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In vitro reconstitution of complexes of stress HliA protein with pigments

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Abstract

Proteins similar to Hli (high light inducible) proteins of cyanobacteria are present in all photosynthetic eukaryotes and are necessary for survival in various stressful conditions, although their exact function is not fully understood. In current study, the recombinant stress-induced protein HliA of cyanobacterium *Synechocystis* was isolated and characterised for the first time. The synthetic gene of HliA protein was created and cloned into plasmid for expression of recombinant protein with Hisx6-tag at the C-terminus in bacteria. Recombinant HliA protein of *Synechocystis* was isolated by metal-affinity chromatography. The HliA protein was reconstituted with chlorophyll a and carotenoids. Using circular dichroism spectroscopy, it was shown that chlorophyll a and carotenoids interact in vitro with the HliA protein. The binding of pigments to the HliA protein favours the protective function of this protein. Apparently, Hli proteins are involved in the coordinated delivery of pigments for the biogenesis of photosynthetic complexes, thereby reducing the risk of accumulation of phototoxic free chlorophyll molecules. Current results are important for understanding the processes of photoprotection in either cyanobacteria or algae and higher plants.

Keywords

Carotenoid, chlorophyll, cyanobacteria, high light inducible proteins, high light stress, *Synechocystis*

Introduction

Cyanobacteria are ubiquitous and inhabit almost all ecosystems, thanks to their effective adaptation to various conditions, including extreme ones. Therefore, these phototrophs are a promising model system for studying mechanisms of resistance and adaptation to various stresses, including one of the most common – light stress.

Under light stress, the photosynthetic apparatus is damaged due to the accumulation of both toxic free chlorophyll molecules and the formation of reactive oxygen species. For the normal functioning of photosynthetic organisms under conditions of light stress, numerous protective mechanisms have emerged in the course of evolution. Protective mechanisms involving light-induced stress proteins are of great importance. These include cyanobacterial Hlips (high light-inducing proteins) with a single transmembrane helix (Chidgey et al. 2014; Hey and Grimm 2020).

Hli proteins are necessary for cell survival under light stress and other stressful conditions, as well as for maintaining normal cell activity. It is assumed that Hli proteins take part in important processes, such as: regulation of chlorophyll biosynthesis; transport and binding of free chlorophyll molecules; quenching of singlet oxygen; assembly and repair of photosystem 2; non-photochemical dissipation of absorbed light energy (Komenda and Sobotka 2016). However, the full picture of the functioning of Hli proteins has not been fully studied.

Four Hli proteins (HliA, B, C and D) were found in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Staleva et al. 2015). These proteins have been isolated as part of small complexes with other proteins and only recently, the first individual HliC protein from this family has been purified (Shukla et al. 2018). There is no such information about other representatives of Hli proteins. Of particular interest is the protein of this family – HliA, since it is important for the survival of *Synechosystis* cells under light stress (He et al. 2001). To protect cells from excessive light, binding of toxic free chlorophyll molecules, which cause photodestruction and oxidative stress, is necessary. The stress protein HliA can serve as such a key agent binding these molecules. Therefore, to understand this process, it is necessary to obtain a recombinant HliA protein and its subsequent complex characterisation and study of its interaction with chlorophyll *in vitro*. Isolation and characterisation of pure HliA protein has not yet been carried out. Until recently, it was not clear whether the pigments (chlorophyll a and beta-carotene) bind to the HliA protein.

The aim of the study was to isolate pure recombinant HliA protein and reconstruct the HliA protein complex with pigments (chlorophyll a and carotenoids).

Materials and methods

HliA gene expression and protein purification

Nucleotide sequence of the *hliA* gene of *Synechocystis* sp. PCC6803 (hereinafter *Synechocystis* is used) was obtained from CyanoBase (http://genome.annotation.jp/

cyanobase/Synechocystis/genes/ssl2542 ID ssl2542). This sequence was optimised for expression in bacteria and the synthetic gene was created. The gene was cloned into the pET28 vector in open reading frame with 6xHis at the C-terminus of resulting protein. *E.coli* BL(DE3) cells were transformed with obtained plasmid. HliA protein synthesis with His6 at the C-terminus (.....HGVIGWLNSL) 6xHis tag) was achieved by induction with IPTG. Recombinant protein was isolated from *E.coli* cells using metal-affinity chromatography on Ni-NTA Resin (Thermo-Fisher, USA) according to the manufacturer's recommendation. Protein was stored at 4 °C in the presence of 1 mM sodium azide. Protein concentration was determined spectrophotometrically at 280 nm, using extinction coefficients from ProtParam programmes in ExPASy for calculation (https://web.expasy.org/protparam/).

