**In Vitro Random Mutagenesis of the D1 Protein of the Photosystem II Reaction Center Confers Phototolerance on the Cyanobacterium Synechocystis sp. PCC 6803**

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The D1 protein of the photosystem II reaction center is thought to be the most light-sensitive component of the photosynthetic machinery. To understand the mechanisms underlying the light sensitivity of D1, we performed *in vitro* random mutagenesis of the *psbA* gene that codes for D1, transformed the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 with mutated *psbA*, and selected phototolerant transformants that did not bleach in high intensity light. A region of *psbA2* coding for 178 amino acids of the carboxyl-terminal portion of the peptide was subjected to random mutagenesis by low fidelity polymerase chain reaction amplification or by hydroxylamine treatment. This region contains the binding sites for Qb, D2 (through Fe), and P680. Eighteen phototolerant mutants with single and multiple amino acid substitutions were selected from a half million transformants exposed to white light at 320 μmol m⁻² s⁻¹. A strain transformed with non-mutagenized *psbA2* became bleached under the same conditions. Site-directed mutagenesis has confirmed that one or more substitutions of amino acids at residues 234, 254, 260, 267, 322, 326, and 328 confers phototolerance. The rate of degradation of D1 protein was not appreciably affected by the mutations. Reduced bleaching of mutant cyanobacterial cells may result from continued buildup of photosynthetic pigment systems caused by changes in redox signals originating from D1.

Although photosynthetic organisms require light for growth, they can suffer damage from high light intensity, especially at low temperatures and reduced concentrations of CO₂ (1). The loss of photosynthetic productivity that occurs when organisms are exposed to visible light quanta above the level required for saturating photosynthetic electron flow is known as "photoinhibition" (2). Under photoinhibitory conditions, the reaction center of photosystem (PS) II, which consists of D1 and D2 proteins (3), is specifically inactivated (4, 5). Although the precise mechanisms of photoinactivation have not been fully elucidated, the process involves several steps, including an initial reversible reduction of electron flow and irreversible damage to the D1 protein (6–8). Recovery of PSII activity can occur when irreversibly photodamaged D1 is replaced by newly synthesized protein (7).

Site-directed or deletion mutagenesis has been employed to investigate the basic mechanisms of the photosensitivity, including the relationships between the structure and function of D1 (9–12). Although site-specific mutagenesis seldom confers new function, this technique has produced some mutants in which PSII and D1 gained partial resistance to high irradiance (9, 10, 12). *In vitro* random mutagenesis, ideally saturation mutagenesis, coupled with appropriate methods for screening mutants, is a useful method to obtain new species of D1 protein that are phototolerant. For these experiments, we have chosen the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. In addition to having plant-like photosynthetic activity, *Synechocystis* can be genetically transformed at high efficiency and can be easily screened as colonies under defined conditions (13).

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions—** *Synechocystis* sp. PCC 6803 strain Cm4A-1 provided by R. J. Debuss (University of California, Riverside, CA) was employed as a host of mutated *psbA2*. In this strain, both *psbA1* and *psbA3* genes (14) have been inactivated by insertion mutagenesis with antibiotic resistance cassettes (15, 16). Cultures were grown on BG-11 agar medium in the presence of 20 μg/ml spectinomycin and 5 μg/ml chloramphenicol at 30 °C under a photon flux density of 50 μmol m⁻² s⁻¹ (13). The Cm4A-1 strain was transformed as reported (13) with plasmid pPSBA2-KM (Fig. 1). This plasmid consists of native *psbA2* with a kanamycin resistance cassette (Kan r) integrated at the StuI site by blunt-end ligation on the backbone of pUC118. The Kan r was excised from pCR1000 (Invitrogen) by digestion with AvoI and SacII, followed by end-blunting. The resulting transformant designated "KC" contained the *psbA2-Kan r" construct integrated in the chromosome by homologous recombination. Strain KC was grown in the presence of 20 μg/ml spectinomycin, 5 μg/ml chloramphenicol, and 20 μg/ml kanamycin under the same conditions as strain Cm4A-1. Liquid cultures were grown under the same conditions as cultures on agar plates, except that air mixed with 1% CO₂ was continuously supplied.

