Alterations in the accumulation of adenylylated nucleotides in heavy-metal-ion-stressed and heat-stressed *Synechococcus* sp. strain PCC 6301, a cyanobacterium, in light and dark

Zsófia PÁLFI, Gyula SURÁNYI and György BORBÉLY*

Institute of Plant Physiology, Biological Research Center, H-6701 Szeged, P.O. Box 521, Hungary

Heavy-metal-ion- (Cd^{2+} , Cu^{2+} , Pb^{2+} , Hg^{2+} and Zn^{2+}) or heat (50°C)-stress treatments of the unicellular cyanobacterium *Synechococcus* sp., strain PCC 6301, under both light and dark conditions led to the accumulation of bis(5'-nucleosidyl)oligophosphates: Ap_4A , Ap_4G , Ap_3A , Ap_3G and Ap_3Gp_2 . Under light regimens, the accumulation of Ap_4A and Ap_4G is more characteristic of heavy-metal-ion-stressed cells, whereas the accumulation of Ap_3A , Ap_3G and Ap_3Gp_2 is the dominant feature of heavy-metal-ion or heat-shock treatment during energy deprivation (i.e. in the dark). This accumulation of bisnucleoside oligophosphates supports a model whereby the adenylylated nucleotides are synthesized by the backward reaction of tRNA-aminoacyl synthetases. These nucleotides may also act to switch or modulate cyanobacterial responses under various environmental stress conditions.

INTRODUCTION

We have previously provided evidence that in heat-shocked *Synechococcus* sp., an obligate photoautotrophic cyanobacterium, the elevated temperature induces a transient synthesis of heat-shock proteins [1]. In addition, heat-shocked cyanobacterial cells accumulated guanosine 3'(2')-diphosphate 5'-diphosphate (p_2Gp_2), a signal molecule known to be involved in the stringent response [2]. We have suggested that the elevated p_2Gp_2 level may have pleiotropic effects on cyanobacterial metabolism under stress conditions [2].

Bisnucleoside polyphosphates are ubiquitous nucleotides found in prokaryotic and eukaryotic cells (reviewed in [3–6]), and in heterotrophic bacteria they accumulate in response to oxidative stresses and/or heat shock. These compounds were proposed as 'alarmones' which signal the onset of an oxidative or heat stress and which trigger the transient synthesis of stress proteins [3,7]. However, it has been shown recently that the pattern of accumulation of the adenylylated nucleotides is more consistent under oxidative stress than with heat shock [8], though the accumulation of bisnucleoside polyphosphates is apparent in heat-shocked cells [7].

Studies of cellular strategies for overall regulation in heterotrophs have revealed the importance of adenylylated nucleotides in this process under stress conditions [9,10]. In spite of their relevance to the regulation of overall cellular metabolism in heterotrophic organisms, no pertinent data are available for cyanobacteria. The present study describes the isolation, identification and characterization of alterations of several adenylylated nucleotides from heavy-metal-ion-stressed or heat-stressed *Synechococcus* sp., strain PCC 6301, an obligate photoautotrophic cyanobacterium.

MATERIALS AND METHODS

Cyanobacterial strain and its growth

Synechococcus sp. strain PCC 6301 (*Anacystis nidulans*, ATCC 27144) was grown in the liquid medium of Allen [11]. Cultures

were kept in glass vessels thermostatically maintained at 39°C and illuminated with warm white fluorescent light ($3.6 \times 10^4 \text{ mW} \cdot \text{m}^{-2}$), except where otherwise stated [12]. Aeration was achieved by bubbling with sterile air containing 2–3% CO_2 (v/v). Growth of the cultures was monitored spectrophotometrically at 800 nm and, when it was necessary, the number of colony-forming units (c.f.u.) per ml was determined to detect any possible changes in the viability of cells [1].

Stress conditions

Exponentially growing *Synechococcus* cultures ($0.3\text{--}0.6 A_{800}$ units) were heat-shocked by transferring cultures from 39°C to 47°C or 50°C . To produce heavy-metal-ion stress, analytical grade salts of cadmium acetate, zinc acetate, lead nitrate, mercury chloride or copper sulphate (Reanal, Budapest, Hungary) were used to prepare sterile 10 mM stock solutions, which were added to the medium to give the required concentration of the metal ions. Concentrations determined by experiments to be growth-inhibitory (between 80 and 100% inhibition of growth), but sublethal as judged by counts of c.f.u. (not more than 15% decrease in c.f.u. $\cdot \text{ml}^{-1}$ at the end of experiment, except with heat shock at 50°C , where 30% of viable cells were lost), were used [1,12]. The pH of the medium was checked and corrected if necessary.

