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Association of High Light-Inducible HliA/HliB Stress Proteins with Photosystem 1 Trimers and Monomers of the Cyanobacterium *Synechocystis* PCC 6803

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Abstract—Hlip (high light-inducible proteins) are important for protection of the photosynthetic apparatus of cyanobacteria from light stress. However, the interaction of these proteins with chlorophyll–protein complexes of thylakoids remains unclear. The association of HliA/HliB stress proteins with photosystem 1 (PS1) complexes of the cyanobacterium *Synechocystis* PCC 6803 was studied to understand their function. Western blotting demonstrated that stress-induced HliA/HliB proteins are associated with PS1 trimers in wild-type cells grown under moderate light condition (40 μmol photons/m^2 per sec). The content of these proteins increased 1.7-fold after light stress (150 μmol photons/m^2 per sec) for 1 h. In the absence of PS1 trimers (Δ*psaL* mutant), the HliA/HliB proteins are associated with PS1 monomers and the PS2 complex. HliA/HliB proteins are associated with PS1 monomers but not with PS1 trimers in *Synechocystis* PS2-deficient mutant grown at 5 μmol photons/m^2 per sec; the content of Hli proteins associated with PS1 monomers increased 1.2-fold after light stress. The HliA/HliB proteins were not detected in wild-type cells of cyanobacteria grown in glucose-supplemented medium at 5 μmol photons/m^2 per sec, but light stress induces the synthesis of stress proteins associated with PS1 trimers. Thus, for the first time, the association of HliA/HliB proteins not only with PS1 trimers, but also with PS1 monomers is shown, which suggests a universal role of these proteins in the protection of the photosynthetic apparatus from excess light.

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Key words: high light-inducible proteins HliA/HliB, light stress, photosystem 1, cyanobacteria

Non-photochemical quenching of excess absorbed energy under high light intensity protects the plant and cyanobacterial photosynthetic apparatus from destruction [1-4]. Light-induced stress proteins Hlip (high light-inducible proteins) or SCPs (small chlorophyll *a/b* Cab-like proteins) play an important role in protecting the photosynthetic apparatus of cyanobacteria from destruction [5, 6]. These proteins, required for the survival of organisms under high light intensity, are found to be similar to light-harvesting chlorophyll *a/b*-binding proteins (Cab) of plants and appear to be their evolutionary predecessors [7, 8]. Hli proteins are located in the thylakoid membrane and contain one transmembrane helix and a chlorophyll-binding domain. They are characterized by a low molecular weight of 6-10 kDa [5-7, 9]. In cyanobacteria, these proteins are coded by light-inducible *hli* genes, which are found in all currently sequenced genomes of cyanobacteria; the number of *hli* gene copies depends on the species and the ecotype of cyanobacteria [9, 10].

Five Hli proteins have been identified for the *Synechocystis* PCC 6803 bacterium (hereinafter referred to as *Synechocystis*), four of which are low molecular weight proteins HliA/HliB, HliC/HliD; the fifth protein is the *C*-terminal fragment of ferrochelatase [11, 12]. The genes encoding HliA-HliD are induced by various stress conditions, including not only high intensity light, but also low temperature as well as nitrogen and sulfur starvation [11, 13], which complicates the elucidation of mech-

Abbreviations: β-DM, *n*-dodecyl-β-D-maltoside; HliA/HliB proteins, high light-inducible proteins; PS1(2), photosystem 1(2).

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anisms behind the induction of the synthesis of these proteins.

Mutant cyanobacteria with inactivated *hliA-hliD* genes were shown to be sensitive to high intensity light and to differ from wild-type cells by their pigment content, and they are incapable of non-photochemical dissipation of absorbed light energy [14]. Hli proteins can prevent generation of reactive oxygen species by binding free chlorophyll molecules formed under stress, which generate singlet oxygen [15]. Besides that, Hli proteins appear to participate in tetrapyrrole biosynthesis regulation, therefore regulating chlorophyll synthesis [15]. HliD was found to bind chlorophyll *a* and β-carotene and to dissipate absorbed energy by transferring energy from chlorophyll *a* (state G_y) to β-carotene (state S_1) [16].

Of particular interest are two proteins from this family, HliA and HliB, since they are critical for the survival of *Synechocystis* cells under light stress [17]. The *hliA* and *hliB* genes were found to be functionally complementary [15] and to be characterized by a high degree of nucleotide sequence similarity (87%) [11], which indicates a relatively recent duplication of the gene [12, 18]. The expression of *hliA* and *hliB* genes and a high degree of sequence similarity suggests that they play a similar role in the cell, revealing close co-regulation [12].

