

## Role of Arg180 of the D2 protein in photosystem II structure and function

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On the basis of sequence comparison with the M subunit of the reaction center of purple bacteria, no residues in photosystem II can be clearly identified that may be predicted to correspond to the His residue that binds one of the accessory bacteriochlorophylls in the purple bacterial reaction center. However, the Arg180 residue of the D2 protein is close to where this residue is predicted to be and could conceivably serve as a chlorophyll ligand. To analyze the function of Arg180, it was changed to nine different amino acids in the cyanobacterium *Synechocystis* sp. PCC 6803. Except for the Arg180→Gln (R180Q) mutant, the resulting strains were no longer photoautotrophic. The properties of photosystem II upon mutation of Arg180 were probed in strains from which photosystem I had been deleted genetically. Mutations at the Arg180 residue affected oxygen evolution capacity and the amount of photosystem II that was present in thylakoids. Surprisingly, in the Arg180 mutants, EPR signals that may originate from the oxidized redox-active Tyr160 of the D2 protein ( $Y_D^{\text{ox}}$ ) were small and generally did not resemble the usual signal  $II_s$ , signifying an effect of the Arg180 mutations on the environment surrounding Tyr160. In addition, in most mutants, the charge recombination kinetics between the primary electron-accepting quinone in photosystem II ( $Q_A^-$ ) and oxidized species on the donor side were faster upon introducing mutations at Arg180 suggesting an increased steady-state concentration of  $P680^+$  in the mutants. However, Arg180 mutations also affected  $Q_A^-$  oxidation by the secondary electron-accepting quinone ( $Q_B$ ). HPLC analysis showed that, in the Arg180 mutants that were assayed, the pheophytin/chlorophyll ratio of photosystem II had not changed, indicating that the mutations did not lead to a pheophytinization of one of the chlorophyll molecules. Even though the results presented do not provide positive evidence that Arg180 of the D2 protein corresponds in function to the ligand to the central Mg in an accessory bacteriochlorophyll in reaction centers of purple bacteria, it is clear that changes in Arg180 greatly affect Tyr160 and P680. Various scenarios are discussed that are compatible with the data presented, and include an apparently close interaction between Arg180, His189, and Tyr160, and the possibility of the involvement of multiple chlorophylls to together form P680.

**Keywords:** cyanobacteria; electron transport; photosynthesis; thylakoids; tyrosine radicals.

Photosystem II (PS II) is a multimeric pigment-protein complex that utilizes light energy to oxidize water and reduce plastoquinone. There is a striking structural and functional similarity between the acceptor sides of PS II and of the reaction center of purple bacteria (Michel and Deisenhofer, 1988), for which a high-resolution crystal structure is available (Deisenhofer et al., 1985; Michel et al., 1986; Chang et al., 1986; Allen et al., 1987,

1988; Deisenhofer and Michel, 1989; Feher et al., 1989). The D1 and D2 subunits of PS II are similar to the L and M subunits of purple bacteria, respectively. On the basis of this similarity, predictions may be made regarding the amino acid residues that are likely to interact with prosthetic groups and cofactors at the acceptor side of PS II. Predictions regarding similarities at the donor side are more tenuous but modeling approaches in some cases have yielded useful information (for example, see Svensson et al., 1990, 1996; Ruffle et al., 1992). Many of these predictions were tested by site-directed mutagenesis and generally supported functional and structural similarity between the reaction centers from purple bacteria and PS II (reviewed by Pakrasi and Vermaas, 1992; Vermaas, 1993; Pakrasi, 1995). However, some questions remained unanswered. One of them pertains to the existence and position of accessory chlorophyll molecules in PS II similar to the bacteriochlorophyll located between the primary donor (P870) and bacteriopheophytin in both the L and M subunit branches. The function of this bacteriochlorophyll is not known but it may be involved in primary charge separation by facilitating electron transfer between the primary donor and bacteriopheophytin, either by a super exchange mechanism (Bixon et al., 1987; Creighton et al., 1988) or by serving as an

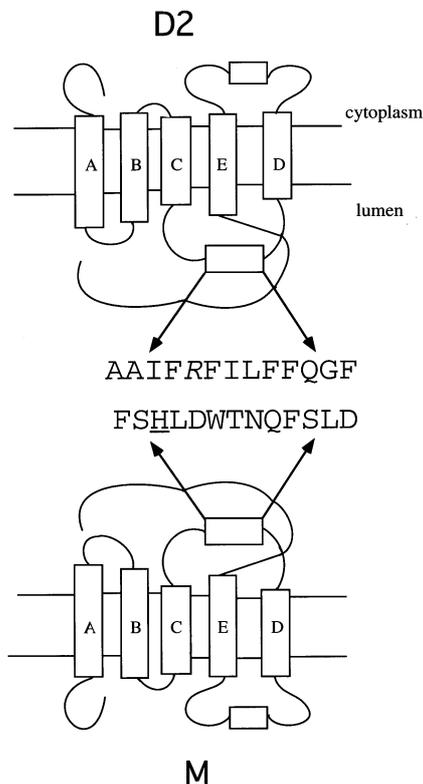
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*Abbreviations.* DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea;  $F_i$ , intermediate chlorophyll fluorescence level;  $F_m$ , maximal chlorophyll fluorescence level;  $F_o$ , chlorophyll fluorescence level after dark adaptation; P680, primary electron donor of photosystem II; *psbDIC*, gene cluster encoding the D2 (*psbDI*) and CP43 (*psbC*) proteins; *psbDII*, second *psbD* gene encoding the D2 protein; PS I, photosystem I; PS II, photosystem II;  $Q_A$ , primary electron-accepting quinone in photosystem II;  $Q_B$ , secondary electron-accepting quinone in photosystem II;  $Y_D$ , redox-active residue Tyr160 of the D2 protein;  $Y_Z$ , redox-active residue Tyr161 of the D1 protein.



**Fig. 1. Comparison of the M subunit of *Rhodobacter capsulatus* with the D2 subunit of PS II.** Boxes represent the membrane-spanning  $\alpha$ -helices (A–E) and arrows indicate the alignment of the amino acid sequence in the *cd* helix parallel to the membrane. The underlined His residue in the projected amino acid sequence from the M subunit serves as a ligand to the central Mg in an accessory bacteriochlorophyll molecule. Note that the amino acid sequence similarity in this region is very poor and the alignment therefore is equivocal. The Arg180 residue of D2 that is the subject of this study has been italicized.

actual electron transport intermediate (Holzapfel et al., 1989). In the reaction center of purple bacteria, the accessory bacteriochlorophylls are ligated by His residues that are located in the *cd* helix of the L and M subunits; this helix is roughly parallel to the membrane and is between transmembrane helices C and D. These His residues are not conserved in PS II. Accessory chlorophyll molecules, however, appear to exist in PS II because the PS II reaction center in its most stable and pure form binds six chlorophyll molecules/pair of pheophytin molecules (Eijkelhoff et al., 1996; Zheleva et al., 1996). There are indications that one of the chlorophyll molecules in the PS II reaction center is oriented similarly to accessory bacteriochlorophyll in the purple bacterial reaction center (van Mieghem et al., 1991); in some of the models for primary charge separation in PS II such a type of accessory chlorophyll is suggested to play a very important role (van Gorkom and Schelvis, 1993; Kwa et al., 1994; Durrant et al., 1995).

Upon simple alignment of the *cd* helix of the M subunit in the purple bacterial reaction center with the corresponding region of D2 in PS II, the His residue that serves as the accessory pigment ligand in purple bacteria corresponds to Ile in PS II. However, the overall amino acid sequence similarity of the D2 subunit of PS II with the M subunit of purple bacteria in this area is very poor (Fig. 1). In D1, the residue lining up with the His residue of the L subunit is Thr. For neither Ile nor Thr is there precedence to serve as ligand to the central Mg in chlorophyll. Conceivably the protein backbone could contribute a ligand. However, in view of the highly equivocal sequence align-

ment, the *cd* region in the D1 and D2 sequences was scanned for other options for potential chlorophyll-binding residues. Two residues further in the sequence of D1 and D2, conserved Asn (D1) and Arg (D2) residues are found. Asn is presumed to be able to act as a chlorophyll ligand in purple bacteria (Wagner-Huber et al., 1988) and in PS II (Shen et al., 1993a), and Arg contains several groups in its side chain that may act as chlorophyll ligand provided that the presumably positive charge on the residue does not interfere.

When the primary donor P680 is oxidized upon primary charge separation, within 20–100 ns (Brettel et al., 1984) it is reduced by the redox-active Tyr161 of the D1 protein,  $Y_z$  (Debus et al., 1988a; Metz et al., 1989).  $Y_z$  in its oxidized form gives rise to a characteristic radical EPR signal called signal  $\Pi_{(v)f}$ , where (v)f stands for (very) fast and represents its oxidation-reduction kinetics (Babcock, 1987; Hoganson and Babcock, 1988). Signal  $\Pi_z$  is an EPR signal with similar spectral shape but with much slower kinetics (may be stable for hours at room temperature) and originates from another oxidized tyrosyl radical (Barry and Babcock, 1987), Tyr160 of the D2 protein (Vermaas et al., 1988; Debus et al., 1988b). Tyr160 of D2 is referred to as  $Y_D$ . It was shown in this work that, even though there is no direct evidence that the Arg180 residue of D2 may serve as an essential ligand to chlorophyll, it seems to be closely associated with  $Y_D$  and P680, and greatly modifies PS II electron transfer properties.

