The Journal of Microbiology, April 2005, p.191-195
Vol. 43, No. 2


NOTE

# Functional Characterization of the Gene Encoding UDP-glucose: Tetrahydrobiopterin α-Glucosyltransferase in *Synechococcus* sp. PCC 7942

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(Received January 18, 2005 / Accepted February 25, 2005)

**In this study, we attempted to characterize the *Synechococcus* sp. PCC 7942 mutant resultant from a disruption in the gene encoding UDP-glucose: tetrahydrobiopterin α-glucosyltransferase (BGluT). 2D-PAGE followed by MALDI-TOF mass spectrometry revealed that phycocyanin rod linker protein 33K was one of the proteins expressed at lower level in the BGluT mutant. BGluT mutant cells were also determined to be more sensitive to high light stress. This is because photosynthetic $O_2$ exchange rates were significantly decreased, due to the reduced number of functional PSIs relative to the wild type cells. These results suggested that, in *Synechococcus* sp. PCC 7942, BH4-glucoside might be involved in photosynthetic photoprotection.**

***Key words***: tetrahydrobiopterin-glucoside, proteomics, phycocyanin rod linker, photosynthesis, photoprotection, cyanobacteria

Tetrahydrobiopterin (BH4)-glucoside is a pteridine glycoside, which is generated by *Synechococcus* sp. PCC 7942 (Choi *et al.*, 2001a). Pteridine glycosides are characterized by a variety of sugars attached to the side chain at the C-6 of the pterin ring. These sugars include biopterin, 6-hydroxymethylpterin, and neopterin, and are found in the cyanobacteria (Forrest & Van Baalen, 1970; Lee *et al.*, 1999; Chung *et al.*, 2000), the anaerobic photosynthetic bacteria *Chlorobium limicola* and *Chlorobium tepidum* (Cho *et al.*, 1998), and the chemoautotrophic archaebacterium, *Sulfolobus solfataricus* (Lin & White, 1988). In contrast to the aglycosidic BH4, which is a well-known cofactor for aromatic amino acid hydroxylation and nitric oxide synthesis in higher animals (Thöny *et al.*, 2000), BH4-glucoside, as well as several other pteridine glycosides, is produced at high cellular concentrations, and its cellular function has, thus far, not been precisely elucidated. Earlier studies have postulated its possible role in photosynthetic electron transport (Maclean *et al.*, 1965). It has also been suggested that it plays a protective role against UV damage, due to findings of increased biopterin-glucoside synthesis in that marine cyanobacterium, *Oscillatoria* sp., upon exposure to UV-A irradiation (Wachi *et al.* 1995). More recently, biopterin-glucoside was demonstrated to stabilize phycocyanin upon exposure to UV light (Noguchi *et al.*, 1999; Saito *et al.*, 2003).

BH4-glucoside is synthesized from BH4 and UDP-glucose by the enzyme UDP-glucose: BH4 α-glucosyltransferase (BGluT), which was initially identified in *Synechococcus* sp. PCC 7942 (Chung *et al.*, 2000). The encoding gene was cloned after protein purification, and was subsequently disrupted in *Synechococcus* sp. PCC 7942 (Choi *et al.*, 2001b). The BGluT mutant produced only aglycosidic BH4 in 8.3% of the wild type cells, with a significantly reduced growth rate (approx. one half of the wild type) (Choi *et al.*, 2001b), thereby suggesting that aglycosidic BH4 plays a role in the light harvesting and utilization process, under visible light growth conditions. Therefore, we attempted to determine whether the BGluT mutant is more sensitive to high degrees of light stress, then analyzed protein expression profiles, in order to identify some of the proteins involved herein.

*Synechococcus* sp. PCC 7942 wild type and mutant strain were grown at 30°C in BG-11 medium in batch cultures under continuous white light (100 ~ 120 μmole photons/$m^2$/s) and air bubbling. Growth was measured by optical density at 730 nm. *Synechococcus* sp. PCC 7942

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wild type and BGluT mutant cells were grown axenically in BG-11 medium supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH (pH 7.5), then bubbled with air at 35°C, under continuous illumination of 60 μmole photons $m^2$/s of white light (Philips, TLD 18W/95O, USA).

