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Heat shock protein synthesis of the cyanobacterium *Synechocystis* PCC 6803: purification of the GroEL-related chaperonin

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Abstract

Synechocystis PCC 6803 cells could be induced to synthesize four major HSPs with apparent molecular sizes of 70, 64, 15 and 14 kDa. Heat stress at 42.5 °C appeared to be the optimum temperature for HSP formation in cells grown at 30 °C.

The relative rate of synthesis of HSP70 and HSP15 reached a maximum at 30 min after the temperature shift-up whereas the capability of cells to accumulate HSP64 and HSP14 continued through 2 h.

The two most abundant HSPs, HSP70 and HSP64, were recognized on western blots by antibodies raised against authentic DnaK and GroEL from *Escherichia coli.* To furnish sufficient evidence for the assumption that HSP64 is a GroEL-related chaperonin, this protein was purified to homogeneity. There was a 76% sequence identity between the amino acid sequence of HSP64 and the corresponding protein in *Synechococcus* PCC 7942. Moreover, the purified HSP64 cross-reacted to anti-*E. coli* GroEL antibody. To our knowledge, this is the first report about the purification and partial protein sequencing of a cyanobacterial chaperonin.

Introduction

All organisms respond to elevated temperatures by protecting mechanisms. Amongst these the induction of the synthesis of a small set of proteins (the heat shock proteins, or HSPs) is the most striking one. In addition to its physiological importance, this phenomenon offers a valuable model system to study gene expression, which has contributed highly to our rapidly growing knowledge about the synthesis of HSPs [18, 19].

Much less is known about the function of the heat shock proteins. Only recent observations point to the role of a few HSPs in protein folding, transport and oligomeric assembly processes. Members of the HSP70 family take part in the transport of proteins into the endoplasmic reticulum [6, 38] and mitochondria [12], and they are also present in the chloroplast [23]. DnaK, the prokaryotic member of this family, has an important role in DNA synthesis and in the regulation of the heat shock response [30].

Members of the HSP60 family, termed chaperonins, are also widely distributed. HSP60 of mitochondria has an important function in protein transport [12] and oligomeric assembly [5]. The corresponding polypeptide of chloroplasts, the Rubisco-binding protein, forms complexes with most of the imported proteins [20] and has a fundamental role in Rubisco assembly [28]. The *Escherichia coli* chaperonin GroEL has also various functions in DNA and RNA synthesis, phage head assembly as well as protein secretion [9, 16, 34]. Its presence is an absolute requirement for bacterial growth at all temperatures [8], and its level could determine the upper temperature limit of survival [15].

High-temperature resistance of plants is of great economic importance. It is commonly accepted that photosynthesis is the most heat-sensitive function of the plant cell [39]. Although the role of HSPs in photosynthetic cells is largely unknown at present, their implication in thermoprotection of photosynthesis was clearly evidenced [29]. Several of the small, nuclear-encoded HSPs were shown to be transferred into the chloroplast of pea, soybean and *Chlamydomonas* cells and some of them have been demonstrated to become associated with the photosynthetic membranes. Heat-induced alteration of the thylakoid was required for the binding of stress proteins. A 22 kDa HSP, reportedly associated with granal thylakoids protects against photoinhibition during heat-treatment.

Cyanobacteria are transformable prokaryotic organisms, possessing a plant-type photosynthetic apparatus, thus offering an ideal model for studying heat shock response [26]. Recently we have initiated a long-term project to study the phenomena of heat shock response and acquired thermotolerance in a well transformable and photoheterotrophic cyanobacterium, *Synechocystis* PCC 6803.

Here we present a detailed characterization of heat stress protein production in this strain. It is shown that HSPs of four size classes, 70, 64, 15 and 14 kDa, can be induced when cells are shifted from their normal growth temperature at 30 °C to 42.5 °C. Pulse labelling analysis of protein synthesis during heat shock will also be demonstrated. Two of the HSPs are identified as analogous to DnaK and GroEL, major HSPs of *E. coli*, by immunological cross-reactivity with antibodies raised against the *E. coli* proteins. This is the first report, however, about the purification and partial protein sequencing of a cyanobacterial chaperonin.