## MATERIALS AND METHODS

### Cell culture, transformation and construction of mutants.

Growth and transformation procedures involving the cyanobacterium *Synechocystis* sp. PCC 6803 were described by Vermaas et al. (1990a). To grow cultures in the presence of exogenous deuterated tyrosine, filter-sterilized L-phenylalanine and L-tryptophan (dissolved in water) were added to the growth medium to a final concentration of 0.50 mM and 0.25 mM, respectively (Barry and Babcock, 1987). L-[Phe-2,3,5,6- $^2H_4$ ]Tyrosine was purchased from Sigma Chemicals. Due to its low solubility in water, [ $^2H_4$ ]tyrosine under sterile conditions was added as a powder to the growth medium to a final concentration of 0.5 mM. For the generation of mutations at residue 180 of D2, oligonucleotide-directed mutagenesis of *psbDI*, the D2 gene that is in a cluster with the CP43-encoding *psbC* gene, was performed as described by Vermaas et al. (1990a). Plasmids carrying appropriate site-directed mutations were introduced into PS-I-containing (Vermaas et al., 1990a) and PS-I-less (Ermakova-Gerdes et al., 1995) *Synechocystis* sp. PCC 6803 strains lacking *psbDIC* and *psbDII*. Therefore, the introduced *psbDIC* gene cluster carrying the site-directed mutation in the *psbDI* gene contains the sole D2-encoding gene in the resulting transformant.

**Quantification of PS II and oxygen evolution.** In PS-I-containing strains, PS II was quantified on a chlorophyll basis by herbicide-binding assays using  $^{14}C$ -labeled 3-(3,4-dichlorophenyl)-1,1-dimethylurea ([ $^{14}C$ ]DCMU; Amersham) (Vermaas et al., 1990b). In PS-I-less strains, where most of the chlorophyll is associated with PS II, the amount of PS II relative to the number of cells was quantified by measuring the amount of chlorophyll (determined as absorbance at 663 nm after methanol extraction) and comparing it to absorbance at 730 nm (cell scattering) of the cell suspension. Cells in logarithmic phase were used for these measurements, and cells of the various PS-I-less strains grew at essentially identical rates. After correction for the amount of chlorophyll remaining in cells after genetic deletion of both PS II and PS I (which is 20–25% of the amount

of chlorophyll found in a PS-I-less strain with normal PS II) the amount of PS II/cell was estimated. The procedure for oxygen evolution measurements was as described by Shen and Vermaas (1994).

**Fluorescence emission spectra.** For a qualitative estimate regarding PS II and the functional organization of pigments in the photosynthetic apparatus, fluorescence emission measurements at 77 K were performed using a Spex Fluorolog 2 instrument. Whole cells of PS-I-less strains were used at a concentration of 2 µg chlorophyll/ml. The samples were mixed with 1 ml 60% glycerol in 25 mM Hepes/NaOH pH 7.0 prior to freezing in liquid nitrogen. To record chlorophyll emission spectra, the excitation wavelength was 440 nm and the excitation and emission bandwidths were 12 nm and 2.4 nm, respectively.

**Measurements of fluorescence kinetics.** Room-temperature chlorophyll *a* fluorescence was monitored in a Walz PAM fluorometer. Cells growing in log phase were harvested from a 100-ml culture and the pellet was resuspended in 25 mM Hepes/NaOH pH 7.0. The cell suspension was kept at room temperature under dim light for 30 min before measuring chlorophyll fluorescence; 500 µl cell suspension containing 2 µg chlorophyll was used. The variable fluorescence yield is indicative of the redox state of the primary donor and the electron acceptor in PS II:  $Q_A$  and  $P680^+$  quench chlorophyll fluorescence whereas the reduced forms,  $Q_A^-$  and  $P680$ , do not. The intensity of the measuring light was sufficiently weak to not have a measurable actinic effect, even in the presence of DCMU.

**Preparation of thylakoid membranes for EPR studies.** Cells of PS-I-less strains growing at late-log phase were harvested from 4–20-l cultures. The pellet was washed with thylakoid buffer [25 mM Hepes/NaOH pH 7.0, 5 mM  $MgCl_2$ , 15 mM  $CaCl_2$ , 15% (by vol.) glycerol and 0.5% (by vol.) dimethyl sulfoxide]. The washed pellet was resuspended in the same buffer at a concentration exceeding 20 µg chlorophyll/ml. Cells were kept on ice for 1 h. To break the cells, large Braun homogenizer bottles were used for large volumes; otherwise screw-cap Eppendorf tubes were used. The cell suspension was mixed with an equal volume of glass beads (0.1 mm diameter) and incubated in ice for another 30 min. Cells were broken by two consecutive 30–50-s shakings (with an incubation on ice for 5 min between the shakings) with the glass beads, using either a mini Bead-Beater or Braun homogenizer. The homogenate was separated from glass beads by centrifuging at low speed ( $\approx 2000$  rpm for 3 min). The supernatant was subjected to another low-speed ( $\approx 4000$  rpm for 5 min) centrifugation to pellet unbroken cells and debris. Finally, to pellet the thylakoid membranes, the supernatant was diluted with up to 20 vol. of ice-cold thylakoid buffer and centrifuged at 18000 rpm in a Sorvall SS34 rotor for 30 min at 4°C. The pelleted thylakoid membranes were resuspended with a paint brush in ice-cold thylakoid buffer containing 3 mM EDTA (to remove loosely associated  $Mn^{2+}$ ).

**EPR measurements.** To study the properties of  $Y_D$  (Tyr160 of D2), X-band EPR spectra were recorded at 100 K with a Bruker ESP 300E spectrometer. EPR tubes were filled with 0.5 ml thylakoid suspension prepared from wild-type and Arg180 mutants in a PS-I-less background. The chlorophyll concentration of the suspensions was between 130–400 µg/ml. In PS-I-less strains, thylakoids at this chlorophyll concentration are very viscous. Stable light-induced radicals such as signal II<sub>s</sub>, which originates from  $Y_D^{\bullet}$ , were generated by illuminating thylakoid membranes in EPR tubes with room light for 2 min before freezing in liquid nitrogen. EPR conditions were: microwave power 500 µW, microwave frequency 9.55 GHz, modulation amplitude 0.313 mT, modulation frequency 100 kHz, time constant 82 ms, scan rate 0.2 mT/s, and temperature 100 K.

**Pigment analysis.** Pigments were extracted from 200 µl thylakoid membranes by sonicating with 800 µl cold 100% acetone in an ice/water bath. Denatured protein was precipitated by centrifugation and the pellet was extracted once more with 100% acetone to assure complete extraction. The extracts were pooled and filtered, after which a 200-µl sample was applied on a reverse-phase HPLC column (Spherisorb C8-5, 250×4.6 mm), equilibrated with 100% methanol (Rathburn, HPLC grade) to separate the pigments [see Eijkelhoff and Dekker (1995) for more details]. For detection, a diode array detector (Waters 990) was used in the wavelength range 280–750 nm with 2-nm resolution. Relative amounts of chlorophyll *a*, pheophytin *a* and  $\beta$ -carotene were determined by normalizing peak areas at 618 nm (chlorophyll *a*), 409 nm (pheophytin *a*) and 450 nm ( $\beta$ -carotene) to the absorption coefficients of these pigments in 100% methanol at the corresponding wavelengths (Eijkelhoff and Dekker, 1995).

## RESULTS

**Generation of mutants.** Site-directed mutagenesis was performed using mixed oligonucleotides, which were designed to change the residue Arg180 of the D2 protein to a variety of different amino acids. Nine clones with *psbDIC* operons carrying point mutations at the Arg180 site of D2 were introduced into PS-I-containing and PS-I-lacking background strains of *Synechocystis* sp. PCC 6803 that lacked *psbC* as well as both copies of *psbD*. The mutants were named according to the amino acid present at residue 180: R180D, R180H, R180I, R180L, R180N, R180Q, R180S, R180V and R180Y, where R180X indicates Arg180→X. The identity of the mutants was confirmed by sequencing of PCR-amplified fragments generated using DNA from the cyanobacterial strains.

**Photoautotrophic competence.** Of the nine mutants, only R180Q was photoautotrophic and had a doubling time of 12 h, which is identical to that of the wild type. The R180H mutant did not die in the absence of glucose, but did not grow significantly either; no photoautotrophic doubling time could be determined for this mutant but it significantly exceeded 60 h. The other mutants were true obligate photoheterotrophs; they could be complemented to a photoautotrophic phenotype by transformation with a 0.4-kb wild-type *psbDI* fragment covering codon 180.

**PS II presence.** To quantify the amount of PS II centers present on a chlorophyll basis in the wild-type and the mutant strains in a PS-I-containing background *in vivo*, herbicide binding assays were performed. In this experiment, binding of atrazine-displaceable [<sup>14</sup>C]DCMU, a PS-II-directed herbicide, was monitored as a function of the free DCMU concentration. As in *Synechocystis* sp. PCC 6803, most chlorophyll (80–90% of the total amount) is associated with PS I (Shen et al., 1993b), in PS-I-containing strains, the amount of DCMU that is specifically bound to PS II [i.e. can be replaced by high concentrations (30–40 µM) of unlabeled atrazine] with respect to chlorophyll at saturating DCMU concentration is approximately proportional to the amount of PS II in cells. Since a stably assembled PS II has one DCMU binding site, a plot of  $[DCMU]_{bound}/$ amount of chlorophyll versus  $[DCMU]_{free}^{-1}$  can provide information both on the number of chlorophyll molecules present/PS II center and on the DCMU dissociation constant ( $K_d$ ). The results of this experiment are shown in Table 1.

