Tryptophan at Position 181 of the D2 Protein of Photosystem II Confers Quenching of Variable Fluorescence of Chlorophyll: Implications for the Mechanism of Energy-Dependent Quenching†

Dmitrii V. Vavilin,* Svetlana Y. Ermakova-Gerdes, Anna T. Keilty, and Wim F. J. Vermaas

Department of Plant Biology and Center for the Study of Early Events in Photosynthesis, Arizona State University, Box 871601, Tempe, Arizona 85287-1601

Received July 7, 1999; Revised Manuscript Received September 9, 1999

ABSTRACT: The lumenal CD loop region of the D2 protein of photosystem II contains residues that interact with a reaction center chlorophyll and the redox-active Tyr_D. Using combinatorial mutagenesis, photoautotrophic mutants of *Synechocystis* sp. PCC 6803 have been generated with multiple amino acid changes in this region. The CD loop mutations were transferred into a photosystem I-less *Synechocystis* strain to facilitate characterization of photosystem II properties in the mutants. Most of the combinatorial photosystem I-less mutants obtained had a high yield of variable fluorescence, F_V. However, in three mutants, which shared a replacement of Phe181 by Trp, the F_V yield was dramatically reduced although a high rate of oxygen evolution was maintained. A site-directed F181W D2 mutant shared similar properties. Picosecond time-resolved fluorescence measurements revealed that in the combinatorial F181W mutants the fluorescence lifetimes in closed and open photosystem II centers were essentially identical and were similar to the fluorescence lifetime in open centers of the control strain. These results are explained by quenching of variable fluorescence in the mutants by charge separation between Trp181 and excited reaction center chlorophyll. This reaction competes efficiently with fluorescence and nonradiative decay in closed photosystem II centers, where the lifetime of the excitation in the chlorophyll antenna is long. Thermodynamic considerations favor the formation of oxidized tryptophan and reduced chlorophyll in the quenching reaction, presumably followed by charge recombination. A possible role of tryptophan−chlorophyll charge separation in the mechanism of energy-dependent quenching of excitations in photosynthesis is discussed.

Photosystem II (PS II)[1] is a part of the photosynthetic apparatus in plants and cyanobacteria that uses light energy to catalyze reduction of plastoquinone by water (reviewed in ref *1*). Light energy absorbed by the light-harvesting pigment molecules migrates to the reaction center and causes electron transfer between the primary donor P680, consisting of chlorophyll (Chl) *a*, and the primary electron acceptor, pheophytin. To stabilize the charge separation, the electron is transferred from pheophytin to the primary electron-accepting plastoquinone Q_A and then to the plastoquinone pool through the Q_B molecule. These cofactors are all bound to the D1 and D2 proteins, which together form a heterodimer. The rereduction of $P680^+$ to P680 occurs by transfer of one electron from Tyr_Z (Tyr161 of D1) or Tyr_D (Tyr160 of D2). Tyr_Z^{OX} oxidizes the Mn-containing oxygen-evolving complex within 60−2000 μs (*2*), whereas oxidized Tyr_D remains stable for many minutes. The formation of Q_A^- and subsequent reduction of $P680^+$ are accompanied by an increase in the PS II Chl fluorescence yield. The fluorescence yield with Q_A in oxidized form is named F_O, whereas the higher yield F_M reflects centers in $P680Q_A^-$ state. Analysis of changes in the PS II variable fluorescence ($F_V = F_M - F_O$) has become one of the most widely used approaches in photosynthesis research and other areas of plant physiology (reviewed in ref *3*−*5*).

The D2 protein of PS II, which together with the D1 protein binds the cofactors crucial for PS II photochemistry, consists of five transmembrane helices. Helices C and D of D2 are connected at the lumenal side by the CD loop (residues 164−195), which plays a key role in the structural environment around Y_D and Chl. His189 is a proton acceptor for Y_D (*6*), Arg180 appears to be closely associated with Y_D and an accessory Chl that contributes to the properties of P680 (*7*), and Phe179 was proposed to provide ring stacking forces to one of the accessory Chl molecules (*8*).

To evaluate the structural requirements for function of redox-active components at the donor side of PS II, we have employed a combinatorial mutagenesis approach to determine the amino acid combinations in the CD loop region of the D2 protein that support stable PS II function and significant photoautotrophic growth. Using this approach, a large number of combinatorial D2 mutants have been generated

† This research was supported by a grant from the National Institutes of Health to W.F.J.V. (GM 51556).

* To whom correspondence should be addressed. Phone: (480) 965-3698. Fax: (480) 965-6899. E-mail: vavilin@imap4.asu.edu.

[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,5-dichloro-1,4-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_M, maximal fluorescence; F_O, constant fluorescence; F_V, variable fluorescence; LHC, light harvesting complex; PS I, photosystem I; PS II, photosystem II.