Materials and methods

Organism and culture conditions

Synechocystis PCC 6803 cells were grown photoautrophically at 30 °C in BG-11 medium [1] supplemented with HEPES-NaOH (pH 7.5) under illumination with cool white fluorescent light (70 μE m^{-2} s^{-2}) with aeration of 1.0% CO_2 in air. Growth rate was monitored by measuring the optical density of the cell suspension at 800 nm. Cultures at the exponential growth phase were used for experiments.

Isotope labelling and sample preparation for SDS-PAGE and fluorography

1 ml aliquots of mid-log phase cultures were incubated under light at various conditions (specified in Results and the figure legends) in the presence of 2 μCi (U-^{14}C) protein hydrolysate (Chemapol, Prague, Czechoslovakia; specific activity 1.3 MBq/mmol). After labelling, cells were harvested by centrifugation at 1500 × *g* for 5 min, resuspended in 50 μl of SDS sample buffer and boiled for 5 min. To determine the amount of radioactivity incorporated in total proteins, 5 μl aliquots of the boiled samples were dropped into Whatman 31 ET Chroma filter paper discs and boiled again in 10% TCA for 5 min. After the removal of TCA, discs were washed in ethanol, dried in air, dropped into toluene cocktail and the amount of radioactivity was measured by a Beckman LS 5000 TD liquid scintillation counter. For separation of proteins 8–15% SDS-polyacrylamide gel was used [17]. Gels were then stained with Coomassie blue R-250 and further

processed for fluorography by the PPO/acetic acid procedure, according to Skinner and Griswold [31]. They were then dried onto Whatman 3 MM paper under vacuum and fluorography was carried out by exposing the dried gels to X-ray films. Quantitation of some of the autoradiograms was performed by a BioRad Model 1650 Scanning Densitometer connected to a HP 3394 A Integrator.

Purification of the 64 kDa HSP

All steps were carried out at 4 °C. Prior to harvest, cells were heat-treated overnight at 42 °C in order to enhance the formation of 64 kDa HSP. Cells were then collected by centrifugation ($1500 \times g$ for 5 min) and resuspended in a small volume of buffer A (750 mM potassium phosphate pH 7.0, 1 mM each of phenylmethylsulphonyl fluoride (PMSF), benzamidine, and ε-amino caproic acid). Cells were broken by vortexing with glass beads three times for 3 min [25], and after removal of the glass beads the homogenate was centrifuged at $15\,000 \times g$ for 15 min. The supernatant was removed carefully, overlayered onto a 10–30 % linear sucrose gradient prepared in buffer A, and centrifuged overnight with a Beckman SW-28 rotor at $100\,000 \times g$. On the next day portions of the 2 ml fractions were precipitated with TCA and analysed by SDS-PAGE. Peak fractions were pooled, desalted in a Sephadex G-25 column equilibrated with buffer B (100 mM potassium phosphate pH 7.0, and 1 mM each of PMSF, benzamidine, and ε-amino caproic acid), and the proteins were precipitated by addition of solid $(NH_4)_2SO_4$ to 60 % saturation. After recovery of the precipitate by centrifugation it was redissolved in 1/10 of the previous volume of buffer B and loaded onto a 1 cm × 90 cm Sephacryl S200 column equilibrated with the same buffer. Fractions were eluted at a flow rate of 0.2 ml/min and analysed by SDS-PAGE. The purified protein was concentrated with an Amicon stirred flow cell (YM-10 membrane) and stored at −20 °C in 50 % glycerol.

Protein sequencing

The purified HSP64 was subjected to SDS-PAGE and transferred electrophoretically onto Immobilon P membrane (Millipore). The membrane was stained with Coomassie blue and dried in air. The stained band was cut out and sequenced according to Moos *et al.* [24].

