higher plant one, indicative of reduced carotenoid content (confirmed by HPLC data; see Table I).

The pigment and cofactor composition of the WT Synechocystis PS II reaction center preparation was determined by combining the results from three different types of analyses, the results of which are given in Table I. It contains approximately seven to eight chlorophyll a, two pheophytin a, one cytochrome b$_{559}$, and one $\beta$-carotene; no quinone was detected.

This pigment and cofactor composition is similar to that for the higher plant PS II reaction center (2), apart from the presence of one to two extra chlorophyll and the carotenoid content, which is reduced by half. The Synechocystis PS II preparation contains no PS I, as confirmed by the lack of the 725 nm fluorescence peak, originating from PS I, in steady state fluorescence measurements at 77 K (data not shown). It does, however, contain some extra polypeptides, over and above those observed for the higher plant PS II reaction center (data not shown), and as also observed by Oren-Shamir et al. (38) for another isolated Synechocystis PS II reaction center preparation. These extra polypeptides may be responsible for the extra one to two chlorophylls found in this preparation compared with higher plant reaction centers. Despite this contamination, it is still possible to photoselectively excite P680, which is a crucial requirement for examining radical pair formation. We have carried out femtosecond transient absorption experiments on higher plant PS II reaction centers, which contain 5, 6, 7, or 8 chlorophylls and have shown that, with photoselective excitation of P680, the kinetics for primary charge separation are not affected greatly by the presence of these extra chlorophylls. The pigment and cofactor composition of the PS II preparations isolated from the D1-Gln130Glu and D1-Gln130Leu mutants was analyzed by the reduced minus oxidized cytochrome b$_{559}$ difference spectrum and pheophytinization. The composition of these PS II Synechocystis mutant preparations was similar to that reported for the WT Synechocystis.

Fig. 2 compares transient absorption data collected in the region of the pheophytin Q$_y$ absorption band for the WT Synechocystis PS II reaction center particle and for the higher plant, P. sativum, PS II reaction center. The pheophytin Q$_y$ band was chosen as it can be most easily assigned (10, 11). Data were collected using pulses centered at 694 nm that achieved photoselective excitation of P680 (12, 13). Global analyses of these data resolved two components, an exponential decay component and a component that did not decay on the detection timescale (0–60 ps). The exponential component had a lifetime of 22 ± 3 ps for the WT Synechocystis PS II reaction center particle and 21.5 ± 1 ps for the higher plant PS II reaction center. (The error margins quoted here are one standard error and result from approximately 20 analyses of independent data sets; it can therefore be concluded that the lifetimes of these two components are indistinguishable.) Spectra of the amplitudes of these components are shown in Fig. 2.

For the higher plant, P. sativum, the minimum at 544 nm in the spectrum of the nondecaying component, which has a lifetime much longer than 60 ps (Fig. 2b, solid line), results from bleaching of the Q$_y$ band of the photoactive pheophytin associated with formation of the radical pair state $P680^+\text{Ph}^-$ (for a full discussion of the assignment of these spectra see Refs. 5, 6, 9, 10, 25). As mentioned earlier, the decay of this radical pair state occurs primarily on the nanosecond time scale (18). The maximum in the spectrum of the higher plant 21.5 ps component (Fig. 2b, dotted line), also at 544 nm, allows us to identify this component with the production of reduced pheophytin. The amplitude of this feature indicates that 40–50% of the final pheophytin Q$_y$ bleach grows in with the 21.5 ps component, indicating that a lower limit of 40% of the total pheophytin reduction occurs with a 21.5-ps time constant.

The spectral features observed in the WT Synechocystis data (Fig. 2a) show similarities with those observed for higher plant reaction centers of P. sativum. As in the higher plant data, the spectrum of the nondecaying component (which has a lifetime

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Gln130Glu mutant), the final absorption change at 60 ps is similar to that of higher plants, i.e. it is negative.