In wild-type *Synechocystis* sp. PCC 6803, one DCMU-binding site is found/680 chlorophyll molecules; thus the chloro-

**Table 1. Oxygen evolution rates, chlorophyll/ $A_{730}$  ratios, chlorophyll/PS II ratios and the  $Y_D$  signal amplitude of R180 mutants in PS-I-containing or lacking backgrounds.** Chlorophyll/PS II ratios in PS-I-containing strains were measured by DCMU-binding assays. In cells at a chlorophyll concentration of 50–125  $\mu\text{g/ml}$ , the amount of [ $^{14}\text{C}$ ]DCMU bound by PS II was calculated by determining the amount of bound [ $^{14}\text{C}$ ]DCMU that could be displaced by atrazine at each [ $^{14}\text{C}$ ]DCMU concentration used. In PS-I-less strains, chlorophyll/cell ratios were determined by comparison of 663-nm absorbance of a methanol extract from cells (chlorophyll content) and absorbance of intact cells at 730 nm (cell scattering). A 100% value of this chlorophyll/cell ratio corresponds to  $0.44 \text{ ng chl} \cdot \text{ml}^{-1} \cdot A_{730}^{-1}$ . If ratios or signal sizes are listed in percentages, these are expressed in comparison with the appropriate control strain (with or without PS I) carrying wild-type PS II (100%). For oxygen evolution, cells were used at a chlorophyll concentration of 2  $\mu\text{g/ml}$  in 25 mM HEPES/NaOH pH 7.0 and oxygen evolution was measured as  $\mu\text{mol O}_2 \cdot \text{mg chl}^{-1} \cdot \text{h}^{-1}$ . To determine the relative amount of the  $Y_D^{\text{ox}}$  radical, signals presented in Fig. 4 were double-integrated and corrected for the chlorophyll concentration in the sample. The results in this table are an average of three independent experiments and were reproducible within 15–30%; n.d., not detectable.

Strain	PS I present		PS I absent			
	chlorophyll/PS II	PS II/chlorophyll	chlorophyll/cell	calculated PS II/cell	oxygen evolution	integrated $Y_D^{\text{ox}}$ signal
	mol/mol	%			$\mu\text{mol mg chl}^{-1} \cdot \text{h}^{-1}$	%
Control	680	100	100	100	1050	100
R180D	2514	27	43	24	240	27
R180H	1608	43	68	58	500	16
R180L	2995	23	84	79	360	35
R180I	2125	32	73	64	450	17
R180N	n. d.	n. d.	50	34	420	21
R180Q	640	106	84	79	590	25
R180S	1785	38	34	12	0	n. d.
R180V	2489	28	74	66	300	22
R180Y	1819	37	49	32	290	41
<i>psbDIC</i> <sup>-</sup> / <i>psbDII</i> <sup>-</sup> /PS I-less	n. d.	n. d.	25	0	0	n. d.

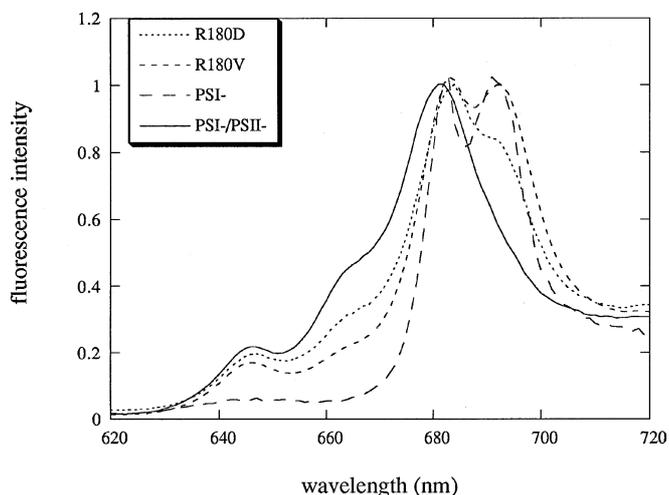
phyll/PS II ratio is 680 (Table 1). This ratio is increased in most Arg180 mutants except in the R180Q strain, indicating a decreased steady-state concentration of PS II in thylakoids from most Arg180 mutants. The effect of the Arg180 mutations on the amount of PS II in the membrane largely depends on the nature of the amino acid that is being substituted. In PS-I-containing strains, the mutant R180H retained approximately 40% of the amount of PS II centers present in wild-type, whereas the R180D, R180L and R180V mutants accumulated only 25% of the wild-type level of PS II centers. For the R180N strain no significant herbicide binding to PS II could be detected, suggesting that the amount of PS II is even lower in this mutant. For all strains with a measurable amount of DCMU binding to PS II, the DCMU affinity was essentially normal (16–20 nM; data not shown).

Most of the results presented in this paper were obtained with strains lacking PS I as these are more amenable to fluorescence and EPR studies using intact cells or isolated thylakoids. The control PS-I-less strain carries a deletion of part of the *psaAB* operon (Shen et al., 1993b) but retains a wild-type PS II. In PS-I-less strains, most of the chlorophyll is associated with PS II. Therefore, determination of the PS II/chlorophyll ratio by DCMU-binding assays is not informative as this ratio will be rather similar even if the amount of PS II is decreased several-fold (the amount of chlorophyll will have decreased by a rather similar amount). Instead, in PS-I-less strains a convenient method to estimate the relative amount of PS II/cell is a comparison of the chlorophyll content of a culture (determined via 663-nm absorbance of methanol extracts from cells) and the cell scattering at 730 nm of this culture. Cell counts of different *Synechocystis* 6803 mutants at the same absorbance are similar (data not shown), indicating the general validity of this approach for *Synechocystis* 6803. The relative  $A_{663 \text{ nm}}/A_{730 \text{ nm}}$  ratio of mutants in a PS-I-less background is indicated as chlorophyll/cell in Table 1. As in the absence of both PS II and PS I a significant amount of chlorophyll remained detectable in the cells (about

20–25% of that present in the PS-I-less strain with normal PS II), conversion of this ratio to PS II/cell requires correction for the amount of chlorophyll not associated with either PS II or PS I (calculated PS II/cell in Table 1). As in PS-I-containing strains, the amount of accumulated PS II/cell was decreased in the Arg180 mutants. However, in a quantitative sense the amount of apparent destabilization of PS II as a function of a mutation is sensitive to the presence or absence of PS I. In several mutants, particularly in those mutants where Arg180 has been replaced by a hydrophobic residue (L, I, V), significantly more PS II was retained in the PS-I-less strains than in the PS-I-containing background (Table 1). The reason for this phenomenon has not yet been clarified but may be related to the redox state of the cell and how this influences PS II degradation and synthesis in particular R180 mutants.

**Oxygen evolution.** Oxygen evolution was measured to determine whether the PS II complex in the Arg180 mutants was functional. The initial rates of oxygen evolution in intact cells of Arg180 mutants in a PS-I-less background in the presence of artificial electron acceptors are presented in Table 1. In all Arg180 mutant strains in a PS-I-less background the amount of oxygen evolution relative to chlorophyll was decreased significantly. Note that in PS-I-less strains retaining a considerable amount of PS II the PS II/chlorophyll ratio is rather similar to that in wild-type (most chlorophyll in such strains is associated with PS II). Therefore, in most Arg180 mutants, PS II reaction centers showed a 2–3-fold decrease in steady-state oxygen evolution rates at saturating light intensity, suggesting either a sluggishness of electron flow in all centers or an inactivation of part of the centers in these strains.

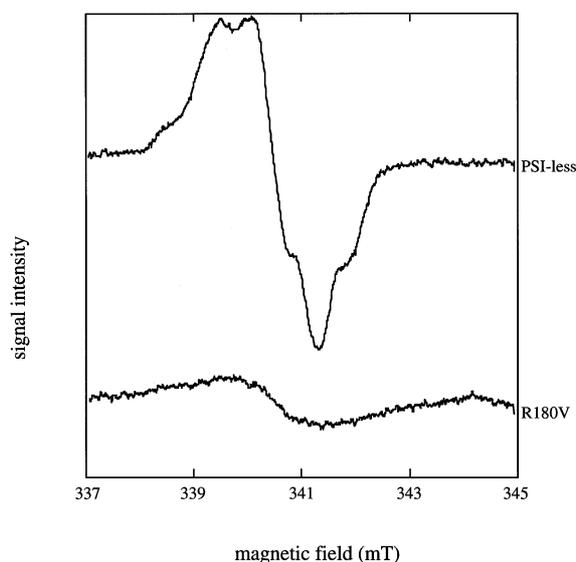
**Fluorescence emission properties.** The data presented above indicate that some mutants retained a substantial amount of PS II, but that oxygen evolution rates were reduced. To monitor whether PS II in these mutants appeared to be assembled nor-



**Fig. 2. Fluorescence emission spectra at 77 K.** Dark-adapted whole cells of the PS-I-less strain (— · — ·), the R180V (---) and R180D (····) mutants in a PS-I-less background, and the PS-I-less/PS-II-less (*psbDIC*<sup>-</sup>/*psbDII*<sup>-</sup>) (—) strain were used at a chlorophyll concentration of 2 µg/ml in 25 mM Hepes/NaOH, 60% glycerol. The spectra were measured upon exciting chlorophyll at 440 nm and normalized to 1 at their maximal emission.