Immunoblotting

Following separation by SDS-PAGE, polypeptides were transferred to nitrocellulose membranes (Schleicher and Schuell) electrophoretically [33], using a BioRad Transblot apparatus at constant voltage (15 mV) for 3 h. The efficiency of the transfer was followed by fluorescein-labelled bovine serum albumin. The nitrocellulose membranes were then probed with (a) an antiserum against *E. coli* GroEL protein (a kind gift of Dr A.A. Gatenby), (b) an antiserum against *E. coli* DnaK protein (a kind gift of Dr H. Weissbach), (c) an antibody against the C-terminal portion of the low-molecular-weight chloroplastic heat shock protein of pea (a kind gift of Dr E. Vierling). Alkaline phosphatase-conjugated goat anti-rabbit IgG was used in 1:7500 dilution as a second antibody (Promega).

Other techniques

Protein concentrations were determined by the Coomassie dye–binding assay [4] with bovine serum albumin as standard.

Results

Effect of elevated temperatures on the protein pattern of Synechocystis *PCC 6803: the kinetics of HSP synthesis*

Aliquots (1 ml) of *Synechocystis* cells were incubated for 1 h with uniformly labelled ^{14}C-protein hydrolysate at different temperatures ranging

from 30 to 47.5 °C. Proteins synthesized during this period were resolved by SDS-PAGE and visualized by fluorography (Fig 1). Mild heat treatment at 30–37.5 °C resulted in the formation of several constitutive polypeptides, including phycobiliproteins. If cells were exposed to higher temperatures (40–45 °C) there was a significant increase in the level of four protein bands, with apparent molecular sizes of 70, 64, 15 and 14 kDa. These proteins are considered as heat shock proteins (HSPs). Heat stress at 42.5 °C appeared to be the optimum temperature for HSP formation, whereas synthesis of proteins normally present in cells was drastically reduced. Exceeding this optimum at 45 °C, radioactivity was detected practically in two HSPs, HSP70 and HSP64. No incorporation was seen at 47.5 °C in the exposure shown.

To investigate the kinetics of HSP formation, aliquots of cell suspensions were pulse-labelled for 30 min at different phases of a heat shock treatment at 42.5 °C, lasting for 3 h. The relative proportion of individual HSPs were calculated by scanning the fluorograms (Fig. 2). Obviously, the rate of formation of all HSPs increased during the first 30 min of heat treatment. But, whereas the level of HSP70 and HSP15 reached a maximum and declined thereafter, synthesis of HSP64 and HSP14 increased for 2 h within the period of temperature shift-up.

Immunological screening of HSPs with heterologous antibody probes

As was highlighted above, kinetics of the induction of HSP64 and HSP14 was strikingly similar in the entire time interval investigated. A resembling pair of heat shock proteins (59 and 16 kDa) constitutively synthesized at normal growth temperatures has been recognized in *Anacystis nidulans* [22]. It is known that in *E. coli* a corresponding couple of HSPs exists. The GroE proteins (GroEL 65 kDa and GroES 15 kDa) are encoded by a common operon. The GroE operon of *Synechococcus* was recently sequenced and

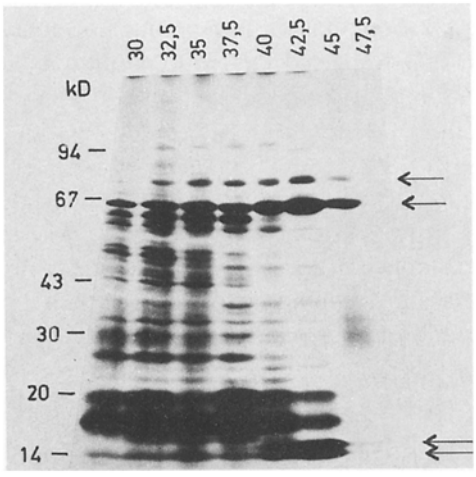

Fig. 1. Effect of temperature on the fluorographic profiles of proteins. *Synechocystis* PCC 6803 cells grown at 30 °C were incubated in the presence of ^{14}C-protein hydrolysate for 1.5 h at the temperatures indicated and treated further as described in Materials and methods. Each lane of the gel was loaded with equal amounts of proteins. Positions of molecular mass markers are given in kDa. Arrows indicate the four major HSPs, HSP70, HSP64, HSP15 and HSP14.

