Activation and pathway of glucosylglycerol synthesis in the cyanobacterium *Synechocystis* sp. PCC 6803

Martin Hagemann and Norbert Erdmann

Author for correspondence: Martin Hagemann. Tel: +49 381 395 353. Fax: +49 381 395 352.

Department of Biology,
University of Rostock, 18051
Rostock, FRG

The biosynthetic pathway of glucosylglycerol (GG), the osmoprotective compound of *Synechocystis* sp. PCC 6803, was found to proceed from ADP-glucose and glycerol 3-phosphate via glucosylglycerol phosphate in a two-step reaction. Using an *in vitro* assay it was shown that the GG-forming enzyme system required activation, which could be initiated *in vivo* by hypertonic salt concentrations and osmotic shock, or *in vitro* by NaCl addition at the stage of enzyme extraction or assay.

Keywords: *Synechocystis*, glucosylglycerol synthesis, osmoprotection, salt activation

INTRODUCTION

Synthesis of an osmoprotective compound represents a fundamental part of the strategy of salt adaptation in plants, algae and micro-organisms. One of about 30 such compounds known today is the heteroside *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-glycerol (glucosylglycerol; GG) (Reed & Stewart, 1988; Trüper & Galinski, 1989). GG accumulates mostly in cyanobacteria in response to external salt concentration (Borowitzka *et al.*, 1980; Erdmann, 1983) and enables the organisms to survive moderate salinities (Mackay *et al.*, 1983; Reed *et al.*, 1984).

GG is absent in unstressed cells and is synthesized immediately after a salt shock (Erdmann *et al.*, 1992) to an intracellular concentration which corresponds to the strength of the stress, and without marked turnover (Hagemann *et al.*, 1987). After a downshock, the necessary rapid depletion of the accumulated GG is achieved by turnover (Warr *et al.*, 1985; Tel-Or *et al.*, 1986) or excretion (Fulda *et al.*, 1990). Although glycogen has been shown to be a possible precursor of GG (Tel-Or *et al.*, 1986), it seems likely that this pathway is of limited value only and may be restricted to the early period of the adaptation course or to darkness (Erdmann *et al.*, 1989). The majority of GG is synthesized by light-dependent CO₂ photoassimilation (Mackay & Norton, 1987).

Little is known about the biochemical pathway leading to GG (Reed & Stewart, 1988). All osmoprotective compounds structurally related to GG (isofloridoside, floridoside, sucrose and trehalose) are synthesized via a unique

pathway (Brunner & Kauss, 1988; Gæver *et al.*, 1988). In each case the respective UDP sugar is combined with the appropriate polyol phosphate, yielding a phosphorylated intermediate which is hydrolysed to the free osmoprotective compound. Reed & Stewart (1988) have proposed that GG is formed in a similar manner from UDP-Glc and glycerol 3-phosphate (G3P) via the intermediary glucosylglycerol phosphate (GGP).

The present work was done to determine the pathway of GG synthesis in the cyanobacterium *Synechocystis* sp. PCC 6803.

METHODS

Culture conditions. Axenic cells of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 were grown in the medium of Allen & Arnon (1955), containing 2 mM NaCl, in continuous light (20 W m⁻²) at 30 °C and aerated by CO₂-enriched air (5%, v/v). Salt-adapted cells were obtained by cultivation of *Synechocystis* for 5 d in Allen & Arnon's medium containing 324 mM NaCl. Salt or osmotic stress experiments were performed by adding the desired osmolyte in solid form to the medium. After the times indicated, cells were harvested and used for the estimation of enzyme activities. Bacterial contamination was checked by spreading 0.2 ml of the cyanobacterial suspension on LB agar plates and incubating at 30 °C for 36 h.