mally, fluorescence emission spectra were measured at 77 K upon exciting chlorophyll at 440 nm in whole cells of wild-type and Arg180 mutants in a PS-I-less background strain (Fig. 2). The PS-I-less wild-type strain showed two major emission peaks around 685 nm and 695 nm, which are characteristic of PS-II-associated chlorophyll molecules; the amplitude of the two peaks was about equal in this strain. Upon genetic deletion of PS II (as occurred in the the *psbDIC*<sup>-</sup>/*psbDII*<sup>-</sup>/PS-I-less strain), the 695-nm peak disappeared, the 685-nm peak (also containing contributions from phycobilisome components and chlorophyll molecules not associated directly with one of the photosystems) shifted to lower wavelength, and a shoulder around 665 nm became more prominent. The spectrum of the R180V mutant, with rather normal amounts of PS II, retained an amplitude ratio of the 685-nm and 695-nm emission peaks resembling that of wild-type. As compared to the PS-I-less strain, the spectrum of the R180V mutant showed an increased intensity at lower wavelength (640–670 nm; possibly attributable to phycobilisome components) which was much more prominent in the mutant lacking PS II. Therefore, the increased intensity at 640–670 nm in R180V presumably is a consequence of a small decrease in the amount of PS II/cell in this strain. Indeed, in the R180D mutant the intensity of 695-nm versus 685-nm fluorescence intensity had decreased (implying a significant loss of PS II (Haag et al., 1993) in agreement with data shown in Table 1) and this was accompanied by an increase in the relative fluorescence emission intensity of 640–670-nm emission in this strain. These results together indicate a much larger loss of PS II in the R180D mutant as compared to R180V, in line with the relative PS II percentages presented in Table 1. The fact that their oxygen evolution rates are not very different is due in large part to the fact that these rates are expressed relative to chlorophyll. Fluorescence emission spectra of the other mutants (data not shown) were also consistent with the PS II percentages shown in Table 1.

**Tyr160 properties.** To determine whether mutations in residue Arg180 affected the Tyr160 ( $Y_D$ ) environment on the donor side of the PS II complex, EPR spectra of  $Y_D^{\text{ox}}$  were recorded. Surprisingly, Arg180 mutants did not show a characteristic signal  $II_s$ ,



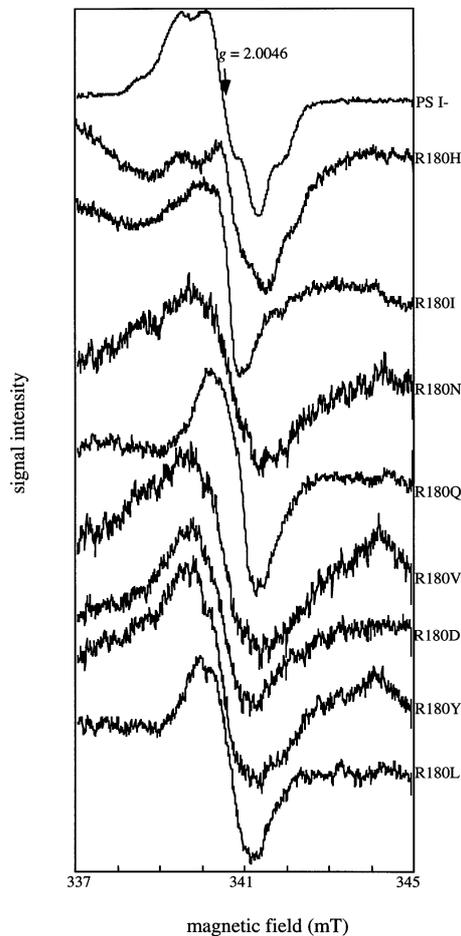
**Fig. 3. EPR spectra of the  $Y_D^{\text{ox}}$  radical in thylakoid membranes from the PS-I-less strain and R180V in the same PS-I-less background.**

The samples were illuminated with room light for 2 min before freezing in liquid nitrogen. The chlorophyll concentration of the PS-I-less sample was 280 µg/ml; that of R180V in a PS-I-less background was 315 µg/ml. EPR conditions were: microwave power 500 µW, microwave frequency 9.55 GHz, temperature 100 K, modulation amplitude 0.313 mT.

that is known to originate from  $Y_D^{\text{ox}}$ . Instead, in most mutants a very small, dark-stable radical was observed. Fig. 3 provides a comparison on the same scale of EPR spectra of thylakoids from PS-I-less strains with wild-type PS II and with the R180V mutation. The remaining radical in R180V seems to be associated with PS II as no such radical was observed in thylakoids from mutants lacking both PS I and PS II (data not shown). Comparing the spectra on differential vertical scales, the signal intensities and shapes were different for the various Arg180 mutants, but in all cases the signal was considerably smaller and generally did not much resemble signal  $II_s$  (Fig. 4). Also, under other illumination conditions (such as illumination with intense light upon freezing), no increased EPR signal could be observed in the mutants (data not shown) and the radical signal was stable for minutes in darkness at room temperature.

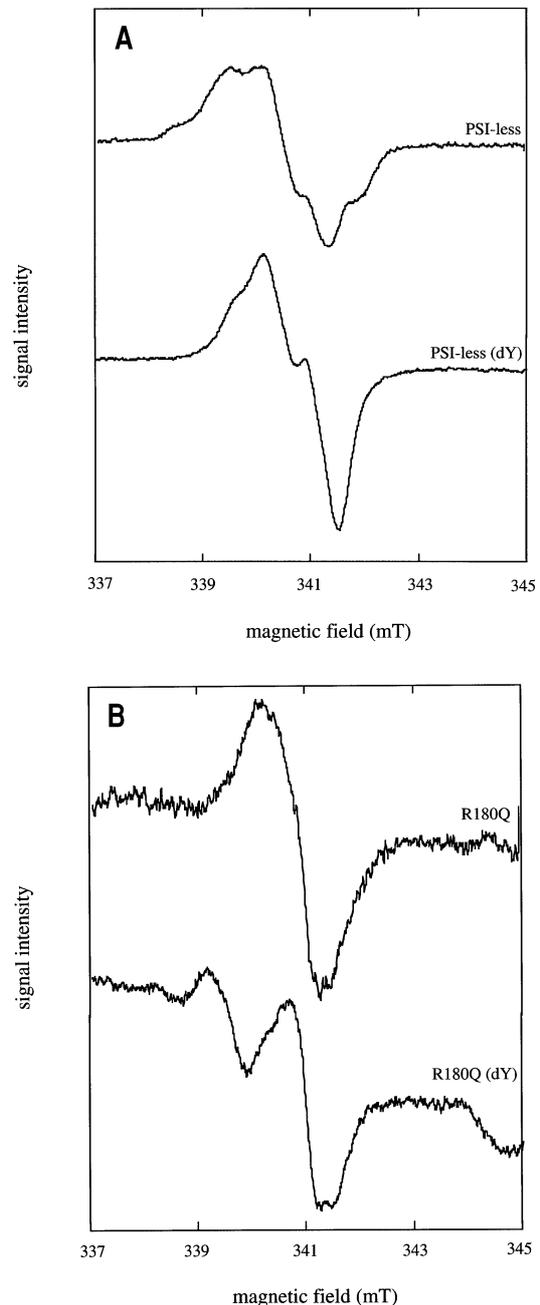
The R180Q and R180H mutants had the largest signal amplitude of the Arg180 mutants and, in the case of R180H, hyperfine features characteristic of signal  $II_s$  were observed. All other mutants exhibited relatively featureless signals with small amplitudes in spite of the presence of significant amounts of PS II in the thylakoids. The EPR signals shown in Fig. 4 are PS-II-related as they are not detectable in the PS-I-less/PS-II-less mutant (data not shown). These results suggest that, due to the mutation of the Arg180 residue of the D2 subunit of PS II, the  $Y_D$  environment was changed and that the effects were least drastic when Arg180 was replaced by His or Gln. This parallels the observations regarding photoautotrophic growth and oxygen evolution.

To establish whether in R180Q (a mutant with a reasonably large EPR spectrum that had little resemblance to signal  $II_s$ ) the detected signal indeed originated from  $Y_D^{\text{ox}}$  and was not due largely to other radicals, cells were grown in the presence of exogenously added [*Phe*-2,3,5,6-<sup>2</sup>H<sub>4</sub>]tyrosine. In the presence of phenylalanine and tryptophan (Barry and Babcock, 1987) this [<sup>2</sup>H<sub>4</sub>]tyrosine can be incorporated into *Synechocystis* sp. PCC 6803 proteins *in vivo*. Using tyrosine deuterated at specific positions, the major hyperfine couplings responsible for the spectral