The cells, after being cultured up to 2.0 at A730, were harvested and sonicated in suspension buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM PMSF). The sonicated extract was centrifuged for 20 min at 4°C, and 15,000 rpm. The supernatant was mixed with 1 mg/ml DNase, 0.25 mg/ml RNase, 50 mM $MgCl_2$, incubated for 2-3 h on ice, dialyzed against 10 mM Tris-HCl (pH 7.5), then freeze dried. The freeze dried sample was dissolved in IEF equilibration solution (8 M Urea, 4% CHAPS, 2% Pharmalyte pH 3 - 10, 2.8 mg/ml DTT) and centrifuged for 10 min at 20°C, and 14000 rpm. The supernatant was diluted in order to yield a 40 μg/μl protein concentration, and stored at -70°C until use.

Isoelectric focusing was performed with IPGphor (GE Healthcare Bioscience, USA). 50 μg of the protein sample was dissolved in 250 μl of rehydration solution (8 M Urea, 2% CHAPS, 0.5% Pharmalyte, 0.002% bromophenol blue, 2.8 mg/ml DTT) and left for 2 h at room temperature. The sample was centrifuged for 10 min at 20°C, and 14000 rpm, and the supernatant was applied to a 13 cm long IPG strip, then electrophoresed at 20°C for 14 h at 30 V, 1 h at 500 V, 1 h at 1000 V, and finally for 3 h at 8000 V. After electrophoresis, the IPG strip was equilibrated for 20 min in 10 ml of solution, containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 2.8 mg/ml DTT, and a further 20 min in the same solution, supplemented with 25 mg/ml iodoacetamide. The equilibrated IPG strip was loaded into the top of a precast 12.5% SDS-PAGE gel, then electrophoresed at a consistent 25 mA current per plate. The displayed proteins were then visualized by silver staining as described elsewhere, and finally stored in 1% (w/v) glacial acetic acid. A Bio-rad low range marker was utilized in the determination of molecular weight and a Bradford reagent was used for protein determination, using bovine serum albumen as a standard. The protein spots were analyzed manually after the 2-D gel images had been copied into a personal computer using an image scanner. Protein spots were manually isolated and sent to KRIBB (Daejeon, Korea) for matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF) analysis. The resulting mass fingerprinting peak values were then analyzed with MASCOT (http://www.matrixscience.com), peptident (http://www.Expasy.ch/cgi-bin/peptident.pl), and MS-Fit (http://prospector.ucsf.edu/uscfhtml4.0/msfit.htm), using the Swiss Prot and NCBInr databases. The peptide mass tolerance value was ±1 Da, and the amount of missed cleavage sites by trypsin was 0-1. The identified proteins were restricted to those exhibiting more than 4 matched peptides. The peptides were fragmented further for sequencing via MS/MS ion searches (MALDI-TOF-TOF).

The maximum extent of P700 oxidation induced by far-red was determined *in vivo,* as described in Ivanov *et al.* (2000), using a PAM-101 modulated fluorometer (Heinz