The data concerning binding these important proteins to chlorophyll–protein complexes of cyanobacterial thylakoid membranes is varied. HliA and HliB in *Synechocystis* were shown to be associated with PS1 trimers, but not monomers, and to be necessary for their stabilization [17]. On the other hand, HliA and HliB proteins in *Synechocystis* were found to be bound to the CP47 protein in PS2, but not PS1 [19]. For a better understanding of HliA/HliB functions, it was necessary to determine which chlorophyll–protein complexes in cyanobacterial thylakoids are associated with them. The aim of this study was to assess the association of light-induced stress proteins HliA and HliB with PS1 trimers and monomers in wild-type *Synechocystis* cells, in a PS2-deficient mutant, and in a mutant unable to form PS1 trimers.

MATERIALS AND METHODS

Cyanobacteria strains and growth conditions. We studied wild-type cells of the cyanobacterium *Synechocystis* PCC 6803, as well as a PS2-deficient (Δ*psbDI*, Δ*psbDII*, Δ*psbC*) mutant [20] and the Δ*psaL* mutant, which is unable to form PS1 trimers due to a *psaL* gene disruption [21]. The cyanobacteria were grown in BG-11 liquid medium [22] at 30°C under constant fluorescent daylight illumination lamps and ambient aeration by a magnetic stirrer until mid-log phase of growth. Wild-type cells and the cells of mutant unable to form PS1 trimers were grown at medium light intensity of 40 μmol photons/m^2 per sec. The PS2-deficient mutant cells were grown at low light intensity (5 μmol photons/m^2 per sec) supplemented with 5 mM glucose and antibiotics (chloramphenicol 20 μg/ml, spectinomycin 20 μg/ml). The Δ*psaL* mutant was grown in the presence of kanamycin (80 μg/ml). To create light stress, wild-type cells and PS2-deficient mutants grown under the aforementioned conditions were illuminated by high-intensity light (150 μmol photons/m^2 per sec) for 1 h. The use of not very high light stress intensity was intended to prevent photodestruction and cell death, especially for the PS2-deficient mutant. In comparative studies of wild-type cells and PS2-deficient mutant cells, the wild-type cells were pre-adapted to the mutant growth conditions.

Thylakoid membrane isolation and chlorophyll–protein complex fractionation. Thylakoid membranes were isolated by the method described in [23, 24]. For extraction of native photosystem complexes from the thylakoid membrane, the mild nonionic *n*-dodecyl-β-D-maltoside (or β-DM) detergent was used. It was added at chlorophyll/detergent ratio of 1 : 15. After incubation at 4°C for 30 min, the lysate was centrifuged at 18,000*g* for 10 min. Chlorophyll–protein complexes were extracted by anion-exchange chromatography on a DEAE-Toyopearl-650 column [23, 24].

Evaluation of chlorophyll content and PS1 complex activity. Chlorophyll *a* content in samples was evaluated in ethanol extract [25].

PS1 trimer fraction activity was determined by the capability of P700 (primary electron donor in PS1 reaction center) photooxidation as a light-induced change in absorption at 810 nm (against 870 nm) under illumination by 730-nm light. The measurements were performed using a DUAL-PAM-101 fluorimeter with the ED-P700 DW-101 device (Walz, Effelrich, Germany). The PS2 activity was estimated by the variable fluorescence, which was measured with the PAM-101 fluorimeter with 680-nm light.

Isolation of proteins, SDS-PAGE, and Western blotting. Proteins were fractionated using PAGE in the presence of SDS [26]. Protein content was measured by the Bradford method [27]. Samples containing 20 μg of protein were applied to a lane. The samples were incubated at 95°C for 10 min before application, and then centrifuged at 18,000*g* for 10 min.