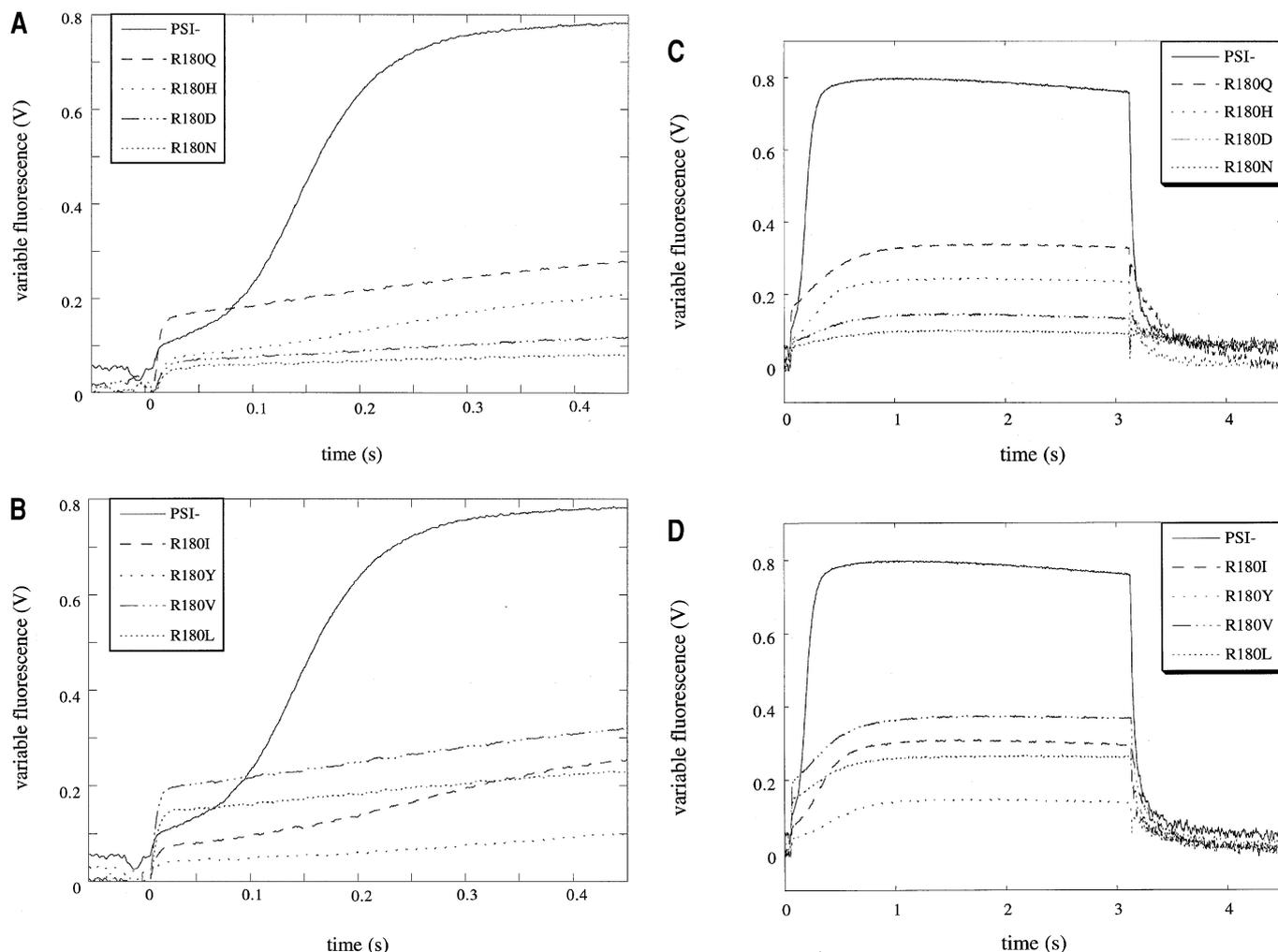
**Fig. 4.** EPR spectra of  $Y_D^{ox}$  radicals in thylakoid membranes of the PS-I-less strain and several Arg180 mutants in the same PS-I-less background. To obtain similar amplitudes in this figure, the spectra presented were recorded on different vertical scales and at slightly different chlorophyll concentration for the different samples. Assuming the vertical amplitude of the signal in thylakoids of the PS-I-less strain to be 100%, after correction for scaling and chlorophyll concentration, the vertical amplitude of the Arg180 mutants at the same chlorophyll concentration is as follows: R180D, 21%; R180H, 22%; R180I, 23%; R180L, 31%; R180N, 14%; R180Q, 45%; R180V, 15%; and R180Y, 29%. For double-integrated amplitudes of  $Y_D^{ox}$ , see Table 1. EPR conditions were as described in Fig. 3.

characteristics of  $Y_D^{ox}$  were shown to be associated with hydrogens associated with carbons at the 3 and 5 positions of the phenyl ring and at the  $\beta$ -methylene position (Barry et al., 1990). In Fig. 5 spectra from this experiment are presented for the PS-I-less strain (Fig. 5A) and for the R180Q mutant (Fig. 5B) in a PS-I-less background. EPR spectra of thylakoids isolated from cells grown on non-deuterated medium are included as controls. In the wild-type samples, deuteration of the ring protons of tyrosine produced a reduction in overall spectral width (Fig. 5A). This is consistent with the replacement of one or more strongly coupled protons by deuterium. In the R180Q mutant, a significant change was also observed (Fig. 5B). Thus, a substantial portion of the R180Q EPR spectrum is indeed due to a tyrosyl radical. However, the spectrum of R180Q after deuteration appeared to have greater overall spectral width than that of R180Q with native tyrosine. This is, of course, not possible. The spectrum of the undeuterated sample must contain very broad components, particularly near its maximum  $g$ -value (2.013), which are difficult to detect in the first-derivative display of the microwave absorption that is used in standard EPR measure-



**Fig. 5.** EPR spectra after deuteration of ring protons of Tyr. Spectra of (top) the non-deuterated and (bottom) deuterated (at Phe ring positions 2, 3, 5 and 6) form of the  $Y_D^{ox}$  radical were measured in thylakoid samples prepared from the PS-I-less strain (A) and the R180Q mutant (B). Thylakoids were isolated from cells grown in the presence of externally added deuterated L-[Phe-2,3,5,6- $^2H_4$ ]tyrosine (0.25 mM). The medium was also supplemented with 0.25 mM L-phenylalanine and 0.5 mM L-tryptophan. EPR conditions were as described in Fig. 3.

ments. These broad components must be due to strong, highly anisotropic tyrosine proton hyperfine couplings. The coupling would appear to be largest near  $g_{max}$ , but it is still rather large in the central part of the spectrum. The improved resolution in the deuterated sample indicates that, if the spectrum is due mostly to a single species, its  $g$ -tensor is rather strongly axial. Perhaps most significantly, the apparent loss of low-field EPR signal due to hyperfine broadening may help to explain discrepancies between the change in the amount of PS II/mutant cell and the



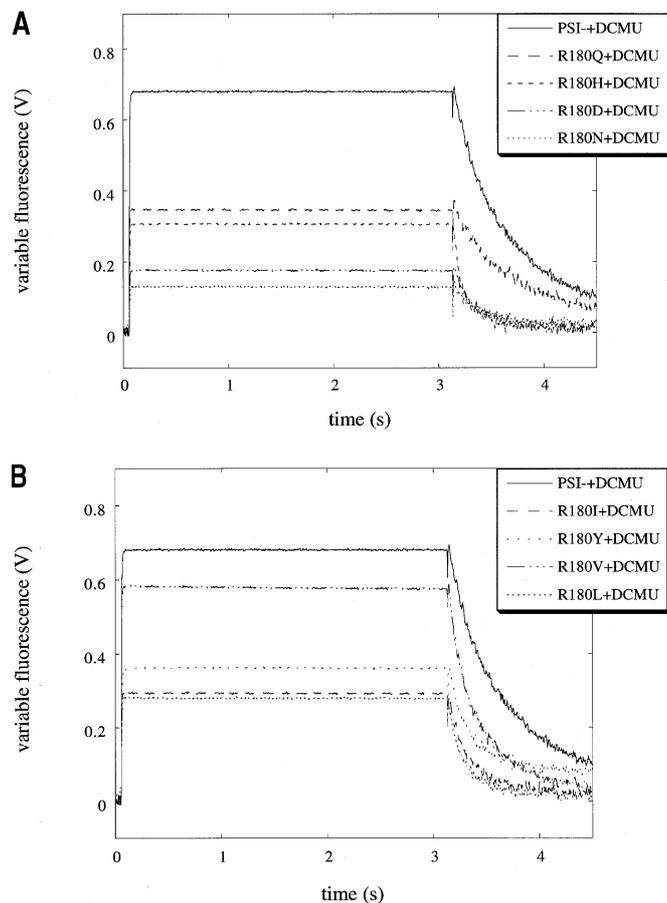
**Fig. 6. Induction kinetics of variable fluorescence.** Variable fluorescence induction kinetics were measured in cells of the PS-I-less strain (—) and of the (A): R180Q (—), R180H (---), R180D (----), R180N (----) and (B) R180I (—), R180Y (---), R180V (----), R180L (----) mutants in a PS-I-less background. To determine the maximal fluorescence yield, data have also been plotted on an extended time scale (C, D). Dark-adapted whole cells were used at a chlorophyll concentration of 2  $\mu\text{g/ml}$  in 25 mM HEPES/NaOH pH 7.0. Actinic illumination (100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of red light) was turned on at  $t = 0$ .

change in double-integrated  $Y_D^{\text{ox}}$  microwave absorption intensity as listed in Table 1 (see Discussion).

**Chlorophyll fluorescence properties.** Comparison of oxygen evolution rates with the amount of PS II that is present suggested a discrepancy for most of the mutants (Table 1). This is even more striking in view of the fact that, because most chlorophyll in PS-I-less strains is associated with PS II, a significant decrease in oxygen evolution on a per chlorophyll basis is not expected even if the PS II content is somewhat decreased. The fact that, in all mutants, the oxygen evolution rate/mass chlorophyll was less than 60% of that measured in the control may mean that PS II complexes in Arg180 mutants have been functionally altered. To investigate this further, fluorescence induction was measured in the PS-I-less strain and in Arg180 mutants in a PS-I-less background. The results of this experiment are presented in Fig. 6. Fluorescence induction in intact cells was changed in the Arg180 mutants in that most mutants showed a fast rise to an intermediate level ( $F_i$ ) followed by a slow increase to the maximum fluorescence level, which generally was lower than expected on the basis of the amount of PS II present. However, as shown in Fig. 7, upon addition of DCMU (blocking  $Q_A^-$  oxidation by  $Q_B$ ) a marked increase in  $F_m$  was observed in

most Arg180 mutants, indicative of an inhibition of electron transport prior to  $Q_A$ . This suggests that one or more of the donor side reactions is impaired in the Arg180 mutants. However, the variable fluorescence level in the presence of DCMU in most cases is still somewhat lower than expected, suggesting that a measurable amount of inactive PS II centers is present in several Arg180 mutants.