10.1021/bi9915622 CCC: $18.00
Published on Web 10/16/1999

and the mutations were introduced into a PS I-less background to facilitate screening of their fluorescence characteristics (*9*). In several mutants, the variable fluorescence was dramatically reduced, although these mutants retained normal PS II activity. The anomalous variable fluorescence yield resulted from substitution of Phe181 of D2 by Trp. This residue apparently can quench the excited state of one or more Chl in the reaction center complex. The concept of a quenching Chl−Trp interaction provides a novel mechanism for energy-dependent quenching, modulating energy transfer efficiency in photosynthetic systems.

MATERIALS AND METHODS

Growth Conditions. PS I-containing strains of *Synechocystis* sp. PCC 6803 were grown in liquid BG-11 medium (*10*) at 28 °C at 40 μmol photons m^{-2} s^{-1}. The PS I-less mutants were grown at 30 °C at 4 μmol photons m^{-2} s^{-1} in a modified liquid BG-11 medium supplemented with 10 mM glucose and 10 mM TES-NaOH buffer (pH 8.2). In the modified BG-11 medium, sodium nitrate was replaced by a combination of 1.12 g/L of sodium nitrate and 0.35 g/L ammonium nitrate. Cultures were cultivated on a rotary shaker (volumes of less than 1 L) or bubbled with air (for larger volumes).

Solid BG-11 medium (standard or modified) used for segregation and maintenance of the mutants was supplemented with 1.5% (w/v) agar, 0.3% (w/v) sodium thiosulfate, and 10 mM TES-NaOH buffer (pH 8.2). Moreover, 10 mM glucose and appropriate antibiotics were added as required.

Combinatorial Mutagenesis. Combinatorial CD loop mutants were constructed essentially as described in ref *11* with the following modifications. A M13/*psb*DIC clone containing part of the *psb*DIC operon (a 2.9 kb *Eco*RV/*Eco*RI fragment) and lacking codons 179−186 of *psb*DI (*9*) was used as a template; the template had been propagated in the *dut*$^-$*ung*$^-$ *E. coli* CJ236 strain and thus contained uracil residues instead of thymines at random positions (*12*). Subsequently, combinatorial mutations were introduced into this region. For this purpose, a mixture of primers with a fully degenerate sequence in the eight codons corresponding to the deleted region and flanked on both sides by sequences of 20 nucleotides long that were identical to *psb*DI sequences immediately adjacent to the deletion was hybridized with the uracilated M13/*psb*DIC template carrying the deletion at codons 179−186. After generation of heteroduplex phage DNA by primer extension and ligation, the mixture was used for transformation of *E. coli*. Upon transformation, the parental uracil-containing M13/*psb*DIC strand was preferentially degraded, thus increasing the yield of mutations (*12*). After amplification of the phage, double-stranded M13 DNA was isolated and used for transformation of the obligatory photoheterotrophic D2ΔCD strain of *Synechocystis* (*9*). This strain lacked *psb*DII, as well as the *psb*DI region coding for the CD loop of the D2 protein (residues G163−P195), and carried a kanamycin-resistance cartridge downstream of *psb*DIC. Photoautotrophic transformants that appeared on BG-11 plates without glucose were selected, and *psb*DI was sequenced in the region coding for the CD loop in order to determine the nucleotide sequences in the mutants that satisfy the requirement for photoautotrophic growth. To allow a more detailed characterization of selected combinatorial mutants, genomic DNA was isolated and used for transformation of the PS I-less *psa*AB$^-$/*psb*DIC$^-$/*psb*DII$^-$ *Synechocystis* sp. PCC 6803 strain (*13*) followed by selection for kanamycin resistance (a kanamycin-resistance marker linked to *psb*DI was present in the D2ΔCD strain and in the photoautotrophic combinatorial mutants). Only those transformants that did not incorporate the *psa*AB operon (distinguished by the blue-green color of the colonies) were used in further experiments.

Site-Directed Mutagenesis. Site-directed mutations were introduced into the D2 protein of *Synechocystis* sp. PCC 6803 by transformation of the obligatory photoheterotrophic *Synechocystis* D2ΔCD strain with M13 clones carrying the desired mutation in *psb*DI (*14*). Transformants were selected for photoautotrophic growth. Genomic DNA was isolated from the resulting site-directed mutants, was sequenced in the appropriate *psb*DI region, and was used for transformation of the *psa*AB$^-$/*psb*DIC$^-$/*psb*DII$^-$ *Synechocystis* strain in order to obtain the desired mutant in a PS I-less background.

Chlorophyll Fluorescence. Fluorescence induction was detected with a PAM fluorometer (Walz, Effeltrich, Germany) and recorded using FIP fluorescence software and an ADC-12 card (Q_A-Data, Turku, Finland). A cell suspension of PS I-less cells, containing about 1 μg of Chl mL^{-1}, was placed in a Walz cuvette, incubated for 5 min in darkness in the presence of 10 μM DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], an inhibitor of electron transfer from Q_A to Q_B, and then illuminated for up to 5 s with red monitoring light pulses delivered at a frequency of 100 kHz (light intensity: 20 μmol photons m^{-2} s^{-1}). NH_2OH (hydroxylamine), which acts as an electron donor to Tyr_Z^{OX}, was added to the samples in the dark simultaneously with the DCMU as indicated.