The reconstitution of complexes of HliA-6xHis protein and pigments

After metal-affinity chromatography, fractions containing HliA protein (0.5–1 mg/ml) were dialysed against the buffer: 15mM NaH_2PO_4 x $2H_2O$, 0.07M KF, 15mM urea, 0.1mM PMSF, pH 4.5 overnight at 4 °C and the protein concentration was measured spectrophotometrically using absorbance at 280 nm. Pigments (total chlorophyll a and carotenoids) were previously isolated from *Synechocystis* cells according to the method by Natali et al. (2014). The pigments were re-suspended in a buffer 15mM NaH_2PO_4 x $2H_2O$, 0.07M KF, 0.015M urea, 0.1mM PMSF, pH 4.5 and were sonicated on ice three times for 20 seconds with an interval of 10 seconds. Before the reconstitution, the HliA solution was subjected to short-term heating (95 °C) for 1 minute. Then the sample was transferred to ice and pigment extract was added in an equimolar ratio (1:1). After incubation on ice for 30 minutes, the sample was used for analysis.

Western blotting

Using SDS electrophoresis and subsequent Western blotting, the content (level) of recombinant HliA protein in *E.coli* cell lysate was evaluated (Akulinkina et al. 2015). Aliquots of HliA protein were separated in 12.5% SDS-polyacrylamide gel. The gel was stained by Coomassie R-250 or was used for immunodetection. The proteins were transferred from gel on to a nitrocellulose membrane (pore size of 45 mkm). Then the membrane with the transferred proteins was incubated for 1h at 4 °C in TBST buffer (50mM Tris-HCl, 200 mM NaCl, 0.1% Tween 20, pH 7.5), supplemented with 5% dry milk and then primary antibodies were added. We used polyclonal rabbit antibodies to HliA (1:4000) (Abcam, USA) or antibody in TBST buffer to 6X His-tag (1:5000) (ab213204, USA). The membrane was incubated with the antibodies overnight at 4 °C. The blots were rinsed and incubated with a secondary antibody – goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000) (Agrisera, Sweden). 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).

Fluorescence spectroscopy

Fluorescence measurements were made on the Spectrofluorophotometer RF-5301 PC (Shimadzu, Japan) at 20 °C. Tryptophan residues make a decisive contribution to protein fluorescence. HliA protein contains two tryptophan residues. Fluorescence spectra of HliA protein (0.5 mg/ml) along with reconstituted complexes of protein with pigments in buffer (15mM NaH_2PO_4 x $2H_2O$, 0.07M KF, 15mM urea, 0.1mM PMSF, pH 4.5) were excited at 295 nm (2 nm slit bandwidth) and were recorded in the range of 610–750 nm (5 nm slit bandwidth) at a speed of 200 nm/min.

Circular dichroism spectroscopy

The CD spectra of HliA protein (0.5 mg/ml) and reconstituted complexes of protein with pigments in buffer: 15mM NaH_2PO_4 x $2H_2O$, 0.07M KF, 15mM urea, 0.1mM PMSF, pH 4.5, were performed on a Chirascan CD dichrograph (Applied Photophysics, UK) equipped with a thermal insert at 13 °C. The CD spectra were recorded in the range of 190–260 nm and 400–740 nm at a speed of 20 nm/min in a 0.02 cm quartz cuvette. Then the signal of buffer was subtracted from the spectra, prescribing its spectrum under identical conditions.

Results

Isolation and purification of HliA-6xHis protein

Purified recombinant HliA protein of *Synechocystis* was isolated by metal-affinity chromatography for the first time (Fig. 1A). The isolated protein had the expected molecular weight 10 kDa (Fig. 1A). The protein was present mainly in monomeric form. Sometimes a small number of HliA protein dimers have been detected. The isolation of HliA protein was confirmed by Western blotting using antibodies to HliA (Fig. 1B).