Nanosecond transient absorption measurements at 820 nm were carried out to obtain information regarding the quantum yield of radical pair formation ($\phi_{RP}$) in the isolated WT and mutant Synechocystis PS II reaction center preparations, compared with the isolated higher plant, P. sativum, PS II. These nanosecond kinetics are shown in Fig. 5. Measurements at 820 nm monitor the presence of P680$^+$Ph$^-$ and triplet P680; contributions from unbound chlorophyll singlet excited states are relatively small (see "Discussion"). Analysis of the decay kinetics in Fig. 5 resulted in the following lifetimes, with absolute amplitudes ($\times 10^{-3}$), being given in parentheses: P. sativum, 48 ns (3.26) and 4.8 ns (3.43); WT Synechocystis, 36 ns (1.07) and 4.8 ns (2.84); D1-Gln130Glu mutant, 45 ns (1.70) and 4.9 ns (2.57); D1-Gln130Leu mutant, 44 ns (0.55) and 6.4 ns (2.39). (The amplitude of the 4.7-$\mu$s component, due to an electronic filter, was of the order of 0.23 $\times 10^{-2}$ for all samples). The 20 ns component resolved in Ref. 18 is not resolved in the data shown in Fig. 5, due to poorer signal to noise, but its amplitude will contribute to both the nanosecond components that are resolved in the data of Fig. 5. These nanosecond components represent charge recombination of the primary radical pair (18), although the faster nanosecond component may also contain a contribution from chlorin singlet excited states formed in active reaction centers. The amplitudes of the nanosecond components can, therefore, be taken to be a measure of the quantum yield of radical pair formation ($\phi_{RP}$) in the sample. Since the optical densities of the samples at the excitation wavelength used were identical and the excitation pulses were non-saturating, it is valid to compare the amplitude of the nanosecond components in the different samples directly. Table II estimates the $\phi_{RP}$ from the nanosecond transient absorption data (i) by comparing the total amplitude of the decay kinetics in Fig. 5 and (ii) by comparing the relative amplitudes of just the long-lived nanosecond component. In both cases the amplitudes are normalized to 100% for $\phi_{RP}$ in isolated higher plant PS II reaction centers. As can be seen in Table II, the relative quantum yield of radical pairs, at 1 ns, is in the following order: higher plant > D1-Gln130Glu mutant > WT > D1-Gln130Leu mutant. This order is, in fact, apparent by inspection of the nanosecond data presented in Fig. 5. Similar results (i.e. lifetimes and amplitudes) were obtained for the nanosecond data when either 630 nm or 680 nm excitation wavelengths were used.

Time-resolved fluorescence studies of the WT Synechocystis PS II reaction center preparation were used to assay the level of uncoupled chlorophyll present in the samples. We obtained similar time-resolved fluorescence data for both WT Synechocystis and higher plant, P. sativum, PS II reaction centers (data not shown). As discussed in Refs. 17 and 18 (in which the higher plant data is interpreted), this indicates that in the WT Synechocystis PS II reaction center preparation, up to 94% of the chlorophyll is energetically coupled to active PS II reaction centers, assuming the equilibration kinetics between the radical pair state and the antenna are similar for Synechocystis and higher plants. Therefore, we can establish that the additional one to two chlorophylls present in the Synechocystis preparation are functionally connected to active reaction centers (also confirmed by the nanosecond data described above; see "Discussion").

**DISCUSSION**

In this work we have compared the primary photochemistry of a PS II reaction center preparation isolated from the WT cyanobacterium Synechocystis PCC 6803 and from two different site-directed mutants of the D1–130 residue in Synechocystis, one in which this residue has been changed from a glutamine to a glutamate (the D1-Gln130Glu mutant), as is the case for higher plants, and another in which it has been changed to
In order for the ultrafast transient absorption measurements to be interpreted in a straightforward manner, it is necessary to photoselectively excite P680. We have shown that this can be done with PS II reaction centers isolated from the higher plant, P. sativum (8, 9) and that if P680 is not photoselectively excited, then slow energy transfer processes interfere with the kinetics observed (10). A criterion, therefore, that had to be satisfied in this study, was that the PS II preparation isolated from the transformable cyanobacterium Synechocystis had to have its chlorophyll content reduced sufficiently to allow photoselective excitation of P680, while retaining maximum stability and activity.

The procedure we have used to isolate a Synechocystis PS II reaction center preparation employed only a single detergent treatment for solubilization of the membranes rather than two, as published previously (30). Further changes to the Gounaris et al. (30) preparation included an extra chromatographic column, to remove further chlorophylls, and exchange of the sample on the second and third columns from Triton X-100 to maltoside, for stabilization of the sample (40).

The Synechocystis PS II reaction center preparation we have isolated from WT and the D1–130 mutants has a pigment and cofactor composition similar to that reported for higher plant PSII reaction centers (2), except for the presence of some extra chlorophyll and the loss of one β-carotene. The chlorophyll content of this preparation has therefore been reduced sufficiently to allow photoselective excitation of P680 and to allow the ultrafast absorption data to be interpreted straightforwardly. The composition of our Synechocystis PS II preparation is similar to that reported recently for another isolated Synechocystis PS II reaction center preparation (38). It is, how-

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**TABLE II**

|                      | Higher plant, P. sativum | Wild-type Synechocystis | D1-Gln130Glu mutant | D1-Gln130Leu mutant |
|----------------------|--------------------------|------------------------|---------------------|---------------------|
| (a) Nanosecond transient absorption data | % | % | % | % |
| (i) total amplitude of decay kinetics | 100 | 60 | 65 | 46 |
| (ii) relative amplitude of long-lived nanosecond component | 100 | 55 | 81 | 36 |
| (b) Simulation of ultrafast data | 100 | 50 | 75 | 20 |

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**Fig. 5.** Comparison of the nanosecond decay kinetics, monitored at 820 nm and following excitation at 680 nm, in PS II reaction centers isolated from higher plants (P. sativum), WT Synechocystis, and the D1-Gln130Glu and D1-Gln130Leu Synechocystis mutants.
ever, different to the Synechocystis PS II particle of Gounaris et al. (30); this particular particle was reported to contain only one pheophytin a/eight chlorophyll a, one cytochrome b$_{563}$, and 0.75 ß-carotene.

