Nitrogen status and heat-stress-dependent differential expression of the cpn60 chaperonin gene influences thermotolerance in the cyanobacterium Anabaena

Hema Rajaram and Shree Kumar Apte

Molecular Biology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085, India

Heat stress caused rapid and severe inhibition of photosynthesis and nitrate reduction in nitrate-supplemented cultures of the cyanobacterium Anabaena sp. strain L-31, compared to nitrogen-fixing cultures. Anabaena strains harbour two hsp60 family genes, groEL and cpn60, respectively encoding the 59 kDa GroEL and 61 kDa Cpn60 chaperonin proteins. Of these two Hsp60 chaperonins, GroEL was strongly induced during heat stress, irrespective of the nitrogen status of the cultures, but Cpn60 was rapidly repressed and degraded in heat-stressed nitrate or ammonium-supplemented cultures. The recovery of photosynthesis, nitrate assimilation and growth in heat-stressed, nitrate-supplemented cultures were preceded by resynthesis and restoration of cellular Cpn60 levels. Glutamine synthetase activity, although adversely affected by prolonged heat stress, was not dependent on either the nitrogen status or Cpn60 levels during heat stress. Overexpression of the Cpn60 protein in the closely related Anabaena sp. strain PCC7120 conferred significant protection from heat stress to growth, photosynthesis and nitrate reduction in the recombinant strain. The data favour a role for Cpn60 in carbon and nitrogen assimilation in Anabaena.

INTRODUCTION

The heat-shock response (HSR) involves de novo synthesis of heat-shock proteins (Hsps) comprising several chaperones (Hsp70, Hsp60) and proteases (Lon, Clp). HSR is a universal stress response that living organisms use to restore homeostasis during short-term temperature up-shifts and other environmental stresses (Morimoto et al., 1994; Yura et al., 2000). Although studied in great detail in several bacteria and higher organisms, the HSR is not so well understood in nitrogen-fixing cyanobacteria. Most bacteria possess a single bicistronic groESL operon harbouring the groES and groEL genes encoding a 10 and a 60 kDa chaperonin, respectively (Yura et al., 2000). Occurrence of additional hsp60 genes in Streptomyces species (Guglielmi et al., 1991), Mycobacterium tuberculosis and M. leprae (Rinke de Wit et al., 1992), and of multiple groEL genes in rhizobia (Rusanganwa & Gupta, 1993) and Bradyrhizobium japonicum (Fischer et al., 1993) has also been reported. All cyanobacteria whose genomes have been completely sequenced exhibit two distinct hsp60 genes, a groEL gene as part of the groESL operon and a solitary cpn60 gene (Chitnis & Nelson, 1991; Lehel et al., 1993; www.kazusa.or.jp/cyano).

Earlier work from our laboratory showed that (a) the two Hsp60 protein-encoding genes are expressed abundantly in the nitrogen-fixing cyanobacterium, Anabaena sp. strain L-31 (hereafter referred to as Anabaena L-31) during exposure to heat and other environmental stresses (Apte et al., 1998; Bhagwat & Apte, 1989), and (b) nitrogen-fixing Anabaena L-31 cultures recover from prolonged exposure to continuous heat stress as a consequence of continuous synthesis, greater stability and accumulation of the two Hsp60 proteins during heat stress (Rajaram & Apte, 2003). In this study, we have cloned the cpn60 gene from Anabaena L-31 and compared its expression with that of groEL (Rajaram et al., 2001; Rajaram & Apte, 2003) in response to nitrogen status and heat stress. Our results demonstrate that loss of Cpn60 during heat stress in nitrate-supplemented cultures of Anabaena correlates with the inactivation of the photosynthetic machinery and nitrate reduction, while Cpn60 overexpression enhances the thermal stability of these vital metabolic processes.

METHODS

Organism and growth conditions. Anabaena L-31 (Thomas, 1970) and Anabaena sp. strain PCC7120 (hereafter referred to as Anabaena...
RESULTS

Nitrogen-status-dependent effect of heat stress on major metabolic activities in Anabaena L-31

Anabaena L-31 cells were unable to grow upon continuous exposure to heat stress at 42 °C, irrespective of the nitrogen status of the growth medium (data not shown). At 27 °C, photosynthetic activity was comparable in the presence or absence of combined nitrogen, but decreased to undetectable levels within 15 h in nitrogen-supplemented (NO$_3^-$ or NH$_4^+$) cultures. In comparison, nitrogen-fixing cultures showed very slow inhibition of photosynthesis (Fig. 1a). The nitrate reductase activity, in the absence (L-NR) or