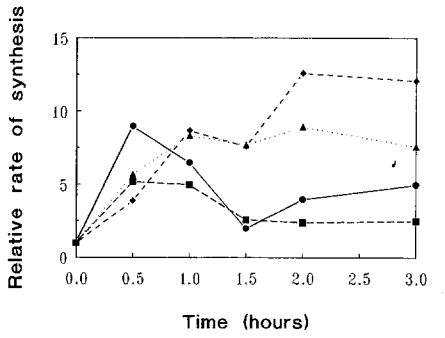

Fig. 2. Kinetics of HSP synthesis during heat stress at 42.5 °C. Aliquots of cell suspensions were pulse-labelled for 30 min at different phases of a heat-shock treatment at 42.5 °C, lasting for 3 h. Fluorograms were scanned and quantitation of authentic peaks was performed as described above. The relative rate of synthesis (in arbitrary units) of major HSPs is shown (●, HSP70; ◆, HSP64; ■, HSP15; ▲, HSP14). 1 unit is equal to proteins synthesized in non-stressed cells.

proved to be highly homologous to that of *E. coli* [37]. Combining these facts we presume that HSP64 in *Synechocystis* PCC 6803 might be a cyanobacterial chaperonin. As has been shown by the fluorographic profile (Fig. 1), another polypeptide which appeared as a distinct band at heat shock was the 70 kDa HSP. By its molecular size, this protein was comparable to the HSP70 homologues. Because of the apparent multifunctional role and relative incidence of HSP70s in other systems, we were interested in determining whether members of this HSP family might also be found in *Synechocystis* PCC 6803.

To verify the above presumptions concerning HSP64 and HSP70, total cell extracts derived from normally grown (30 °C) as well as heat-stressed (42 °C, 1.5 h) cells were separated on SDS-PAGE (Fig. 3A), blotted onto nitrocellulose membrane and further processed as described in Materials and methods. Western blots were probed with antibodies of anti-*E. coli* GroEL (Fig. 3B) and anti-*E. coli* DnaK (Fig. 3C). Both the presence of the GroEL-related chaperonin as well as the 70 kDa DnaK homologue can be demonstrated in *Synechocystis* PCC 6803. In accordance with our previous findings, these two proteins accumulated to higher levels during heat shock. It is noteworthy that a similar attempt to reveal the existence of a plant-type, low-molecular-weight HSP in this strain of cyanobacteria by using an antibody against the C-terminal portion of the 21 kDa HSP of pea [35] was not successful (data not shown). It can be explained either by the lack of such proteins in cyanobacteria or by the low level of similarity between the corresponding low-molecular-weight HSPs of cyanobacteria and chloroplasts (E. Vierling, personal communication).

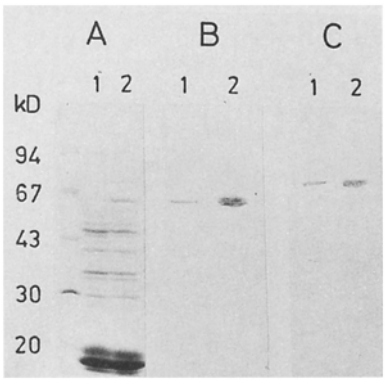

Fig. 3. Identification of HSP70 and HSP64 by using heterologous antibodies. Total proteins extracted from non-stressed (lanes 1) and heat-exposed (42.5 °C, 1.5 h; lanes 2) cells were separated by SDS-PAGE (panel A) and transferred to nitrocellulose membranes. Filters were probed with anti-*E. coli* GroEL (panel B) and anti-*E. coli* DnaK (panel C).

Purification and N-terminal amino acid sequence determination of the 64 kDa heat shock protein

It has been established that members of the chaperonin family form oligomeric structures, which can be isolated by sucrose gradient centrifugation [9]. Our attempts to isolate the oligomeric form of the 64 kDa HSP in low-ionic-strength buffers (10 mM Tris-HCl pH 7.5, or 100 mM potassium phosphate pH 7.0) were not successful (data not shown). Instead, a 750 mM potassium phosphate (pH 7.0) buffer was used. As judged by refractometric measurements, in the peak fractions (Fig. 4) the concentration of sucrose was 18–20 % (w/v).