Obtaining purified HliA protein is of interest due to the possibility of studying its spectral properties, as well as studying its functions and interaction *in vitro* with pigments. This would serve as a direct proof of the binding of the pigment by protein. Most analyses, with the exception of structural analysis using X-ray crystallography or NMR, do not require the removal of the His-tag. Its presence does not have a significant impact on the result of the analysis. The method of circular dichroism spectroscopy was used to characterise the secondary structure of the HliA protein. The data of CD spectroscopy of HliA protein were processed using the DichroWeb programme (http://dichroweb.cryst.bbk.ac.uk/). It should be noted that there are relatively few spectra of membrane proteins in the CD database, which is due to their low solubility.

The CD spectrum in the far UV of the HliA protein preparation shows the presence of a secondary structure (Fig. 2) and corresponded to the alpha-helical structure of the protein. According to calculations, the alpha helix segments make up 61%. The crystallographic structure of the HliA protein and other Hli proteins has not yet been studied.

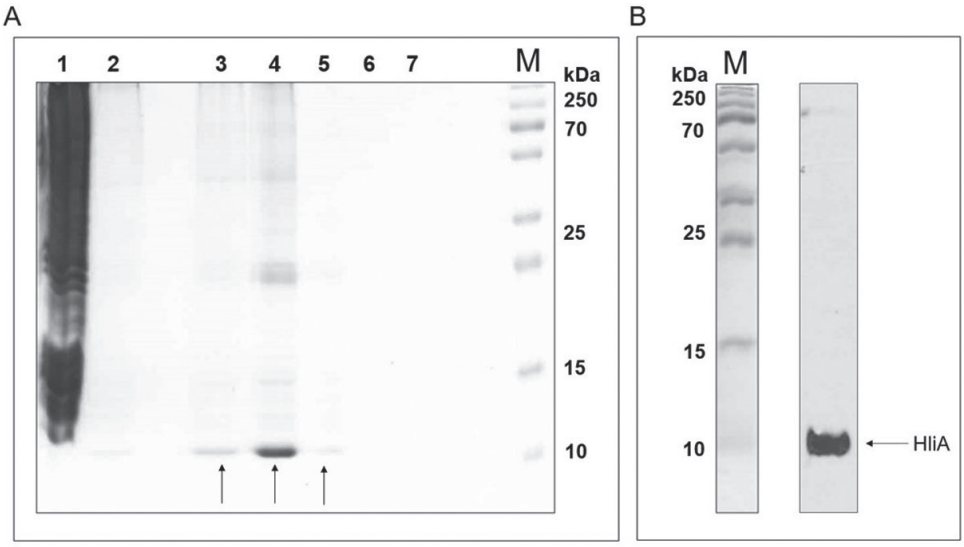

Figure 1. SDS-PAGE (**A**) and immunoblot analysis of the HliA eluate **A** fraction collected during His-tag affinity chromatography (separation) of HliA eluate were further separated by SDS-electrophoresis and were stained with Coomassie brilliant blue. The numbers at the top of the gel indicate elution fraction numbers from the column. The arrows show the location of the HliA protein in the gel **B** the gel was blotted and the HliA protein immunodetected.

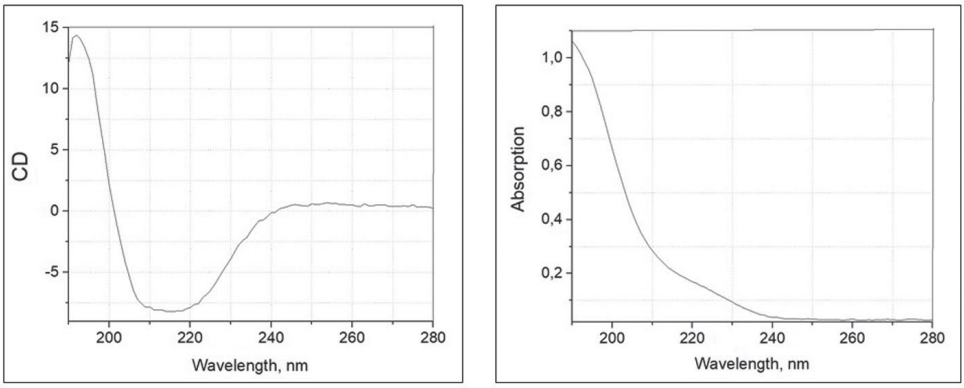

Figure 2. Circular dichroism **A** and absorption **B** spectra of the HliA protein.