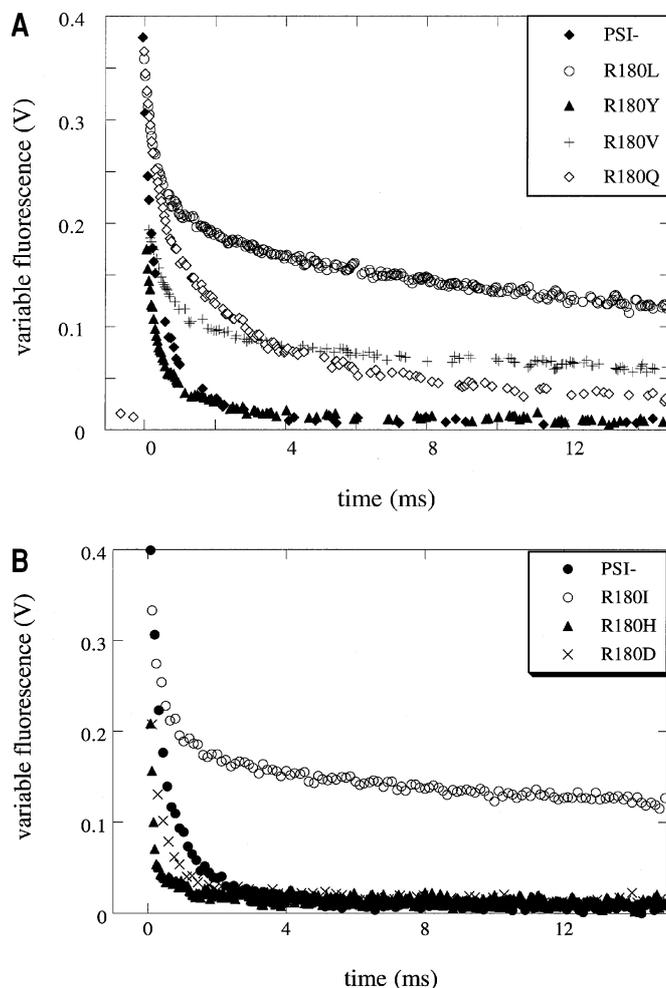
These fluorescence experiments suggest that the functional effects of the Arg180 mutations are complex: the increase in the  $F_i$  level upon fluorescence induction suggests that electron transfer at the acceptor side has been modified, the decreased  $F_m$  in the absence of inhibitors implies donor-side impairment, and the larger-than-expected decrease of  $F_m$  in the presence of DCMU suggests the presence of some inactive PS II centers. To investigate further the effects of Arg180 mutations on PS II function, first  $Q_A^-$  oxidation by  $Q_B$  was probed by following the decay of the variable fluorescence yield after single-turnover flashes; this provides information on the rate of electron transfer to  $Q_B$  or  $Q_B^-$  as well as on the position of the  $Q_A^- \cdot Q_B \rightleftharpoons Q_A \cdot Q_B^-$  equilibrium. As indicated in Fig. 8, particularly with the Arg180 mutants replaced with a hydrophobic residue (R180I, R180V, R180L), a significant slow phase in the decay of the variable fluorescence yield after a flash was ob-



**Fig. 7. Induction kinetics of variable fluorescence in the presence of DCMU.** Fluorescence induction kinetics were measured in PS-I-less cells and several Arg180 mutants in a PS-I-less background (—) in the presence of 50  $\mu\text{M}$  DCMU; (A) R180Q (---), R180H (---), R180D (---), R180N (---), and (B) R180I (—), R180Y (---), R180V (---), R180L (---). Dark-adapted whole cells were used at a chlorophyll concentration of 2  $\mu\text{g}/\text{ml}$  in 25 mM Hepes/NaOH pH 7.0. Actinic illumination (100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of red light) was turned on at time  $t = 0$ .

served. In R180Q,  $Q_A^-$  decay was slower than in the control strain with wild-type PS II, but was relatively monophasic, in contrast to the situation in R180I, R180L and R180V. Decay in Arg180 mutants replaced with other hydrophilic residues (R180Y, R180D, and R180H) was similar to that in the control, except that in R180H no clear slow phase could be detected. The relative amplitudes of the slow and fast phases and the corresponding half-times of decay are presented in Table 2. These results are in good agreement with the relative  $F_i$  levels observed in the various mutants: R180V and R180L, the two mutants with the most significant slow phase of  $Q_A^-$  decay, and R180Q, which also has a considerable slow phase, showed the highest  $F_i$  level.

To investigate possible functional changes on the donor side, the decay of the yield of variable fluorescence after a flash was followed in the presence of DCMU. These conditions allow charge recombination between  $Q_A^-$  and the donor side of PS II to be monitored. Recombination became faster in all the mutants except R180Q (Fig. 9). This could be due to a decrease in the midpoint redox potential of the  $Q_A/Q_A^-$  couple or to changes at the donor side leading to an increased steady-state  $P680^+$  concentration after the flash. However, a decrease in the  $Q_A/Q_A^-$  midpoint potential is not consistent with the smaller apparent semiquinone equilibrium constant. However, the explanation of



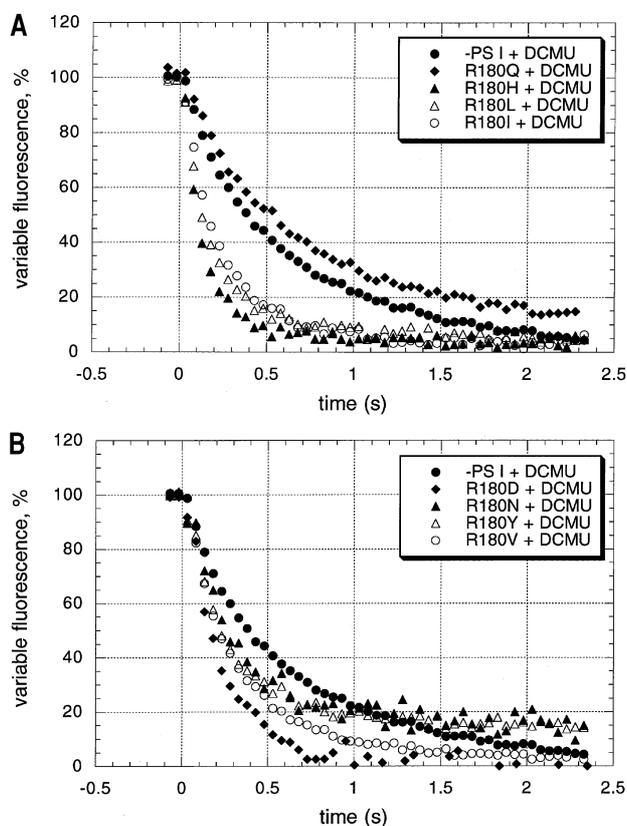
**Fig. 8. Decay kinetics of the variable fluorescence after single-turnover flashes.** Decay kinetics were measured in intact cells of the PS-I-less ( $\blacklozenge$ ,  $\bullet$ ) strain and of (A) R180L ( $\circ$ ), R180Y ( $\blacktriangle$ ), R180V ( $+$ ), R180Q ( $\diamond$ ); and (B): R180I ( $\circ$ ), R180H ( $\blacktriangle$ ) and R180D ( $\times$ ). Dark-adapted whole cells were used at a chlorophyll concentration of 2  $\mu\text{g}/\text{ml}$  in 25 mM Hepes/NaOH pH 7.0.

an increased steady-state concentration of  $P680^+$  is plausible as it is consistent with a donor-side effect suggested from fluorescence induction measurements.

**Pigment composition.** As indicated earlier, the reason why the Arg180 codon initially was selected for introduction of a site-directed mutation was to test the hypothesis that Arg180 serves as a ligand to an accessory chlorophyll. To determine whether Arg180 mutations actually led to an altered pigment composition of PS II, thylakoid preparations were analyzed by reverse-phase HPLC. The results of this analysis are presented in Table 3. In the PS-I-less strain and in the analyzed Arg180 mutants in the same PS-I-less background, in the PS II fraction about 42 molecules chlorophyll were found per two molecules of pheophytin. These results exclude the possible exchange of chlorophyll into pheophytin in any of the Arg180 mutants that were analyzed because, if so, the chlorophyll/pheophytin ratio would drop from 21 to 14, which is clearly outside the error margin of the HPLC experiments. His and Gln are known to be able to serve as chlorophyll ligands in other proteins, but Leu and Ile are not expected to do so. The results presented in Table 3 therefore suggest that no irreplaceable ligand to Mg was removed upon

**Table 2. Decay half-times and relative amplitudes of variable fluorescence with and without DCMU.** The half-times of decay of variable fluorescence and the relative amplitudes of the slow and fast phases were determined in several Arg180 mutants in a PS-I-less background upon single-turnover flashes in the absence of DCMU, as well as in continuous light in the presence of 50  $\mu$ M DCMU. The results presented in this table are an average of three independent experiments with ten single-turnover flashes each time. The decay curves obtained in the absence of DCMU (see Fig. 8) were fitted with two components assuming an exponential decay;  $t_{1/2s}$ , half-time of the slow phase;  $t_{1/2f}$ , half-time of the fast phase. In the PS-I-less strains R180D, R180H, R180L, and R180Y the half-time of fluorescence decay in the presence of DCMU was also monitored after single-turnover flashes and yielded essentially the same half-time.