The stability of our Synechocystis PS II reaction center preparation, during the course of these experiments, was the same as the P. sativum PS II reaction center (see “Materials and Methods”).

We have shown, in our ultrafast transient absorption studies, that formation of the primary radical pair state P680$^+$/Ph$^-$ occurs mainly with a time constant of 20–30 ps (see Fig. 4) in the WT and both D1–130 mutants of Synechocystis; this time constant is very similar to that observed in the higher plant, P. sativum.

The residue at D1–130, however, does affect the wavelength of the active branch pheophytin Q$^-$(transition (Fig. 3). The peaks are at 541.5 ± 0.5 nm for WT Synechocystis, at 544 ± 0.5 nm for the D1-Gln130Glu mutant, and at 540 ± 0.5 nm for the D1-Gln130Leu mutant. These peaks are to be compared with the 544 ± 0.5 nm peak observed in the higher plant, P. sativum. Hence, only when the residue at position D1–130 is the same as in higher plants (i.e. the D1-Gln130Glu mutant) is the peak of the functional pheophytin Q$^-$ absorption band located at the same wavelength as that of higher plants. Shifts in the peak of the pheophytin Q$^-$ absorption band, when comparing WT and mutant Synechocystis and higher plants, have also been observed in steady state pheophytin anion photoaccumulation experiments (39). Recent Fourier transform infrared data indicate that the residue at position D1–130 probably lies close to the binding site of the pheophytin electron acceptor$^4$, as predicted from sequence similarities to the bacterial reaction center (34).

In studies of R. capsulatus mutants (29), a change in the residue at position L-104 (equivalent to the higher plant position D1–130) was also reported to cause a shift in the position of the pheophytin Q$^-$ absorption peak (see the Introduction). Furthermore, it was observed (29) that the primary electron transfer event in the glutamine mutant was only slightly slower than in the wild type (4.6 ± 0.7 ps compared with 3.4 ± 0.5 ps, respectively). These observations of Bylina et al. (29) using purple bacterial mutants, therefore, show some similarity to those reported here.

Also observed in our ultrafast absorption data, are differences in the overall absorption change associated with the spectrum of the nondecaying component (Fig. 3). The difference in the spectrum of the nondecaying component observed for WT Synechocystis, when comparing it with that for the higher plant, P. sativum, can be reversed by changing the D1–130 residue (to a glutamate) so that it is the same as in higher plants. This variability in overall absorption change associated with the nondecaying component, for the isolated WT and mutant Synechocystis PS II preparations, may be related to their quantum yield for radical pair formation ($\phi_{RP}$). This quantum yield has been estimated (a) from the nanosecond transient absorption data in two different ways and (b) by simulation of the spectrum of the nondecaying component resolved in the ultrafast data (Fig. 3). The results are presented in Table II.

$\phi_{RP}$ Estimated from Nanosecond Transient Data—For the nanosecond data, the $\phi_{RP}$ in the different samples was estimated by analyzing the nanosecond decay kinetics in Fig. 5, as described under “Results.” The results of these analyses are given in Table II. The same results were obtained for either 630 or 680 nm excitation. Using 630-nm excitation, all chlorophylls present, whether they be uncoupled or bound to active reaction centers, will be excited; using 680-nm excitation, only chlorophyll bound to active reaction centers will be excited preferentially. Since the nanosecond absorption data is similar for either excitation wavelength, it can be concluded that very little uncoupled chlorophyll is present in these samples. This is confirmed, at least for the WT Synechocystis PSII preparation, by the time-resolved fluorescence measurements. From Table II, therefore, it appears that, independent of the way in which the quantum yield of radical pair formation in isolated PS II reaction center preparations is estimated from the nanosecond data, the relative quantum yield of radical pairs, at 1 ns, is in the following order: higher plant > D1-Gln130Glu mutant > WT > D1-Gln130Leu mutant.

