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The random mutational analysis of the structure and function of photosystem II reaction center targeting to the *psbA*II gene of *Synechocystis* sp. PCC 6803

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Introduction

In oxygenic photosynthesis, the photosystem II (PSII) provides a high redox potential to utilize water molecules as the electron donor. In the photosystem, the D1 subunit plays central roles in the structure and function (Nanba and Satoh 1987), and thus site-directed mutagenesis has been targeted to this subunit to analyze the function of specific amino acid residues in the PSII reaction center (e.g., Chu et al. 1995). However, a limitation of the method is that the targeting is basically depends on the established evidence. In the present study, we have intended to create mutants of *Synechocystis* sp. PCC 6803 having random amino acid substitution(s) in the targeted wide region of the D1 subunit by random mutagenesis of the *psbA*II gene, in order to discover unexpected structure–function relationships in the PSII reaction center. For this purpose, a cyanobacterium *Synechocystis* sp. PCC 6803 was used because of its advantages in the efficiency of transformation/homologous double-recombination and the capacity for heterotrophic growth (Williams 1988). The random mutation was targeted to a wide region of the *psbA*II gene, corresponding to the Ser148-Ala357 (210 amino acids) segment of the D1 protein comprising the region of functional importance. The random mutants were subjected to screening based on the loss of photoautotrophy, either by systematic nitrofurantoin selection under illumination (Yamasato and Satoh 2001) or by direct inspection of the growth on agar plates. By this procedure, we have isolated 146 photosynthesis-deficient mutants impaired in the function of PSII caused by nucleotide substitution(s) or insertion(s) on the *psbA*II gene. The present paper describes overall features of the random mutagenesis, mostly based on the nucleotide sequence information, and provides a preliminary characterization of some mutants established by this method. The analysis of these random mutants is expected to provide entirely new aspects in understanding the structure–function relationships in PSII, which will complement the result obtained by crystallographic analysis.

Materials and methods

Random mutagenesis and screenings

Organisms and culture conditions used in this study are detailed in Yamasato and Satoh (2001). The host strain, Cm4Δ-1, of *Synechocystis* was provided by Dr. R. J. Debus (UC Riverside). *In vitro* random mutagenesis using PCR, as described by Cadwell and Joyce (1992), was applied to the cloned *psbA*II gene of *Synechocystis* in the pBS-psbA2 plasmid provided by Dr. M. Ishiura (Nagoya Univ., Japan). The screening method for photosynthesis-deficient mutants by nitrofurantoin is described in Yamasato and Satoh (2001). Some mutants were isolated by directly inspecting the growth on BG11 agar plates with or without 5 mM glucose.

Nucleotide sequencing

The *psbA*II gene of random mutants was amplified by PCR using cells as the template. The PCR products were purified and then subjected to the sequencing. The sequencing reaction was carried out using psbA2 E primer [5'- TTC CAC TTC CTC ATC GGC AT -3'] or psbA2 B primer [5'- CAA TCA GAA GAT TAC TCA GT -3']. Nucleotide sequences were analyzed by an ABI 373S sequence analyzer (Applied Bio Systems, CA).

Measurement of thermoluminescence

The thermoluminescence was measured for whole cells suspended in BG11 medium containing 10 mM HEPES (pH 7.5). The sample, which was dark adapted for more than 15 min in the presence or absence of 20 μM DCMU, was excited by a saturating flash at -10 ˚C (+DCMU) or 0 ˚C (-DCMU), and then rapidly cooled in liquid N_2. The light emission during sample warming (rate about 1.0 ˚C sec^{-1}) was recorded against the sample temperature. These measurements were carried out in collaboration with Drs. Y. Inoue and J.-R. Shen (RIKEN, Harima, Japan).

