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Photosynthetic electron transport in genetically altered photosystem II reaction centers of chloroplasts

(Chlamydomonas/chloroplast DNA/membrane protein/psbA gene/tyrosine)

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Communicated by Lawrence Bogorad, July 16, 1991 (received for review February 15, 1991)

ABSTRACT Using a cotransformation system to identify chloroplast transformants in Chlamydomonas reinhardtii, we converted histidine-195 of the photosystem II reaction center D1 protein to a tyrosine residue. The mutants were characterized by a reduced quantum efficiency for photosynthetic oxygen evolution, which varied in a pH-dependent manner, a reduced capacity to oxidize artificial donors to photosystem II, and P680-redox sensitive reduction kinetics (microsecond) that were essentially similar to wild type. In addition, a dark-stable radical was detected by ESR in mutant photosystem II particles but not in wild-type particles. This radical was similar in g value and lineshape to chlorophyll or carotenoid cations but could have arisen from a tyrosine-195 cation. The ability of the photosystem II trap (P680+) to oxidize tyrosine residues suggests that the mutant tyrosine residue could be used as a redox-sensitive probe to investigate the environment around the photosystem II trap.

It is generally accepted that the photosystem II (PSII) reaction center of chloroplasts is structurally and functionally similar to the bacterial photosynthetic reaction center (1–5). Both reaction center types have two core polypeptides that coordinate the primary electron donor, presumably a chlorophyll (Chl) special pair, and the primary electron acceptors, phoephtin and the plastoquinone QA. These polypeptides, the L and M subunit of the bacterial reaction center and the D1 and D2 subunits of PSII, have significant levels of sequence identity (25%) (6). In addition, the D1 and D2 polypeptides have the same number and orientations of transmembrane domains as the L and M subunits (3). These and other similarities suggest that the protein domains involved in the coordination of the PSII trap and binding of electron acceptors are structurally similar to those of the L and M polypeptides of the bacterial reaction center (1–3). The bacterial and PSII reaction centers, however, differ with respect to the organization of secondary electron donors and the redox potential of the reaction center traps. The Chl special pair of the bacterial reaction center is reduced by a cytochrome, whereas the PSII trap, P680+, is reduced by a peptidyl tyrosine residue that in turn is reduced by electrons derived from the oxidation of water. Since the redox potential of a Chl dimer in solution is not sufficiently positive to drive the oxidation of water, it has been generally assumed that the redox potential of the PSII trap is determined by interactions between the chromophores and proteins. Modified Chl molecules containing point charges or the presence of charged residues near Chl may alter spectral properties or the redox potential of Chl (7–9). A survey of the amino acid sequences of the PSII core polypeptides D1 and D2 was conducted to identify conserved and potentially charged residues that could influence the electronic properties of the PSII trap.

Identified a potentially ionizable residue, histidine-195 (H195) of the D1 protein, which is projected to be adjacent to the Chl special pair at the amino terminus of the fourth transmembrane span (1, 3, 4). To determine whether H195 influences the redox potential of the PSII trap and/or charge-transfer processes, we converted H195 to a tyrosine (Y195) residue in the chloroplast-encoded psbA gene of Chlamydomonas reinhardtii. This substitution alters the charge and electron density of the amino acid side chain as well as its pK (assuming Y195 is ionizable). In addition, the mutation introduces a potentially redox-sensitive electron donor into the vicinity of the PSII trap.

METHODS

C. reinhardtii strains (CC-125, wild type; CC-1848, atrazine-resistant) were provided by the Chlamydomonas Stock Culture Collection (Duke University). A wild-type 20-kilobase (kb) psbA clone encoding the D1 protein was provided by Laurie Mcts (University of Chicago). A 16S rRNA clone encoding spectinomycin resistance, plasmid p228, was provided by Elizabeth Harris (Duke University) (10).

A 3.0-kb XbaI fragment of psbA containing exons 4 and 5 of the D1 protein was cloned into pBS+ (pW7) (Fig. 1). Site-directed mutagenesis (11) was carried out using a 22-base oligonucleotide primer. A cytochrome-to-thymine replacement converted the H195 codon into a tyrosine codon (Y195) and generated a Rsa I restriction site (Rs+) in Fig. 1).