Strain	Amplitude of		Half-time		
	fast phase	slow phase	$t_{1/2f}$	$t_{1/2s}$	$t_{1/2} + \text{DCMU}$
	%		$\mu$ s		ms
PS-I-less	64	36	150	690	395
R180D	86	14	180	1100	180
R180H	100	—	160	—	90
R180I	69	31	330	5300	170
R180L	52	48	500	>10000	125
R180Q	55	45	380	2580	540
R180V	66	34	660	>10000	210
R180Y	79	21	180	1030	220



**Fig. 9. Decay kinetics of the variable fluorescence in the presence of DCMU.** Decay kinetics were measured in the PS-I-less strain (●) and of (A) R180Q (◆), R180H (▲), R180L (△), R180I (○); and (B): R180D (◆), R180N (▲), R180Y (△), R180V (○) mutants in the same background in the presence of 50  $\mu$ M DCMU. Dark-adapted whole cells were used at a chlorophyll concentration of 2  $\mu$ g/ml in 25 mM Hepes/NaOH pH 7.0 in presence of 50  $\mu$ M DCMU. The variable fluorescence  $[(F-F_0)/(F_m-F_0)]$  values were normalized to 100%.

mutation of Arg180. However, we cannot exclude the possibility that in one or more of the Arg180 mutants one chlorophyll molecule has been lost. The difference of only one chlorophyll molecule/PS II complex is within the error margin of the HPLC experiments.

**Table 3. Pigment composition of thylakoid membranes.** Membranes were isolated from the PS-I-less strain, and from the R180H, R180I, R180L, and R180Q mutants in the same PS-I-less background. The pigments were extracted from the thylakoids with 80% acetone and analyzed by HPLC.

Strain	Relative ratio of chlorophyll a/ pheophytin a/ $\beta$ -carotene
PS I-less	43 $\pm$ 2: 2: 8 $\pm$ 1
R180H	40 $\pm$ 2: 2: 12 $\pm$ 1
R180I	44 $\pm$ 3: 2: 10 $\pm$ 1
R180L	41 $\pm$ 3: 2: 9 $\pm$ 1
R180Q	46 $\pm$ 3: 2: 13 $\pm$ 1

## DISCUSSION

The effects of Arg180 mutations are numerous, and include (a) large changes in the shape and amplitude of the dark-stable EPR signal presumably originating from  $Y_2^S$ , (b) altered electron transfer parameters on the donor side of photosystem II, and (c) modified electron transfer kinetics between  $Q_A$  and  $Q_B$  (possibly due to a changed semiquinone equilibrium) at the acceptor side of PS II. However, the pheophytin/chlorophyll ratio in the reaction center of the two mutants analyzed in this respect remained normal, indicating that no extra pheophytin had been introduced as a consequence of the Arg180 mutations.

**Similarity between the M subunit and the D2 protein.** According to a simple line-up between the sequences of the M subunit of purple bacteria and the D2 subunit of PS II, the Arg180 residue would be expected to be in a position similar to the Asp two residues down from the His that serves as ligand to the central Mg in an accessory bacteriochlorophyll. This Asp residue is located in the *cd* helix with its side group pointing out toward the periplasmic space. By analogy, the Arg180 residue would be expected to point towards the lumen. However, this is unlikely as a consequence of the various functional effects that Arg180 mutations have. Many other charged residues have been altered in the D2 protein with very little functional consequence (reviewed by Pakrasi and Vermaas, 1992), suggesting that changes in charged residues are rather innocuous unless they play a direct role in PS II structure or function. Therefore, the

Arg180 residue most likely points inward, possibly toward  $Y_D$  and/or P680 components. This implies that the arrangement of the *cd* helix in the D2 protein (assuming that this helix actually exists in this protein) is different from what has been assumed thus far on the basis of a simple alignment with the M subunit. Two possibilities exist to explain this apparent difference between D2 and M: (a) the beginning and end of the putative *cd* helix may be at similar positions in the two proteins, but the helix as a whole may have rotated in the D2 protein as compared to the *cd* helix in the M subunit; or (b) the start of the helix may be at a different position in D2 as compared to M, thus shifting the orientation of residues in the helix relative to those in the membrane. As illustrated in Fig. 1, the similarity between the M subunit and the D2 protein is very poor, and an  $\alpha$ -helical configuration in this region of the D2 protein seems likely on the basis of its amino acid sequence. Therefore, both possibilities mentioned above seem plausible.

Assuming that Arg180 points inward rather than toward the lumen, it is interesting to consider what cofactors and residues may be close by. In the M subunit, several hydrophobic and often aromatic residues in the *cd* helix that point inward are close to the accessory bacteriochlorophyll on the M side and, to a lesser extent, close to one of the primary donor bacteriochlorophyll molecules [see El-Kabani et al. (1991) for approximate distances]. A similar concentration of hydrophobic and often aromatic residues is found around Arg180 of D2 (also see Fig. 1) with appropriate spacing between residues (assuming an  $\alpha$ -helical arrangement) to point in the same general direction as Arg180. In addition, in the D2 protein selected residues in the *cd* helix may be in the immediate vicinity of  $Y_D$  and His189 (Svensson et al., 1990, 1996), with which  $Y_D^{ox}$  appears to be in close interaction (Tommos et al., 1993; Tang et al., 1993). Even though, according to the model proposed by Svensson et al. (1990, 1996), Phe185 of the D2 protein was the residue in the presumed *cd* helix thought to be in close contact with  $Y_D$ , in view of the probable differences in orientation and/or start of the *cd* helix in the D2 protein as compared to the M subunit (see above) other residues in the *cd* helix would be expected to be in close vicinity of  $Y_D$  and His189 instead.

**Residue 180 and  $Y_D^{ox}$ .** One of the most striking effects of mutations in Arg180 is the change in shape and amplitude of the dark-stable EPR spectrum (Figs 3 and 4). The EPR signal in R180Q can be assigned to originate from a Tyr radical because of deuteration results, but for even smaller and more distorted EPR signals shown in Fig. 4 an origin of these signals from radicals other than  $Y_D^{ox}$  cannot be rigorously excluded. However, the exact origin of the very small radicals is not important; what is important is that apparently much less  $Y_D^{ox}$  can be generated in most strains than would be expected on the basis of the amount of PS II present.

In earlier work, signal  $II_s$  had been observed to be sensitive to some but not all mutations in residues in its close vicinity (Vermaas et al., 1988; Tommos et al., 1993, 1994; Tang et al., 1993). Most significantly, signal  $II_s$  was found to be altered to a much narrower signal upon mutations in His189, and results were interpreted in terms of a hydrogen bond between  $Y_D$  and His189; upon oxidation of  $Y_D$  the radical would deprotonate and  $H^+$  would reside mostly on His189 (Tommos et al., 1993; Tang et al., 1993). Proton ENDOR spectra of His189 mutants confirmed this interpretation (Tang et al., 1993). From these observations, a specific interaction between Tyr160 and His189 was inferred. The results presented here indicate that Arg180 mutations lead to even larger changes in the dark-stable, photosystem-II-associated radical than mutations in His189 and, at least in the case of R180Q where the EPR signal was shown to origi-

nate from a Tyr radical, the Arg180 mutations also lead to a loss of the hyperfine features of signal  $II_s$ .

The decreased  $Y_D^{ox}$  signal amplitude is particularly striking for several mutant strains, including R180L, R180V and R180Y, in a PS-I-less background, where the maximum loss in the amount of PS II in the membrane is only up to 50–60% (Table 1) whereas the  $Y_D^{ox}$  signal amplitude is decreased at least 5–7-fold (Figs 3 and 4), and even more if this EPR signal is not due entirely to  $Y_D^{ox}$  in these mutants. We observed that the EPR signal in the Arg180 mutants could not be increased in intensity by prolonged illumination, illumination during freezing, etc., suggesting that the amount of the signal observed in these mutants is maximal. Indeed, measurements of formation and decay kinetics of the signal in Arg180 mutants suggest that the rate of formation upon illumination is comparable to that of signal  $II_s$  in wild-type whereas the rate of decay is not fast (data not shown). During broad magnetic field scans (440 mT), no unusual signals were observed in the Arg180 mutants (data not shown). Therefore, either part of the  $Y_D^{ox}$  spectrum in the Arg180 mutants is too broad to detect, or only part of  $Y_D$  can be oxidized. The former possibility is certainly plausible because, upon Tyr deuteration of R180Q, a feature outside the usual  $Y_D^{ox}$  spectrum in R180Q becomes visible. The amplitude of the broad EPR spectrum in R180Q relative to that of the narrow spectral feature that changes upon Tyr deuteration and is therefore  $Y_D^{ox}$  cannot be determined at this point, and so only a lower limit regarding the amount of  $Y_D^{ox}$  relative to the number of active PS II centers can be determined.