Protein electrophoresis was performed in Tris-glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 7.5). The proteins were transferred from gel to a nitrocellulose membrane in a blotting chamber with Tris-glycine transfer buffer (25 mM Tris, 250 mM glycine, 20% ethanol, 0.02% SDS, pH 7.5) over 1 h at 200 mA. Then the membrane with the transferred proteins was put for 1 h at 4°C in TBST blocking buffer (50 mM Tris-HCl, 200 mM NaCl, 0.1% Tween 20, pH 7.5) supplemented with 5% dry skim milk, and then primary antibodies were added. We used polyclonal rabbit antibodies to HliA/HliB

(1 : 4000) (Abcam, USA). The membrane was incubated with the antibodies overnight at 4°C under constant stirring. As secondary antibodies, we used goat anti-rabbit IgG conjugated to horseradish peroxidase (1 : 10,000) (AgriSera, Sweden). Each step was accompanied by repeated washing of membranes with TBST buffer. Immune complexes on a membrane were detected with the ECL fluorescent detection system (GE Healthcare, England), and the signals were registered on X-ray film (Retina, Germany). The film was scanned and the data were processed with the Image J program (http://rsbweb.nih.gov/ij/).

RESULTS

Solubilization of thylakoid membranes and extraction of chlorophyll–protein complexes. Thylakoid membranes were lysed with the mild nonionic detergent β-DM, using 1 : 15 chlorophyll/detergent ratio. In preliminary experiments, various β-DM concentrations were used for thylakoid membrane solubilization (with chlorophyll/detergent ratio of 1 : 15; 1 : 18; and 1 : 20). For further work, the chlorophyll/detergent ratio of 1 : 15 was chosen since this concentration allowed a sufficient yield of chlorophyll–protein complexes together with the lowest formation of free pigments. The same β-DM concentration was used in [17].

Fractionation of chlorophyll–protein complexes from *Synechocystis* cyanobacterium thylakoid membranes with anion-exchange chromatography yielded three separate peaks, characterized by absorption spectroscopy as well as by protein composition and PS1 activity. We isolated a PS1 trimer fraction, a fraction containing PS1 monomers together with PS2 complex, and a fraction of free chlorophylls and carotenoids (Fig. 1a). Chlorophyll content in PS1 trimers of wild-type cells averaged 68 ± 4% of the total chlorophyll, meaning that most of thylakoid membrane chlorophyll of this cyanobacterium is located in PS1 trimers, like in *Arthrospira platensis* studied earlier [28].

The comparison of light-induced change in P700 absorption in PS1 trimers and photosynthetic membranes from wild-type cells indicated activity of isolated PS1 trimers. PAGE revealed the fact that the PS1 trimer fraction from wild-type cells is intact according to their protein content, since it contains all typical protein compo-