Reconstitution of complexes of stress HliA protein with pigments

Pigments (chlorophyll a and carotenoids), isolated from *Synechocystis*, were used in HliA protein-binding experiments. It was planned to find out whether the HliA protein monomer has the ability to bind pigments.

Fluorescence spectra of HliA protein and HliA protein with pigments were studied (Fig. 3). As follows from the Figure, more intense fluorescence was shown for the sample of HliA protein with pigments then pure HliA protein. This may be due to the binding of the pigment to the formation of the complex.

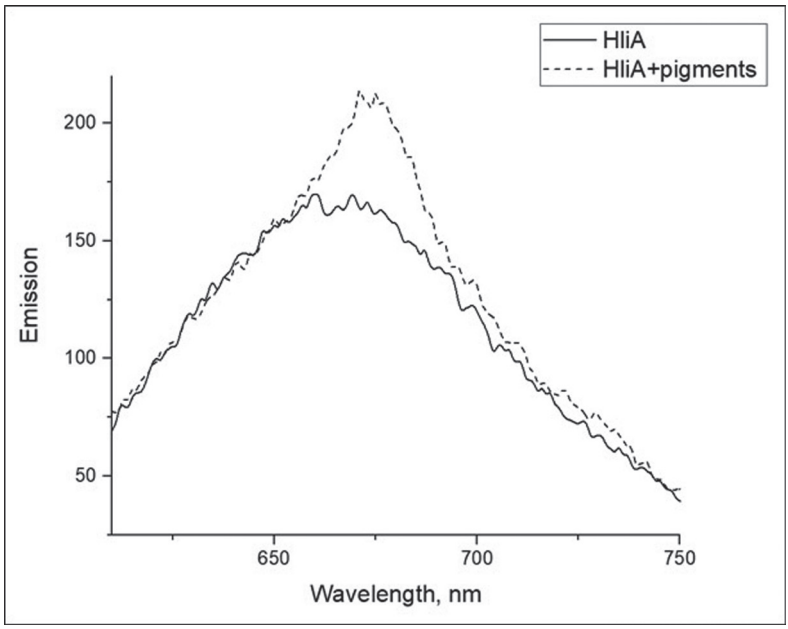

Figure 3. Fluorescence emission of reconstituted pigment-HliA protein (---) and HliA protein (—) samples.

Then the CD spectra of pigments (chlorophyll and carotenoids), HliA protein and their mixture were studied. A change in the CD spectra in the far red (640–740 nm) region of the HliA protein, incubated with pigments in comparison with the control pigments sample, was detected. This result indicates the interaction of chlorophyll with HliA protein. Small signal changes in the CD spectra in the 480–507 nm region were also detected, indicating the interaction of HliA protein with carotenoids (https://conf.icgbio.ru/plantgen2021/en/2021/06/06/detection-of-the-binding-the-stress-hlia-protein-synechocystis-sp-with-pigments/). Apparently, no more than 1–2 molecules of chlorophyll and carotenoids bind to the protein. Analysis of CD spectra of the HliA protein with pigments showed that the sample contained the monomeric chlorophyll. Thus, it has been shown experimentally that HliA protein molecules have the ability to bind pigments.

Discussion

In previously published papers, the HliA protein was studied in complex with other Hli proteins. In this work, the pure recombinant protein HliA *Synechocystis* was isolated for the first time. The pigment-binding capacity of the HliA protein was studied and the reconstruction of the HliA protein with pigments was carried out. CD spectroscopy has shown that chlorophyll a and carotenoids interact *in vitro* with the HliA protein.

The data obtained by us are consistent with the fact that Hli proteins contain conserved amino acid residues that are important for the binding of chlorophyll in

all proteins of light-harvesting complexes (Shukla et al. 2018). The HliA protein also contains a chlorophyll binding domain.