The data presented here suggest that mutations in Arg180 caused an alteration in the environment and properties of  $Y_D$ , which thus far was supposed to be distant from the Arg180 residue. The large change in the shape of signal  $II_s$  in most mutants is compatible with alterations in hydrogen-bonding potential, similar to the phenotype observed for His189 mutants. This suggests that Arg180 (most likely via His189) can accept a proton upon oxidation of  $Y_D$ , or can stabilize the proton on His189. Upon mutation of Arg180 to most other groups (including side groups that could form hydrogen bonds if their position was appropriate) this apparent proton-accepting/proton-stabilizing property was lost. If Arg180 can serve as (indirect) proton acceptor, then the midpoint redox potential of  $Y_D/Y_D^{ox}$  is expected to move closer to that of P680/P680<sup>+</sup> in the Arg180 mutants; this might lead to a decrease in the amount of  $Y_D^{ox}$  that accumulates upon illumination. Therefore, the reason for a lack of a significant signal  $II_s$  in many of the Arg180 mutants may also be thermodynamic, at least in part.

A protonation event with a  $pK = 7.0$ – $7.5$  appears to influence the redox equilibrium between  $Y_D$  and the  $S_2$  and  $S_3$  states of the water-oxidizing complex (Vass and Styring, 1991; Deák et al., 1994), presumably involving also P680 and  $Y_Z$  as redox intermediates connecting  $Y_D$  and the water-oxidizing complex. The  $pK$  of this protonation was assigned to His189 of the D2 protein and is thought to be associated with the pH-dependent kinetics of  $Y_D$  oxidation (Deák et al., 1994). However, in view of the highly hydrophobic environment of  $Y_D$  (Svensson et al., 1991) a nearly normal  $pK$  value of His would be somewhat surprising. Another possibility is that this  $pK$  reflects protonation of R180.

**Electron transfer properties.** The Arg180 mutations appeared to significantly affect electron transfer properties within PS II. The amount of variable fluorescence in the presence of DCMU (roughly indicative of the number of PS II centers (relative to chlorophyll) that can lead to stable charge separation) was somewhat smaller than expected (Fig. 7, Table 1) suggesting the presence of some inactive centers. However, oxygen evolution rates

in most Arg180 mutants were even lower than expected from the maximal amount of variable fluorescence (see Table 1; note that the oxygen evolution rates are expressed relative to chlorophyll and have been measured on PS-I-less cells where most chlorophyll is associated with PS II) suggesting an impairment of PS II function. Moreover, the lack of full fluorescence induction in PS-I-less strains unless DCMU was added (Fig. 6) suggests that electron transport on the donor side has been impaired in the mutants. The combination of these effects suggests that Arg180 mutations do not lead to an overall and unspecific conformational change in the PS II complex as this would most likely result in a major destabilization of the PS II complex and in less specificity of the phenotypic effects that are observed.

In the presence of DCMU the charge recombination between  $Q_A^-$  and the donor side has become faster in all mutants except R180Q. As the charge recombination rate presumably is proportional to the steady-state concentration of  $P680^+$  (Metz et al., 1989; Chu et al., 1994), the increased recombination rate observed in most mutants suggests an increased concentration of  $P680^+$  after a flash. This could reflect altered redox properties of  $P680$ ,  $Y_Z$  and/or the S states. As the Arg180 residue presumably is closer to  $P680$  than to  $Y_Z$  or the water-splitting complex, and as a modification of the  $Y_D/P680$  equilibrium is inferred from the decreased accumulation of  $Y_D^{ox}$ , a change in the redox characteristics of  $P680/P680^+$  (stabilizing the oxidized form of  $P680$ ) seems most likely. As Arg180 is not expected to be in immediate contact with the homologs of the special pair of purple bacteria, an effect of Arg180 on  $P680$  properties may provide support for the idea that there are excitonic interactions among all porphyrins in the PS II complex (Tetenkin et al., 1989) and that  $P680$  may be a multimer of (more than two) pigments (Durrant et al., 1995).

However, the effects on electron transfer are not limited to donor-side phenomena as a higher fluorescence level immediately at the start of illumination indicates a higher steady-state  $Q_A^-$  concentration. This indication was confirmed by measuring  $Q_A^-$  decay kinetics after a flash in the absence of inhibitors. How a change at the donor side of PS II affects  $Q_A^-$  oxidation by  $Q_B$  is not well understood, but in some other systems with impaired donor-side activity [such as upon  $Ca^{2+}$  depletion (Andréasson et al., 1995) but not upon  $Cl^-$  depletion (Krieger and Rutherford, 1997)]  $Q_A^-$  oxidation by  $Q_B$  has also been found to be impaired. The reason for this impairment of acceptor side electron transfer upon  $Ca^{2+}$  depletion is a 150-mV shift in the  $Q_A/Q_A^-$  midpoint redox potential (Krieger et al., 1995). Another example of processes on the donor side that affect electron transfer at the acceptor side is provided by photoactivation: dark-grown cells of *Scenedesmus obliquus* lacking the oxygen-evolution complex showed a shift in the  $Q_A/Q_A^-$  midpoint redox potential from +100 to -80 mV when transferred to light (Johnson et al., 1995). Similar observations were made in cells that are unable to assemble the oxygen-evolving complex and after  $NH_2OH$  treatment of spinach PS II preparations (Johnson et al., 1995). Our observation on the inhibition of  $Q_A^-$  oxidation by  $Q_B$  upon introduction of modifications at the donor side matches conceptually with these previous studies.

**Chlorophyll binding.** Work on the Arg180 residue was initiated to probe its potential functional correspondence to the His residue in the M subunit of purple bacteria that binds an accessory bacteriochlorophyll. In *Chloroflexus aurantiacus* this His residue is replaced by Leu, and reaction centers of this organism appear to have one bacteriochlorophyll molecule replaced by a bacteriopheophytin one (Ovchinnikov et al., 1988; Shiozawa et al., 1987). None of the data presented here confirm a corresponding role of Arg180 in chlorophyll binding. PS II from the

R180L mutant has the same chlorophyll/pheophytin ratio as PS II from wild-type or R180H. It should be noted that the ratios presented in Table 3 imply 40–45 chlorophyll molecules/PS II center in thylakoid membranes from PS-I-less strains. However, on the basis of DCMU-binding experiments in these strains, the chlorophyll/PS II ratio is found to be about 100 (Shen et al., 1993b). Of these chlorophyll molecules, some are not associated with PS II as chlorophyll remains present in systems lacking both *psaAB* and *psbDIC/psbDII* or *psaAB* and *psbB/psbC*. The reason for this apparent discrepancy unknown at present but has no major implications for our findings regarding Arg180.

The effects of the Arg180 mutations on the charge recombination rate are indicative of decreases in the  $P680/P680^+$  midpoint potential of up to 40 mV (in R180H). The size of these changes is roughly comparable to that observed upon mutating His198 of D1 or His197 of D2, the putative ligands to the central Mg in what are thought to be the  $P680$  chlorophyll molecules (see Coleman et al., 1995). This suggests that the Arg180 residue affects  $P680$  properties just as much as removing a putative ligand to one of the special pair analogs. Indeed, only very few mutations in His198 of D1 lead to a loss of the PS II reaction center, and  $P680$  difference spectra are not changed by more than 2.5 nm in these His198 mutants (Coleman et al., 1995). These data are interpreted as meaning that other PS II components close to the special pair analog (possibly including water) take over the function of serving as ligand to the chlorophyll molecules that are homologous to the special pair. Other molecules have been shown to be able to serve as ligand to bacteriochlorophyll molecules if space is available (Goldsmith et al., 1996). Therefore, the lack of change in the PS II pigment composition upon changing Arg180 by no means rules out the possibility that Arg180 might serve as chlorophyll ligand, but does not support it either.

However, support for a direct effect of Arg180 on  $P680$  properties is provided by the implication that the  $P680/P680^+$  redox potential has been changed significantly, to an extent comparable to that when His198 of D1 or His197 of D2 are mutated. The question, however, is why Arg180 should affect  $P680$  properties. This in turn raises the question of what constitutes  $P680$ . By analogy with the purple bacterial reaction center,  $P680$  is commonly thought of as a dimer associated with His198 of D1 and His197 of D2. However, chlorophyll triplets originating from charge recombination reside on a chlorophyll molecule with an orientation similar to that expected for the accessory chlorophyll (van Mieghem et al., 1991). One option to interpret this may be that the accessory chlorophyll on the D1 side (the active branch) is part of  $P680$ . By the same token it could be argued that the accessory chlorophyll associated with D2 is part of the  $P680$  complex (Durrant et al., 1995). Removal of the positive charge from a possible ligand to this accessory chlorophyll is likely to affect the charge distribution over chlorophyll molecules when the primary donor of PS II is in its oxidized ( $P680^+$ ) state, leading to an increased stability of  $P680^+$  by moving the delocalized charge more toward the accessory chlorophyll on the D2 side. Another possibility is that the removal of the positive charge on residue 180 stabilizes  $P680^+$  for purely electrostatic reasons. This remains speculative at this moment, but the apparent effects of Arg180 mutations on  $P680$  properties certainly are consistent with a direct correlation of residue 180 of the D2 protein with the primary donor of PS II.

Thus, Arg180 of the D2 protein appears to play a prominent role in PS II. The effects appear to be specific as other mutations in charged D2 residues on the luminal side of the membrane either have little effect or lead to a major destabilization of the PS II complex in the membrane (see Pakrasi and Vermaas, 1992). With an apparent function in determining both the prop-

erties of  $Y_D^{ox}$  and the stability and charge distribution in  $P680^+$ , the data presented here seem most compatible with the hypothesis that one active group in Arg180 is involved in stabilizing  $Y_D^{ox}$  through direct or indirect hydrogen bonding or proton acceptance, whereas another active group in Arg180 may be close to an accessory chlorophyll.

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