Results and discussions

1) Classification of random mutants

One hundred forty-six random mutants obtained in this study include 141 strains screened by nitrofurantoin treatment from nearly 7000 colonies of transformants and additional 5 strains by direct inspection of the loss of photoautotrophy for 42 colonies on agar plates. The photosynthetic capacity was impaired in all of these mutants, although 20 strains could grow photoautotrophically on BG11 agar plates without glucose (Weakly autotrophic mutants). Early termination in the translation of D1 protein was predicted in 53 mutants (Stop codon and Codon shift mutants); 14 out of them accompanied with codon-shifted regions (Codon shift mutants). Heterotrophic mutants having 1 to 9 amino acid substitution(s) with variegated combination in the targeted region constituted

Table I Classes of random mutants

Mutants	Number
Heterotrophic mutants	126
Substitution mutants	73
Single-point	(14)
2-points	(16)
3-points	(21)
4-points	(12)
5-points	(7)
6-points	(2)
9-points	(1)
Stop codon mutants	39
Codon shift mutants	14
Weakly autotrophic mutants	20
Total	146

the majority, i.e., 73 strains. The average number of substitution in these mutants in the targeted region was calculated to be 2.9; this number is reasonably small to pursue further systematic analysis by site-directed mutagenesis. The nucleotide sequencing was repeated more than twice for 16 mutants with perfect reproducibility. Moreover, the complementation analysis of the *psbA*II gene as well as site-directed mutagenesis conducted for these mutants confirmed that the mutation(s) in the targeted region is responsible for the observed loss of photoautotrophy.

2) Amino acid substitutions

The superimposed illustration of amino acid substitutions in 93 mutants (Substitution and Weakly autotrophic mutants) shown in Fig. 1, demonstrates that the mutated points are distributed more or less evenly all over the targeted region of the D1 protein as intended. However, closer inspection reveals that the frequency of mutations is high at specific positions; some of which have established or proposed to be important in the PSII function, such as Y161 (secondary donor), H198 (ligand for P680), H272 (ligand for non-heme Fe) and E333 (ligand for Mn-cluster), proving the effectiveness of present method in identifying the amino acid residue(s) of functional importance. The high frequency of mutation is also observed on some other sites previously not reported, such as Q165, M172, Q187, I192, W278, I283, W284, N296, I314 etc., suggesting the importance of these non-charged residues in the structure and function of PSII complex. The importance of some novel sites in the PSII was substantiated absolutely by the presence of single point mutants, for example Q165L, Q187P, W278R and W284R (Fig. 1). A remarkable fact is that the substitution sites in these single point mutants and the residues substituted with high frequency in Fig 1 are located mostly on the donor side of the putative secondary structure of D1 protein. This may suggest the importance of a rigid protein structure supported by non-charged residues in the organization of the water oxidizing machinery.

