S35-023

Heat shock proteins play a role in both high and low temperature stresses in cyanobacteria

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Keywords: Cyanobacterium, Small heat shock protein, HtpG, Thermotolerance, Photoinhibition

Introduction

Cyanobacteria, like other organisms, synthesize a diverse range of heat-shock proteins (Hsps) upon exposure to high temperatures. In order to demonstrate a specific contribution of a Hsp in thermotolerance in cyanobacteria, mutants of the *clpB*, *hsp16.6* (small Hsp homolog), and *htpG* genes have been constructed by gene-targeting (Eriksson and Clarke 1996, Tanaka and Nakamoto 1999, Lee et al. 2000). Interestingly, all these disruptants showed much more striking thermosensitive phenotypes than corresponding mutants of other heterotrophic prokaryotes, suggesting that those Hsps are particularly important for photoautotrophic organisms. Here, we will show that HspA, a small Hsp homolog, and HtpG, a homolog of Hsp90, play roles in the acquisition of high and low temperature tolerances in cyanobacteria. Compared with GroEL (Hsp60 homolog) and DnaK (Hsp70 homolog), functions of small Hsp and HtpG are enigmatic. HspA from *Synechococcus vulcanus* and HtpG from *Synechococcus* sp. PCC 7942 form a homo-oligomer consisting of 24 subunits (Roy et al. 1999) and a homo-dimer (unpublished data), respectively. Both Hsps prevent the aggregation of model substrates at high temperatures (Roy et al. 1999 and unpublished data). The in vivo substrate(s) for none of the Hsps has been identified yet. Upon heat shock, small Hsp accumulates as a major Hsp in cyanobacteria (Roy et al. 1999), while HtpG appears to be a minor one (unpublished data).

Materials and methods

Organisms and culture conditions. The *Synechococcus* sp. PCC 7942 cells were cultured photoautotrophically in BG-11 inorganic medium (Tanaka and Nakamoto 1999). The liquid culture in a flat glass vessel was continuously aerated. Unless otherwise indicated, cultures were grown at 30^{o}C with a light intensity of 30 to 40 μE/m^2/s. In order to express the *hspA* gene from *Synechococcus vulcanus* constitutively in *Synechococcus* sp. PCC 7942, it was transformed with an expression vector (pECAN8) carrying the *hspA* coding sequence, resulting in the ECT16-1 strain (Nakamoto et al. 2000). The HspA protein was overexpressed under the control of the *tac* promoter. The reference strain, ECT, was obtained by transforming the cyanobacterium with the vector containing no *hspA* gene. A knockout mutant of the *htpG* gene was constructed by insertion (Tanaka and Nakamoto 1999).

Viability assays and measurements of whole cell absorbance were performed as described previously (Nakamoto et al. 2000).

Measurements of oxygen evolution. Rates of oxygen evolution by cells were measured either in the presence of 10 mM $NaHCO_3$ for whole-chain electron transport or 1 mM 1,4-

benzoquinone and 1 mM $K_3Fe(CN)_6$ for photosystem II activity with a DW1 oxygen electrode unit (Hansatech) at 30°C at a photosynthetic photon flux density of 1,200 μE/m²/s.

Protein extraction from Synechococcus sp. PCC 7942 cells. Cells harvested by centrifugation were suspended in 25 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and 1 mM each of phenylmethylsulfonyl fluoride, benzamidine and caproic acid, mixed with glass beads, and disrupted by vortexing vigorously for 3 min. This process was repeated three times with a 3-min interval on ice between the vortexing, and the resulting suspension was centrifuged at 16,000 g for 30 min. The supernatant fraction was used for the non-denaturing polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate (SDS)-PAGE, and Western blot analysis (Roy et al. 1999).

Results and Discussion

Small Hsp confers cellular thermotolerance. The transformant (ECT16-1) which was shown to constitutively express HspA displayed improved viability compared with the reference strain (ECT) upon transfer from 30°C to 50°C in the light (Table 1). In contrast to our results, overexpression of IbpA and IbpB (small Hsp homologs) in *E. coli* led to 5- to 10-fold reductions in viability (Thomas and Baneyx 1998). When the heat shock was given in darkness, the survival rate in the reference strain increased greatly, exceeding the level for the HspA expressing strain after heat shock in the light (Table 1). These results indicate that heat stress produces a much severer effect on the cellular survival in the light than in the dark, and small Hsp plays an important role especially under the photoautotrophic conditions.

Table 1. Survival rate, photosystem II (PSII) activity, and phycocyanin level of the ECT and ECT16-1 strains after a direct shift to 50°C either in the light (30 - 40 μE/m²/s) or in the dark.