Unfortunately, cyanobacteria have additional high-molecular-weight oligomeric structures, the most abundant ones being Rubisco and the phycobilisome. The subunits of phycobiliproteins [13] (the very abundant 16–20 kDa proteins in Fig. 4) possess a pI value (between 5.5 and 6.0) and hydrophobic behaviour very similar to our 64 kDa HSP. Therefore, further purification, performed either by ion-exchange chromatography (Servacel DEAE 52) or by reversed-phase chromatography (Phenyl Sepharose) was still not efficient (data not shown). Instead, selective dissociation of the oligomer of the 64 kDa HSP in relatively low ionic strength buffer (100 mM potassium phosphate pH 7.0), coupled with gel filtration by a Sephacryl S200 column proved to be a powerful tool to gain the 64 kDa HSP in high purity (Fig. 5). Since the other, contaminating oligomeric structures did not dissociate into mono-

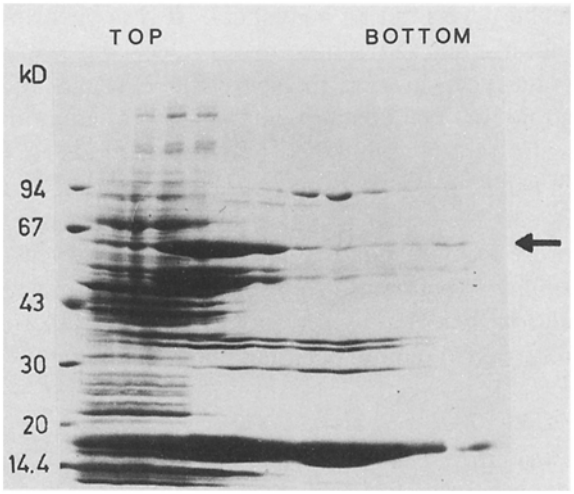

Fig. 4. Purification of the 64 kDa heat shock protein on linear sucrose gradient. 15000 × *g* supernatant was overlayered onto a 10–30% linear sucrose gradient in buffer A and was centrifuged in a Beckman SW-28 rotor overnight at 100000 × *g*. 2 ml fractions were collected and analysed by SDS-PAGE. The arrow indicates the position of the 64 kDa HSP.

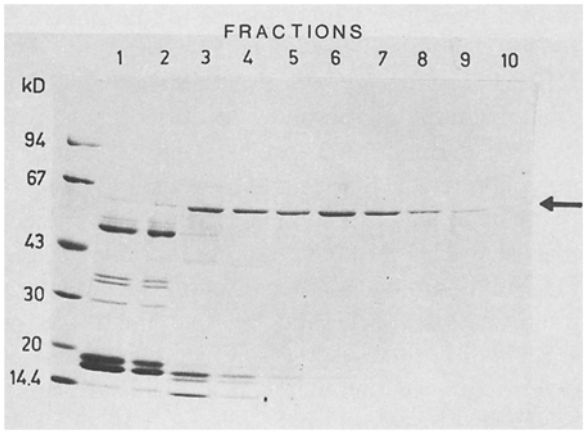

Fig. 5. Further purification of the 64 kDa HSP on Sephacryl S200 column. Fractions of the linear sucrose gradient enriched in 64 kDa HSP (Fig. 4) were gathered and concentrated as described in Materials and methods, and applied onto a 1 cm × 90 cm Sephacryl S200 column. 4.5 ml fractions were collected and analysed by SDS-PAGE. Position of the 64 kDa HSP is marked by an arrow.

mer subunits in this buffer, their separation from the monomer 64 kDa HSP could be achieved. Most of the contaminating proteins were eluted in the exclusion volume (fractions 1 and 2), whereas the 64 kDa HSP was the major protein entered into the separation range of the column (from fraction 6). In fractions 6–8 the purity of our protein was at least 90 %.