Fluorescence Decay Kinetics. To monitor the lifetime of excited states in the pigment bed, time-resolved fluorescence was measured at room temperature in a single-photon-timing-spectrometer as described in ref *15*. Fluorescence was excited by a cavity-dumped dye laser system (Coherent), synchronously pumped by an Nd:YAG laser (Coherent Antares). The half-width of the output pulses was 5−15 ps at a repetition rate of 4 MHz with a mean power of less than 5 mW on the sample. Emission was detected with a microchannel plate photomultiplier tube (Hamamatsu R2809U-01) through a double monochromator with 4 nm band width (Jobin-Yvon DH-10). The full width at half-maximum of the instrument response function was 30−40 ps. Cyanobacterial cells were diluted to a concentration of about 1 μg of Chl/mL and stored in a 2 L reservoir. Cells were pumped through a flow-through cuvette (dimension of the window 1.5 × 10 mm) at a rate of 15 mL/s. To measure fluorescence decay in open PS II centers, the reservoir and tubes leading to and from the cuvette were covered with black cloth. If closed PS II was desired, 50 μM DCMU was added to the suspension and the reservoir was continuously illuminated with white light.

Oxygen Evolution. To determine the oxygen evolution rate of PS I-less cells, samples taken from liquid cultures (Chl concentration 0.8−1.2 μg mL^{-1}) were illuminated (5000 μmol photons m^{-2} s^{-1}) at 25 °C in the presence of 1 mM $K_3Fe(CN)_6$ and 0.2 mM DCBQ (2,5-dichloro-1,4-benzoquinone), and the oxygen level was monitored using a Clark-type electrode.

Oxygen Evolution in Single Turnover Flashes. Flash-induced oxygen evolution was measured at 20 °C using a Joliot-type oxygen electrode equipped with a programmed flash illumination and data acquisition systems as described in ref *16*. A suspension of PS I-less cells of cyanobacteria was first concentrated by centrifugation and then resuspended in a small volume of fresh BG-11 containing 50 μM DCBQ and concentrated again. The resulting paste was spread on the electrode and kept in darkness for at least 15 min before applying saturating flashes.

RESULTS

Photosynthetic Characteristics. Mutants with combinatorial sequences at residues 179−186 of the D2 protein were generated by transformation of the D2ΔCD strain with a mixture of M13 DNA carrying combinatorial sequences of codons 179−186 of *psb*DI. Transformants capable of photoautotrophic growth were selected. A total of 14 different photoautotrophic mutants with combinatorial mutations in this region has been obtained (*9*). Subsequently, DNA from the mutants was introduced into a *psa*AB$^-$/*psb*DIC$^-$/*psb*DII$^-$ strain to generate these mutants in a PS I-less background to facilitate PS II analysis. Three of the 14 mutants (C7-3, C7-4, and C7-10) showed a very low variable fluorescence yield (F_V) in the presence of DCMU, with $F_V/F_O = 0.2-0.5$ as compared to 1.6−2.1 in the other mutants and 2.1−2.3 in the control (Figure 1A). The F_V/F_O ratio was also low in the C7-3, C7-4, and C7-10 mutants (but not in the control strain) when the fluorescence induction was recorded in the absence of DCMU even when the cells received additional illumination with red light at 100 μmol photons m^{-2} s^{-1}. NH_2OH, added to the samples simultaneously with DCMU, partially restored the yield of F_V in the C7-4, but not in C7-3 and C7-10 mutants (Figure 1B).

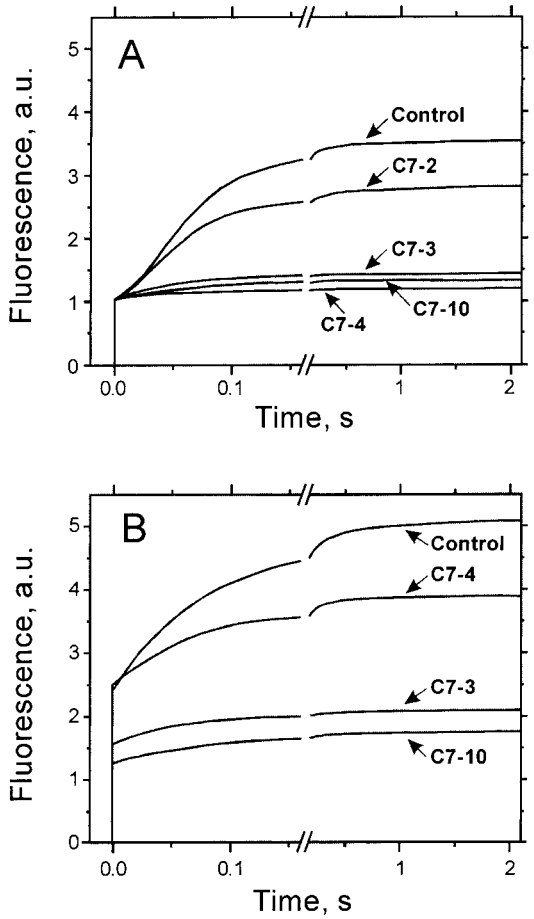

FIGURE 1: Induction kinetics of variable fluorescence measured in the presence of 50 μM DCMU (A) or 50 μM DCMU and 0.5 mM NH_2OH (B) in the control strain and the C7-2, C7-3, C7-4, and C7-10 mutants. All mutants and the control strain are in a PS I-less background. The C7-2 mutant carried the sequence FVIVRIF at codons 179−186 of D2. The changes in the amino acid sequences in the other mutants are listed in Table 1. The fluorescence intensity of every mutant was normalized to the F_O value of DCMU-treated samples.

