Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–8, 2018

www.elsevier.com/locate/jbiosc

Characterization of mutants expressing thermostable D1 and D2 polypeptides of photosystem II in the cyanobacterium *Synechococcus elongatus* PCC 7942

Norihisa Haraguchi, Jun Kaseda, Yasumune Nakayama, Kazuhiro Nagahama, Takahira Ogawa, and Masayoshi Matsuoka*

Department of Applied Microbial Technology, Faculty of Biotechnology and Life Science, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan

Received 18 December 2017; accepted 23 April 2018
Available online xxx

Photosystem II complex embedded in thylakoid membrane performs oxygenic photosynthesis where the reaction center D1/D2 heterodimer accommodates all components of the electron transport chain. To express thermostable D1/D2 heterodimer in a cyanobacterium *Synechococcus elongatus* PCC 7942, we constructed a series of mutant strains whose *psbA1* and *psbD1* genes encoding, respectively, the most highly expressed D1 and D2 polypeptides were replaced with those of a thermophilic strain, *Thermosynechococcus vulcanus*. Because the C-terminal 16 amino acid sequences of D1 polypeptides should be processed prior to maturation but diverge from each other, we also constructed the *psbA1*ΔC-replaced strain expressing a thermostable D1 polypeptide devoid of the C-terminal extension. The *psbA1*/*psbD1*-replaced strain showed decreased growth rate and oxygen evolution rate, suggesting inefficient photosystem II. Immunoblot analyses for thermostable D1, D2 polypeptides revealed that the heterologous D1 protein was absent in thylakoid membrane from any mutant strains with *psbA1*, *psbA1*ΔC, and *psbA1*/*psbD1*-replacements, whereas the heterologous D2 protein was present in thylakoid membrane as well as purified photosystem II complex from the *psbA1*/*psbD1*-replaced strain. In the latter strain, the compensatory expression of *psbA3* and *psbD2* genes was elevated. These data suggest that heterologous D2 polypeptide could be combined with the host D1 polypeptide to form chimeric D1/D2 heterodimer, whereas heterologous D1 polypeptide even without the C-terminal extension was unable to make complex with the host D2 polypeptide. Since the heterologous D1 could not be detected even in the whole cells of *psbA1*/*psbD1*-replaced strain, the rapid degradation of unprocessed or unassembled heterologous D1 was implicated.

[**Key words:** Photosystem II; Gene replacement; Cyanobacteria; D1/D2 heterodimer; Thermostability; C-terminal processing]

Photosystem II (PSII) complex catalyzes light-enhanced electron transfer from water to plastoquinone (PQ) in thylakoid membrane of chloroplasts and cyanobacterial cells. PSII complex is linked with cytochrome b_6f and photosystem I complexes in the membrane, constituting a so-called Z-scheme for electron transport chain from water to $NADP^+$ (1). The PSII complex in a monomeric unit is composed of more than 20 protein subunits, some of which contain bound cofactors such as chlorophyll, β-carotene, and metal ions (2) Combined biochemical and spectroscopic data indicated that photon energy absorbed by antenna chlorophylls in chloroplasts or by phycobilisome in cyanobacteria is transferred through CP43 and CP47 internal antenna proteins to a specialized chlorophyll *a* dimer called P680 bound to D1/D2 heterodimer proteins at the center of PSII complex. Exited P680 donates an electron to pheophytin and finally to PQ. The oxidized P680 ($P680^+$) quickly returns to P680 by accepting an electron from the nearby tyrosine 161 (Y_Z) residue of D1 protein. The oxidized $Y_Z^{\cdot}$ radical is a strong catalyst to abstract electrons from water molecules associated with a manganese/calcium cluster which is built in the luminal side of D1 protein.