Measurement of nucleotide pools

The cellular nucleotide pool was labelled by the addition of carrier-free [^{32}P]P_i (Izinta, Budapest, Hungary) to the cultures for three doubling times (specific radioactivity $4.0 \text{ MBq} \cdot \text{ml}^{-1}$; [13–15]). For identification and measurement of the bisnucleoside polyphosphates ATP, ADP and AMP, the methods described by Lee *et al.* [15] were used with minor modifications. For the preparation of formic acid extracts, 100 μl samples of the control and treated cultures were each rapidly mixed with 50 μl of 98% formic acid in Eppendorf plastic centrifuge tubes. The suspensions were kept on ice for at least 30 min and then centrifuged (12000 g, 3 min). Portions of the supernatants were separated by two-dimensional chromatography on polyethyleneimine (PEI)-cellulose t.l.c. plates (Macherey and Nagel,

Abbreviations used: Ap_4A , diadenosine 5',5''- P^1P^4 -tetraphosphate; Ap_4G , adenosine 5- P^1 -tetraphospho- P^4 -5''-guanosine; Ap_3A , diadenosine 5',5''- P^1P^3 -triphosphate; Ap_3G , adenosine 5'- P^1 -triphospho- P^3 -5''-guanosine; Ap_3Gp_2 , adenosine 5',5''-triphosphoguanosine-3''-diphosphate; p_2Gp_2 , guanosine 3'(2')-diphosphate 5'-diphosphate; DCMU, 3'-(3,4-dichlorophenyl)-1,1-dimethylurea; PEI, polyethyleneimine; c.f.u., colony-forming unit.

* To whom correspondence should be addressed.

Fig. 1. Separation of adenylylated nucleotides in heavy-metal-ion- and heat-stressed *Synechococcus* sp.

(a) After 45 min of exposure to various stressors, autoradiograms were produced showing the separations of cellular nucleotides of illuminated Zn^{2+} ($25 \mu\text{M}$), Cu^{2+} ($25 \mu\text{M}$), Pb^{2+} ($100 \mu\text{M}$), Hg^{2+} ($2 \mu\text{M}$) ion-treated and illuminated untreated (control, L) *Synechococcus* sp. cells by optimized two-dimensional t.l.c. (see the Materials and methods section and [15]). (b) Autoradiograms from extracts of cells treated with Cd^{2+} ($25 \mu\text{M}$) for 45 min and heat-shocked for 30 min at 50°C under light (L) and dark (D) regimes (L refers to all autoradiograms on the left and D to those on the right). The schematic representation showing the identities of nucleotides resolved is as follows: spot 1, AMP; 2, ADP; 3, ATP; 4, GMP; 5, GDP; 6, GTP; 7, p_2Gp_2 ; and a, Ap_4A ; b, Ap_3G ; c, Ap_4A ; d, Ap_4G ; e, Ap_3Gp_2 . f is an unidentified spot.

Fig. 2. Separation of reaction products of snake venom phosphodiesterase-digested adenylylated nucleotides of *Synechococcus* sp.

(a) Extracts of spots of presumed bisnucleoside polyphosphates, prepared from cells stressed by Cd^{2+} ($25 \mu\text{M}$) for 90 min in the dark, were digested by venom phosphodiesterase at pH 8.4; the reaction products were separated on PEI thin layers using $3 \text{ M}-(\text{NH}_4)_2\text{SO}_4/2\% \text{ Na}_2\text{EDTA}$ (pH 5.5) as solvent. Uniformly ^{32}P -labelled ATP, GTP and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($\text{ATP}\gamma$) were digested under identical conditions. Lane a corresponds to undigested sample, and lanes b, c and d correspond to 10 min, 1 h and 12 h of digestion respectively. (b) A nucleotide extract, isolated from cells subjected to simultaneous light-to-dark shift and Cd^{2+} treatment, was digested and chromatographed as above. Lane a corresponds to the purified and undigested sample and lane b corresponds to samples digested for 12 h at 37°C . ^{32}P -labelled ATP, $\text{ATP}\gamma$ and GTP were digested under identical conditions. Autoradiograms (a) and (b) also contained radioactive P_i .