**In Vitro Random Mutagenesis—** Mutations were randomly introduced into the 3′ half of *psbA2* by polymerase chain reaction (PCR) under low fidelity conditions (17). This region occurs between nucleotide numbers 538 (KpnI) and 1071 (HincII) in the coding frame (534 base pairs) (Fig. 1) and corresponds to amino acid residues Phe-180 to Ala-357 of D1. PCR was performed using 1 ng of the DNA template and

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1 The abbreviations used are: PS, photosystem; PCR, polymerase chain reaction; TE, Tris-HCl/EDTA.

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primes enclosing the entire coding region (5'-GCCGAAGCTTTAGG
GAATTATACCAATAAGGACCTCT-3' and 5'-AGGGAAACGGTAC
CAAGGAATTACACGTAGCAGGAGC-3'). The reaction was carried
out in presence of 1 mM each of dCTP, dGTP, and dTTP, 0.2 mM
dATP, 6.1 mM MgCl₂, and 0.5 mM MnCl₂ for 25 cycles to induce nu-
cleotide transition from A to G and T to C as reported (17). The PCR
products were digested with KpnI and HindII and ligated into pPSBA2-
KM. A mutant library of D1 proteins was produced by transforming Synec-
chocystis sp. PCC 6803 strain Cm4A-1 with this construct as de-
scribed (13) except that ligated DNA was used directly without ampli-
fication in Escherichia coli since constructs harboring psbA likely in-
terfere with the growth of E. coli cells.

Random mutagenesis with hydroxylamine as reported, to introduce the mutations C → T and G → A (18), was also performed. Fifty μg of the KpnI-HindII fragment of ppsA2 was incubated in a reaction mixture (200 μl) containing 0.5 mM hydroxylamine HCl and 5 mM EDTA in 0.1 mM sodium phosphate (pH 6.0) at 70 °C for different periods. An aliquot (20 μl) of the mixture was then diluted 10 times with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to terminate the reaction. The treated DNA was precipitated with ethanol two times and resuspended in 20 μl of TE buffer. The DNA fragments treated with hydroxylamine for 90 min were inserted into pPSBA2-KM and used to transform strain Cm4A-1 as described for PCR mutagenesis.

Site-directed Mutagenesis—Point mutations were introduced into wild-type ppsA2 by site-directed mutagenesis using a modification of the PCR method (19). To achieve higher fidelity nucleotide incorpora-
tion, DNA polymerase (Stratagene) was used instead of Taq DNA
polymerase. Mutagenesis experiments were performed on a KpnI-Hin-
dIII fragment covering the 3' portion of ppsA2 and its 3' downstream
region subcloned into pUC118. Mutagenized DNA species were ampli-
ﬁed by PCR using the primer 5'-GCTTGATAGCAGGATACAGGCTC-3'
(the italicized sequence for disrupted EcoRI site in the vector adjacent to the HindIII site on the fragment described above). The mutated KpnI-EcoRI fragment was ligated with corresponding sites in pBlue-
script KS+ (Stratagene) and cloned into E. coli JM109 to conﬁrm the mutation by DNA sequencing. The mutagenized KpnI-HindII fragment was returned into pPSBA2-KM and used to transform strain Cm4A-1.