Strain	Survival rate		PSII activity		Phycocyanin	
	Time after a shift to 50°C		Time after a shift to 50°C		Time after a shift to 50°C	
Heat shock	0	15 min	0	15 min	0	15 min
in the light						
ECT	100%	2%	100%	ND	100%	~30%
ECT16-1	100%	28%	100%	33%	100%	~80%
in the dark						
ECT	100%	32%	100%	ND	100%	~100%
ECT16-1	100%	47%	100%	9%	100%	~100%

In each heat-shock treatment shown, the value obtained before the shift to 50°C was taken as 100%. ND, not detectable. Phycocyanin level was approximated from a decrease at 625 nm in the whole cell absorbance after the shift to 50°C (see Nakamoto et al. 2000).

Identification of the possible sites of action of the small Hsp in vivo to be the photosystem II (PSII) complex and the light-harvesting phycobilisomes. In the ECT strain, PSII was heat-inactivated both in light and darkness, while the inactivation of phycocyanin took place only in the light. Expression of HspA increased thermal resistance of both PSII and phycocyanin (Table 1). Although PSII was completely inactivated in darkness as well as in the light, the survival rate increased markedly in darkness as shown above. Thus, PSII inactivation may not be the primary cause for the cell death at elevated temperatures. We propose that the

phycobilisomes may interact with PSI when PSII is inactivated. The PSI with the light-harvesting apparatus produces ATP through the cyclic electron flow, which may be used to restore PSII and other functions, and thus contribute for the increase in survival rate.

Small Hsp plays a role in the protection of the physical order of thylakoids under high temperatures. The ECT and ECT16-1 cells after exposure at 50°C for 15 min were examined by transmission electron microscopy. Significant differences were observed in cell ultrastructure between these strains. While the integrity of thylakoid membranes was disrupted in heat-shocked ECT cells, concentric layers of thylakoids at the periphery of cells were still observed in heat-shocked ECT16-1 cells (data not shown). Thus, constitutive expression of the HspA protein increased the physical order of thylakoid membranes, thus stability at elevated temperatures. The association of HspA with thylakoid membranes may serve as a membrane protection mechanism. Experiments such as immunogold localization of HspA using transmission electron microscopy are in progress.

HtpG is essential for the thermal stress management. We inactivated the *htpG* gene in the cyanobacterium, *Synechococcus* sp. PCC 7942 by gene-targeting to elucidate the role of HtpG *in vivo* (Tanaka and Nakamoto 1999). The mutant cells lost both basal and acquired thermotolerances, indicating that HtpG plays an essential role for the thermotolerance in cyanobacteria. These results with *Synechococcus* are in contract to those with *E. coli* (Bardwell and Craig 1988, Thomas and Baneyx 1998) and *B. subtilis* (Versteeg et al. 1999). Inactivation of the *htpG* gene in these heterotrophic organisms did not cause a fatal effect on cells at elevated temperatures. The photosynthetic oxygen evolution by whole-chain electron transport in the mutant was more sensitive to high temperatures than that in the wild type (data not shown), indicating that the photosynthetic apparatus may be one of the targets which HtpG may interact with.

HtpG plays a role in the acclimation to low temperatures. The inactivation of the *htpG* gene resulted in severe inhibitions of cell growth and the photosynthetic activity when the *htpG* mutant was shifted to 16°C from 30°C (data not shown). After the shift, the photosynthetic activity of the mutant continued to decrease with only negligible activity remaining after 3 days at 16°C, while the wild type kept approximately 20% of the level at 30°C even after 5 days at 16°C. The wild-type cells were able to resume growth without a lag period when shifted to 30°C after 5 days at 16°C, while the mutant displayed a detectable lag (data not shown). Electrophoresis in the absence of SDS showed that a novel high-molecular-weight (450-kDa) complex containing a 48-kDa polypeptide, GroEL, and DnaK accumulated in the wild-type cells grown at 16°C for 5 days, but the accumulation was strongly inhibited in the *htpG* mutant (Fig.1). In *E. coli*, GroEL is an 800 kDa assembly, a tetradecamer of 57 kDa subunits arranged in two stacked seven-membered rings. In cyanobacteria, GroEL may also be assembled into a tetradecamer (Lehel et al. 1992). Thus, it is unexpected that the GroEL protein exists in the 450-kDa complex. GroES was not detected immunochemically in this complex (data not shown). Since the amount of the 450-kDa protein complex was reduced in the mutant cells, the HtpG protein may be involved in the cold-induced assembly of this novel multiprotein complex. The complex may play a role in the cold acclimation. Under low temperatures, the assembly of the GroEL tetradecamer and further binding of the GroES heptamer to GroEL may be inhibited. Instead, the GroEL protein may form a novel multiprotein complex with DnaK and the 48-kDa protein.