Even though the C7-3, C7-4, and C7-10 mutants had a very low F_V yield, they showed close to normal rates of oxygen evolution and significant photoautotrophic growth rates in PS I-containing cells (Table 1). Moreover, the oscillation pattern of oxygen evolution upon excitation of the cells by saturating single-turnover flashes was normal (Figure 2). The steady-state 77 K fluorescence emission spectra (excitation at 420 nm) were almost identical in the PS I-less control and the C7 strains with low F_V (data not shown). These results indicate that PS II function was essentially unaltered in the three mutants despite the very low yield of F_V. The reason for the decreased growth rate of C7-3, C7-4, and C7-10 (Table 1) may be a somewhat decreased quantum yield of PS II. As shown in Figure 3, the amount of light needed for half-maximal oxygen evolution is about twice higher for the C7-4 mutant than for the control.

The most obvious common feature of the three mutants virtually lacking variable fluorescence was that they carried a Trp residue at position 181 (a Phe is present in the wild-type) (Table 1). Amino acid residues that were adopted at position 181 in the other photoautotrophic mutants were Ile, Leu, Tyr, and Cys or the wild-type Phe. To determine whether the unusual fluorescence characteristics of the C7-3, C7-4, and C7-10 mutants were explicitly due to the Phe181 substitution by Trp, two site-directed mutants were constructed. In one mutant, designated F181W, Phe181 of the D2 protein was replaced by Trp, whereas other amino acid residues in this region remained unchanged. The amino acid sequence of the second mutant (C7-4/W181F) was identical to that of C7-4 except that Trp181 was replaced by Phe, the residue present at this position in the wild-type. As shown in Table 1, the single amino acid replacement in the F181W mutant was enough to quench F_V considerably, whereas the reverse W181F substitution in the C7-4 mutant restored an almost normal F_V yield.

Picosecond Fluorescence Decay. To further investigate the excitation energy transfer and utilization by PS II in the CD loop mutants, the picosecond fluorescence decay kinetics were compared in the PS I-less C7-4 mutant and the PS I-less control. Figure 4 shows the decay of fluorescence recorded at 690 nm with PS II reaction centers open (Q_A oxidized) and closed (Q_A reduced). The excitation wavelength was set at 595 nm (phycobilisome excitation). When PS II centers are open, the decay curves were similar in the control and the C7-4 mutants. In this case, the decay can be satisfactorily fitted to a sum of two components, with $\tau_1 = 250-260$ ps and $\tau_2 = 1.4$ ns with relative amplitudes of 93 and 7%, respectively. The major 250−260 ps component of the fluorescence decay is likely to stem from energy transfer

Table 1: Selected CD Loop PS II Mutants and Their Photosynthetic Characteristics

strain	amino acid sequence (residues 179-186)	PS I-containing doubling time (% of control)[a]	PS I-less F_v/F_O	PS I-less O_2 evolution[b] [μmol of O_2/(mg of Chl h)]
wt (control)	FR**F**ILFLQ	100	2.33 ± 0.18	1620 ± 380
C7-3	FR**W**WFFVL	140 ± 24	0.36 ± 0.09	1330 ± 290
C7-4	LR**W**MLFAH	158 ± 15	0.18 ± 0.06	1150 ± 180
C7-10	FR**W**LLFFL	132 ± 26	0.24 ± 0.14	1110 ± 290
C7-4/W181F	LR**F**MLFAH	134 ± 16	1.98 ± 0.22	1090 ± 280
F181W	FR**W**ILFLQ	128 ± 25	0.36 ± 0.17	1490 ± 200

[a] The doubling time of the wild type *Synechocystis* strain was 10.5 ± 1.0 h. [b] Rate of oxygen evolution was measured upon illumination with continuous saturating light (5000 μmol photons m^{-2} s^{-1}) in the presence of 0.2 mM DCBQ and 1 mM $K_3Fe(CN)_6$.

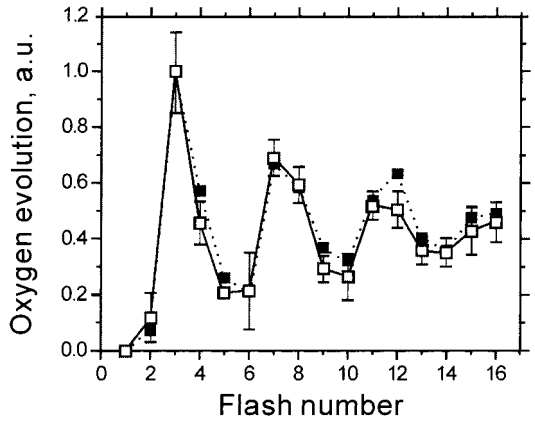

FIGURE 2: Oscillation pattern of oxygen evolution induced by a sequence of saturating single-turnover flashes in the control strain (□) and in the C7-4 (■) mutant. The mutant and the control strain are in a PS I-less background. The time interval between flashes was 0.4 s. Each point represents an average of three to four measurements. Error bars are shown for the control. Data obtained for the C7-4 mutant had similar error bars.