Table 1. List of primers used for PCR amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>(*Underlined sequences are cut by the restriction enzyme shown in parentheses.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpn60F2</td>
<td>GTATCCCTACATGAGCATCAAG</td>
<td></td>
</tr>
<tr>
<td>cpn60R1</td>
<td>ACGATCGTCTCGGTTG</td>
<td></td>
</tr>
<tr>
<td>cpn60OE</td>
<td>GGCAATGGCATAATTTCAT (Ndel)</td>
<td></td>
</tr>
<tr>
<td>cpn60OERev</td>
<td>GCCCTCGAGAAC ATACCCATACCACC (Xhol)</td>
<td></td>
</tr>
<tr>
<td>cpn60OERev1</td>
<td>CCCTGCTTAGAACAATACCATC (BamHI)</td>
<td></td>
</tr>
<tr>
<td>CgroELFwd</td>
<td>GCCCATATGGCAAGGCGATATTTCAT (Ndel)</td>
<td></td>
</tr>
<tr>
<td>CgroELRev</td>
<td>CGCGATCCCTAGTTAATCGAAGTACCCGACC (BamHI)</td>
<td></td>
</tr>
</tbody>
</table>

DNA isolation and dot blot hybridization. DNA was isolated from Anabaena cultures as described previously (Rajaram et al., 2001) and treated with DNeasy for removing any DNA contamination. Purified DNA (5 μg per spot) was spotted onto nylon membranes (Roche Diagnostics), cross-linked with UV and hybridized with specific DIG-labelled DNA probes as described previously (Rajaram et al., 2001).

Generation of antibodies against purified Cpn60 and GroEL proteins of Anabaena L-31. The 1.7 kb Ndel/Xhol fragment of the cpn60 gene, amplified from pH302 using cpn60OE and cpn60OERev primers (Table 1), was cloned at identical sites in the overexpression vector pET29a. The 1.6 kb Ndel/BamHI fragment of the groEL gene, amplified from Anabaena L-31 chromosomal DNA using primers CgroELFwd and CgroELRev (Table 1), was cloned at identical sites in the overexpression vector pET16b. The resulting constructs, pETcpn60 and pETgroEL, were transformed into Escherichia coli BL21(pLysS) cells. The Cpn60 and GroEL proteins were overexpressed by induction with 1 mM IPTG at 37 °C for 1 h and purified under denaturing conditions (8 M urea) using NiNTA affinity chromatography (Qiagen). Purified Cpn60 and GroEL proteins of Anabaena L-31 were used to generate the corresponding polyclonal antibodies in rabbit.

Western blotting and immunodetection. Proteins were extracted, resolved by 10% linear SDS-PAGE, or by 2-D IEF/SDS-PAGE and electroblotted onto positively charged nylon membranes (Roche Diagnostics), as described previously (Alahari & Apte, 1998). Immunodetection was carried out with anti-GroEL antiserum raised against the purified GroEL protein of E. coli (anti-EcGroEL) or with antiserum raised against the purified Anabaena L-31 Cpn60 (anti-AnCpn60) or GroEL (anti-AnGroEL) proteins, respectively. Cpn60 levels were quantified using a densitometer (Syngene Biotech).

Overexpression of Cpn60 in Anabaena 7120. The cpn60 ORF was PCR-amplified from pH302 using primers cpn60OE and cpn60OERev (Table 1), restriction-digested with Ndel/BamHI and ligated to an identically digested Anabaena 7120 vector pFPN, developed in our laboratory (GenBank accession no. EF468631), to obtain plasmid pFPNcpn. Plasmid pFPN allows integration of the transgene between positions 4 654 700 and 4 655 900 in the Anabaena 7120 genome and its expression from a strong light-inducible promoter, PpsA. Plasmid pFPNcpn was electroporated into Anabaena 7120 as described by Thiel & Poo (1989) and electro-transformants were selected on BG-11, N’ Neo$^{25}$ plates.

Measurement of photosynthetic, nitrate reductase and glutamine synthetase activities. Light-dependent photosynthetic oxygen evolution was measured using the Oxygen Monitoring System (OxyLab, Hansatech Instruments). Oxygen evolution measurement involved three alternating cycles of light and dark for 5 min duration each and calculation of the average rate. Cellular nitrate reductase activity was estimated as extracellular nitrite released either in light as natural reductant (L-NR) (Hageman & Hubblesy, 1971) or with reduced methyl viologen (MV$_{2-}$-NR) (Herrero et al., 1981). The results from both assays were similar. Glutamine synthetase activity was assayed by the transferase assay described by Martin et al. (1997).