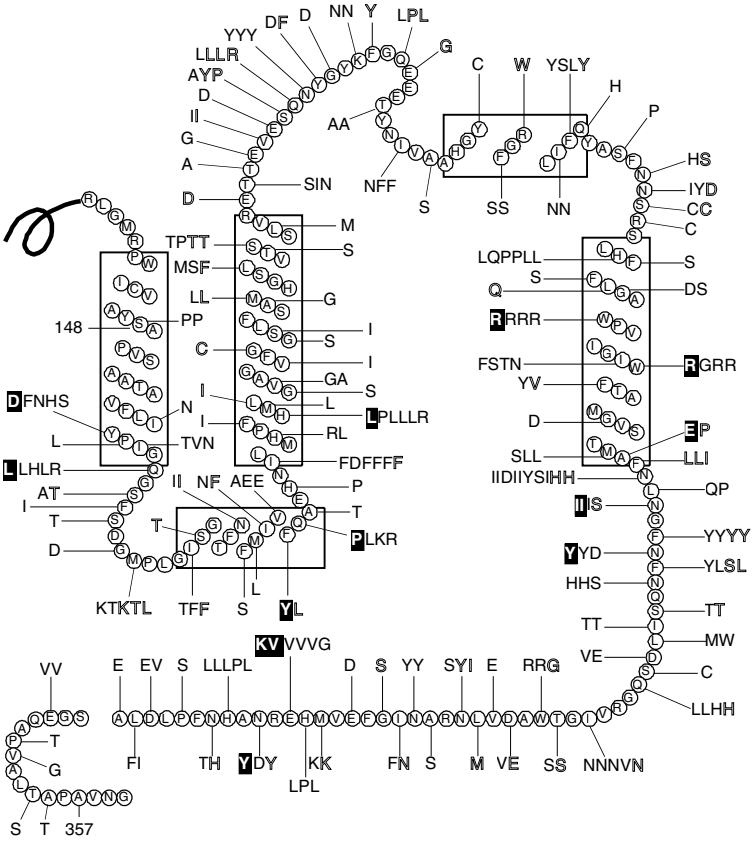

Fig.1. Amino acid substitutions in random mutants. The superimposed expression of amino acid substitutions in 73 substitution mutants and 20 weakly autotrophic mutants (Table I). Boxed letters indicate single-point mutations.

3) A preliminary characterization of mutants

A preliminary characterization of random mutants demonstrated that almost a half of these mutants are impaired in the accumulation of stable PSII complexes for as yet unknown reasons, but the other of them accumulate substantial amounts of PSII with altered properties. For example, thylakoid membranes prepared from N298I mutant, which accumulates 20-30 % of D1/D2 proteins as compared with that of the control strain on a chlorophyll basis, exhibited the activity of DCIP (2,6-dichlorophenol-indophenol) photoreduction supported by DPC (1,5-diphenylcarbohydrazide) as high as the control on a D1 protein basis. However no oxygen evolution nor thermoluminescence emission, Q- and B-bands, were detected from the mutant cells. On the other hand, N296I mutant, which was site-directly constructed based on the information in Fig. 1, was able to grow photoautotrophically, but exhibited modified thermoluminescence bands (Fig. 2) and slower rates of oxygen evolution. The above result may suggest that the environment around the Mn-cluster in PSII is supported by an Asn rich region, i.e., N296-N303 of D1 protein (see, Fig. 1). For further analysis of random mutants containing H272 substitution, see accompanying paper (S15-027, Kamada et al.).

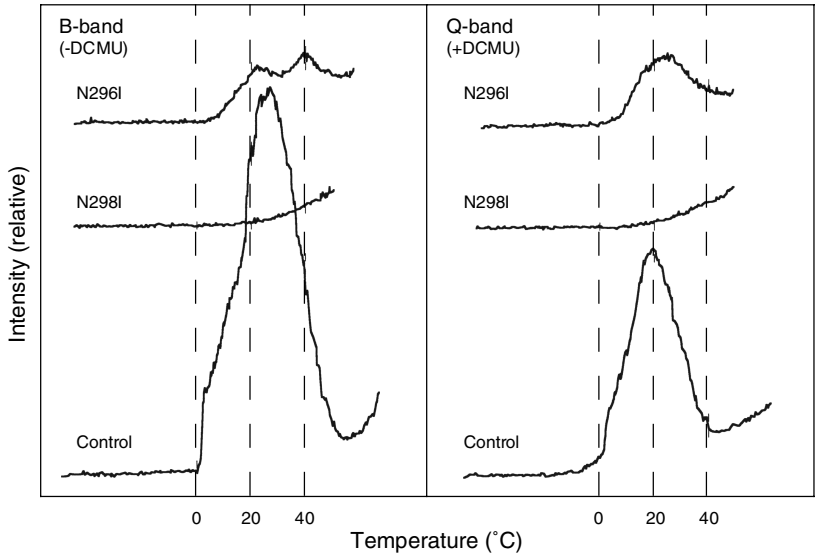

Fig.2. Thermoluminescence emission of two mutants (N296I and N298 mutants)

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