Atrazine-resistant Chlamydomonas (CC-1848) cultures were grown in Tris/acetate/phosphate (TAP) medium to a density of 2–5 × 10^6 cells per ml and treated with 0.5 mM fluorodeoxyuridine 48 hr prior to transformation (12). Cells (10^8) were transformed via particle bombardment (13) using 1.1-μm tungsten particles coated with 2.25 μg of p11 and 1.0 μg of p228 plasmid DNA per 25 μl of suspension. Following bombardment, cells were resuspended in TAP medium and spread at a density of 5 × 10^6 cells per plate on TAP plates containing spectinomycin at 35 μg/ml. Spectinomycin-resistant colonies appeared after 2–3 weeks and were maintained on TAP plates containing spectinomycin at 50 μg/ml (14).

Small-scale DNA preparations were obtained from 1.5 ml of Chlamydomonas cells grown to a density of 2–5 × 10^6 per ml. Cells were pelleted in a microcentrifuge, washed with 10 mM Tris, pH 8.0/1 mM EDTA (TE), resuspended in 120 μl of 150 mM sodium citrate, pH 7.0/50 mM EDTA, and frozen.

Abbreviations: Chl, chlorophyll; cpDNA, chloroplast DNA; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethyureas; DBMQ, 2,6-dimethylbenzquinone; PSII, photosystem II; QA, primary plastoquinone electron acceptor; D, redox-active tyrosine-160 of the D2 protein; Z, redox-active tyrosine-161 of the D1 protein.

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in liquid nitrogen. One volume of 2% (wt/vol) SDS was added to the cells and they were incubated at 60°C for 15 min. After two extractions with phenol/chloroform, DNA was precipitated with 1.75 M ammonium acetate and 2.5 volumes of ethanol at −20°C. Southern blots of Rsα1-digested DNA were probed with random primer-labeled pW7 DNA.

Chloroplast DNA was isolated from 1 liter of cells. Cells were washed and resuspended in 20 ml of 50 mM Tris, pH 8.0/50 mM EDTA, frozen in liquid nitrogen, and ground to a fine powder. SDS (20%) was added to a final concentration of 1% and the mixture was incubated at 60°C for 30 min. Cesium chloride was added (1.1 g/ml) and the mixture was incubated at 60°C for 15 min and then centrifuged at 4000 × g for 10 min to remove cell debris. The supernatant was removed from below the pellet, bisbenzimide was added (0.1 mg/ml), and the solution was centrifuged at 196,000 × g in a VTi50 rotor for 24 hr. The chloroplast DNA (cpDNA; uppermost fluorescent band) was removed and dialyzed vs. 2 liters of TE at 4°C overnight. The cpDNA was then precipitated with 2 M ammonium acetate and 2.5 volumes of ethanol, resuspended in TE, and extracted with phenol/chloroform prior to reprecipitation. A 200-bp psbA fragment (bases 532–728) was amplified by PCR using cpDNA from the Y195 mutant and cloned into pBS+ for dyeoxy DNA sequencing (15).

Chlamydomonas thylakoids were prepared according to Chua and Bennoun (16). Digitonin/Triton PSII particles were prepared according to Diner and Wollman (17) and stored at −80°C in DEAE-Sephadex column elution buffer before use. Chl was quantified according to Arnon (18). Light-dependent rates of oxygen evolution were determined with a Hansatech oxygen electrode, using thylakoids (1.5 μg of Chl per ml) in 0.3 M sucrose/20 mM Hepes, pH 7.5/1 mM MgCl2/20 mM methylene/200 μM 2,6-dimethylbenzoquinone (DMBQ)/2 mM K3Fe(CN)6. For measurement of pH-dependent rates of oxygen evolution, PSII activity was assayed in 0.3 M sucrose/3 mM KCl/0.5 μM valinomycin/2 μM nigericin/200 μM DMBQ/2 mM K3Fe(CN)6/20 mM Tricine (pH 7–8) or Mes (pH 5.5–6.5). Rates of hydroxylation oxidation were determined by the rate of oxygen reduction in 0.3 M sucrose/25 mM Hepes, pH 7.5/2 mM MgCl2/3 mM KCl/0.5 μM valinomycin/2 μM nigericin/100 μM methyl viologen/1 mM KCN. Chl-a fluorescence was measured in the presence of 1% μM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) and 1 mM dimethylsulfoxide (DMSO). Excitation was with blue light (Corning 4-96 filter, 30 μEinstein−2s−1; 1 E (einstein) = 1 mol of photons), and fluorescence was detected by a photodiode protected by a Corning 2-58 filter.