Preparation of crude enzyme extract. Aliquots of the cyanobacterial suspension (10 ml; OD₇₅₀ approx. 1.5) were centrifuged at 4000 g for 3 min. The pellet was resuspended in 400 μ l ice-cold homogenization buffer containing the same amount of NaCl or organic osmolyte used in the shock experiments (50 mM HEPES, pH 7.8, 10 mM MgCl₂, 2 mM EDTA, 0.1% human serum albumin and, as required, 324 mM NaCl, 684 mM NaCl or 1.3 M sucrose). Cells were broken by sonication (2 min at 30 W) under permanent ice cooling. After

Abbreviations: ADP-Glc, adenosine diphosphate glucose; GG, glucosylglycerol; GGP, glucosylglycerol phosphate; G3P, glycerol 3-phosphate; UDP-Glc, uridine diphosphate glucose.

Fig. 1. Dependence of the *in vitro* synthesis of GG on the glucosylation agent and activation procedures. Crude homogenates of *Synechocystis* sp. PCC 6803 were assayed with respect to GG accumulation from [^{14}C]G3P. Activation was performed *in vivo* (hyperosmotic stress of whole cells with 324 mM NaCl; A) or *in vitro* (addition of crystalline NaCl to the homogenization buffer (B) or assay buffer (C) to final concentrations of 324 and 162 mM, respectively). Reaction products were separated by TLC and visualized by autoradiography. Positions of authentic substances are indicated by arrows (1, glycerol; 2, GG; 3, sucrose; 4, G3P).

NaCl supply to:	1	2	3	4	5	6	7	8	GG
A	+	-	-	-	+	+	+	+	-
B	+	-	-	+	+	-	-	+	-
C	+	-	+	-	-	-	+	-	-
Glucose source:									
UDP-glucose	-	-	-	-	-	-	-	+	-
ADP-glucose	-	+	+	+	+	+	+	-	-

centrifugation (5000 *g* at 4 °C for 10 min) the supernatant was used directly as a crude enzyme extract. The enzyme assays were started approximately 20 min after harvesting the cells. There were no differences in enzyme activities if the cells or the enzyme extracts were frozen and stored at -20 °C prior to the enzyme assays.

GGP synthase/phosphatase assay. The assay mixture contained 5 μl assay buffer (250 mM HEPES, pH 7.8, 50 mM MgCl_2 , 10 mM EDTA and 0.5% human serum albumin), 5 μl ADP-Glc (6 mM), 5 μl L-[U- ^{14}C]G3P (18.5 kBq, specific activity 3.7 GBq mmol^{-1} , Amersham, containing 3 mM G3P [dicyclohexylammonium salt]), 5 μl benzamidinium chloride (50 mM) and, as required, 5 μl NaCl (1.7 M). The volume was brought to 50 μl with water. The reaction was started by the addition of the crude enzyme extract (20 μl) and terminated after 10 min at 30 °C by the addition of 400 μl acetone. Protein was precipitated at -20 °C for at least 3 h. The sample was centrifuged (14000 *g*, 4 °C, 15 min) and the supernatant lyophilized. The residue was treated with 25 μl ethanol (80%, v/v) and an aliquot (5 μl) of the alcoholic solution spotted onto a thin-layer plate (usually HPTLC KG 60; Merck). TLC was performed in acetonitrile/water (85:15, v/v) and repeated three times with careful drying after each run.

For the determination of the whole spectrum of reaction products, aliquots of the enzyme assays were additionally separated by two-dimensional TLC (see Erdmann, 1983). Authentic ^{14}C -labelled GG was obtained from ^{14}C -labelled salt-adapted cells of *Microcystis firma* after purification by ion-

exchange chromatography (Hagemann *et al.*, 1987). Labelled compounds were detected by autoradiography.

GG. The amount of [^{14}C]GG synthesized *in vitro* was estimated by densitometry or liquid scintillation counting. In the former case autoradiogram spots were quantified by means of a video densitometer (Bio-Profil; Vilber Lourmat) using brightness and area of the spots. For liquid scintillation counting the spots were marked on the chromatogram according to their position on the autoradiogram, scraped off and placed into a scintillation vial with 5 ml cocktail [toluene/Triton X-100 (2:1, v/v), PPO (0.4%), dimethyl-POPPOP, (0.012%)]. Results were expressed as radioactivity incorporated into GG ($\mu\text{g protein}^{-1}$). Protein was estimated according to Bradford (1976). All experiments were performed at least three times.