Constitutive expression of small Hsp in the htpG mutant. The *htpG* mutant in which the HspA protein was constitutively expressed was constructed in the same manner as the construction of ECT16-1. In vitro studies have shown that small Hsp and HtpG function similarly as molecular chaperones. They keep a heat-denatured model substrate in a soluble folding-competent state which may be refolded by an ATP-dependent molecular chaperone machine such as GroES/GroEL or DnaK/DnaJ/GrpE. Thus, we thought that the thermolabile

htpG mutant may be complemented with a return to the wild-type phenotype by the constitutive overexpression of the small Hsp. However, it did not alleviate the inhibition of growth at 45°C (see Tanaka and Nakamoto 1999) in the *htpG* mutant (data not shown).

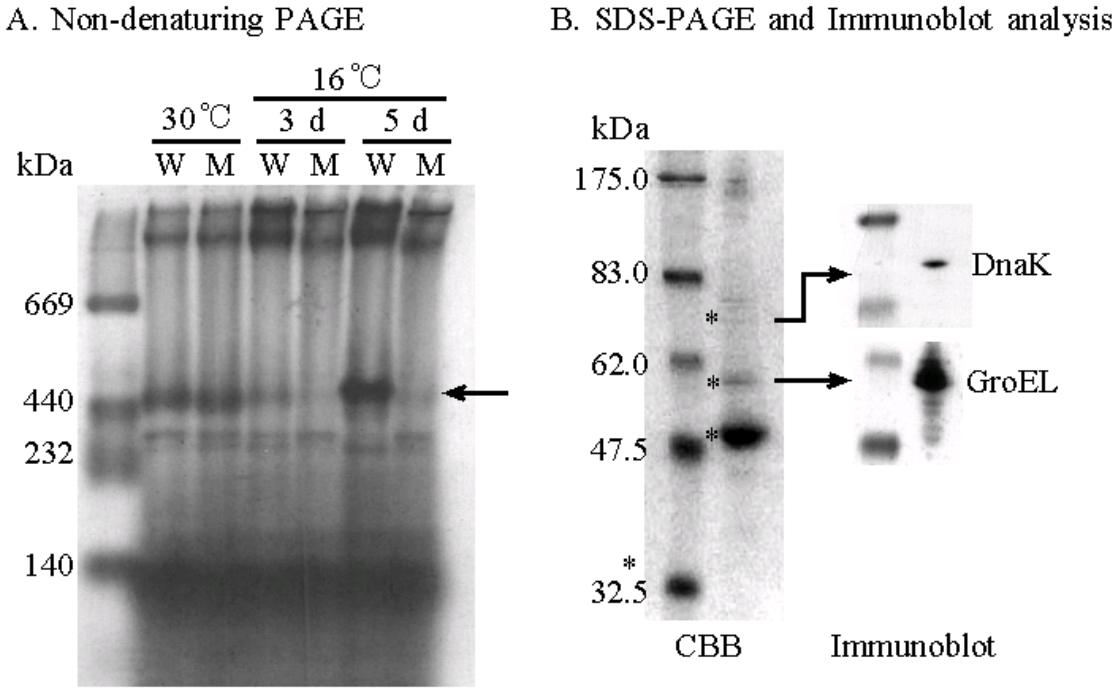

Fig. 1. Detection of a 450-kDa protein complex (indicated by the arrow) by non-denaturing PAGE (A) and detection of its components by SDS-PAGE and immunoblot analysis (B). In A, equal amounts of crude soluble proteins extracted from the wild type (indicated by 'W') or the *htpG* mutant (indicated by 'M') cells grown at 30°C, and then shifted down to 16°C for 3 days, or 5 days were separated by PAGE containing no SDS and the gel was stained by coomassie brilliant blue (CBB). In B, a CBB-stained gel strip containing the 450-kDa band indicated by the arrow in A was excised from the non-denaturing gel, applied to a sample well of a SDS-polyacrylamide gel and electrophoresed. Then, the gel was either stained by CBB, or the GroEL and DnaK proteins were specifically detected by immunoblot analysis using polyclonal antibodies raised against *Synechococcus vulcanus* GroEL and DnaK. The CBB-stained bands corresponding to DnaK, GroEL, and a 48-kDa polypeptide are indicated by asterisks.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research (C) (no. 11640641 and 13640640) to H.N. from the Ministry of Education, Science, Sports and Culture, Japan. M.M.H. is a recipient of the Japanese government (MONBUKABAKUSHO) scholarship.

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