The elucidation of mechanism for water oxidation reaction has been one of the most challenging problems in photosynthesis for a long time. A classical Kok's model defines S_0 to S_4 states for water-oxidizing complex (WOC), each state advancing to the next state via consecutive photon-energy activation, which results in the accumulation of positive charges in WOC (3). Recently, a wealth of structural information about PSII complex from thermophilic cyanobacteria has been obtained from X-ray crystallographic studies (4–6). The static structure of Mn_4CaO_5 cluster with a distorted chair shape was revealed as a dark-stable S_1 state of WOC (7). In the cluster, four manganese atoms and one calcium atom are coordinated by six amino acid residues from D1 (Asp170, Glu189, His332, Glu333, Asp342, and Ala344) and one from CP43 (Glu354). This unique structure of manganese/calcium cluster has not only shed light on the mechanism of water oxidation but also enhanced the prospect for developing artificial water-oxidation catalysts capable of utilizing photon energy.

In relation with the static structure of Mn_4CaO_5 cluster, the D1-Ala344 deserves a special notion. This residue is the C-terminal residue formed after proteolytic cleavage of a short C-terminal extension of a precursor D1 polypeptide (pD1). The C-terminal extension of pD1 varies in length among different organisms; 16 amino acids in all cyanobacteria (8,9) and 9 amino acids in chlorophytes including higher plants (9,10). Proteolytic cleavage of

* Corresponding author at. Department of Applied Microbial Technology, Faculty of Biotechnology and Life Science, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan. Tel./fax: +81 96 326 3937.
E-mail address: matsuoka@bio.sojo-u.ac.jp (M. Matsuoka).

1389-1723/$ – see front matter © 2018, The Society for Biotechnology, Japan. All rights reserved.
https://doi.org/10.1016/j.jbiosc.2018.04.015

pD1 is catalyzed by a specific endopeptidase, CtpA (11,12), which acts in an early step of WOC formation (13). Only after cleavage of pD1, the C-terminal carboxylate of Ala344 can be coordinated to the Mn_4CaO_5 cluster. Thus, when C-terminal processing of pD1 is blocked, the WOC with active oxygen evolution cannot be formed (11,12). In addition, it has been well known that D1 protein is turned over rapidly by removal of photo-damaged D1 protein followed by reinsertion of newly synthesized D1 polypeptide into D1-less PSII complex, a phenomenon called a repair cycle (14,15).

In this study, we propose a genetic approach to study water oxidation reaction using genetically engineered cyanobacterial strains. Since D1 and D2 proteins carry out all the redox reactions from water to PQ, the D1/D2 heterodimer can be considered as a minimal entity on which various cofactors and Mn_4CaO_5 cluster are assembled. If purified D1/D2 heterodimer could be obtained in a reasonable quantity, biochemical and spectroscopic analyses for the WOC would become much simpler and straightforward. However, such a preparation of pure D1/D2 heterodimer has never been obtained so far. Only the best purified PSII reaction center preparations contained D1/D2/cytchrome b_{559}, which was often contaminated with peripheral subunits (16,17), because it is difficult to remove the peripheral protein subunits of the PSII complex using available detergents.

We postulated, if D1/D2 heterodimer is thermostable while other PSII subunit proteins are thermolabile, that the D1/D2 heterodimer could be separated from the peripheral proteins by differential heat denaturation. Ideally, heat treatment of such chimeric PSII complex would denaturalize the peripheral subunits, while the thermostable D1/D2 heterodimer should remain intact. The host cyanobacterium *Synechococcus elongatus* PCC 7942 engineered in this work normally grows at 28−30°C and cannot grow above 50°C (18), whereas a gene donor species, *Thermosynechococcus vulcanus*, grows optimally at 57°C and is tolerant to heat treatment as high as 72°C (19). Both cyanobacterial strains contain three *psbA* genes for D1 and two *psbD* genes for D2 polypeptides (20, 21, and our unpublished results). At normal growth condition with moderate light and optimal temperatures, only *psbA1* and *psbD1* genes are highly expressed in both cyanobacteria. Thus, replacement of *psbA1* and *psbD1* genes of *S. elongatus* with those from *T. vulcanus* would permit us to engineer the recombinant strains of *S. elongatus* expressing thermostable D1/D2 proteins. To maintain the host control on expression of the replaced genes, we changed only the coding region of each gene (open-reading frame; ORF). We describe here the characterization of such mutant strains harboring the interspecific genes for thermostable D1 and D2 polypeptides.