Absorption transients due to P680+ turnover were measured at 820 nm with a single-beam spectrophotometer consisting of a 30-mW laser diode source (model DC25F; Spindler & Hoyer, Milford, MA) and a silicon photodiode detector (PIN 10D; United Detector Technology, Santa Monica, CA) located 3 m from the sample cuvette. For nanosecond studies, a 400-MHz-bandwidth transimpedance amplifier (United Detector Technology) was used; for microsecond studies, the photocurrent was converted to a voltage across a 50-Ω resistor and amplified 10-fold with a 70-MHz-bandwidth voltage amplifier (model 115; EG & G Vactec, St. Louis). The signal was amplified further with a 600-MHz-bandwidth plug-in (Tektronix T11A52) and digitized with a Tektronix DSA 401 oscilloscope. The samples were flashed with a 600-ps-pulsewidth nitrogen laser (model 2300; PTI, London, ON, Canada) at a frequency of 1 Hz. At a sample concentration of 50 μg of Chl per ml and a flash energy of 1.4 mJ (= 337.1 nm), the flash intensity was 90% saturating.

PSII particles suspended in DEAE-Sephadex column elution buffer plus 1 mM K3Fe(CN)6 were used for ESR analyses. ESR spectra were recorded in a conventional flat cell using the following instrument settings: 9.722-GHz microwave frequency, 2.0-mW microwave power, 200-G field sweep (1024 data points), 3450-G center field, 5.0-G field modulation, and 100-kHz modulation frequency. All spectra were normalized on the basis of Chl concentration and a seven-point second-order polynomial smoothing function was applied to the spectra. Irradiated samples were exposed to white light for 1 min while in the cavity and spectra were acquired for 14 min during illumination. After illumination, samples were dark-adapted for 1 min prior to initiation of data acquisition for the dark-readapted samples.

**RESULTS**

Since the phenotype of the Y195 mutant was unknown, it was necessary to select for cpDNA transformants on the basis of a nonphotosynthetic trait. *Chlamydomonas* cells were cotransformed with two separate plasmids, one containing the mutagenized psbA fragment (p11) and the second a 16S rRNA gene (p228) conferring spectinomycin resistance. Chloroplast DNA cotransformants were first selected on TAP medium containing spectinomycin and subsequently screened by Southern blot analysis for a diagnostic Rsα1 restriction site corresponding to the Y195 point mutation (Figs. 1 and 2A). Southern blot analyses indicated that ~1 in 20 spectinomycin-resistant colonies was cotransformed with p11 DNA (Fig. 2A), corresponding to a cotransformation frequency of 10−7.