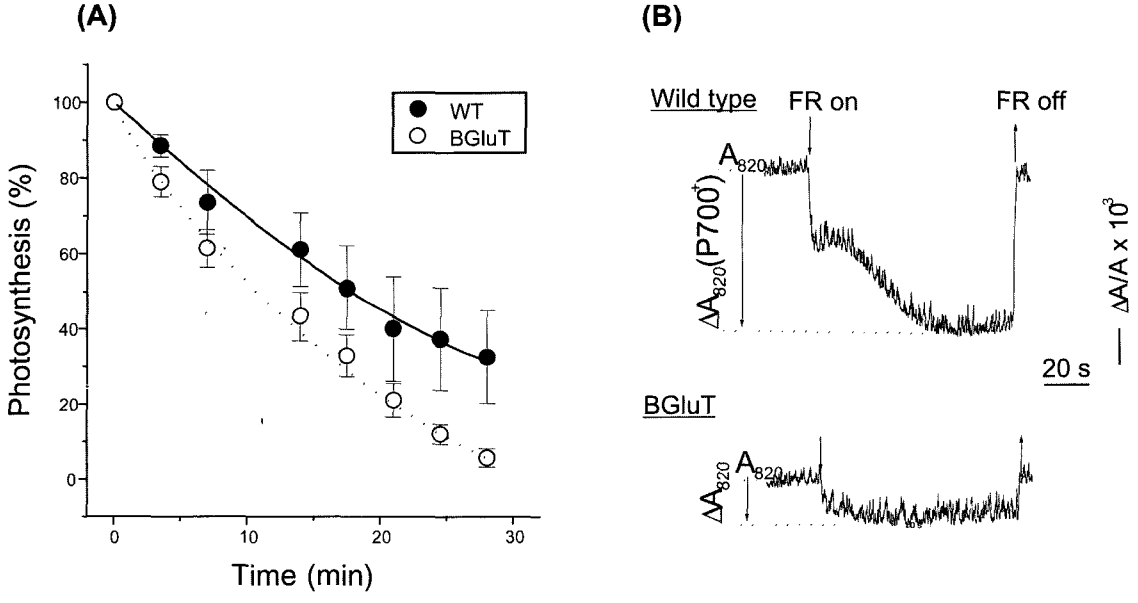


**Fig. 1.** *In vivo* photosynthetic functions measured by photosynthetic $O_2$ exchange rates and the redox state of P700. (A) Changes in the photosynthetic $O_2$ exchange rates (% of initial) as a function of saturating light treatment (744 μmole photons/$m^2$/s) in *Synechococcus* sp. PCC 7942 wild type (WT) and mutant (BGluT, ) cells. To block the repair of damaged reaction center D1, lincomycin (1 mg/ml) was added prior to the onset of illumination. Cells at the exponential phase (3.5 μg Chl) were used. (B) *In vivo* measurements of the redox state of P700 in *Synechococcus* sp. PCC 7942 wild type (WT) and mutant (BGluT) cells. Data obtained from cells at the exponential growth phase (60 μg Chl) represent typical traces.

Waltz GmbH, Germany) equipped with an ED P700DW emitter-detector and a PAM DAT Acquisition System (PDA-100). Far-red light (FR, transmitted by a Schott RG 715 filter) was provided by a saturation light source. The cells (60 μg of chlorophyll) were filtered onto a cellulose nitrate membrane (3.0 μm of pore size) using a vacuum system, then placed in front of the fiber optic light source. The light response curves of photosynsthetic $O_2$ evolution were determined with a liquid phase $O_2$ electrode unit (DW3), a white light source (LS2), and an electrode control unit (Oxygraph system, Hansatech, UK).

We investigated the *in vivo* photosynthetic functions of the mutants by measuring photosynthetic $O_2$ exchange rates, as well as the redox state of P700 (Fig. 1). The resistance to high light stress was assessed in the cells enclosed in the $O_2$ electrode chamber, which was illuminated under a saturating light intensity (744 μmole photons/$m^2$/s). The photosynthetic $O_2$ exchange rates were measured continuously, as a function of illumination time. Lincomycin (1 mg/ml) was included to inhibit the repair of the damaged PSII reaction center D1 protein. Photoinhibition of photosynthesis was observed as a decrease in the $O_2$ exchange rates. Compared to the wild type cells, photosynthesis declined rapidly in the BGluT mutant, resulting in a lesser degree of resistance to high light stress (Fig. 1A). This decline in photosynthetic $O_2$ evolution is not attributable to PSII inhibition, as the photochemical efficiencies of PSII during steady photosynthesis, estimated as the Chlorophyll (Chl) fluorescence parameter Fv/Fm, were comparable between the two strains (data not shown). Instead, the number of photooxidizable PSI reaction centers ($P700^+$) and redox kinetics of $P700^+$, as estimated by recording absorbance changes at 820 nm upon far-red (FR) illumination, decreased significantly. As shown in Fig. 1B, the inactivation of BGluT reduced the numbers of functional PSI ca. 30%, relative to the wild type cells. Wild type cells exhibited a rapid oxidation of P700 to $P700^+$, followed by a further slower oxidation via far-red light treatment. Interestingly, BGluT cells exhibited only the initial rapid oxidation, without further oxidation. However, the reduction of $P700^+$ to P700 upon the turning off of the far-red light was not disrupted by the inactivation of the BGluT gene. These results clearly demonstrated that BGluT is more susceptible to light stress due to PSI inhibition, suggesting BH4-glucoside's protective role against high light stress.