MATERIALS AND METHODS

Bacterial strains and growth condition All the cyanobacterial mutant strains were derived from *S. elongatus* PCC 7942 R2-SPc obtained from RIKEN (Wako, Saitama, Japan). A thermophilic cyanobacterial strain *T. vulcanus* was obtained from NIES (Tsukuba, Ibaraki, Japan). Cyanobacteria were routinely grown in liquid BG-11 medium or solid BG-11 agar plates supplemented with appropriate antibiotics under the continuous illumination by white fluorescent lamps (4W/m^2 or 20 μmol m^{-2} sec^{-1}) at 28°C except that *T. vulcanus* was grown at 50°C as described in the standard methods in photosynthesis (22). For middle scale cultivation, cyanobacterial cells were grown in 1 L of BG-11 medium in a glass bottle with continuous bubbling of 5% (v/v) carbon dioxide-enriched air (0.2 L/min). The glass bottles were immersed in an acrylic transparent water bath maintained at constant temperature and illuminated by white fluorescent lamps (7.25 W m^{-2} at the surface of the water bath).

Escherichia coli strains JM109, JM110, XL1-Blue, XL10, and TOP10 were grown in LB medium at 37°C (23). All the media were sterilized by autoclaving at 121°C for 15min. 1.5% (w/v) agar was used for solid media. After autoclaving the media, filter-sterilized antibiotics were added to the media at the following concentrations; ampicillin at 50 μg/ml, kanamycin (Km) at 20 μg/ml and streptomycin (Str) at 25 μg/ml.

Genetic manipulation The *rps12*-mediated gene replacement in cyanobacteria is schematically represented in Fig. S1. Km^R and Str^R show resistant phenotypes, while Km^S and Str^S show sensitive phenotypes. The cyanobacterial mutants for gene replacements of PSII (GRPS strains) were constructed as described previously (24,25). A series of plasmid constructed in this study are shown in Table 1 and Fig. S2. Construction of plasmids in *E. coli* was performed by the standard methods (23). The plasmids pUC19 conferring ampicillin resistance (*amp*) and pUC4K carrying kanamycin resistance (*kan*) gene were purchased from TaKaRa-Bio (Kusatsu, Shiga, Japan) and GE Healthcare (formerly Pharmacia) (Little Chalfont, Bukinghamshire, UK), respectively. The plasmids pEXR91 and pEXE1 were constructed previously (25,26). Transformation of cyanobacteria was carried out by electroporation (24).

Oligodeoxynucleotides used in this study are shown in Table S1. They were purchased from Hokkaido System Science, Co. (Sapporo, Hokkaido, Japan). Polymerase chain reaction (PCR) was carried out with DNA polymerases and reagents obtained from TaKaRa-Bio, Toyobo (Osaka, Japan), Agilent Technologies (formerly Stratagene) (La Jolla, CA, USA), and Applied Biosystems (Foster City, CA, USA) following the manufacturers' protocols. Blunt-ended PCR cloning kits with pMOS*Blue* and pCAP^S were purchased from GE Healthcare and Roche Diagnostics (Tokyo, Japan), respectively. TA cloning kit with pCR8/GW/TOPO was obtained from Invitrogen (Carlsbad, CA, USA). Site-directed mutagenesis was conducted with reagents from Takara-Bio-Clontech and Agilent Technologies. DNA sequencing was performed with BigDye terminator cycle sequencing kit from Applied Biosystems.

RT-PCR analysis Comparative transcriptome analysis was performed on cDNA that had been prepared from total RNA from recombinant cyanobacteria. For this purpose, approximately 5×10^8 cells grown in BG11 medium were suspended in RNAprotect Bacteria Reagent purchased from Qiagen (Hilden, Germany) and pelleted by centrifugation. Total RNA was purified from the cells treated with lysozyme using RNeasy Mini Kit and RNase-Free DNase Set (Qiagen). The purified RNA was quantitated by E_{260} measurement, and cDNA was synthesized with QuantiTect Whole Transcriptome Kit (Qiagen).