DNA Sequencing—The full-length ppsA2 (1.1 kilobase pairs) was amplified from a single colony of Synecchocystis or E. coli by PCR with the same primers used for PCR random mutagenesis. The nucleotide sequences of the PCR products were determined using a 373A DNA Sequencer (Applied Biosystems, Perkin-Elmer). The samples were pre-
pared by the cycle sequence method (20) using Taq DNA polymerase
and one of four kinds of primer labeled with fluorescent markers ac-
cording to the manufacturer's instructions (5'-Aminoink2-CAGGACC
ATTGTCAGGATCCATCTTG-3', 5'-Aminoink2-CAGGACTACAATCC
CGGATCCATCTTG-3', 5'-Aminoink2-CAGGAGCTAGGCTTTGACAT
TAC-3', and 5'-Aminoink2-CAGGATGCGATTACAGGAGCC-
3'). Samples were separated on sequencing gels containing 8 M urea and 6% acrylamide. Both strands of DNA were sequenced to conﬁrm the nucleotide sequences.

Turnover of D1 Protein—Pulse-chase labeling of cyanobacterial cells with [35S]Met was performed essentially as reported (12). Washed cells were resuspended in sulfur-depleted BG-11 medium (BG-11-S) in which MgSO₄, ZnSO₄, and CuSO₄ were replaced with MgCl₂, ZnCl₂, and Cu(NO₃)₂, respectively. The chlorophyll concentration of each suspen-
sion was adjusted to 25 μg/ml, and the cells were exposed to 50 μmol
m⁻² s⁻¹ white light for 30 min at 30 °C. [³⁵S]Met (>37 MBq/mmôl, ICN) was added to achieve a final activity of 37 kBq/ml, and light treatment continued for another 30 min. Radiolabeled cells were collected by centrifugation and resuspended in the same volume of BG-
11-S medium containing 2 mM non-radioactive Met. Cells were sub-
jected to high irradiance (500 μmol m⁻² s⁻¹), and 10-ml aliquots were withdrawn at appropriate intervals for preparation of thylakoid mem-
branes (12). Thylakoid membrane proteins were electrophoresed in 15% polyacrylamide gels containing 6 M urea and 0.1% SDS (21). Thylakoid membranes containing 5 μg of chlorophyll were loaded in each lane. Autoradiograms were recorded using a BAS2000 Bio-Imaging Analyzer (Fujix, Tokyo).

RESULTS

Screening of Phototolerant Mutants—To determine suitable conditions for screening phototolerant mutants, Synecchocystis sp. PCC 6803, its progeny strain Cm4A-1 in which pssA1 and pssA3 were disrupted (15, 16), and a derivative of Cm4A-1 transformed with the pssA2-Kan' construct (strain KC), were cultured under 50–640 μmol m⁻² s⁻¹ white light. Although recent information compiled at Cyanobase² indicates that the Stul site where Kan' was introduced lies within one of two copies of speA encoding arginine decarboxylase present in the chromosome (1), the phenotype of strain KC was indistinguis-
huishable from wild-type PCC 6803 and strain Cm4A-1. When exposed to 320 to 640 μmol m⁻² s⁻¹ light, the pigment contents of all three strains decreased dramatically with incubation time, changing from deep blue-green to yellow-green (data not shown). Spectroscopic analysis of pigments demonstrated that, under high irradiance, the levels of phycobiliproteins decreased rapidly, followed by a decrease in the level of chlorophyll a. In contrast, carotenoids were stable under the same conditions (data not shown). Therefore, the screening of phototolerant transformants with mutagenized ppsA2 was conducted on agar plates exposed to continuous irradiance at 320 μmol m⁻² s⁻¹ for 10 days. A region of ppsA2 coding for 178 amino acids from the carboxyl-terminal portion of D1 was subjected to random mutagenesis by PCR under low fidelity conditions and by hy-
droxylamine treatment. The peptide encoded by this DNA frag-
ment, including the binding sites for Qb, D2 (through Fe), and P680, as well as the proposed cleavage sites affected by photo-
degradation. Thirty-five colonies that did not bleach at this light intensity were selected from approximately one half million transformants harboring randomly mutagenized ppsA2 species. Southern blot analysis confirmed that the endogenous ppsA2 was replaced with one copy of pssA2-Kan' per chromo-
some in each mutant (data not shown).