It is known that free chlorophyll molecules in a cell are dangerous for cells due to the threat of the formation of damaging reactive oxygen species. In this regard, the binding of pigments to the HliA protein revealed by us indicates the protective role of this protein. Apparently, Hli proteins can participate in the coordinated delivery of pigments in the biogenesis of photosynthetic complexes, reducing the risk of accumulation of phototoxic unrelated chlorophyll molecules (Shukla et al. 2018).

We found a smaller number of bound chlorophyll molecules (1–2) per protein than was hypothetically assumed (4–5 molecules). Perhaps this is due to the fact that a complex with other protein molecules is needed to bind more chlorophyll molecules. Apparently, the association of HliA protein with lipid components of the membrane is necessary for the binding of a large quantity of pigment molecules. It is possible that the binding of pigments depends on the formation of HliA/HliB heterodimers or HliA protein homodimers. It is assumed that Hli proteins are important for the re-utilisation of free chlorophyll molecules (Shukla et al. 2018).

The study of the constitution of the recombinant HliA protein with chlorophyll a and carotenoids *in vitro* allows us to expand knowledge about the photoprotective functions of light-induced Hli proteins. The results are important for understanding photoprotection processes in both cyanobacteria and algae and higher plants. Knowledge about the mechanism of functioning of stress proteins can also be used in the creation of artificial photosynthesis systems.

Conclusion

Pure recombinant protein HliA *Synechocystis* was isolated by Ni-affinity chromatography. The binding of pigments to HliA was shown, which confirms the protective function of this protein. Apparently, HliA proteins are involved in the coordinated delivery of pigments in the biogenesis of photosynthetic complexes, reducing the risk of accumulation of phototoxic free chlorophyll molecules. The results are important for understanding photoprotection processes in both cyanobacteria and algae and higher plants.

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References

Akulinkina DV, Bolychevtseva YuV, Elanskaya IV, Karapetyan NV, Yurina NP (2015) Association of high light-inducible HliA/HliB stress proteins with photosystem 1 trimers and monomers of the Cyanobacterium *Synechocystis* PCC 6803. Biochemistry (Moscow) 80(10): 1254–1261. https://doi.org/10.1134/S0006297915100053

Chidgey JW, Linhartová M, Komenda J, Jackson PJ, Dickman MJ, Canniffe DP, Koník P, Pilný J, Hunter CN, Sobotka R (2014) A cyanobacterial chlorophyll synthase-HliD complex associates with the Ycf39 protein and the YidC/Alb3 insertase. The Plant Cell 26(3): 1267–1279. https://doi.org/10.1105/tpc.114.124495

He Q, Dolganov N, Bjorkman OAR, Grossman AR (2001) The high light-inducible polypeptides in *Synechocystis* PCC6803. Expression and function in high light. The Journal of Biological Chemistry 276(1): 306–314. https://doi.org/10.1074/jbc.M008686200

Hey D, Grimm B (2020) One-Helix Protein1 and 2 form heterodimers to bind chlorophyll in photosystem ii biogenesis. Plant Physiology 183(1): 179–193. https://doi.org/10.1104/pp.19.01304

Komenda J, Sobotka R (2016) Cyanobacterial high-light-inducible proteins– Protectors of chlorophyll-protein synthesis and assembly. Biochimica et Biophysica Acta 1857(3): 288–295. https://doi.org/10.1016/j.bbabio.2015.08.011

Natali A, Roy LM, Croce R (2014) In vitro reconstitution of light-harvesting complexes of plants and green algae. Journal of Visualized Experiments 92(92, e51852): 1–13. https://doi.org/10.3791/51852

Shukla MK, Llansola-Portoles MJ, Tichý M, Pascal AA, Robert B, Sobotka R (2018) Binding of pigments to the cyanobacterial high-light-inducible protein HliC. Photosynthesis Research 137(1): 29–39. https://doi.org/10.1007/s11120-017-0475-7

Staleva H, Komenda J, Shukla MK, Šlouf V, Kaňa R, Polívka T, Sobotka R (2015) Mechanism of photoprotection in the cyanobacterial ancestor of plant antenna proteins. Nature Chemical Biology 11(4): 287–291. https://doi.org/10.1038/nchembio.1755