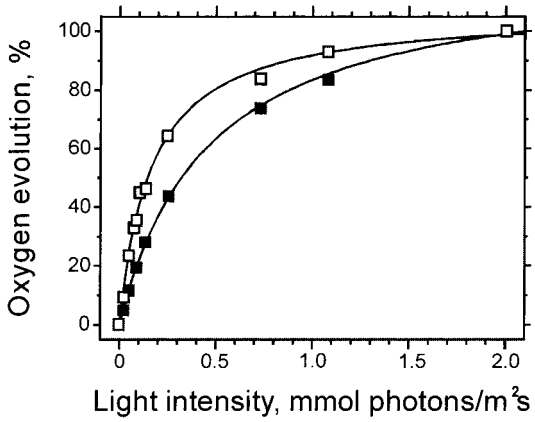

FIGURE 3: Light-response curves of oxygen evolution measured in the PS I-less control strain (□) and C7-4 mutant (■) in a PS I-less background. The maximum oxygen evolution rate was 1460 μmol O_2/(mg of Chl h) for the C7-4 mutant and 1840 μmol O_2/(mg of Chl h) for the control. 0.2 mM DCBQ and 1 mM $K_3Fe(CN)_6$ were added to the cells as electron acceptors.

to the terminal emitter together with Chl fluorescence originating from the PS II core (*15*). As expected, closure of PS II centers resulted in a much slower fluorescence decay in the PS I-less control strain. This decay can be deconvoluted into two components with $\tau_1 = 430$ ps and $\tau_2 = 1.6$ ns; both phases have similar amplitudes. In contrast, closure of PS II centers in the C7-4 mutant induced only minor

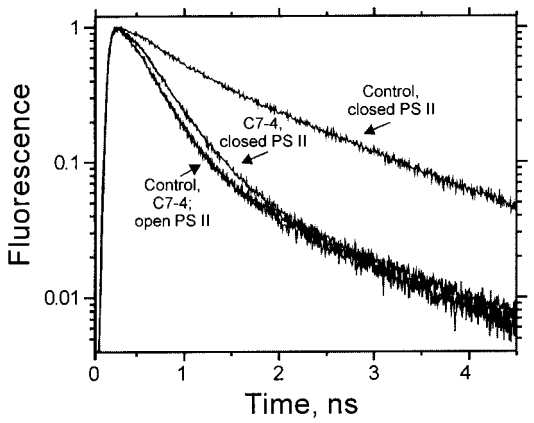

FIGURE 4: Fluorescence decay kinetics measured in intact cells of the control and C7-4 PS I-less strains with PS II reaction centers open or closed.

changes in the fluorescence decay kinetics as compared to the decay in open centers: the lifetime of the fast component increased slightly ($\tau_1 = 320$ ps) whereas the slow component remained essentially unaffected ($\tau_2 = 1.6$ ns); the relative amplitudes of the two phases were identical to those found in the case of open centers (93 and 7%, respectively). The sum of the amplitudes of fluorescence decay components multiplied by their respective characteristic lifetimes ($\sum A_i\tau_i$) reflects the yield of Chl fluorescence. Therefore, replacement of Phe181 by Trp in the D2 protein reduces the F_M yield but leaves the F_O yield essentially unaffected.

DISCUSSION

Substitution of Phe with Trp at the 181 position of the CD loop in the D2 protein of *Synechocystis* sp. PCC 6803 resulted in a 5−10-fold quenching of F_V and a 2-fold decrease in the quantum yield of PS II, but otherwise PS II function was normal. The mutants with Cys, Ile, Leu, Tyr, or the wild-type Phe have normal F_V, indicating a specific correlation between the presence of Trp at position 181 and the large decrease in F_V.

To understand this effect, first the location and orientation of residue 181 should be discussed. On the basis of sequence and functional similarity of the PS II reaction center with the photosynthetic reaction center of purple bacteria, it was proposed that amino acid residues 177−188 in the CD loop of the D2 protein form an α-helix almost parallel to the membrane plane, with the residues at one side of the helix facing toward an accessory Chl in the reaction center (*17*).

Experimental support for the helical configuration of this part of the CD loop comes from the observation that Arg180, which is present in the wild-type and which is important for the normal PS II charge separation and Tyr_D function (*7*), can be shifted to position 184, or one turn of the helix away, without loss of photosynthetic capacity of the resulting mutants (*9*). The Arg180 residue appears to be in close contact with Y_D and with a Chl that is part of the reaction center (*7*). Orientation of residue 180 toward the membrane is clear on the basis of experimental evidence, but had not been expected on the basis of homology modeling (*18*). However, sequence similarity between PS II and the reaction center of purple bacteria is very poor in the CD loop region. As Arg180 is expected to interact with Y_D and the reaction center Chls, it is plausible that the indole group of the neighboring residue is also close to the reaction center pigments.