The purified 64 kDa HSP was applied onto SDS-PAGE, blotted onto Immobilon P membrane, the Coomassie-stained protein was cut out and sequenced [24]. The first 25 amino acids of the protein were compared to the corresponding region of other known GroEL-related proteins. Their amino acid sequence alignment is presented in Fig. 6. Obviously, the deduced protein sequence from *Synechococcus* PCC 7942 exhibited the highest homology with the 64 kDa HSP.

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S. 6803   A K S I I Y N D E A T R A L E + G M D I L A E A V
S. 7942   * * R * * * * E N * R * * * * K * I * * * * * * *
E. coli   * * D V K F G N D * R V K M L R * V N V * * D * *
Wheat     * * E * A F D Q K S R A * * Q A * V E K * * N * *

                    Homology

          S. 6803- S. 7942 : 76 %
          S. 6803- E. coli : 33 %
          S. 6803- Wheat   : 42 %
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Fig. 6. N-terminal sequence comparison of some chaperonin proteins. S. 6803, *Synechocystis* 6803 64 kDa HSP; S. 7942; *Synechococcus* PCC 7942 GroEL [38]; *E. coli*, GroEL [11]; Wheat, wheat Rubisco-binding protein [11]. HSP64 derived from S. 6803 was sequenced as described in Materials and methods. An asterisk denotes an identical amino acid, a + an existing but unidentified amino acid.

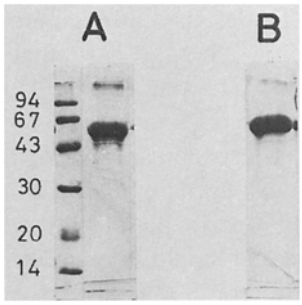

Fig. 7. Immunological evidence of the identity of HSP64 as a GroEL-related chaperonin. HSP64 was purified to homogeneity and was applied to SDS-PAGE (lane A). The protein was recognized on western blot (lane B) by antibody raised against authentic GroEL, from *E. coli*.

The purified 64 kDa protein was also identified by using immunoblotting technique. Cross-reactivity of the HSP64 to anti-*E. coli* GroEL antibody fully supported our assumption that this protein is a cyanobacterial chaperonin (Fig. 7).

Discussion

Synechocystis PCC 6803 cells can be induced to synthesize abundant quantities of HSPs, of which those with molecular sizes of 70, 64, 15 and 14 kDa are the most abundant ones. When cells grown at 30 °C are shifted to elevated temperatures, 42.5 °C appeared to be the optimum temperature for HSP induction, which was accompanied with a sharp reduction in total protein synthesis (Fig. 1).

The kinetics of HSP formation was closely resembling the one demonstrated by Borbély *et al.* for *Synechococcus* PCC 6301 [3]. The relative rate of synthesis of HSP70 and HSP15 transiently reached a maximum at about 30 min after the temperature shift-up and decreased afterwards, whereas the capability of the cells to accumulate the other set of HSPs (64 kDa and 14 kDa) continued through 2 h, and diminished slightly at the final phase of heat treatment (Fig. 2).

In conclusion, the heat shock response involved the synthesis of two relatively large HSPs (70 and 64 kDa) and two small HSPs (in the molecular range of 14–15 kDa), and is in *Synechocystis* very much comparable to the heat shock response observed in other cyanobacteria [3, 22] or some plant cells [19].

We made an effort to identify the two high-molecular-weight HSPs (HSP64 and HSP70) found in heat-exposed *Synechocystis* cells. These proteins were recognized on western blots by antibodies raised against GroEL and DnaK from *E. coli*, respectively (Fig. 3). As a part of the screening of our HSPs with heterologous antibody probes, our attempt with an antibody against the C-terminal portion of a plastid low-molecular-weight HSP was not successful, not even at high antigen and antibody concentrations. Obviously, this failure is not a proof for the lack of plant-type, low-molecular-weight HSPs in cyanobacteria. But considering the fact that we were unable to demonstrate abundant HSPs in a range of 20–30 kDa, it suggests the lack of these proteins in cyanobacteria.