$\phi_{RP}$ Estimated by Simulation of Ultrafast Transient Data—Simulations were made of the spectra of the nondecaying component resolved in the ultrafast data (Fig. 3) to determine whether differences in these spectra were also indicative of differences in radical pair concentrations. These spectra come from a combination of radical pair states and chlorin singlet excited states. For the higher plant, P. sativum, data from recent time-resolved emission experiments$^2$ have determined that the spectrum at 60 ps has an approximate 20% contribution from chlorin singlet states and therefore there will be a 80% contribution from the radical pair state P680$^+$/Ph$^-$. For higher plants, the difference spectrum associated with chlorin singlet excited states corresponds to the spectrum before charge separation, i.e. the spectrum at 250 fs (7). By changing the contribution of the 60 ps and 250 fs spectra obtained with the higher plant PS II reaction center and comparing with the spectra obtained for the Synechocystis PS II reaction center preparations, an estimate of the relative proportions of chlorin singlet states and radical pairs contributing to each spectrum in Fig. 3 can be obtained. The results of such simulations, normalized to 100% for the radical pair states in higher plants, are also shown in Table II. Reasonable simulations of the Synechocystis experimental data were obtained in all cases. By carrying out such simulations, it becomes apparent that the more positive spectrum observed for the D1-Gln130Leu mutant (Fig. 3) can be attributed to the presence of a higher concentration of singlet chlorin States and a lower quantum yield of radical pair formation. From Table II, it is apparent that such simulations of the ultrafast data gives rise to the observation that the relative quantum yield of radical pairs in isolated PS II reaction centers, at 60 ps, is also in the following order: higher plant > D1-Gln130Glu mutant > WT > D1-Gln130Leu mutant. It should be noted that, for the relative quantum yield of radical pairs, only qualitative agreement can be expected between the ultrafast and nanosecond data, since it is not possible to distinguish between chlorin singlet states and radical pairs in the nanosecond data.

$\phi_{RP}$ estimated in isolated Wild Type and Mutant PS II Reaction Centers—From Table II it can be seen that when we estimate the radical pair concentration by two completely independent means, we come to the same conclusion, namely that, in terms of quantum yield of P680$^+$/Ph$^-$ formation, in isolated PS II reaction centers, $\phi_{RP}$ higher plant > $\phi_{RP}$ D1-Gln130Glu mutant > $\phi_{RP}$ WT > $\phi_{RP}$ D1-Gln130Leu mutant. Both analyses give the rather surprising conclusion that PS II isolated from the D1-Gln130Glu mutant has a higher quantum yield of radical pair formation than that isolated from WT Synechocystis. Also, the relative quantum yield of radical pair formation in the isolated WT Synechocystis PS II is only about half of that in PS II isolated from the higher plant, P. sativum. For the isolated higher plant PS II reaction center, the quantum yield of radical pair formation has previously been shown to be 1.1 ± 0.2 (18).

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$^4$ P. J. Nixon and J. Breton, unpublished data.
It is interesting to note that Oren-Shamir et al. (38) observe that the quantum yield for the photoaccumulation of Ph− in their WT Synechocystis PS II reaction center preparation, is about half of that activity observed in PS II reaction centers of higher plants. At first sight, it seems unlikely that the reduced quantum yield that we have observed in wild-type Synechocystis PS II reaction center reflects the situation in vivo. It is widely believed that a reaction center should be optimized for maximum quantum yield, but this expectation is somewhat naive. The cyanobacterium Synechococcus sp. PCC 7942 has two forms of PS II that differ by 25 amino acids in the D1 polypeptide (27). One form is expressed under low light and one under high light, and it has been shown that the high light form is less easily photoinhibited than the low light form (8). Moreover, it was also demonstrated that the high light form of PS II exhibits a 25% higher quantum yield than the low light form (8). What makes this observation particularly relevant is that one of the amino acids that changes between the two forms of Synechococcus PS II reaction center is in fact the D1–130 residue; in the low light form it is a glutamine, while in the high light form it is a glutamate. We have shown here that the mutation D1-Gln130Glu increases the quantum yield in the isolated Synechocystis PS II reaction centers by approximately 20–30%. It is therefore tempting to conclude that the quantum yield in Synechocystis is controlled largely by the D1–130 residue and that the PS II reaction center isolated from wild type Synechocystis probably does show a reduced quantum yield even in vivo.

In conclusion, we have shown that the time constant observed for the formation of the radical pair state P680′Ph− is similar for WT Synechocystis, for the D1–130 Synechocystis mutants, and for the higher plant, P. sativum. However, while the rate of radical pair formation is relatively insensitive to the residue at position D1–130, a mutation at this site can result in a shift in the peak of the Qx band of the acceptor pheophytin in a change in the quantum yield for the formation of P680′Ph−. It is remarkable that the quantum yield of radical pair formation in isolated Synechocystis PS II reaction centers can be increased by mutating the D1–130 residue from a glutamine to a glutamate, in a manner similar to that found naturally in Synechococcus.
Comparison of Primary Charge Separation in the Photosystem II Reaction Center Complex Isolated from Wild-type and D1-130 Mutants of the Cyanobacterium *Synechocystis* PCC 6803

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