RESULTS

In vitro assay for GGP synthase/phosphatase

The biosynthesis of GG requires the glucosylation of glycerol. UDP-Glc and ADP-Glc were compared as possible substrates for glucosylation of G3P. GG was only synthesized in those tests which contained ADP-Glc (Fig. 1, lanes 3–7); substitution of ADP-Glc by UDP-Glc under otherwise optimal conditions prevented GG-synthesis (compare lanes 8 and 5 in Fig. 1).

On the basis of this result, a radiochemical assay for the

Fig. 2. Identification of the reaction products from the *in vitro* synthesis of GG. The assay was performed as in Fig. 1. Separation of the reaction products was done by two-dimensional TLC. Black spots, labelled reaction products; open circles, non-labelled marker amino acids. 1, GGP; 2, G3P; 3, aspartic acid; 4, glutamic acid; 5, GG; 6, citrulline; 7, alanine. The origin is not shown.

Fig. 3. Activation of the GG-forming enzyme system by NaCl *in vivo* and *in vitro*. NaCl was added to the culture medium (A; 324 mM), homogenization buffer (B; 324 mM) and assay (C; 170 mM) buffer, respectively, to yield the concentrations indicated. The assay was performed as in Fig. 1. GG was estimated by densitometry (□) and by liquid scintillation counting (■); the mean value of experiment 8 was set to 100%.

GG-forming enzyme system was developed, using ADP-Glc and [14 C]G3P as substrates. GGP should be the intermediary product of GG biosynthesis. When assay samples were analysed by two-dimensional TLC, only three spots were detected (Fig. 2). Two of these were identified as GG and G3P by rechromatography and

Fig. 4. Activity of the GG-forming enzymes after application of salt or organic osmolytes. The osmolytes were added to the culture medium [final concentration 324 mM NaCl (■), 648 mM NaCl (□) and 1.3 M sucrose (◆), respectively]; cells were harvested and homogenized after the times indicated. Homogenization, but not the assay, was performed in the presence of the osmolytes. GG was estimated by liquid scintillation counting. The values shown are means of four experiments.

comparison with authentic substances. The third spot yielded only GG following treatment with alkaline phosphatase and rechromatography (data not shown), suggesting its likely identity to be GGP. Usually, GGP formation was weak or undetectable.

In vivo and *in vitro* activation of the GG-synthesizing enzyme system

Enzyme extracts made from unstressed cells of *Synechocystis* sp. PCC 6803 were unable to synthesize GG (Fig. 1, lane 2). An extract of salt-stressed cells showed only poor activity (Fig. 1, lane 6). Activity could be markedly increased when NaCl was added to the homogenization or assay buffer using both unstressed (Fig. 1, lanes 3 and 4) and salt-stressed (Fig. 1, lanes 5 and 7) cells.

Activation of the GG-synthesizing enzyme achieved by *in vivo* salt shock (30 min, 324 mM NaCl) was substantially lost *in vitro* during NaCl-free manipulations (Fig. 3, column 5). However, activation could be retained when NaCl was present at the homogenization stage (Fig. 3, column 7), or restored when NaCl was included in the assays (Fig. 3, column 6), and was at a maximum when NaCl was present throughout the procedure (Fig. 3, column 8). GG synthesis could also be activated in unstressed cells *in vitro* by NaCl addition at the stage of homogenization, assay, or both (Fig. 3, columns 3, 2 and 4, respectively).

Enzyme activation achieved at one stage was not completely lost during subsequent NaCl-free stages. This can be seen from tests in which NaCl was omitted from the assay (Fig. 3, columns 3 and 7) or homogenization (Fig. 3, columns 2 and 6) stages. When NaCl was omitted from both homogenization and assay stages, a low, but detectable, enzyme activity was found (Fig. 3, column 5).