Two-dimensional IEF/SDS-PAGE. The cell-free protein extract was prepared by repeated freezing–thawing of the cells resuspended in 10 mM Tris buffer, pH 8.0, followed by centrifugation (10 000 g, 5 min) to collect the supernatant. Proteins were subjected to isoelectric focussing (IEF) using ampholines (Amersham Biosciences) of two pH ranges, 3–10 and 3.5–5, mixed in a 2:1 ratio in tube gels followed by resolution using 10% SDS-PAGE (Bhagwat & Apte, 1989).

Genomic DNA isolation, PCR amplification and electrophoresis of DNA fragments. Anabaena genomic DNA was isolated as described previously (Apte & Haselkorn, 1990). PCR amplification of genomic DNA (100 ng) was carried out using Taq DNA Polymerase (Roche Biochemicals). DNA samples were electrophoretically resolved on 0.7% agarose gels in TBE (Tris-Borate-EDTA) at 80 V for 2 h.

Cloning of Anabaena L-31 cpn60 gene and groEL operon. The 1.7 kb cpn60 gene was amplified from Anabaena L-31 chromosomal DNA using the cpn60F2 and cpn60R1 primers (Table 1), designed on the basis of the genome sequence of Anabaena 7120. The PCR product was end-filled with dNTPs and Klenow enzyme and ligated to EcoRV-digested plasmid vector, pBluescript SKII. The insert from this construct, designated pH302, was sequenced and the sequence has been submitted to GenBank (accession no. AY328922). The groEL operon from this cyanobacterium was cloned and sequenced previously (Rajaram et al., 2001; accession no. AF324500).

7120) were grown in BG-11 liquid medium, pH 7.0 (Castenholz, 1988) with (BG-11, N$^{+}$) or without (BG-11, N$^{-}$) combined nitrogen (17 mM NaNO$_3$, or 3 mM NH$_4$Cl with 5 mM MOPS) under continuous illumination (30 μm$^{-2}$s$^{-1}$) and aeration (3.1 min$^{-1}$) at 27 °C. The transgenic Anabaena 7120 strain AnFPNcpn was maintained in BG-11, N$^{+}$ supplemented with 25 μg neomycin ml$^{-1}$ (hereafter referred to as BG-11, N$^{+}$Neo$^{25}$). Heat stress involved exposure to 42 °C, other growth conditions being identical. For thermotolerance and recovery experiments, the heat-stressed cultures were inoculated into fresh, BG-11 medium at 1 μg chlorophyll (chl) a ml$^{-1}$. Growth was measured as chl a content in methanolic extracts as described by Mackinney (1941).

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The presence of an artificial reductant (MV₇,N₇-NR), similarly decreased to very low levels within 15 h of heat stress (Fig. 1b). Inhibition of another assimilatory enzyme, glutamine synthetase, was observed only on prolonged exposure to heat stress, but showed no relationship with the nitrogen source in the growth medium (Fig. 1c).

Immunodetection techniques showed that a 59 kDa GroEL protein was undetectable at 27°C, but was strongly induced during heat stress, irrespective of nitrogen status (Fig. 1d). Cpn60 protein was higher in both nitrate or ammonium-supplemented Anabaena cultures compared to nitrogen-fixing cultures, but was undetectable after 6 h of heat stress in nitrogen-supplemented cultures, unlike its accumulation in nitrogen-deficient cultures (Fig. 1d).

**Effect of nitrogen status on expression of hsp60 genes**

Detailed analysis of hsp60 expression in nitrogen-deficient (BG-11, N⁻) and nitrate-supplemented (BG-11, NO₃⁻) cultures confirmed the results shown in Fig. 1(d). The identities of the 59 kDa (GroEL) and 61 kDa (Cpn60) Hsp60 proteins were confirmed by 2-D electrophoretic resolution (Fig. 2a) and MALDI-TOF-MS-based peptide
mass fingerprinting analysis (data not shown). The absence of Cpn60 protein during prolonged heat stress in the presence of combined nitrogen (Fig. 2b) correlated well with the rapid inactivation of photosynthetic machinery and nitrate reduction observed previously (Fig. 1a, b). Heat stress strongly and rapidly repressed the transcription of the cpn60 gene (Fig. 2c), and also caused degradation of the pre-synthesized 61 kDa Cpn60 protein (Fig. 2a, b) during nitrate-supplemented growth. In comparison, cpn60 transcription was significantly enhanced by heat stress in nitrogen-fixing cultures (Fig. 2c). Preincubation of nitrate-grown cultures with transcriptional (rifampicin) or translational (chloramphenicol) inhibitors showed that Cpn60 protein was quite stable in controls up to 3 h, but was rapidly degraded during heat stress (Fig. 2d).

