S15-027

Characterization of a heterotrophic mutant of *Synechocystis* sp. PCC 6803 created by random mutagenesis targeted to the *psbA*II gene

T Kamada[1, 2], A Yamasato[1, 3], and K Satoh[1]

[1]*Okayama University, Okayama 700-8530, Japan. kimiyuki@cc.okayama-u.ac.jp*
[2]*Present address; National Institute for Basic Biology, Okazaki 444-8585, Japan. kamada@nibb.ac.jp*
[3]*Present address; Institute of Low Temperature Science, Hokkaido University, Sapporo 060-0818, Japan.*

Keywords: D1 protein, non-heme Fe, oxygen evolution, photosystem II, Y_D – signal

Introduction

The photosystem II (PSII) is unique in that it can generate a high redox potential to oxidize water molecules. To elucidate molecular architectures behind the unique function, the structure of PSII complex has been analyzed extensively during the past two decades. However, the structural information provided by x-ray crystallographic analysis at this moment is insufficient to locate amino acid residues in the complex and to understand the molecular details (Zouni A. et al. 2001). The mutational analysis, on the other hand, has a potential to provide specific information on the function of particular amino acid residues and the structure of complex. Thus we have been engaged in random mutational analysis targeted to the D1 subunit, in order to discover unexpected structure-function relationships in the PSII reaction center, both in static and dynamic aspects. By this method, we have created more than one hundred random mutants impaired in the capacity of photoautotrophic growth caused by 1-9 amino acid substitution(s) in the targeted 210 amino acid (Ser148-Ala357) region of the D1 protein (See, Yamasato et al., S22-029). This article provides a preliminary characterization of a mutant (a triple mutant named npRK07) obtained by this kind of random mutagenesis, which carries 3 amino acid substitutions on the cytosolic side of D1 protein, i.e., F255I, Q261P and H272R. We have generated 3 single-point mutants, each possessing one of three substitutions in the triple mutant, and demonstrated that the H272R substitution at the putative ligand for non-heme Fe on the acceptor side dramatically affects the structure and function on the donor side in the PSII complex and is responsible for the observed loss of photoautotrophy in the random mutant.

Materials and Methods

Organisms and culture conditions
Synechocystis sp. PCC 6803 strain with inactivated *psbA*I and III genes (Cm4Δ-1 strain) was provided by Dr. Debus (1988). The control strain (KC) carries a kanamycin-resistant cassette downstream of the *psbA*II gene. Cells were grown on BG11 medium (Rippka 1988) supplemented with 5mM glucose, 5μg ml^{-1} chloramphenicol, 5μg ml^{-1} spectinomycin and 20μg ml^{-1} kanamycin at 30˚C under white-light of 20-30μ$Em^{-2}s^{-1}$, with air bubbling.

Preparation of thylakoid membranes and PSII core complexes
Thylakoid membranes and PSII core complexes were prepared according to the method of Tang and Diner (1994). In the column chromatography, PSII core complexes from the H272R mutant were eluted by 50mM MES-NaOH buffer (pH 6.0 at 4˚C) containing 15mM $MgSO_4$.

Photochemical activity
The kinetics of DCIP (2,6-dichlorophenolindophenol) photoreduction was measured by a 356 spectrometer at 580 nm (Model 356; Hitachi, Ibaragi, Japan), providing saturated actinic light through a VR-67 red filter by a slide-projector ($600\mu Em^{-2}s^{-1}$). Five-μg chl ml^{-1} of thylakoid membranes were suspended in the 50mM MES-NaOH buffer (pH 6.0 at 4˚C) containing 80μM DCIP with or without 0.5mM DPC (1,5-diphenylcarbohydrazide).

EPR spectroscopy
The EPR spectrum was measured for the dark adapted PSII core complexes from KC (control) and H272R strains at 77K. To measure light-induced signal, the samples were illuminated by a saturating light at 253K and then immediately cooled down to 77K.

Results and Discussion

The npRK07 strain of *Synechocystis* sp. PCC 6803 created by random mutagenesis of *psbA*II gene possesses 3 amino acid substitutions on the cytosolic side of D1 protein in the putative secondary structure, i.e., F255I, Q261P and H272R. This mutant cannot grow photoautotrophically in the absence of glucose. However, if glucose is supplemented to the culture medium, cells can grow heterotrophically and accumulate D1/D2 proteins in the amounts of 20-40 % of the control (KC strain) on a chlorophyll basis, although they do not evolve oxygen even in the presence of artificial electron acceptors, DMBQ/DCBQ. We have generated 3 site-directed mutants, each one possessing one of three substitutions in the triple mutant, in order to examine the responsibility of each amino acid substitution to the observed properties of the triple mutant. Characterization of these 3 site-directed mutants confirmed that the H272R substitution, at the site of putative non-heme Fe binding, is primarily responsible to the major phenotype of the triple strain, i.e., loss of photoautotrophy, no fluorescence variables (Fv/Fm) and no oxygen evolution (Table 1). The H272R mutant cells accumulated substantial amounts of D1/D2 proteins as detected by the western blotting analysis using specific antibodies raised against D1 proteins, although the level on a chlorophyll basis was appreciably lower than that of the control (10-30 %) (Table 1). A remarkable fact, however, was that thylakoid membranes prepared from heterotrophically grown mutant cells were highly active in the photochemical activity of 2,6-dichlorophenolindophenol (DCIP) reduction supported by an artificial electron donor, 1,5-diphenylcarbohydrazide. This indicates that the water oxidizing machinery is impaired in the H272R mutant. Consistent with the result, thermoluminescence emissions reflecting the charge recombination between Q_A and S_2 or Q_B and S_2/S_3 (Q- or B-bands) could not be detected in the mutant cells (data not shown). On the other hand, the sensitivity of DCIP photoreduction supported by 1,5-diphenylcarbohydrazide towards DCMU was markedly reduced (a hundred times), as anticipated from the site of mutation in the PSII complex (Table 1), as in the case of an equivalent mutant for the D2 subunit (Vermaas et al. 1994).