A low yield of F_V may be caused by (i) a long lifetime of the quencher $P680^+$ (for example, due to limitations in electron transfer at the donor side of PS II), (ii) presence of other oxidized Chls, such as Chl_Z^+, a redox-active Chl associated with His118 in the D1 subunit of PS II that can be accumulated upon illumination at 77 K (*19*) but not necessarily at room temperature, and (iii) close proximity of other fluorescence quenchers. We do not favor the idea that oxidized Chl ($P680^+$ or Chl_Z^+) persists in mutants carrying Trp181 as the flash-induced pattern of oxygen evolution is normal and the F_V yield remains low in most mutants even in the presence of DCMU and NH_2OH (the effect of NH_2OH on F_V intensity in the C7-4 mutant will be discussed later). Moreover, EPR measurements performed at 77 K with thylakoids of the C7-3 and C7-4 mutants in a PS I-less background showed no signs of Chl_Z^+, regardless whether the samples were frozen under illumination or in darkness shortly after a light exposure (data not shown). Instead, we explain the quenching phenomenon by a direct interaction of excited Chl with Trp. It has been shown that Trp can efficiently quench the singlet excited state of fluorescein (*20*). The mechanism of the excited fluorescein quenching is likely to involve electron transfer that leads to formation of a charge-separated state between the two molecules. Recombination of radical pair charges regenerates fluorescein and Trp in the ground state. A similar phenomenon may occur in the Trp181 mutants but then involving charge separation and recombination between Chl and Trp.

The potential of the $Chl/Chl^{\bullet-}$ couple in dimethylformamide is -0.87 V for Chl monomers and -0.60 V for Chl aggregates (*21*) (potentials listed in this paper are relative to the Nernst hydrogen electrode). This potential can be considerably less negative for the $P680/P680^-$ couple as the protein environment increases the redox potential of the $P680^+/P680$ couple by 0.65 V compared to free Chl in apolar media. The reported oxidation potentials of Trp in solution at neutral pH range from 0.64 to 1.08 V (see ref *22*), and the estimated midpoint potential of redox active Trp191 in cytochrome *c* peroxidase ($E_{M,7} = 0.65$ V) (*23*) falls within this range. Taking into account that the electrochemical potential for Chl in the singlet excited state rises by 1.84 V, the formation of a short-lived complex of oxidized Trp and reduced Chl is feasible. On the other hand, reduction of Trp by P680* is unlikely as the electrochemical potential for the reduction of the indole group of Trp was estimated to be about -1.75 V (*24*), which is well below the potential of the $P680^+/P680^*$ couple (~ -0.8 V).

Thermodynamic considerations may also explain the absence of considerable F_V quenching in the C7-2 mutant, in which Phe181 was replaced by Tyr, another aromatic molecule often involved in electron-transfer reactions in proteins. When measured under the same conditions, the midpoint potential of Tyr^+/Tyr at pH 7.0 was concluded to be approximately 0.2 V higher than that of the Trp^+/Trp couple (*25*). Such a difference in the oxidation potentials is enough to prevent or at least considerably decrease the efficiency of Chl fluorescence quenching by Tyr compared to Trp. In addition, orientation and/or distance factors may also contribute to the large difference in the quenching efficiency of the two amino acid residues at position 181 of D2.

Interestingly, addition of 0.5 mM NH_2OH in the presence of DCMU had differential effects on the various mutants (Figure 1B). The C7-3 and C7-10 mutants maintained a low F_V yield, whereas the yield of C7-4 increased significantly to levels close to those observed in the control strain. We exclude the possibility that NH_2OH addition alleviates a limitation of electron transfer at the donor side of PS II in the C7-4 strain because the flash-induced oxygen evolution in the C7-4 is indistinguishable from that in control cells (Figure 2) and the oxygen evolution rate of this mutant in continuous light is considerable (Table 1). Instead, we interpret the differential NH_2OH effect by a differential NH_2OH-induced change in the orientation of Trp181 with respect to Chl in the different combinatorial mutants. The NH_2OH treatment causes a loss of extrinsic PS II proteins of the oxygen-evolving complex (*26*), which is likely to lead to a conformational rearrangement of PS II. A small change in the orientation of Trp181 with respect to Chl is expected to have a large effect on electron transfer parameters: an increase of only 0.5 Å in the edge-to-edge distance between the donor and acceptor is predicted to lead to a 2-fold decrease in the electron-transfer rate constant for a typical protein system (*27*).

Other than in C7-3, C7-4, and C7-10, in none of the combinatorial mutants obtained in this study, a new Trp residue has been introduced into combinatorial regions of the CD loop. Therefore, the question whether the observed Chl fluorescence quenching effect is specific for Trp181 cannot yet be addressed. However, in C7-3, a Trp has also been introduced at position 182, and the slightly higher F_V/F_O ratio in the C7-3 strain compared to C7-4 and C7-10 suggests that Trp182 in C7-3 is unlikely to contribute to F_V quenching.