Nucleotide Substitutions—Nucleotide sequences of the mu-
tagened region of ppsA2 from all the phototolerant colonies were determined, and amino acid sequences were deduced from them (Fig. 2). A total of 35 phototolerant colonies were assigned to 18 different species of mutated D1 protein. Some mutants were not unique because some mutagenized cells apparently divided during the 5-h transformation before being spread on

² Cyanobase is available via the World Wide Web (http://www.
kausura.or.jp/cyano/cyano.orig.html).

FIG. 1. Physical maps of the region covering pssA2 of Synechco-
ystis sp. PCC 6803 subjected to in vitro random mutagenesis. A, the HindIII-HindIII fragment of the chromosomal DNA of the cyanobacterium cloned in pUC118. The arrow indicates the region and direction of the coding frame of pssA2. The gene speA codes for arginine decarboxylase (http://www.kazuasa.or.jp/cyano/cyano.orig.html). B, the insert in pPSBA2-KM, HindIII-HindIII fragment harboring the kana-
mycin resistance cassette (Kan'). The region enclosed by KpnI and HindII sites and subjected to random and site-directed mutagenesis is shown by the solid bar.
Mutagenized D1 Proteins Increase Phototolerance

Amino Acids in photosynthetic organisms so far examined (25).

Fig. 2. Amino acid substitutions in D1 proteins generated by in vitro random mutagenesis and selection under high irradiance. Amino acids in large capitals and nucleotide sequences of codons in small capitals are presented along amino acid residues numbers: Wild indicates amino acids of wild-type D1 protein of Synechocystis sp. PCC 6803; A1, A2, and other designations on the left are mutated D1 protein species in selected transformants. Two mutants designated H2 and H7 were those generated by hydroxylamine treatment. All other mutants were generated by PCR random mutagenesis. Mutations of transformants possessing the same amino acid substitutions are shown on the right. Defined secondary structures are indicated along with the amino acid residues of wild-type D1 as reported (22–24).

Wild

| Amino acid residue no. | D-helix | PEST-like sequence | QEET motif | DE-helix | E-helix | C-terminal processing |
|------------------------|---------|--------------------|------------|----------|---------|-----------------------|
|                        | 208 211 221 226 228 232 234 239 241 243 245 248 250 253 254 256 258 260 262 266 268 273 274 277 288 302 304 307 316 322 329 331 335 348 351 |
| Wild                   |         |                    |            |          |         |                      |
| A1                     |         |                    |            |          |         |                      |
| A2                     |         |                    |            |          |         |                      |
| A8                     |         |                    |            |          |         |                      |
| A9                     |         |                    |            |          |         |                      |
| B2                     |         |                    |            |          |         |                      |
| E8                     |         |                    |            |          |         |                      |
| F1                     |         |                    |            |          |         |                      |
| F4                     |         |                    |            |          |         |                      |
| F8                     |         |                    |            |          |         |                      |
| F9                     |         |                    |            |          |         |                      |
| G1                     |         |                    |            |          |         |                      |
| G3                     |         |                    |            |          |         |                      |
| G5                     |         |                    |            |          |         |                      |
| G6                     |         |                    |            |          |         |                      |
| G8                     |         |                    |            |          |         |                      |
| H2                     |         |                    |            |          |         |                      |
| H7                     |         |                    |            |          |         |                      |
| I6                     |         |                    |            |          |         |                      |

The overall results led us to conclude that substitution of one or more amino acids at residues 234, 254, 260, 267, 322, 326, or other loci. The KpnI-HincII fragment of psbA2 in each mutant was prepared by PCR and restriction enzyme digestion, ligated with corresponding sites of pPSBA2-KM, and introduced into strain Cm4A-1. The resulting transformants tolerated 320 μmol m⁻² s⁻¹ light just as the original mutants did (data not shown), indicating that the phenotypes were due to mutation of psbA2.