**Effect of nitrogen status on the recovery of Anabaena L-31 cells from heat stress**

Previous studies showed that nitrogen-fixing cultures of Anabaena L-31 were capable of a remarkable recovery even after 7 days of continuous heat stress at 42 °C (Rajaram & Apte, 2003). When compared to their respective controls, the recovery of nitrogen-fixing cultures was relatively more rapid than that of the nitrate-grown cultures (Fig. 3a), as would be expected if Cpn60 was essential for nitrate-supplemented growth and metabolism. Rapid recovery of photosynthetic activity in the heat-stressed nitrogen-fixing cultures (Fig. 3b) did not transform into equivalent growth recovery (Fig. 3a), since nitrogenase activity takes much longer to recover under such conditions (Rajaram & Apte, 2003). Recovery of nitrate reductase (L-NR) activity followed kinetics very similar to that of recovery of photosynthetic activity in nitrate-grown cultures (Fig. 3b).

The correlation between Cpn60 levels, photosynthesis and nitrate reduction was carefully monitored during recovery of nitrogen-supplemented cultures from a short-term (1 day) heat stress (Fig. 4). About 25% of the Cpn60 protein was synthesized by 2 h of recovery (Fig. 4a) and measurable recovery of photosynthetic and nitrate reductase (MVr-NR) activities commenced subsequently (Fig. 4b). Thus the presence of a threshold level of Cpn60 may be a prerequisite for restoration of photosynthesis and nitrogen assimilation in nitrate-supplemented cultures following heat stress.
Attempts were made to overexpress Cpn60 in *Anabaena* 7120, since *Anabaena L-31* is not amenable to genetic manipulation, such attempts were made in *Anabaena* 7120, which shows an Hsp60 expression profile similar to that in *Anabaena L-31* (Figs 1d and 5a). For this, plasmid pFPNcpn was introduced into *Anabaena* 7120 by electro-transformation. Immunodetection with anti-AnCpn60 antiserum confirmed 2.4-fold overexpression of Cpn60 protein in recombinant *Anabaena* 7120 cells (hereafter referred to as AnFPNcpn) (lane C, Fig. 5b) compared to *Anabaena* 7120 (lane C, Fig. 5a). Significantly higher levels of Cpn60 were observed in nitrate-grown AnFPNcpn cultures after 3 days of heat stress (Fig. 5b), unlike in *Anabaena* 7120 (Fig. 5a), and decreased thereafter (Fig. 5b). As expected, the GroEL levels remained similarly enhanced during heat stress in both cultures (Fig. 5a, b).

**Effect of overexpression of Cpn60 on metabolic activities in *Anabaena* 7120**

The AnFPNcpn cells also showed faster recovery than the wild-type *Anabaena* 7120 cells when exposed to 1 day of heat stress (Fig. 6e). After 4 days of heat stress the difference in recovery was less impressive (Fig. 6f). This correlated well with much higher levels of Cpn60 in AnFPNcpn, compared to *Anabaena* 7120, after 1 day of heat stress than after 4 days (Fig. 5a, b).

**DISCUSSION**

Information available from genome sequences (www.kazusa.or.jp/cyano) and the limited heat-shock response studies in cyanobacteria have revealed the presence of two *hsp60* genes, the *groESL* operon and the *cpn60* gene, in all of them. Both genes have been cloned and sequenced from the nitrogen-fixing cyanobacterium *Anabaena* sp. strain L-31 by our laboratory (GenBank AF324500 and AY328922). In the unicellular adiazotrophic cyanobacterium *Synechocystis sp. PCC6803*, the two *hsp60* genes show differential expression in response to heat stress and light–dark cycles (Asadulghani *et al.* 2003; Glatz *et al.*, 1997; Kovacs *et al.*, 2001; Mary *et al.*, 2004) as well as photosynthetic electron transport (Glatz *et al.*, 1997), or during exposure to UV or oxidative stress (Chitnis & Nelson, 1991). Functional differences in the two Hsp60 proteins have been observed in the complementation of the...
**E. coli groEL44** mutant by the groESL operon and cpn60 gene of *Synechocystis* sp. PCC6803 (Kovacs *et al.*, 2001), but their individual roles have remained enigmatic.