To furnish sufficient evidence of our assumption of the 64 kDa HSP as a GroEL-related chaperonin, we purified and sequenced the N-terminal region of this heat shock protein. Although the presence of a HSP with an approximate molecular weight of 60–65 kDa has been demonstrated in different strains of cyanobacteria [2, 3, 22], and the gene of *Synechococcus* PCC 7942 was sequenced [37], this is the first report on purification and partial sequencing of the protein concerned. Although the N-terminal portion is not a highly conserved region of the chaperonin proteins, our sequence shows a marked similarity to authentic chaperonin sequences. According to our expectations the homology was the highest between the two cyanobacterial proteins.

Whereas HSP64 could be purified to homogeneity, special characteristics, revealed during its purification may possess functional significance. Since the oligomer of the 64 kDa HSP is very sensitive to the ionic strength of the buffer, and an apparently non-physiological salt concentration was required to maintain this structure *in vitro*, it is questionable whether the protein exists in the

cell as a monomer, or as an oligomer. It should be mentioned, however, that intact phycobilisomes can be isolated in a fully organized state only in the presence of 750 mM phosphate buffer [13]. It seems very likely, therefore, that the 64 kDa HSP exists also as an oligomer in vivo and its high sensitivity to the ionic strength of the buffer may reflect its propensity for easy dissociation even within the cells. Consequently, a sensitive balance might exist between the different forms of the 64 kDa HSP in vivo.

It is of special interest to compare the above supposed feature of the cyanobacterial chaperonin with the proposed model of action of the *E. coli* GroEL protein, and of the chloroplast chaperonin. It was shown for the *E. coli* chaperonin that the oligomeric form does not dissociate even during the release of the folded protein [9]. Results are also present, however, demonstrating that the GroES protein, MgATP and K^+ facilitated dissociation, coupled with the removal of the folded polypeptide from the surface of the chaperonin [10]. The oligomeric form of *E. coli* GroEL is stable in low-ionic-strength buffers in water, and can be dissociated into dimers in metrizamide, in the presence of ATP [9].

Slightly different characteristics have been reported for chaperonins in plastids [28]. Although its presence is strongly suggested, especially after the discovery of the authentic mitochondrial protein [21], a GroES-like protein has not been demonstrated so far in chloroplasts. Furthermore, an oligomeric form of a chaperonin derived from plastids dissociates easily and reversibly in the presence of ATP. It is proposed that chaperonins in chloroplasts take cycles between the monomeric and oligomeric forms, allowing the removal of the folded polypeptides during the dissociation of the complex in the presence of ATP.

If our previously described assumption is correct concerning the intracellular behaviour of the chaperonin of *Synechocystis* PCC 6803, the HSP64 could also take cycles between the two forms. Our attempts, however, to induce the reassociation of the monomer by incubation in high-ionic-strength buffer were unsuccessful (data not shown). Perhaps a GroES-like protein is required for this process, whose presence in other cyanobacteria has already been documented [37].

The cyanobacterial chaperonin may possess various functions. It may participate in protein secretion, similar to the *E. coli* GroEL protein [9]. Its assistance in the assembly of the Rubisco holoenzyme is also conceivable [20, 28]. Additional functions, specific to the cyanobacterial chaperonin, like participation in the assembly of the phycobilisomes could be suggested. The presence of this protein in the carboxysomes (whose characteristic protein is the Rubisco) seems very likely, too [27]. Recently, we have found that a pool of 64 kDa chaperonin becomes thylakoid-associated during exposure of cyanobacteria to high temperature [36]. We supposed that the GroEL analogue 64 kDa HSP is directly involved in conferring thermoprotection to thylakoid membrane in cells subjected to heat stress. This chaperonin may assist at the insertion of some protein into membranes, or may itself become bound to the thylakoid in order to repair membrane proteins, partially denatured by heat shock (E. Kovács *et al.*, submitted).

So far, only one paper presents data on HSP synthesis in plastids in higher plants [14], demonstrating the induction of four, membrane-bound HSPs. This report, however, does not contain information about the basic regulation of HSP synthesis, or about the autonomy retained by the chloroplast in this respect. In *E. coli* the key element of this regulation is the sigma 32 subunit of the RNA polymerase [32]. Its presence in cyanobacteria is very likely, but in the case of plastids it is an intriguing and fully open question.

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