Düren, Germany) using 1.75 M -morpholine/ 0.1 M -boric acid/ 1.4 M -HCl (pH 8.7) in the first dimension and 3 M -ammonium sulphate/ 2% disodium EDTA (pH 5.5) in the second

dimension as solvent systems. The nucleotides were detected by autoradiography using intensifier screens (Lightning Plus; DuPont) for 2–10 h at -70°C , and were then excised and

measured by counting for radioactivity in a scintillation counter (Isocap Delta Searle 300). The spots were identified by two-dimensional co-chromatography with authentic *Escherichia coli* nucleotides in the same solvent systems and by enzymic digestion of eluted samples with bacterial alkaline phosphatase and snake venom phosphodiesterase (both from Sigma) as described by Bochner & Ames [13]. To verify their identity, the spots were eluted from chromatograms loaded with samples from both heat-shocked and Cd²⁺-treated cultures and subjected to enzymic analyses. The concentrations of labelled nucleotides were calculated by correcting for a unit absorption at 800 nm of the culture at the time of sampling. The pool sizes of the nucleotides were calculated by converting the ³²P counts into pmol, which can be achieved by the knowledge of the specific radioactivity of the [³²P]P_i in the culture medium. Experiments were done at least three times; results were reproduced with variations of no more than 10%.

RESULTS

Chemical identity of oligonucleoside polyphosphate compounds induced by heavy-metal-ion or heat stresses

In the acid-soluble extracts of heavy-metal-ion-treated cells,

unusual spots were observed on PEI-cellulose chromatograms. To determine the identity of these spots, extracts of *Synechococcus* sp. cells treated with Cd²⁺ were mixed with extracts containing bisnucleoside polyphosphates from heat- or Cd²⁺-treated *E. coli* cells (*E. coli* K-12 wild-type, kindly provided by Dr. Peter Putnoky, Biological Research Center); these were analysed by the optimized two-dimensional t.l.c. system of Lee *et al.* [15]. Since nucleotides isolated from stressed *Synechococcus* sp. cells co-chromatographed with bisnucleoside polyphosphates of stressed *E. coli* (results not shown), it appeared that these nucleotides might be bisnucleoside polyphosphates.

Next we analysed, as shown in Fig. 1, heavy-metal-ion-stress-induced and extreme-heat-stress-induced formation of the presumed bisnucleoside polyphosphates in illuminated and darkened *Synechococcus* sp. cells. To verify the identity of those spots with a cyanobacterial origin, they were extracted and subjected to bacterial alkaline phosphatase and snake venom phosphodiesterase digestion [13]. All of the spots (Fig. 1, spots a, b, c and d), with the exception of spot e, were resistant to the non-specific exonucleolytic activity of bacterial alkaline phosphatase. The bacterial alkaline phosphatase digestion of spot e resulted in adenosine 5'-P¹-triphospho-P³-5'''-guanosine (Ap₃G; results not shown). By snake venom phosphodiesterase digestion the spots

Fig. 3. Kinetics of adenylylated nucleotide pool changes during stress under light and dark regimens

An illuminated exponentially growing *Synechococcus* sp. culture was divided at time zero into subcultures and heat-stressed (50 °C) or heavy-metal-ion-stressed as indicated under light (a) and dark (b) regimens. For the measurement of adenylylated nucleotide pools, cell extracts were chromatographed using optimized two-dimensional PEI t.l.c. (see the Materials and methods section, [15] and Fig. 1). Pools of Ap₄A (▲), Ap₄G (●), Ap₃A (■), Ap₃G (◆) and Ap₃Gp₂ (▼) are indicated.