Photosynthesis is inactivated during heat stress in cyanobacteria (Eriksson & Clarke, 1996) and the Hsp60 proteins have been found to be associated with carboxysomes (Jager & Bergman, 1990). In *Synechocystis* sp. PCC6803, the cpn60 gene is not transcribed during heat stress in the dark nor in the presence of DCMU [3-(3,4-dichlorophenyl)-1, 1-dimethylurea] (Glatz *et al.*, 1997), suggesting that it may be required for the assembly of multimeric photosynthetic complexes in light. The non-availability of the Cpn60 protein in nitrogen-supplemented *Anabaena* cultures during heat stress may thus be responsible for the faster inactivation of photosynthesis compared to that observed under nitrogen-fixing conditions (Figs 1, 2, 5 and 6). Overexpression of Cpn60 does confer a higher thermostability of photosynthesis to *Anabaena* (Fig. 6).

This study is the first in which nitrogen-status-dependent regulation of the hsp60 genes has been studied. Combined nitrogen-supplemented unstressed *Anabaena* cultures exhibit significantly higher levels of Cpn60 compared to nitrogen-fixing cultures. This is perhaps indicative of a higher requirement of Cpn60 for photosynthesis and/or nitrate reductase. Both Hsp60 proteins accumulate during prolonged exposure to heat stress under nitrogen-fixing conditions with GroEL being more strongly induced by heat stress than Cpn60 (Fig. 2; Rajaram & Apte, 2003). Novel features elucidated by the present study are (i) the repression of cpn60 expression and degradation of Cpn60 by heat stress selectively during nitrogen-supplemented growth of *Anabaena* (Fig. 2) and (ii) apparent correlation between the Cpn60 levels and photosynthetic and nitrate reductase activities (Figs 1, 4, 5 and 6). The data presented clearly show that in nitrogen-supplemented cultures, Cpn60 levels during heat stress are determined by inhibition of transcription (Fig. 2c) and enhanced degradation of the Cpn60 protein (Fig. 2a, b and d). Cpn60 is stable in nitrogen-supplemented cultures grown at 27°C (Fig. 2d). Using a pulse–chase technique, we have shown previously that the Cpn60 protein is stable up to 24 h of heat stress under nitrogen-fixing conditions (Rajaram & Apte, 2003). The instability during heat stress in nitrogen-supplemented cultures may, therefore, be due to specific proteolytic degradation of the protein.

In cyanobacteria, photosynthesis and nitrate reduction are closely related, since nitrate reductase requires a photo-reduced ferredoxin (Flores *et al.*, 2005). However, nitrate reductase activity was inhibited during heat stress, even when provided with an artificial reductant, MV-3-NR,
indicating that heat stress influenced nitrate reductase and photosynthesis independently. Thermal inactivation of nitrate reductase has been reported in yeast (Siverio et al., 1993). Interestingly, loss or overexpression of Cpn60 did not directly influence glutamine synthetase activity in Anabaena (Figs 1 and 6). In E. coli, GroES and GroEL are...
reportedly required for the assembly of glutamine synthetase (Fisher, 1994). The decrease in glutamine synthetase activity on prolonged exposure to heat stress in Anabaena may be a generic effect of heat stress, which causes denaturation and repression of synthesis of several proteins. The higher thermosensitivity of nitrate-supplemented Anabaena cultures may be due to complete loss of two vital assimilatory processes (Fig. 1) in the absence of Cpn60 (Fig. 2), compared to that of only nitrogen fixation in heat-stressed nitrogen-fixing cultures. The rapid recovery of heat-stressed diazotrophic cultures may be due to the accumulation of both GroEL and Cpn60 chaperonins in them, while the absence of Cpn60 in heat-stressed nitrate-supplemented cultures seems to limit their recovery until adequate Cpn60 is resynthesized. Our attempts to mutagenize cpn60 did not yield viable mutants (data not shown), but this clearly showed that the cpn60 gene is essential for normal growth of Anabaena. The over-expression of the Cpn60 protein in Anabaena 7120 (Fig. 5), on the other hand, resulted in superior thermal stability of both photosynthesis and nitrate reduction compared to the wild-type strain (Fig. 6). The Cpn60 chaperonin thus appears to play a major role in cellular metabolism, especially during nitrogen-supplemented growth of Anabaena.

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REFERENCES


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