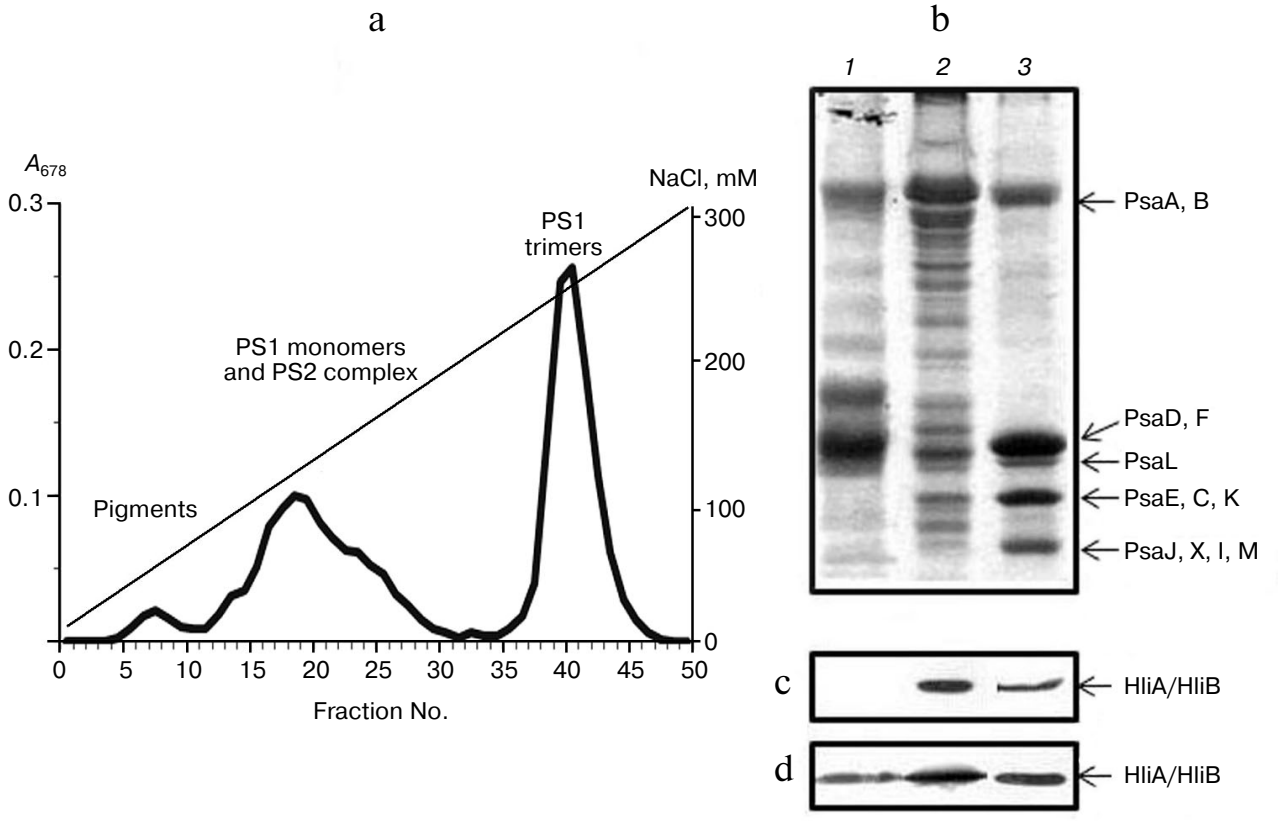

Fig. 1. Association of stress-induced proteins HliA/HliB with chlorophyll–protein complexes from thylakoid membranes of *Synechocystis* wild-type cells. a) Chromatographic profile of chlorophyll–protein complexes fractionation on a DEAE-Toyopearl 650M anion-exchange column; b) electrophoretogram of photosystem proteins after anion-exchange chromatography in fractions 17-20 (*1*), 23-26 (*2*), and 40-43 (*3*). Proteins HliA/HliB in the fractions were detected by Western blotting in cells grown under normal conditions (c) or in cells exposed to light stress (d).

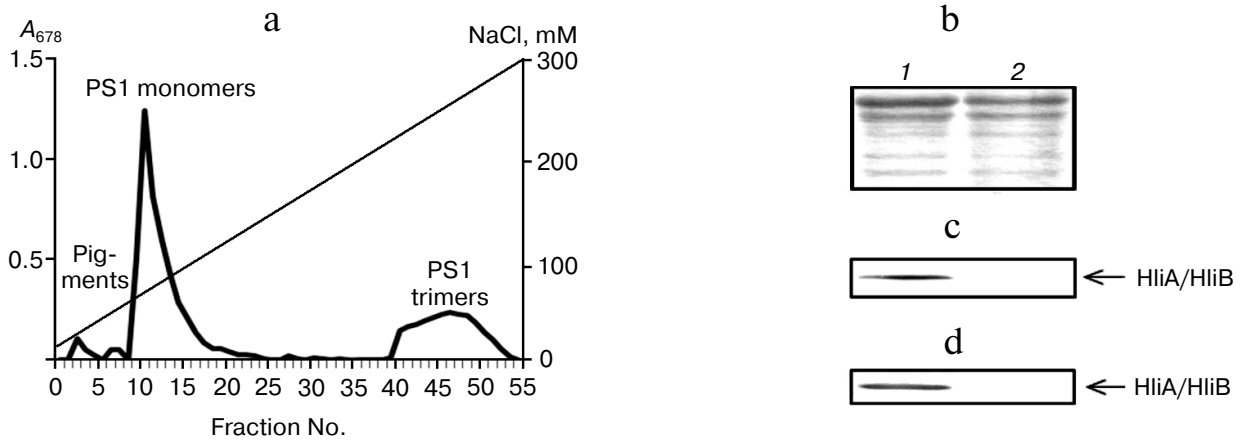

Fig. 2. Association of HliA/HliB proteins with PS1 monomers in PS2-deficient mutant *Synechocystis* cells. a) Fractionation profile of chlorophyll–protein complexes of mutant cells; b) electrophoretogram of photosystem proteins after anion-exchange chromatography and staining with Coomassie R-250, used for application of equal protein aliquots. Lanes: *1*) fractions 10-14; *2*) fractions 45-49. HliA/HliB proteins in the fractions were detected by Western blotting in cells grown under normal conditions (c) or in cells exposed to light stress (d).

nents of PS1 (Fig. 1b): high molecular weight PsaA and PsaB and low molecular weight proteins (PsaD,F; PsaL; PsaE,C,K; and PsaJ,X,I,M). Fractions containing PS1 monomers and PS2 complex were represented by proteins from both photosystems. From PS2-deficient *Synechocystis* cells, PS1 monomers were isolated completely free of PS2.