In order to investigate changes in protein expression profiles due to the malfunction of *BGluT*, the soluble proteins extracted from the mutant and wild type cells were separated by a pH gradient of 3 - 10 and 12.5% SDS-PAGE, and were visualized via silver staining (Fig. 2). We consistently found some proteins, which had been up-regulated or down-regulated in the mutants (dotted circles). Among these, eight down-regulated proteins in the mutants (indicated by arrows in the wild type 2D

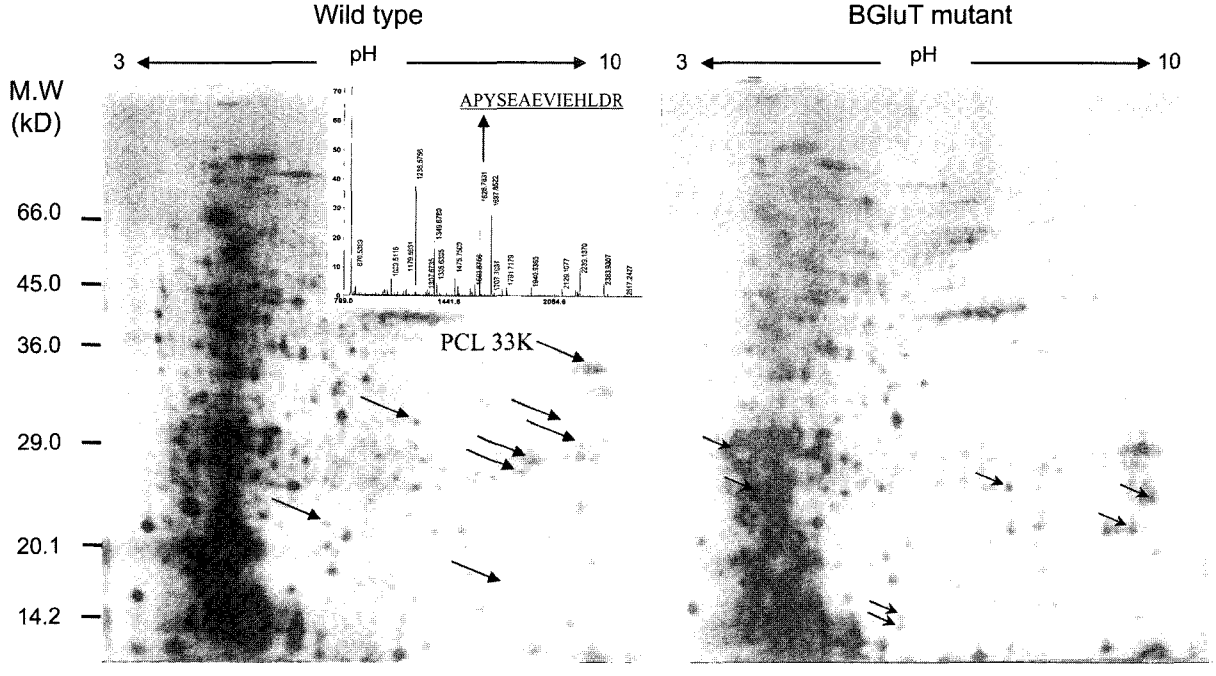


**Fig. 2.** 2D-PAGE of soluble proteins from wild type and BGluT mutant cells. Proteins separated by 2-dimensional electrophoresis were detected by silver-staining. Bio-rad low range marker proteins are indicated. The protein spots which were subjected to MALDI-TOF analysis are marked by arrows pointing at the images: The spots in wild type 2D gel are down-regulated proteins in the mutant, while those in BGluT gel are up-regulated. The protein spot, which was identified as the pycocyanin 33K linker protein (PCL 33K), was fragmented by mass spectrometry and one of them (observed mass of 1628.78) was further fragmented to determine the APYSEAEVIEHLDR amino acid sequence, as shown in the inlet.