A detailed survey of the *in vivo* activation was obtained by time-response studies using different shock-times, strengths and osmolytes (Fig. 4). A rapid activation of the GG-synthesizing enzymes was observed immediately

after applying the osmolytes (324 mM NaCl, 684 mM NaCl and 1.3 M sucrose, respectively). In all cases the enzyme activities achieved the same order of magnitude during the first minutes after each shock treatment. The rapid activity increase was followed by a moderate increase up to 1–2 h which was more distinct for the lower NaCl concentration and was absent in the case of sucrose (Fig. 4).

Properties of the GG-forming enzyme system

Among three salts tested, NaCl had the most pronounced activating effect; KCl was less effective (approximately 50 % of the activity of NaCl) and NaNO₃ induced a small activation only (less than 10 % of the activation obtained by NaCl). Iso-osmolar concentrations of organic osmolytes were unable to induce GG synthesis *in vitro* (not shown); sucrose was effective *in vivo* (Fig. 4), but not *in vitro* (not shown). The enzyme system was cold resistant and could be extracted from lyophilized cells. Among three pH values tested, activity was highest at pH 8.5 and was reduced at lower pH values (78 % at pH 7.8; 50 % at pH 7.1).

To study the localization of the enzyme system in the cell, the homogenate was centrifuged at high speed (46 000 g). Apart from some loss of activity, caused perhaps during centrifugation, the activity of the soluble fraction was similar to that of the crude homogenate, while it was much smaller or absent in the membrane fraction (not shown).

DISCUSSION

In the present work, it has been shown that the precursor of GG synthesis in *Synechocystis* sp. PCC 6803 is ADP-Glc and not UDP-Glc. This contrasts with, for example, isofloridoside synthesis in *Poterioochromonas malhamensis* (Kauss, 1987) and trehalose synthesis in *Escherichia coli* (Gjæver *et al.*, 1988) and *Ectothiorhodospira halochloris* (Lippert *et al.*, 1993), which are strictly dependent on UDP-sugars (UDP-galactose and UDP-Glc, respectively), no trehalose being formed using ADP-Glc. However, in a few cases, ADP-Glc-dependent trehalose synthesis has been reported (Lippert *et al.*, 1993).

As expected, the reaction products were GG and GGP, although the latter did not accumulate in appreciable amounts. Obviously, GGP was rapidly dephosphorylated to GG, the massive accumulation of which was essential for adaptation to enhanced salt concentrations. Thus, the reaction proceeded in the following manner:

Enzymes: 1, ADP-glucose:glycerol-3-phosphate 2-glycosyltransferase (GGP synthase; EC 2.4.1.-); 2, glucosylglycerol-3-phosphate phosphohydrolase (GGP phosphatase; EC 3.1.3.-).

The *in vitro* assay developed here involved both enzyme activities, but reflected almost completely the synthase reaction. The phosphatase reaction was not rate limiting

since addition of alkaline phosphatase did not significantly enhance the yield of GG (data not shown). The occurrence of the phosphatase reaction could be shown indirectly by addition of the phosphatase inhibitor NaF, which suppressed GG formation (data not shown). For trehalose synthesis in bakers' yeast, it has been shown that the same protein has both enzyme activities (Londesborough & Vuorio, 1991).

The GG-forming enzyme system seems to involve a soluble protein which is present in a preformed, inactive state in unstressed cells and requires activation. This was achieved mainly *in vivo* by stressing intact cells of *Synechocystis* sp. PCC 6803 with NaCl-containing media, but also *in vitro* by adding NaCl to the homogenization buffer or assay buffer. KCl and, especially, NaNO₃ were less effective; organic osmolytes failed to stimulate the enzyme system at all (glucose, glycerol; data not shown), or showed only a stimulatory effect *in vivo* (sucrose). The activation was reversible, since activation attained at one stage was progressively lost if the next step was without NaCl, but could be restored by the addition of NaCl. The low enzyme activity found in extracts from salt-stressed cells under NaCl-free homogenization and assay conditions (see Fig. 3, column 5) might be due to NaCl remaining as a result of insufficient dilution during extract preparation (3.5-fold dilution at the step from homogenization to assay, and about 3-fold dilution at the suspension of the cell pellet prior to homogenization).