Real-time PCR (RT-PCR) was carried out in the reaction mixture (total volume 25 μl) consisting of 5 μl of diluted cDNA (1/250 dilution with nuclease-free water), each 3.75 μl of 5 μM oligonucleotide primers, and 12.5 μl of 2x GoTaq qPCR Mastermix obtained from Promega (Madison, WI, USA) using MiniOpticon RT-PCR system from BioRad (Hercules, CA, USA). After optimization of annealing and extension temperature, PCR cycles were set as 1 cycle of 95°C, 2min, 40 cycles of 95°C, 15sec and 62°C or 68°C, 60 sec, followed by 1 cycle of gradient from 60°C to 95°C. The data were analyzed by CFX Manager v2.0 software (BioRad) to obtain a quantification (threshold) cycle (Cq), and the relative gene expression profile was expressed by ΔΔCq method using *rpoB* gene encoding β-subunit of RNA polymerase as a reference. Nucleotide sequences of primers used for RT-PCR analysis of *rpoB*, *psbA1*, *psbA1**, *psbA2*, *psbA3*, *psbD1*, *psbD1**, and *psbD2* genes are shown in Table S1.

Biochemical analysis and oxygen evolution measurements Preparation of thylakoid membrane was carried out as essentially described in the standard methods (22). Cultured cyanobacterial cells were harvested by centrifugation and suspended at the concentration of 1 mg-dry cells/ml (OD_{730} equals 4.0) in buffer A composed of 40 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 25% (w/v) glycerol (pH 6.0). Cell suspension was stored at −80°C until cell disruption. Frozen cell suspension was thawed on ice, and when required, glycinebetaine was added at the final concentration of 1M. The subsequent procedure was operated at 4°C. Cells were disrupted by mixing with glass beads (0.1 mm diameter) using Bead-Beater from BioSpec (Bartlesville, OK, USA). The homogenate was centrifuged at 5000 g for 10 min to remove the cell debris, and the supernatant was centrifuged at 100,000 g for 10 min with a Beckman 70Ti rotor. The pellet was suspended in buffer A containing 1M glycinebetaine when required, and the suspension was used as thylakoid membrane.

For solubilization of membrane proteins, 0.8% (w/v) *n*-dodecyl-β-D-maltoside (DM) was added to the thylakoid membrane suspension, and stirred gently on ice for 20 min. The suspension was centrifuged at 35,000 g for 10min, and the supernatant was saved as solubilized membrane proteins.

Oxygen evolution was measured with a Clark-type oxygen electrode using Oxygraph 9 from Central Kagaku Corp (Bunkyo-ku, Tokyo, Japan) equipped with a circulating water bath. The reaction buffer for thylakoid membrane proteins or PSII complex was buffer A containing 1.5M glycinebetaine. Potassium ferricyanide (FeCN) and 2,6-dichloro-p-benzoquinone (DCBQ) were added at the final concentration of 1 mM and 0.5 mM, respectively, as electron acceptors. The reaction chamber (1.1 ml) was illuminated by halogen lamp with an infrared-cut filter at light-saturating condition. Light intensity was measured with a radiometer Model 4090 from Springfield Jarco Instruments (Yellow Springs, OH, USA).

For extraction of total cellular proteins, the cells grown in BG-11 medium were collected by centrifugation and suspended in the denaturing buffer containing 172 mM Tris, 40 mM dithiothreitol, 5.2% (w/v) lithium dodecyl sulfate, 6M urea, and 0.5M sucrose. The cells were disrupted by ultrasonic treatment, and the cell-free extract was obtained by centrifugation and assayed for chlorophyll concentration. Appropriate volume of the extract was precipitated by trichloroacetic acid, and finally suspended in the denaturing buffer containing 0.01% (w/v) bromophenol blue prior to electrophoresis.

Protein concentration was assayed with DC protein assay (BioRad). Chlorophyll (Chl) was extracted with 80% acetone and quantitated by measuring absorbance at 646.6, 663.6, and 750 nm as described in the standard methods (22). Analysis of membrane proteins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Please cite this article in press as: Haraguchi, N., et al., Characterization of mutants expressing thermostable D1 and D2 polypeptides of photosystem II in the cyanobacterium *Synechococcus elongatus* PCC 7942, J. Biosci. Bioeng., (2018), https://doi.org/10.1016/j.jbiosc.2018.04.015