The next question to address is why quenching occurs almost exclusively with regard to F_V yield, whereas F_O remains virtually unaffected. The picosecond fluorescence lifetime measurements corroborate a normal F_O and a virtual absence of F_V in the Trp181 mutants (Figure 4). For the discussion, first several points should be taken into consideration: (i) the laser excitation used in our fluorescence lifetime experiments ($\lambda = 595$ nm) is absorbed predominantly by phycobilin pigments of cyanobacteria, and not by Chl, (ii) both Chl and allophycocyanin-B contribute to the fluorescence at 690 nm, and (iii) the rate of exciton migration from phycobilin pigments to the PS II core Chl is slower than the rate of exciton trapping by the open reaction centers

but faster than the rate of exciton decay in closed PS II (*15*). In the case of open PS II centers in the wild-type and of open and closed centers in the Trp181 mutants, the detected fluorescence comes mainly from pigments associated with phycobilisomes and from "detached" Chls that are not in functional proximity to PS II centers. Therefore, the contribution of open PS II centers to the overall fluorescence yield is minor.

For quenching by Trp, direct adjacency between the excited state and Trp is essential, and therefore, only excitations at the PS II reaction center can be quenched by Trp181 of D2. The F_O yield in the Trp181 mutants is not expected to be affected, as only a negligible fraction of this fluorescence originates from the PS II reaction center. However, the 2-fold decrease in PS II quantum yield in C7-4 (see Figure 3) indicates that even in open centers, quenching by Trp181 competes effectively with energy trapping in the open reaction center. The relative contribution of quenching by Trp181 becomes much more prominent upon closure of PS II centers, when the lifetime of excitations in the PS II core becomes much longer, and the contribution of reaction center Chls and core antenna to the overall fluorescence yield in F_M state increases drastically.

On the basis of the fluorescence lifetime and quantum yield measurements, the rate constant k_Q of the reaction leading to fluorescence quenching can be estimated. Replacement of Phe181 by Trp reduced the fluorescence lifetime of closed reaction centers to essentially the same level of that in open centers (Figure 4), and the PS II quantum yield in open centers was reduced by a factor of 2 (Figure 2). This indicates that k_Q should be similar to the intrinsic rate constant of primary charge separation between P680 and Pheo, which ranges from 3×10^{11} s^{-1} (*28*) to 5×10^{10} s^{-1} (*29*).

The fact that the Chl fluorescence quenching due to Chl−Trp interaction is about as efficient as trapping of excitations in the open reaction center provides an interesting mechanistic explanation for energy-dependent quenching of excited states that is assumed to play an important regulatory role for energy dissipation in vivo (*30*). The energy-dependent quenching qE, which develops in response to illumination, causes a decrease in the percentage of excitation that is used for photochemistry and fluorescence. A site that is widely accepted to be associated with qE in plants is the PS II antenna, with the minor Chl−protein complexes CP29 and CP26 possibly playing a key role (*31*, *32*). The currently favored mechanism of qE involves the light-induced formation of a pH gradient across the thylakoid membrane, H^+ binding to one or more light harvesting complex II (LHCII) polypeptides, and a conformational transition in LHCII resulting in formation of a quencher (*30*). According to this concept, the quenching may be due to Chl−Chl or Chl−zeaxanthin interaction (reviewed in ref *30*); aggregates of LHCII complexes formed in vitro as well as very concentrated solutions of Chl *a* have much lower fluorescence yield compared to trimeric LHCII (*33*) or a diluted Chl solution (*34*), respectively. Quenching by a Chl−zeaxanthin interaction is supported by a good correlation between the rise of zeaxanthin content in thylakoids and development of qE upon illumination (*35*; see, however, ref *36*). In addition, analysis of energy levels in carotenoids predicts that the Chl excited state can be quenched via the zeaxanthin low-energy 2^1A_g state (*37*).

Our observation that Trp acts as a very efficient trap of excitations on Chl suggests an alternative mechanism for qE quenching: protonation of an amino acid residue of a minor LHCII protein in plants (such as Glu166 of CP29, see ref *38*) may move a Trp residue closer to a Chl and cause efficient quenching. The change in fluorescence quantum yield in C7-4 upon NH_2OH addition illustrates the large effect that small rearrangements may have. It is interesting to note in this regard that sequence analysis of different LHCII proteins reveals a number of highly conserved Trp residues. On the basis of the results presented in this paper, quenching resulting from formation of Chl^-Trp^+ and charge recombination without reformation of Chl* can compete with trapping of excitation in open reaction centers and provides an attractive explanation for energy-dependent fluorescence quenching.

ACKNOWLEDGMENT

We thank Dr. Alain Boussac for his help with flash-induced oxygen evolution measurements.