Table 1 Characteristics of mutants

Strains	Growth [1]	Proteins [2]		Fv/ Fm [3]	DCIP photoreduction [4]		Sp. activity [5]	DCMU inhibition (I_{50}, μM)
		D1	D2		H_2O	DPC		
KC	+	100	100	0.47	229	287	100	5
npRK07	-	36	24	0	6	68	64	248
H272R	-	20	10	0	5	43	61	493
F255I	+	81	48	0.38	85	177	69	26
Q261P	+	92	64	0.42	140	214	66	7

1) Photoautotrophic growth.
2) Protein contents on chlorophyll basis (per cent of KC).
3) Fluorescence induction.
4) DCIP photoreduction (μmoles / mg chl / hr) in the absence (H_2O) or presence (DPC) of DPC.
5) Activity of DCIP photoreduction in the presence of DPC (per cent of KC) on D1 protein basis.

An unexpected finding, although it is only preliminary, was that the PSII core complexes prepared from the H272R mutant exhibited no EPR Signal II (Y_D) originating from Tyr-160 radical on the D2 protein (Fig.1). This indicates that a single amino acid substitution on the acceptor side in a constituent subunit of PSII dramatically affects the environment of a functional residue (Tyr-D) on the donor side residing on the other subunit in the multi-subunit complex. This, together with the fact that H272 mutation on the cytosolic side seriously affects the function of PSII on the lumenal side, i.e., water oxidation, suggests a close cooperation between the donor and the acceptor sides.

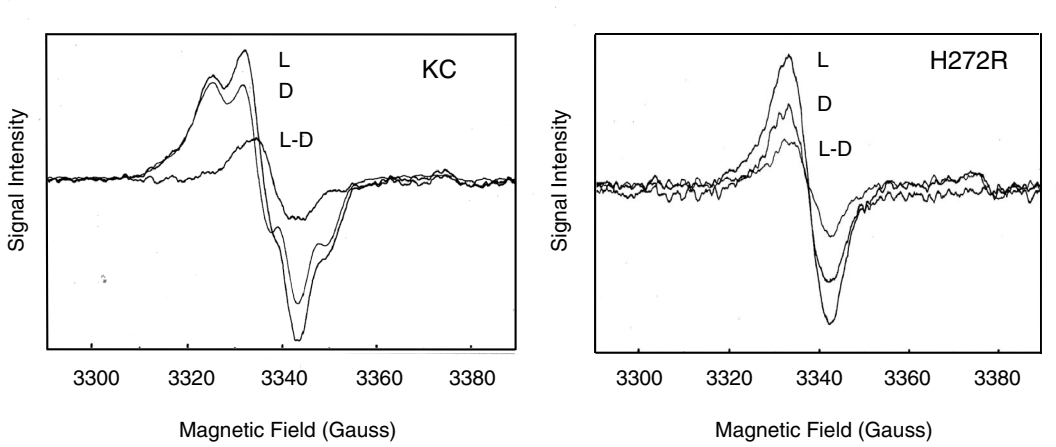

Fig. 1 : EPR spectra of PSII complexes
EPR spectra were measured before (D) and after (L) illumination. The difference spectra (L-D) are also shown.

Acknowledgments

We thank Dr. R. J. Debus (UC Riverside, USA) for providing Cm4Δ-1 strain of *Synechocystis* sp. PCC 6803, Drs. J. R. Shen and Y. Inoue (RIKEN Harima, Japan) for the help in thermoluminescence measurement and Dr. Y. Takahashi (Okayama University, Japan) for the use of fluorescence spectrometer. We are grateful to Dr. A. Kawamori (Kwansei Gakuin University, Japan) for her help in EPR measurements.

References

Debus RJ, Barry BA, Sithole I, Babcock GT and McIntosh L (1988) *Biochemistry* **27**, 9071-9074.
Rippka R (1988) *Methods in Enzymology* **167**: 3-27.
Tang XS and Diner BA (1994) *Biochemistry* **33**: 4594-4603.
Vermaas W, Vass I, Eggers B and Styring S (1994) *Biochimica et Biophysica Acta* **1184**: 263-272.
Xiong J, Hutchison RS, Sayre RT and Govindjee (1997) *Biochimica et Biophysica Acta* **1322**: 60-76.
Yamasato A and Satoh K (2001) *Plant and Cell Physiology* **42** (4): 414-418.
Zouni A, Witt HT, Kern J, Fromme P, Krauss N, Saenger W and Orth P (2001) *Nature* **8**: 409 (6821), 739-743.