Site-directed Mutagenesis—To identify the residues responsible for the observed phototolerance, site-specific mutations were introduced into amino acid residues having a high frequency of displacements by random mutagenesis (Asn-234, Tyr-254, Phe-260, Asn-266, Asn-267, Phe-273, Phe-274, Asn-322, Ile-326, and Phe-328) (Table I). Site-directed mutagenesis has revealed that a single mutation at Tyr-254 or Asn-267 is involved in phototolerance and that mutations at Asn-234 or Phe-260 also contribute to phototolerance. Single mutations at other positions have less effect on the phenotype. In contrast, a double mutation at Asn-234 and Phe-260 has a dramatic cumulative effect on phototolerance (Table I). It is remarkable that Asn-234 is located in the area of a “PEST-like sequence,” which is proposed to be involved in photodamage (26), and that is the most frequently mutated residue in the 18 phototolerant mutants (Fig. 2). A single mutation at Asn-322 or a double mutation at Ile-326 and Phe-328 on the luminal extrusion near the COOH terminus did not contribute to phototolerance, but a triple mutation at these residues conferred high phototolerance (Table I). Similarly, mutations at Phe-260 or at Ile-326 and Phe-328 did not affect phototolerance, but a triple mutation at these positions produced phototolerant transformants (Table I).

The overall results led us to conclude that substitution of one or more amino acids at residues 234, 254, 260, 267, 322, 326, or...
328 plays a role in phototolerance. A single mutation at Tyr-254 or Asn-267, double mutation at Asn-234 and Phe-260, and triple mutation at Asn-322, Ile-326, and Phe-328 confer the highest level of phototolerance.

Visible Characteristics of Phototolerant Mutants—Fig. 3 shows examples of mutant A9 on agar medium (panels A and B) and A2 in liquid culture (panel D). There was no significant difference in pigmentation between the control strain KC and mutant A9 grown under 50 μmol m⁻² s⁻¹ of light for 5 days. However, under 320 μmol m⁻² s⁻¹ light, the control was bleached in 1 week, while the A9 mutant retained its deep green color (Fig. 3B). In liquid culture, mutant A2 retained its color during 36-h exposure to 750 μmol m⁻² s⁻¹ light (Fig. 3D), but the control strain KC was completely bleached under these conditions (Fig. 3C). All mutants generated by random mutagenesis exhibited pigmentation profiles equivalent to those of A9 and A2 (data not shown).

Turnover of D1 Protein—The rapid turnover of D1 in vivo may be due to a PEST-like sequence located between residues 225 and 238 (26). Pulse-chase experiments using [³⁵S]Met were performed to determine the turnover rate of D1 in phototolerant mutants. D1 protein bands were identified in separate experiments by immunoblotting with an antibody against D1 (data not shown). At 500 μmol m⁻² s⁻¹ light, the turnover rate (both synthesis and degradation) was similar in KC and mutants N254D-P260S (NDFS) and I6 (Fig. 4). Similar results were obtained with mutants G3, G6, and H7 (data not shown). Therefore, it is concluded that the mechanism of phototolerance observed in the mutants is distinguished from that proposed as “damage-repair cycle” (7).

**DISCUSSION**

What events occur prior to bleaching in cyanobacterial cells under irradiance? The cell-doubling times of the control strain KC and all mutants were nearly identical at approximately 24 h under irradiance at 500 μmol m⁻² s⁻¹ (data not shown), when the control strain gradually bleached. Therefore, bleaching is an event primarily independent of cell growth. Superoxide anion radical known as the primary product of oxygen photoreduction in thylakoids (27) may degrade photosynthetic pigments. Absorption spectra of the control strain KC and mutant G3 as determined time-sequentially after exposure to irradiance indicate that chlorophyll a content diminishes specifically during bleaching in the control strain (28). This observation is contrary to the phenomenon of sodB mutant of the cyanobacterium Synechococcus sp PCC 7942, which is deficient in functional iron superoxide diamutase (29), where the carotenoid-to-chlorophyll ratio strikingly decreased when cultured in the presence of methyl viologen. Active oxygen species have also been reported to degrade D1 protein (30), in relation to “damage-repair cycle of D1 protein” (26, 31, 32). In the present investigation, the rate of degradation of D1 protein did not change in the wild-type and mutant strains under irradiance (Fig. 4). Therefore, the possibility that generation of oxygen radicals is reduced in the mutants may be excluded.