Fig. 4. ATP, ADP and AMP pool and viability changes in heavy-metal-ion- or heat-stressed *Synechococcus* sp. cells under light and dark regimes

An illuminated exponential-phase *Synechococcus* sp. culture (39 °C) was divided into subcultures and at time zero shifted to the dark, or heat-shocked at 50 °C in the light (open symbols) or the dark (filled symbols), or Cd²⁺-stressed (25 µM) in the light or the dark (symbols as above). Growth was measured as described in the Materials and methods section. The cellular nucleotides were analysed as shown in Fig. 1 and as described in the Materials and methods section. Shown are ATP in the light (○) and dark (●), ADP in the light (△) and dark (▲), and AMP in the light (□) and dark (■). The viability of cells is given in 10⁻⁷ × c.f.u. ml⁻¹ in the dark (◆) and light (◇).

were identified as follows: spot a, Ap₃A; spot b, Ap₃G; spot c, Ap₄A; spot d, Ap₄G (Fig. 2). The identification of the molecular structure of spot e was done under identical conditions, and it was found to be Ap₃Gp₂ (results not shown). Apparently identical compounds were detected in cells subjected to heat stress, but only when 50 °C rather than 47 °C was the stress temperature; p₂Gp₂ was found at both stress temperatures, but it accumulated only to low levels at 50 °C (Z. Pálfi, G. Surányi & G. Borbély, unpublished work).

Bisnucleoside polyphosphate pool changes in stressed *Synechococcus* sp. under light and dark regimens

The time courses of accumulation of the bisnucleoside polyphosphates identified are shown in Fig. 3. High levels of Ap₃A, Ap₄A, Ap₃G, Ap₄G and Ap₃Gp₂ were induced by heat shock at 50 °C in the light and the dark. Heavy-metal-ion stresses also triggered the expansion of the bisnucleoside polyphosphate pool, but in a somewhat different way. In heavy-metal-ion-stressed cells under light regimens the accumulation of bisnucleoside tetraphosphates is a more characteristic feature of the response than is the expansion of the bisnucleoside triphosphate pool (Fig. 3a). In contrast, when cells were treated with heavy metal ions in the dark, the accumulation of bisnucleoside triphosphates was the dominant response (Fig. 3b). Under the latter conditions the expansion of the Ap₃Gp₂ pool was substantially greater than that noted in illuminated cells treated with the heavy metal ions (Figs. 3a and 3b). However, a low concentration of Hg²⁺ ions induced a substantial elevation in the Ap₃Gp₂ pool, even in the light, which might be the consequence of differences in the uptake of various toxic metal ions and/or the heavy-metal-ion-sensitivity of the cells, but this requires further investigation. It seems noteworthy that higher concentrations of heavy metal ions resulted in a faster cellular response in accumulation of the adenylylated nucleotides than was observed at lower concentrations (results not shown). No change in adenylylated nucleotides was detected in cells treated with 1 mM-L-methionine-DL-sulphoximine [16], with a tempera-

ture shift from 39 to 47 °C, or on treatment with 0.1 mM-3'-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a photosynthesis inhibitor (for 40 min), 1 mM-iodoacetamide (for 30 min), 500 µg of *N*-ethylmaleimide/ml (for 30 min) or 10 % ethanol (for 50 min) (results not shown).

In the experiments for the analysis of the bisnucleoside polyphosphates, the pool sizes of ATP, ADP and AMP were also measured. Stress conditions (heavy-metal-ion treatments or heat shock) dramatically decreased the ATP pool size but increased AMP levels under both light and dark conditions (Fig. 4). However, heat shock in the light induced a transient expansion of the ADP pool size, whereas in heat-shocked cells in the dark and in heavy-metal-ion-stressed cells (both in the light and in the dark), no dramatic alteration of this kind was observed.

DISCUSSION

Growing cyanobacteria are strongly influenced by their nutritional, chemical and physical environments, and they respond to various stresses (heat shock, toxic chemicals), energy starvation (darkness), nutritional deprivation (nitrogen starvation), etc., by a decrease in their growth rate, an induction of stress proteins and a concomitant accumulation of guanosine tetraphosphate, the compound implicated as a metabolic signal for the curtailment of several cellular processes ([2,14,17–22], reviewed in [23,24]). Since the heat-shock phenomenon and its regulation in *Synechococcus* sp. may have much in common with the heterotrophic stress regulations, one would also expect a role for bisadenylylated nucleotides in the heavy-metal-ion-induced perturbation of cyanobacterial metabolism [1,2].