Association of HliA/HliB proteins with PS1 trimers of wild-type *Synechocystis* cells. Association of HliA/HliB proteins with PS1 trimers from wild-type cells grown under moderate illumination (40 μmol photons/m^2 per sec) was studied with Western blotting. HliA/HliB proteins were found to be associated with the PS1 trimers fraction, as well as with the fraction containing PS1 monomers and the PS2 complex (Fig. 1c). Wild-type cyanobacterial cells were exposed to moderate light stress. For this, cells grown under normal light (40 μmol photons/m^2 per sec) were illuminated by high-intensity light (150 μmol photons/m^2 per sec, 1 h). HliA/HliB proteins were detected in the fraction containing PS1 monomers and the PS2 complex (fractions 17-20 and 23-26), as well as in the fraction containing PS1 trimers (Fig. 1d). HliA/HliB content in PS1 trimers (fractions 40-43) was found to increase by 1.7-fold after light stress compared to unstressed cells.

Association of HliA/HliB proteins with PS1 monomers in PS2-deficient mutant. To demonstrate the association of HliA/HliB with PS1 monomer, a PS2-deficient *Synechocystis* mutant containing only PS1 in thylakoid membranes was used. Fractionation of the mutant chlorophyll–protein complexes on an anion-exchange column yielded three peaks: PS1 trimers, PS1 monomers, and free pigments. As indicated in Fig. 2a, PS1 content in the mutant is decreased as compared to PS1 monomer content in wild-type cells grown under normal conditions (Fig. 1a). Western blotting showed HliA/HliB proteins to be associated only with the PS1 monomer fraction and to be absent in the PS1 trimer fraction (Fig. 2, b and c). PS2-deficient mutant cells were exposed to light stress (150 μmol photons/m^2 per sec, 1 h). After light stress, the HliA/HliB proteins were also detected in the fraction containing PS1 monomers (fractions 10-14), with HliA/HliB content increased by 1.2-fold (Fig. 2d) compared to cells grown under normal illumination (40 μmol photons/m^2 per sec). HliA/HliB proteins were not detected in mutant PS1 trimer fraction after light stress (Fig. 2d). Therefore, the study conducted on PS2-deficient mutant *Synechocystis* cells showed that HliA/HliB proteins can be associated with PS1 monomers and that their synthesis is induced by moderate light stress.

Association of HliA/HliB proteins with pigment–protein complexes in Δ*psaL* mutant (without PS1 trimers). To determine whether HliA/HliB proteins can associate with PS1 monomers and PS2 complex in the absence of PS1 trimers, PS1 trimer-deficient mutant was studied. The study of mutant cells grown under normal conditions showed HliA/HliB proteins to be present in fractions containing PS1 and PS2 monomers (Fig. 3). After light stress, the concentration of HliA/HliB proteins bound to PS1 monomers and PS2 complex increased 2-fold. Therefore, HliA/HliB proteins associate with PS1 monomers and PS2 complex in the absence of PS1 trimers.

Do cell growth conditions affect HliA/HliB protein association with PS1 trimers? Growth conditions for PS2-deficient mutant differ from those for wild-type cells. As photosynthesis in PS2-deficient mutant is disrupted, these cells were cultivated in medium containing glucose as an energy source, under low light intensity (5 μmol photons/m^2 per sec). To determine whether mutant growth