Unambiguous phenotypic characterization of the Y195 mutants requires that all psbA copies be mutagenized (12). Under normal growth conditions each cell may have as many as 200 copies of the psbA gene (19). One copy of psbA is present in each of the inverted-repeat regions of the chloroplast genome and there are ~80 or more copies of the chloroplast genome per cell. To determine whether all psbA copies were equivalent (homoplasmic), we rescreened single colony isolates from the original transformant by Southern blot analysis. [It was not apparent from the original Southern blot whether all psbA copies were equivalent (Fig. 2A).] Five of 10 subclones from the original transformant were determined to be homoplasmic for the Y195 mutation, whereas the others were either heteroplasmic or homoplasmic for the wild-type H195 restriction fragment pattern (Fig. 2B). To determine whether the Y195 point mutation observed in the original isolate was the product of mixed copies of the chloroplast genome or the result of reversion of the mutant to wild-type genotype, we rescreened single colony isolates from an apparent homoplasmic isolate (no. 14, Fig. 2B) by Southern blot analysis. All single colonies isolated from homoplasmic isolate 14 remained homoplasmic (Fig. 2C). (Homoplasmic lines have been maintained for over 1 year.) Thus it was apparent that the heterogeneity observed in Fig. 2B was due to the presence of both wild-type and mutant.
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Fig. 2. Identification of psbA Y195 point mutants by Southern blot analysis. (A) Chlamydomonas cells were cotransformed by particle bombardment with plasmid p11 and plasmid p228, containing the 16S rDNA gene conferring spectinomycin resistance. Transformed cells were primarily identified on the basis of spectinomycin resistance and subsequently screened by Southern blot analysis for the presence of the unique Rsa I site diagnostic of the psbA mutant. Lanes pWT and p11, Rsa I-digested wild-type and mutant plasmid DNA probed with radiolabeled pWT plasmid DNA; lanes 1-9, Rsa I-digested total DNA isolated from spectinomycin-resistant Chlamydomonas transformants. (B) Identification of heteroplasmic and homoplasmic isolates from the original psbA point mutant by Southern blot analysis of Rsa I-digested total DNA from subclones of Chlamydomonas cotransformant no. 2 (A) following several generations of growth. Blot was probed with radiolabeled pWT DNA. Lanes pWT and p11, as in A; lanes 1-9, Rsa I-digested total DNA from single colony isolates derived from isolate no. 2 in A. Positions of wild-type (WT) and mutant bands are indicated. (C) isolation of homoplasmic Y195 mutants. Rsa I-digested total DNA was isolated from single colonies of the homoplasmic Chlamydomonas transformant no. 14 (B) and probed with pWT. Lanes pWT and p11, as in A; lanes 1-9, Rsa I-digested DNA from single colonies derived from isolate no. 14.

Fig. 3. Light-dependent rates of PSII electron transport in wild-type (•) and Y195 mutant (○) thylakoids. Rates of oxygen evolution with DMBQ as an acceptor were measured at various light intensities as described in Methods. Coincident points on right axis show effect of 10 nM atrazine.

rate of electron transfer was observed when hydroxylamine (2 mM) was used as an artificial electron donor. At saturating light intensities the rates of hydroxylamine oxidation for wild-type and mutant thylakoids were 248 and 138 microequivalents per mg of Chl per hr for wild-type and mutant thylakoids, respectively. These rates correspond to 34% and 38%, respectively, of the rate of electron transfer with water as a donor. In addition, analyses of fluorescence induction kinetics indicated that the time required to reach 0.5 Fv (variable Chl fluorescence; i.e., the difference between the maximum and initial fluorescence) in the presence of 10 μM DCMU was relatively unaffected by the addition of 5 mM hydroxylamine (27 ms with hydroxylamine vs. 21 ms without hydroxylamine) for wild-type cells. In contrast, the time required to reach 0.5 Fv was longer for the mutant than for wild type with (52 ms) or without (30 ms) hydroxylamine. These results indicate (i) that there is a reduction in the rate of charge transfer between the redox-active tyrosine-161 of D1 (Z) and Qa in the mutant or (ii) that the mutant has a lower rate of electron donation from hydroxylamine to Z than wild type. Since both oxygen evolution and hydroxylamine oxidation are inhibited in the mutant, the former explanation is preferred.

To determine the cause of the rate limitation imposed by the mutation, we determined the average number of Chl molecules per P680+ (based on absorption of P680+ at 820 nm) and P680+ reduction kinetics in mutant and wild-type non-oxygen-evolving PSII particles. There was no difference in the P680+ (+200 Chl molecules per P680) or Qa− content (as determined by the ratio of variable to initial fluorescence levels) of wild-type and mutant PSII particles (data not shown), indicating that the Y195 mutation does not result in the generation of inactive reaction centers. Unexpectedly, the nanosecond and microsecond P680+ reduction kinetics were essentially identical for mutant and wild-type non-oxygen-evolving particles (data not shown). Since electron transfer between Z or the redox-active tyrosine-160 of D2 (D) and P680+ is relatively slow (5-7 μs) in non-oxygen-evolving PSII particles compared with oxygen-evolving PSII particles (30 ns), and since the Chlamydomonas PSII particles were missing a portion of Qa, it could not be resolved whether there were differences in the rate of electron transfer between Z and Qa (23-25).