gel) and seven up-regulated proteins in the mutants (indicated in the mutant 2D gel) were isolated, digested with trypsin, and subjected to MALDI-TOF mass spectrometry. The measured masses, when compared with those in the Swiss Prot and NCBInr databases, found several proteins: the down-regulated proteins included phycocyanin rod linker protein 33K, and the acridine efflux pump and elongation factor Tu. The upregulated proteins included the ATP synthase F1 gamma subunit and DNA helicase II. Unfortunately, *Synechocystis* sp. PCC 7942 genome sequencing was not yet finished, and therefore peptide mapping was not sufficient to identify genuine proteins. However, the discovery of the phycocyanin rod linker protein 33K was felt to warrant more detailed analysis. The protein spot indicated by the arrow PCL 33K, shown in Fig. 2 (wild type) was fairly weak in the corresponding position of the BGluT mutant gel, and yielded peptide fragments which matched well with those from the phycocyanin rod linker protein 33K of *Synechococcus* sp. PCC 6301., *Synechococcus* sp. PCC 6301 is very closely related to, and considered to be a member of the same species as, Strain PCC 7942. One of the peptide fragments (with an observed mass of 1628.78) was further fragmented, exhibiting an amino acid sequence of APYSEAEVIEHLDR, which coincided exactly with an internal sequence seen in the PCC 6301 protein. The linker protein 33K consists of 289 amino acid residues, and has a theoretical mass of 31698.69 and a pI value of 9.06, coinciding closely with the values determined from the spot position in the 2D-gel. It was, therefore, fairly certain that the phycocyanin rod linker protein 33K was one of several proteins which are downregulated in the BGluT mutant.

The phycocyanin rod linker proteins are components of phycobilisome, which is a large multiprotein complex, and is predominantly responsible for the harvesting of light energy transferred to both photosystems in the cyanobacteria. The phycobilisome consists of a core structure from which rods fan out. These rods change in length depending upon growth conditions, thereby regulating the amount of light energy harvested by PS II (Kehoe & Grossman, 1994). It has also been reported that most genes which encode for the phycobilisome structural subunits are down-regulated upon exposure to UV-B and white light (Huang *et al.*, 2002). It is also interesting, maybe even more so, that biopterin-glucoside was reported to provide the photostabilization of phycocyanin under 254 nm UV light (Noguchi *et al.*, 1999; Saito *et al.*, 2003). Thus, our finding of reduced 33 kD rod linkers in the BGluT mutant cells was speculated to be a result of phycobilisome dysfunction, which might have resulted from poor photoprotection, due to low pteridine production. Pteridine glycosides are produced in abundance in the cyanobacteria: in *Synechocystis* sp. PCC 6803, the intracellular concentration of cyanopterins was comparable to that of chlorophyll a, suggesting a quantitative role, rather than a cofactor function (Lee *et al.*, 1999). Therefore, BH4-glucoside's putative protective role in light stress seems highly probable.

In summary, a proteomic analysis of the soluble proteins from the wild type and BGluT mutants revealed that the phycocyanin rod linker protein 33K is one of the proteins which is downregulated in the mutants compared to the wild type cells, thereby suggesting BH4-glucoside's role in the function of phycobilisomes in *Synechococcus* sp. PCC 7942. Functional analysis of photosynthesis in the mutant cells also revealed photosynthetic dysfunctions associated with increased susceptibility to high light stress, coupled with the inhibition of PSI, which appeared to have been mediated via poor phycobilisome function. A future in-depth investigation of phycobilisome function in the BGluT mutant is required, in order to elucidate the direct role of BH4-glucoside in *Synechococcus* sp. PCC 7942.

This work was supported by a Korea Research Foundation Grant (KRF-2003-015-C00595).

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