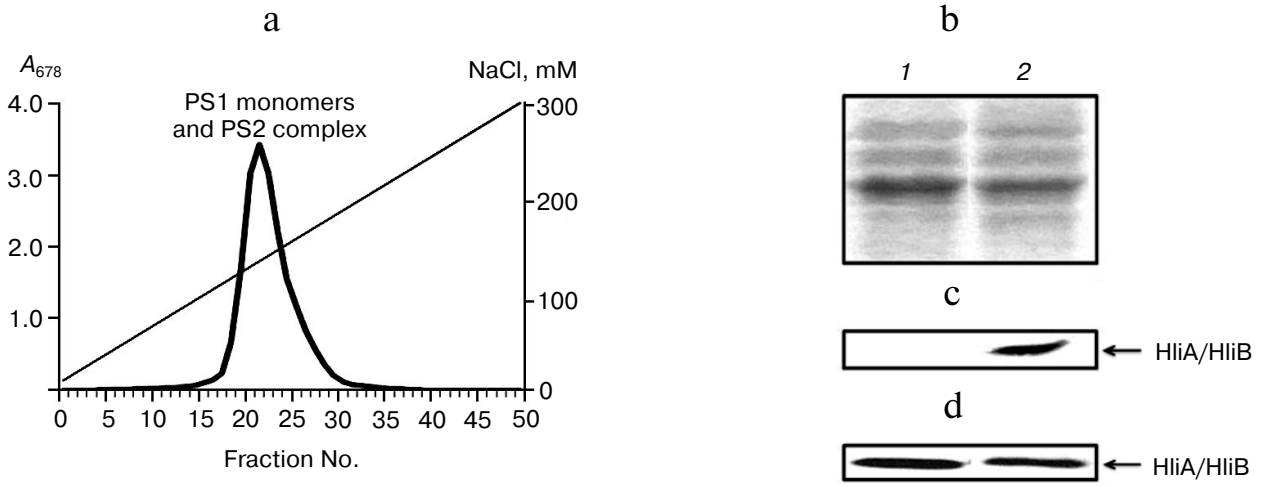

Fig. 3. Association of HliA/HliB proteins with PS1 monomers in Δ*psaL* mutant cells of *Synechocystis* lacking PS1 trimers. a) Fractionation profile of mutant cell chlorophyll–protein complexes; b) electrophoregram of photosystem proteins after anion-exchange chromatography and staining with Coomassie R-250, used for application of equal protein aliquots. Lanes: *1*) fractions 22-24; *2*) fractions 26-30. HliA/HliB proteins in the fractions were detected by Western blotting in cells grown under normal conditions (c) or in cells exposed to light stress (d).

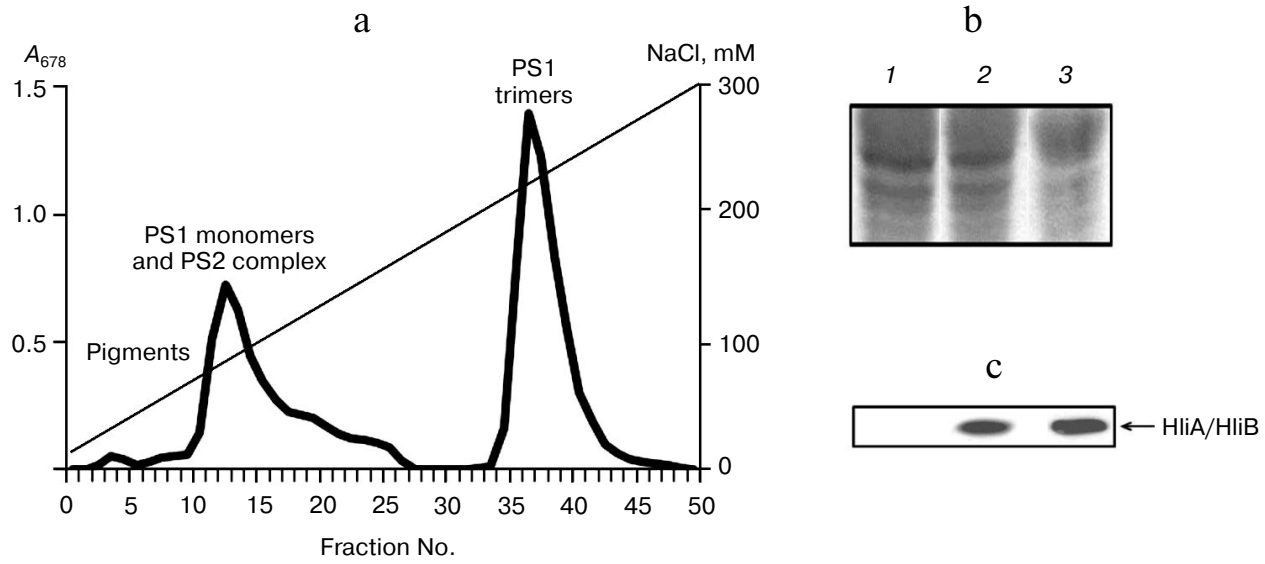

Fig. 4. Association of HliA/HliB proteins with chlorophyll–protein complexes of wild-type *Synechocystis* cells grown in glucose medium under low light (5 μmol photons/m^2 per sec). a) Fractionation profile of chlorophyll–protein complexes from cells exposed to light stress; b) electrophoregram of proteins stained with Coomassie R-250, used for application of equal protein aliquots. Lanes: *1*) fractions 10-14; *2*) fractions 17-22; *3*) fractions 36-40. c) HliA/HliB proteins in the fractions were detected by Western blotting in cells exposed to light stress.