The salt-dependent activation may involve two mechanisms. This can be concluded from the two-phase activation kinetics found after a salt shock on cells of *Synechocystis* sp. PCC 6803 (*in vivo* activation; see Fig. 4). The rapid activation immediately after the salt shock may represent post-translational modification. This mechanism could also be responsible for the rapid activation of GG-synthesis *in vitro*. A similar post-translational activation mechanism has been reported for trehalose synthesis in *E. coli* after an osmotic shock. Trehalose-6-phosphate synthase is activated by high amounts of KCl and other ions by direct stimulation of the enzyme, and also by the induction of transcription (Gjæver *et al.*, 1988; Boos *et al.*, 1990). Altered gene expression can also be postulated for GG synthesis in *Synechocystis* sp. PCC 6803, since the *in vivo* activation process involves a long-term component (2.5-fold increase of enzyme activity within 2 h; see Fig. 4), which might be due to *de novo* protein synthesis. However, this seems to be of less importance, since earlier results have indicated that *de novo* protein synthesis is not necessary for the activation of GG synthesis (Hagemann *et al.*, 1990; Erdmann *et al.*, 1992).

The mechanism of post-translational modification is unknown. The related isofloridoside-phosphate synthase, which shows a similar activation/deactivation behaviour to the GG-forming enzyme system, is activated post-translationally by limited proteolysis with a membrane-associated protease which can be activated *in vivo* (NaCl) and *in vitro* (Ca²⁺ or detergents; Brunner & Kauss, 1988).

Other glucosyl transferases, such as β -glucan synthase and sucrose-phosphate synthase, are known to be regulated by

their phosphorylation/dephosphorylation status (Paliyath & Poovaiah, 1988; Huber & Huber, 1992). Covalent modification by protein phosphorylation is now thought to be a major mechanism for the control of cytoplasmic enzyme activities. It is therefore possible that the GG-synthesizing enzyme system is regulated in the same manner. This is supported by the identification of protein phosphorylation/dephosphorylation during salt adaptation of *Synechocystis* sp. PCC 6803 and its dependence on NaCl treatment in the same manner as GG synthesis (Hagemann *et al.*, 1993).

ACKNOWLEDGEMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft, FRG. We thank Mrs Ilse Dörr and Chem.-Ing. Karin Sommerer for their excellent technical assistance.