The goal of this study was to show whether or not inhibitory concentrations of heavy metal ions (Cd²⁺, Zn²⁺, Cu²⁺, Hg²⁺ and Pb²⁺ ions) and heat shock induce the synthesis of adenylylated nucleotides, compounds which are suspected of being metabolic signals of various stresses [3,4,7,8]. The analysis of extracts of stressed cells demonstrates for the first time the occurrence of these nucleotides, i.e. Ap₃A, Ap₄A, Ap₃G, Ap₄G and Ap₃Gp₂, in

a cyanobacterium (Figs. 1 and 2). Heat shock of *Synechococcus* sp. cells at 50 °C induces accumulation of all of the adenylylated nucleotides to a detectable level, with a dominant accumulation of bisnucleoside triphosphates (Fig. 3). However, at 47 °C, a temperature which is otherwise optimal for the synthesis of heat-shock proteins [1], these nucleotides are undetectable (results not shown). In this respect the cyanobacteria are similar to *E. coli* [8]. The synthesis of the bisnucleoside polyphosphates in heavy-metal-ion-challenged cells varied with energy (light) availability. Under light regimens the accumulation of bisnucleoside tetraphosphates (Ap_4A , Ap_4G) was a more characteristic feature of heavy-metal-ion treatments than was the rise in the bisnucleoside triphosphate (Ap_3A , Ap_3G) pool. Heavy-metal-ion treatment in the dark favoured the accumulation of Ap_3A , Ap_3G and Ap_3Gp_2 (Fig. 3).

Since the synthesis of the adenylylated dinucleotides by the reverse reactions of tRNA-aminoacyl synthetases *in vitro*, and presumably *in vivo*, has been shown [6], we suggest on the basis of our biochemical evidence that the alterations in the pool sizes of these adenylylated nucleotides might be, among others, via interaction with aminoacyl-tRNA synthetases. On the basis of genetic evidence this type of interaction has been proposed for the heterotrophic bacterium *Salmonella typhimurium* [25]. Consistent with this model is the observation that the level of each adenylylated nucleotide is related to the level of its precursor (Fig. 4). However, alterations in bisnucleoside oligophosphate pools showed a slight difference in illuminated and heat-shocked cells (Fig. 3). This is not necessarily inconsistent with the suggested model, since in illuminated and heat-shocked cells the alterations in the AMP/ADP pools show slight differences if they are compared with heavy-metal-ion-stressed cells in both the light and the dark or with cells heat-shocked in the dark (Figs. 3 and 4). Nevertheless, under stress conditions there are some discrepancies between the changes in the bisnucleoside oligophosphate pools and their suggested precursor pools. Therefore one may conclude that the pool sizes of these dinucleotides are influenced not only by their precursor level but also by other stress-dependent modulating processes, such as enzymic synthesis and/or degradation of adenylylated nucleotides (reviewed in [26]). To sum up, it is significant that the bisnucleoside polyphosphates accumulate in an obligate photoautotrophic cyanobacterium in a manner very similar to that described for heterotrophic bacteria such as *E. coli* and *S. typhimurium* [3,4,7,8,15]. Therefore our detailed physiological and biochemical analysis of cyanobacterial stress responses at the nucleotide level extends the previous findings that the adenylylated nucleotides might be metabolic signals for stresses in photoautotrophic cyanobacterial systems as well.

These findings are in accordance with various interpretations of bacterial stress regulons and may be explained by the oxidative stress theory proposed for heterotrophic organisms such as *E. coli* and *S. typhimurium*, in that heavy metal ions such as Cd^{2+} induce the oxidation-stress (*oxyR*-controlled) regulon [3,4,7,8,27,28]. Although the presence of regulatory nucleotides is now well established in cyanobacteria, only a few of the individual reactions in the cyanobacterial global regulatory network are known and/or understood. In addition, it might be predictable that cross-talk between signalling pathways is es-

sential in order to develop and/or regulate the specific light-dependent control processes in these organisms [9,10]. The accumulation of adenylylated nucleotides in *Synechococcus* sp. strain PCC 6301 is therefore the first clear example of the probable involvement of these nucleotides in a light-dependent metabolic control of stress responses in an obligate photoautotrophic cyanobacterium, and would appear to be a system of choice for detailed studies of stress processes in these organisms.

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