conditions affect HliA/HliB association with chlorophyll–protein complexes, wild-type cells were grown under the same conditions as PS2-deficient mutant cells (Fig. 4) and then exposed to light stress. HliA/HliB proteins were detected in fractions containing PS1 and PS2 monomers, as well as in the fraction containing PS1 trimers (Fig. 4c). Growing cells in glucose-containing medium under low illumination was proven not to cause HliA/HliB absence in wild-type PS1 trimers. Therefore, the absence of HliA/HliB in PS1 trimers of a PS2-deficient mutant is not determined by growth conditions.

DISCUSSION

In contrast to higher plants, up to 80% of chlorophyll in cyanobacterial cells is located in PS1 complex [28], which is present in thylakoids predominantly in the form

of trimers [20, 29-31]. The presence of PS1 trimers is supposed to be essential for higher complex stability and protection from photodestruction [17-30]. Chlorophyll–protein complexes from thylakoid membranes contain, in addition to such core proteins as PsaA, PsaB, and low molecular weight proteins, also additional proteins, such as IsiA and Hli. In this study, we analyzed HliA/HliB localization in chlorophyll–protein complexes of PS1 trimers and monomers in *Synechocystis* PCC 6803 cyanobacteria thylakoid membranes. Most articles devoted to studies of Hli proteins localization in chlorophyll–protein complexes are associated with analyzing their localization in PS2. Light-induced HliA/HliB proteins were shown to be localized in cyanobacterial PS2 [19, 32-35]. HliA/HliB proteins are supposed to participate in chlorophyll stabilization for its re-use in repairing damaged PS2 complex and in biogenesis of newly synthesized PS2 [16, 33, 34]. Contradictory data was obtained on HliA/HliB localization in PS1. For example, HliA/HliB proteins were found not to be associated with *Synechocystis* PS1 [33]. On the other hand, HliA/HliB proteins were shown to be present in PS1 trimers and to be absent in cyanobacterial PS1 monomers [17]. We determined that HliA/HliB proteins are associated with PS1 trimers from cells grown under normal conditions and that their concentration increases 1.7-fold under light stress. Our results correlate with data of Wang et al. [17] on the presence of Hli proteins in PS1 trimers from wild-type cells under stress. Wang et al. [17] did not detect Hli proteins in the fraction containing PS1 monomers and PS2 complex, which may be caused by photodestruction under severe light stress (200-400 μmol photons/m^2 per sec for 12 h). Data similar to ours on the presence of HliA/HliB proteins in cells grown under low intensity light was obtained in an earlier study [19].

Our study of PS2-deficient *Synechocystis* cells has shown for the first time that HliA/HliB proteins in cyanobacterial cells grown under normal conditions are associated with PS1 monomers and that their concentration increases under light stress. This data correlate with the study carried out on higher plant cells. The α-helical protein Ohp from *Arabidopsis thaliana*, which is evolutionarily homologous to Hlip, was shown to be bound to a PS1 monomer [10].

Conflicting data may be explained by the fact that the PS1 protein turnover time greatly exceeds that of PS2 proteins [36]. Because of this, the concentration of additional HliA/HliB proteins participating in chlorophyll salvaging and associated with PS1 is less than in PS2. Their concentration may be lower than the detection limit for the methods used in those studies. Moreover, the differences may be caused by different cell growth conditions [36]. The connection of Hli proteins with PS1 monomers and trimers does not seem surprising since they may apparently assist in chlorophyll accumulation and storage, and function as carrier proteins for chlorophyll in cases of PS1 breakdown and the synthesis of nascent PS1 complexes, as proposed for PS2. Hli proteins are believed to participate in the coordinated pigment and newly synthesized apoprotein delivery system during the biogenesis of PS1 and PS2 photosynthetic complexes, decreasing the risk of phototoxic unbound chlorophyll accumulation [37].