The introduction of the Y195 mutation would be expected to alter the charge density and possibly the pK of the side
chain at position 195. As a result we considered the possibility that PSI electron transport rates could vary as a function of pH. Rates of photosynthetic oxygen evolution in the mutant were reduced by as much as 40% compared with wild type at pH values ranging from 6.0 to 8.0 (Fig. 4). At lower pH (<5.5), however, the rate of photosynthetic oxygen evolution was equivalent to the wild-type rate, suggesting that the increase in hydrogen ion concentration may compensate for the loss of H195 (Fig. 4 and data not shown). Unfortunately, rates of hydroxylamine oxidation measured using methylviologen as an acceptor were substantially inhibited in both wild-type and mutant thylakoids at pH ≅ 6.0. As a result, it was not possible to determine the effect of pH on hydroxylamine oxidation.

The oxidized PSI trap is reduced by peptidyl tyrosine residues of the reaction center core polypeptides (26-29). In non-oxygen-evolving PSI particles, the oxidation of these residues gives rise to the ESR-detectable radicals Z and D, corresponding to the tyrosine cations 161 and 160 of the D1 and D2 proteins, respectively. The Z tyrosine, however, competes more effectively for re-reduction of the oxidized PSI trap than does the D tyrosine (30). Our interest was to determine whether the PSI II trap could oxidize Y195. The tyrosine radicals corresponding to Z* and D* were generated in mutant and wild-type PSI particles and had similar steady-state levels (Fig. 5A). The quality of the Z* and D* signals was less than optimal, possibly due to the loss of Qa and the large antenna size (200 Chl per P680*) of the PSI subchloroplast particle. However, no additional tyrosine signal having a line shape similar to Z* or D* was identified in mutant PSI particles (Fig. 5) having equivalent or 3-fold higher Chl concentrations or in hydroxylamine-treated thylakoids. In addition to the Z* and D* radicals, another light-generated radical, giving rise to a symmetrical ESR signal, was observed in mutant PSI particles. A similar radical has been detected in wild-type membrane preparations, but always at substantially reduced (<0.5× the Y195 value) amounts. The radical detected in PSI particles from the Y195 mutant is similar in linewidth, symmetry, and g value to Chl or carotenoid radicals that are generated during illumination of PSI particles (31-33). Alternatively, the radical present in mutant PSI particles could arise from the oxidation of Y195. A symmetrical tyrosyl ESR lineshape, unlike that of the Z* or D* radicals, could result from changes in the distribution of the unpaired spin density on the phenol ring or alterations in the orientation of the phenol ring with respect to the protein environment (26, 30).

**DISCUSSION**

We have introduced a mutagenized 3-kb psbA gene fragment into the chloroplast genome of *Chlamydomonas* by the "biolistics" DNA delivery system (34, 35). Since we could not predict the phenotype of the Y195 mutant, cpDNA transformants were identified on the basis of a nonphotosynthetic chloroplast-encoded trait. By cotransforming the chloroplast genome with two plasmids, one containing the 16S rRNA gene conferring spectinomycin resistance and the second containing the mutagenized *psbA* fragment with a unique restriction endonuclease recognition site, we were able to identify cpDNA and *psbA* transformants. We obtained spectinomycin-resistant colonies at a frequency of 10⁻⁶ and *psbA* cotransformants at a frequency of 10⁻⁷. Southern blot analyses showed that both *psbA* copies encoding the D1 protein were mutagenized. It is probable that recombination initially occurs between the plasmid *psbA* insert and one genomic *psbA* copy. Subsequent intramolecular recombination between the two copies would generate equivalent *psbA* genes. This copy-correction mechanism eliminates the need to mutagenize each *psbA* copy (12). However, mutant and wild-type genomes must be segregated during cell division in order to obtain homoplasmic strains (12).