REFERENCES

- Allen, M. B. & Arnon, D. I. (1955). Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *Plant Physiol* **30**, 366–372.
- Boos, W., Ehmann, U., Forkl, H., Klein, W., Rimmel, M. & Postma, P. (1990). Trehalose transport and metabolism in *Escherichia coli*. *J Bacteriol* **172**, 3450–3461.
- Borowitzka, J. L., Demmerle, S., Mackay, M. A. & Norton, R. (1980). Carbon-13 nuclear magnetic resonance study of osmoregulation in a blue-green alga. *Science* **210**, 650–651.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Brunner, G. & Kauss, H. (1988). Deaggregation and proteolytic modification of a galactosyltransferase of *Poterochromonas malhamensis*. *Physiol Plant* **74**, 708–714.
- Erdmann, N. (1983). Organic osmoregulatory solutes in blue-green algae. *Z Pflanzenphysiol* **110**, 147–155.
- Erdmann, N., Berg, C. & Hagemann, M. (1989). Missing salt adaptation of *Microcystis firma* (cyanobacterium) in the dark. *Arch Hydrobiol* **114**, 521–530.
- Erdmann, N., Fulda, S. & Hagemann, M. (1992). Glucosylglycerol accumulation during salt acclimation of two unicellular cyanobacteria. *J Gen Microbiol* **138**, 363–368.
- Fulda, S., Hagemann, M. & Libbert, E. (1990). Release of glucosylglycerol from the cyanobacterium *Synechocystis* spec. SAG 92.79 by hypoosmotic shock. *Arch Microbiol* **153**, 405–408.
- Giæver, H. M., Styrvold, O. B., Kaasen, I. & Strom, A. R. (1988). Biochemical and genetic characterization of osmoregulatory trehalose synthesis in *Escherichia coli*. *J Bacteriol* **170**, 2841–2849.
- Hagemann, M., Erdmann, N. & Wittenburg, E. (1987). Synthesis of glucosylglycerol in salt-stressed cells of the cyanobacterium *Microcystis firma*. *Arch Microbiol* **148**, 275–279.
- Hagemann, M., Wölfel, L. & Krüger, B. (1990). Alterations of protein synthesis in the cyanobacterium *Synechocystis* sp. PCC 6803 after a salt shock. *J Gen Microbiol* **136**, 1393–1399.
- Hagemann, M., Gollack, D., Biggins, J. & Erdmann, N. (1993). Salt-dependent protein phosphorylation in the cyanobacterium *Synechocystis* sp. PCC 6803. *FEMS Microbiol Lett* **113**, 205–210.
- Huber, S. C. & Huber, J. L. (1992). Role of sucrose-phosphate synthase in sucrose metabolism in leaves. *Plant Physiol* **99**, 1275–1278.
- Kauss, H. (1987). Some experiments of calcium-dependent regulation in plant metabolism. *Annu Rev Plant Physiol* **38**, 47–72.
- Lippert, K., Galinski, E. A. & Trüper, H. G. (1993). Biosynthesis and function of trehalose in *Ectothiorhodospira halochloris*. *Antonie Leeweenhoek* **63**, 85–91.
- Londesborough, J. & Vuorio, O. (1991). Trehalose-6-phosphate synthase/phosphatase complex from bakers' yeast: purification of a proteolytically activated form. *J Gen Microbiol* **137**, 323–330.
- Mackay, M. A. & Norton, R. S. (1987). ¹³C nuclear magnetic resonance study of biosynthesis of glucosylglycerol by a cyanobacterium under osmotic stress. *J Gen Microbiol* **133**, 1535–1542.
- Mackay, M. A., Norton, R. S. & Borowitzka, L. J. (1983). Marine blue-green algae have a unique osmoregulatory system. *Mar Biol* **73**, 301–307.
- Paliyath, G. & Poovaiah, B. W. (1988). Promotion of β -glucan synthase activity in corn microsomal membranes by calcium and protein phosphorylation. *Plant Cell Physiol* **29**, 67–73.
- Reed, R. H. & Stewart, W. D. P. (1988). The response of cyanobacteria to salt stress. In *Biochemistry of the Algae and Cyanobacteria*, vol. 12, pp. 217–231. Edited by L. J. Rogers & J. R. Gallon. Oxford: Clarendon Press.
- Reed, R. H., Richardson, D. L., Warr, S. R. C. & Stewart, W. D. P. (1984). Carbohydrate accumulation and osmotic stress in cyanobacteria. *J Gen Microbiol* **130**, 1–4.
- Tel-Or, E., Spath, S., Packer, L. & Mehlhorn, R. J. (1986). Carbon-13 NMR studies of salt shock-induced carbohydrate turnover in the marine cyanobacterium *Agmenellum quadruplicatum*. *Plant Physiol* **82**, 646–652.
- Trüper, H. G. & Galinski, E. A. (1989). Compatible solutes in halophilic phototrophic procaryotes. In *Microbial Mats: Physiological Ecology of Benthic Microbial Communities*, pp. 342–348. Edited by Y. Cohan & E. Rosenberg. Washington DC: American Society for Microbiology.
- Warr, S. R. C., Reed, R. H., Chudek, J. A., Foster, R. & Stewart, W. D. P. (1985). Osmotic adjustment in *Spirulina plantensis*. *Planta* **163**, 424–426.

Received 2 August 1993; revised 2 November 1993; accepted 15 December 1993.