As our data shows, HliA/HliB proteins can be associated with both PS1 monomers and trimers. This suggests that the binding site for these proteins is located on the surface of PS1 monomer and is not shielded during oligomerization. A similar localization on the PS1 surface is typical for the additional protein IsiA [38]. Our studies of PS2-deficient mutants determined that HliA/HliB proteins are not associated with PS1 trimers. The fact that the PS1 trimer relative content in PS2-deficient mutant cells is decreased compared to wild-type cells (Figs. 1a and 2a) supports the hypothesis on the importance of the presence of Hli proteins for PS1 trimer stabilization [17]. PS1 trimers isolated from wild-type cells and PS2-deficient mutants were photochemically active.

It is possible that significant alterations in structure of chlorophyll–protein complexes from PS2-deficient mutant thylakoid membrane structure [39] prevent HliA/HliB binding to PS1 trimers. Apparently, PS2 deletion causes a disruption in the structure of thylakoid membrane chlorophyll–protein complexes and/or an alteration in PS1 trimer structure. This causes HliA/HliB to no longer associate with PS1 trimers in PS2-deficient mutant. Changes in the functional megacomplex consisting of phycobilisome antenna complex, PS1, and PS2 [40] are demonstrated by data on phycobilisome content (absorption maximum at 625 nm). According to absorption spectra of wild-type cyanobacterial cells and PS2-deficient mutant cells normalized at the absorption maximum of chlorophyll, phycobilisome content in PS2-deficient mutant is two times greater than that in wild-type cyanobacterial cells (Fig. 5). Apparently, in the case of PS2 deletion, the structure of thylakoid membrane megacomplex is severely disrupted, which may also cause alterations in PS1 trimer structure, preventing association of HliA/HliB proteins.

To test the hypothesis that cell growth conditions do not cause the deletion of HliA/HliB proteins from PS1 trimers, wild-type cells were grown under the same conditions as the PS2-deficient mutant. HliA/HliB proteins were detected in PS1 trimers of the wild-type cells after light stress. Thus, growing cells in glucose-containing medium under low illumination does not affect the association of HliA/HliB proteins with chlorophyll–protein complexes. Only disruptions caused by the mutation (PS2 deficiency) cause alterations in Hli association with PS1 trimers. As follows from Fig. 2c, the PS2-deficient mutant expresses HliA/HliB proteins even under low illumination (5 μmol photons/m^2 per sec), in contrast to wild-type cells, which do not possess HliA/HliB proteins under these conditions (data not shown). This indicates

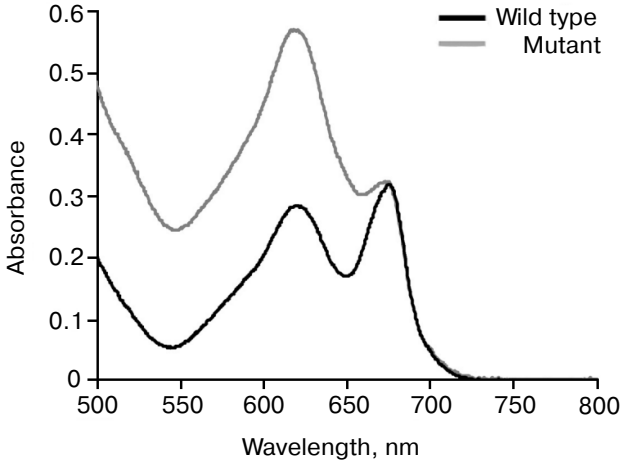

Fig. 5. Absorption spectra of wild-type *Synechocystis* cells and PS2-deficient mutant. The spectra are normalized at the maximum absorption of chlorophyll (680 nm).

the fact that the mutant is more susceptible to light stress, and that HliA/HliB stress protein expression is induced in the mutant even under low illumination. Similar HliA/HliB expression under low illumination was shown for PS1-deficient mutants [17, 33].

To reveal possible interdependence of HliA/HliB association with PS1 monomers and trimers, the Δ*psaL* mutant was studied, which lacks PS1 trimers. Absence of PS1 trimers was found to have no effect on HliA/HliB binding to PS1 monomers and PS2 complexes. Apparently, alterations in thylakoid membrane cause by the absence of PS1 trimers are not so great and do not cause changes in HliA/HliB protein binding to other thylakoid chlorophyll–protein complexes.

Thus, our results show that HliA/HliB proteins can bind to basic chlorophyll–protein complexes of cyanobacterial thylakoid membranes: to PS1 monomers, PS2 complex [19], and PS1 trimers. The association of HliA/HliB with PS1 as well as with PS2 indicates their universal role in protection of the cyanobacterial photosystems.

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