In the presence of DCBQ or DMBQ as an electron acceptor, the rate of oxygen evolution in uncoupled wild-type thylakoids was pH insensitive at pH 5.5-8.0. The conversion

![Fig. 4. Effect of pH on photosynthetic oxygen evolution by wild-type (○) and Y195 mutant (●) thylakoids. Results are the average of three determinations ± SE. Rates were determined at 90% saturating light intensities and are expressed as a percentage of the wild-type rate at pH 7.0 (185 μmol of O₂/hr per mg of Chl).](image1)

![Fig. 5. Room-temperature ESR spectra of wild-type and mutant PSI reaction center particles. (A) Wild-type and mutant particles in elution buffer plus 1 mM K₃Fe(CN)₆ at Chl concentrations of 0.167 and 0.254 mg/ml, respectively. All spectra were normalized to a Chl concentration of 0.254 mg/ml. From top to bottom the spectra are for dark-adapted, irradiated, and dark-readapted samples. (B) Light-minus-dark difference spectra for mutant and wild-type samples.](image2)
of H195 to Y195 resulted in a pH-dependent reduction in the rate of oxygen evolution at pH > 5.5. We have not identified the mechanism by which Y195 could alter the pH dependence of oxygen evolution. However, the presence of an ionized tyrosine residue near the PSII trap may be expected to reduce the rate of reduction of P680+ by Z. Alternatively, other groups may regulate charge transfer in a pH-dependent manner. Both acidic and basic groups having pK values near 5.5 have been implicated in the regulation of charge-transfer rates in oxygen-evolving PSII particles (36, 37). However, the reduction in the rate of oxygen evolution can be compensated by reduced pH in the mutant.

In addition to a decrease in the rate of oxygen evolution, rates of hydroxylamine oxidation were decreased 45% in the mutant. Furthermore, mutant cells had slower fluorescence induction kinetics than wild type with or without hydroxylamine. These results indicate that electron transfer between Z and Qa is inhibited in the mutant. However, analyses of the kinetics of P680+ reduction by Z indicated little difference between wild type and mutant in non-oxygen-evolving particles. Since nonoxygenic PSII particles have substantially altered kinetics relative to oxygenic PSII particles, it will be necessary to analyze primary charge-transfer processes in oxygen-evolving PSII particles before it is possible to fully resolve the mechanism by which PSII electron transport is decreased in the mutant.

The introduction of a tyrosine residue (Y195) adjacent to the PSII trap would presumably break the symmetry between the Z and D tyrosines and the PSII trap. Therefore, it was of interest to determine whether Y195 could compete with Z and/or D for reduction of the PSII trap. We were unable to detect an ESR signal similar in lineshape to the Z+ or D+ radicals. However, mutant PSII particles photoaccumulated an ESR-detectable radical in addition to the Z+ and D+ radicals. There are three likely origins for this radical: i.e., a Chl or carotenoid cation or Y195+. Previous studies have demonstrated that Chl or carotenoid radicals having ESR lineshapes similar to that detected in the mutant are generated during illumination (31). If the ESR-detectable radical arises from these species and not Y195+, then it is apparent that the distance between P680+ and Y195 is not the primary determinant of whether Y195 or another tyrosine residue (e.g., Z or D) is oxidized. If Y195+ gives rise to the ESR-detectable radical, then it is apparent that the tyrosyl side chain can be used as a redox-sensitive probe to investigate the effects of distance and orientation of peptide tyrosine residues on reduction of P680+.

We thank G. Cheniae, C. Yocum, E. Harris, L. Bogorad, Govindjee, and L. Mets for helpful discussions and E. Wrobbl-Boerner for technical assistance. This work was supported by grants from the National Institutes of Health (GM40703) and the Colleges of Biological Sciences and Agriculture to R.T.S. and from the National Science Foundation (DMB-8905065) to J.H.G.