Phototolerance associated with PSII in photosynthetic organisms has been suggested to occur by one or more mechanisms: (i) an efficient energy dissipation or leakage at the stage of excitation energy transfer in the pigment system (33–36) capable of being detected by the fluorescence emission spectra at low temperatures (37–39), and (ii) an efficient electron leakage via some kind of cyclic electron floor around the PSII in the electron transport system due to instability or redox potential change of the primary or secondary reactants as detected by shifts in thermoluminescence bands (40, 41). It is hypothesized that redox signaling can regulate gene expression (42, 43) and physical linkage that facilitates energy transfer between light-harvesting complex II and PSII/PSI (44–46) through protein phosphorylation. The redox sensor is postulated to be an electron carrier located between PSI and PSI (42), and a kinase is activated by a reduced cytochrome b/f complex interacting with a plastoquinol at the Qb site (46).

Our preliminary analysis of the thermoluminescence profiles indicates that changes by the mutagenesis of D1 in the equilibrium between QA and QB due to (i) changes in their stability including redox-potential and (ii) low efficiency of electron transport in PSII, could explain the observed phototolerance (47). Analysis of the fluorescence measurements indicates that these mutants perform low quantum-yield electron flow in PSII (47). Therefore, it is possible that mutated D1 proteins influence redox signaling and in this way interfere with down-regulation of the biogenesis of the photosynthetic apparatus. This may explain the retention of cell pigmentation in these mutants under high irradiance. These hypotheses in turn suggest the existence of an intrinsic protection mechanism in wild-type cells that copes with photoinhibition by down-regulating phycobilisome formation.

In phototolerant mutant species of D1, most amino acid conversions involved changes from nonpolar species to polar or charged ones, i.e. to Ser (32%), Asp (14%), Arg (7%), and Tyr (5%); the conversion of Phe to Ser occurred most often. This may indicate the increase in the hydrophilic or hydrogen-bonding interactions in the D1 protein is responsible for the enhanced phototolerance of the organism. Interestingly, Ser → Phe substitution in the luminal loop of the D2 protein (48) appears to endow PSII with photosensitivity. Further biophysical analysis of cyanobacterial cells harboring the mutated species of D1 is needed to prove this proposal.
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FIG. 3. Phototolerance of Synechocystis sp. PCC 6803 transformed with mutagenized psbA2. The control strain KC possessing wild-type D1 and the phototolerant mutant A9 expressing randomly mutated D1 were cultured on a standard agar medium at 50 μmol m⁻² s⁻¹ of light for 5 days (A), then exposed to high irradiance of 320 μmol m⁻² s⁻¹ for 1 week (B). The strain KC (C) and the phototolerant mutant A2 (D) were cultured in liquid medium under 750 μmol m⁻² s⁻¹ light for 36 h.

FIG. 4. Protein turnover in strains possessing the wild-type (KC) or mutated phototolerant (NDFS and I6) D1 protein species under high irradiance (500 μmol m⁻² s⁻¹). Aliquots were taken at times indicated during pulse-chase experiments, and thylakoid membranes were isolated as described under “Experimental Procedures.” Thylakoid membranes containing 5 μg of chlorophyll were loaded in each lane, electrophoresed in 15% polyacrylamide gels containing 6 M urea and 0.1% SDS (21), and subjected to autoradiography.
Mutagenized D1 Proteins Increase Phototolerance