REFERENCES

1. Diner, B. A., and Babcock, G. T. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Ort, D. R., and Yocum, C. F., Eds.) pp 213−247, Kluwer Academic Publishers, The Netherlands.
2. Dekker, J. P., Plijter, J. J., Ouwehand, L., and van Gorkom, H. J. (1984*) Biochim. Biophys. Acta 767*, 176−179.
3. Krause, G. H., and Weis, E. (1991) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 42*, 313−349.
4. Dau, H. (1994) *Photochem. Photobiol. 60*, 1−23.
5. Lavergne, J., and Trissl, H.-W. (1995) *Biophys. J. 68*, 2474−2492.
6. Campbell, K. A., Peloquin, J. M., Diner, B. A., Tang, X.-S., Chisholm, D. A, and Britt, R. D. (1997) *J. Am. Chem. Soc. 119*, 4787−4788.
7. Manna, P., LoBrutto, R., Eijckelhoff, C., Dekker, J. P., and Vermaas, W. (1998*) Eur. J. Biochem. 251*, 142−154.
8. Xiong, J., Subramanian, S., and Govindjee (1996) *Protein Sci. 5*, 2054−2073.
9. Ermakova-Gerdes, S., Vavilin, D., Keilty, A., and Vermaas, W. (1998) in *Photosynthesis: Mechanisms & Effects* (Garab, G., Ed.) Vol. IV, pp 2859−2864, Kluwer Academic Publishers, Dordrecht.
10. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., and Stainer, R. Y. (1979) *J. Gen. Microbiol. 111*, 1−61.
11. Tichy, M., and Vermaas, W. (1998) *Biochemistry 37*, 1523−1531.
12. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
13. Ermakova-Gerdes, S., Shestakov, S., and Vermaas, W. (1995) in *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) Vol. I, pp 483−486, Kluwer Academic Publishers, Dordrecht.
14. Vermaas, W. F. J., Charité, J., and Shen, G. (1990) *Biochemistry 29*, 5325−5332.
15. Bittersmann, E., and Vermaas, W. (1991) *Biochim. Biophys. Acta 1098*, 105−116.
16. Boussac, A., Maison-Peteri, B., Vernotte, C., and Etienne, A.-L. (1985) *Biochim. Biophys. Acta 808*, 225−230.
17. Michel, H., and Deisenhofer, J. (1988) *Biochemistry 27*, 1−7.
18. Svensson, B., Etchebest, C., Tuffery, P., van Kan, P., Smith, J., and Styring, S. (1996) *Biochemistry 35*, 14486−14502.
19. Stewart, D. H., Cua, A., Chisholm, D. A., Diner, B. A., Bocian, D. F., and Brudvig, G. W. (1998) *Biochemistry 37*, 10040−10046.
20. Periasamy, N., Bicknese, S., and Verkman, A. S. (1996) *Photochem. Photobiol. 63*, 265−271.
21. Kisilev, B. A., Kozlov, Yu. N., and Yevstegneev, V. B. (1970) *Biophysics 15*, 620−628.

22. Jovanovic, S. V., Steenken, S., and Simic, M. G. (1991*) J. Phys. Chem. 90*, 684−687.
23. Miller, M. A., Han, G. W., and Kraut, J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11118−11122.
24. Plato, M., Michel-Beyerle, M. E., Bixon, M., and Jortner, J. (1989) *FEBS Lett. 249*, 70−74.
25. Jovanovic, S. V., Harriman, A., and Simic, M. G. (1986) *J. Phys. Chem. 90*, 1935−1939.
26. Tamura, N., and Cheniae, G. (1985) *Biochim. Biophys. Acta 809*, 245−259.
27. Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S., and Dutton, P. L. (1992) *Nature 355*, 796−802.
28. Leibl, W., Breton, J., Deprez, J., and Trissl, W.-H. (1989) *Photosynth. Res. 22*, 257−275.
29. Durrant, J. R., Hastings, G., Hong, Q., Barber, J., Porter, G., and Klug, D. R. (1992) *Chem. Phys. Lett. 188*, 54−60.
30. Horton, P., Ruban, A. V., and Walters, R. G. (1996*) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47*, 655−684.
31. Bassi, R., Pineau, B., Dainese, P., and Marquardt, J. (1993) *Eur. J. Biochem. 212*, 297−303.
32. Walters, R. G., Ruban, A. V., and Horton, P. (1994) *Eur. J. Biochem 226*, 1063−1069.
33. Mullineaux, C. W., Pascal, A. A., Horton, P., and Holzwarth, A. R. (1992*) Biochim. Biophys. Acta 1141*, 23−28.
34. Beddard, G. S., and Porter, G. (1976) *Nature 260*, 366−367.
35. Demmig-Adams, B. (1990) *Biochim. Biophys. Acta 1020*, 1−24.
36. Niyogi, K. K., Bjorkman, O., and Grossman, A. R. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 14162−14167.
37. Frank, H. A., Cua, A., Chynwat, V., Young, A., Gosztola, D., and Wasielewski, M. R. (1994) *Photosynth. Res. 41*, 389−395.
38. Pesaresi, P., Sandona, D., Giuffra, E., and Bassi, R. (1996) *FEBS Lett. 402*, 151−156.

BI9915622