Glucosylglycerol accumulation during salt acclimation of two unicellular cyanobacteria

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(Received 26 June 1991; revised 14 October 1991; accepted 7 November 1991)

A turbidostat culture technique was used to study the effects of different salt shocks on the freshwater cyanobacteria *Synechocystis* sp. strain PCC 6803 and *Microcystis firma*. Shocks were performed either suddenly or gradually, on both unacclimated cultures and those pre-acclimated to 0.77 M-NaCl. All suddenly shocked cultures exhibited a decline in growth after a few hours, characterized by severely decreased metabolic activities (e.g. photosynthesis, respiration, glucose-6-phosphate dehydrogenase activity) and a time course of restoration which coincided with the accumulation of glucosylglycerol. Additionally, all untreated cultures had a late (after a few days) growth depression, distinguished by the stagnation of cell division. This was overcome by physiological adaptation of the whole cells or selection of cells with superior salt tolerance. The different types of growth depressions and the unique pattern of glucosylglycerol accumulation led to the conclusion that glucosylglycerol was necessary to maintain metabolic processes, but that this alone cannot account for successful salt acclimation.

Introduction

Salt acclimation is a complex process important to all organisms living in brackish-water habitats. To survive such unfavourable conditions, several different physiological strategies have evolved, such as salt-resistant enzymes, the exchange of K⁺ for toxic Na⁺, and the accumulation of compatible solutes. The latter is commonly found in micro-organisms, algae and higher plants and includes at least four subprocesses.

Firstly, activation of a plasma-membrane-bound ion pump to export Na⁺. In cyanobacteria, a Na⁺/H⁺ antiport has been characterized (Molitor *et al.*, 1990), which is accompanied by enhanced respiration, increased cytochrome oxidase activity (Jeanjean *et al.*, 1990; Molitor *et al.*, 1990) and glucose-6-phosphate dehydrogenase activity (G6PDH) which provides the respiratory substrate (Hagemann *et al.*, 1989).

Secondly, the accumulation of compatible solutes (low molecular mass hydrophilic compounds) which substitute ions for their osmotic function and are more or less nontoxic, even at high concentrations (recent reviews: Reed & Stewart, 1988; Trüper & Galinski, 1989; Csonka 1989; Kirst 1990).

Thirdly, ultrastructural changes, especially a reduction of the permeability of the plasma membrane (Apte *et al.*, 1987; Rivière *et al.*, 1990). The change in permeability is brought about by enhanced lipid content (Gimmler & Hartung, 1988) and/or alteration of lipid composition (Russell, 1989).

Fourthly, altered gene expression leading to modifications in the pattern of protein synthesis and the appearance of stress proteins (Apte & Bhagwat, 1989; Hagemann *et al.*, 1990). Sadka *et al.* (1989) have, however, questioned whether differential gene expression is really involved.

Although temporal and isotonic substitutions of ions as well as compatible solutes have been reported (Reed *et al.*, 1985), little is known about other temporal or any causal relations of the adaptive subprocesses. The present study was conducted to assess the role of the accumulation of glucosylglycerol, a compatible solute found in moderately salt-tolerant cyanobacteria (Reed & Stewart, 1988). In simultaneous acclimation experiments, cyanobacteria were subjected to different salt shocks and the time course of glucosylglycerol accumulation and associated physiological parameters were measured. For such parallel experiments, the turbidostat is an appropriate tool. It enables continuous growth of micro-organisms under constant and optimal culture conditions, and allows sampling without adversely interfering with growth.

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Abbreviations: Chl *a*, chlorophyll *a*; G6PDH, glucose-6-phosphate dehydrogenase.

Methods

Organism and growth conditions. Axenic cultures of *Synechocystis* sp. strain PCC 6803 (a gift of the Department of Genetics, Moscow University) were grown in a modified ($\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 110 mg l^{-1} ; NaCl , 117 mg l^{-1} ; Tris, 485 mg l^{-1}) medium of Allen & Arnon (1955) in a turbidostat fitted with an intermittent pigment- and adherence-independent control (Erdmann & Schiewer, 1978). *Microcystis firma* strain Gromow 398 (Leningrad State University), was used in one experiment. Cultures were maintained at 29°C , under continuous light (20 W m^{-2}) and aerated with CO_2 -enriched air (5%, v/v). After several days, a constant specific growth rate was reached (steady state). The culture was then subjected to a severe salt shock, as described in Results.

The specific growth rate μ was calculated according to the equation: $\mu = D \ln^{-1} V^{-1} [D = \text{effluent (ml) during time } t \text{ (h), } V = \text{suspension volume (700 ml)}]$.

The increase in NaCl concentration in the culture vessel during the shock experiment was calculated from the dilution rate, according to the equation: $S = S_R(1 - e^{-Dt}) + S_0$. Symbols: S , actual salinity (g l^{-1}); S_R , salinity in the reservoir vessel; S_0 , initial salinity in the culture vessel; D , flow rate (ml per culture volume); t , time (h).

Glucosylglycerol and glycogen-like polysaccharide. The cyanobacterial suspension (2 ml) was centrifuged at $2000 g$ for 5 min. The resultant pellet was extracted three times with 5 ml boiling 80% (v/v) ethanol for 3 min. Pooled ethanolic extracts (the residues were used for glycogen assay) were dried on a rotary evaporator (40°C) and the residue redissolved in 2 ml water. A 1 ml aliquot was hydrolysed by adding 2 M-HCl (0.3 ml) and heating at 100°C for 2 h. The clear solution was cooled, and neutralized with 4 M-NaOH. A 1 ml sample was taken for enzymic glucose estimation using a glucose oxidase kit (Fermognost, VEB Arzneimittelwerk, Dresden). Apart from glucosylglycerol, negligible amounts of glucose and glucose-containing soluble material would have been included in these measurements.

Glycogen-like polysaccharide was estimated using the method of Warr *et al.* (1985). The cyanobacterial pellet (after ethanolic extraction) was suspended in 5 ml 30% (w/v) KOH and heated at 100°C for 1 h. After centrifugation at $2000 g$ for 5 min, 1 ml supernatant was mixed with 1.3 ml 96% (v/v) ethanol, boiled for 2 min and placed in a refrigerator overnight. The sample was centrifuged at $2000 g$ for 10 min, and the pellet dissolved by heating at 100°C in 2 ml water. After cooling and centrifugation, 1 ml supernatant was mixed with 2 ml anthrone reagent (100 mg anthrone in 50 ml H_2SO_4), heated to 100°C for 15 min and then cooled. Absorbance was measured at 625 nm using a spectrophotometer (Zeiss).

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49). Cyanobacterial suspension (15 ml) was centrifuged at $2000 g$ for 5 min, and the pellet washed with 50 mM-Tris-maleate, pH 7.2, containing 2 mM- Na_2EDTA and 3 mM-2-mercaptoethanol. The pellet was homogenized for 2×1 min using an ice-cooled cell mill (Bühler, Tübingen) after adding 1 ml of glass beads (diam. 0.1–0.2 mm). After centrifugation at $22000 g$ for 15 min, the supernatant was used for measurements of enzyme activity.

The enzyme assay mixture contained 0.1 ml of crude protein extract, 0.5 ml buffer (50 mM-Tris/maleate, pH 6.8, 2 mM- Na_2EDTA), 0.1 ml NADP^+ solution (11 mg ml^{-1}). The reaction was initiated by adding 0.05 ml of glucose 6-phosphate solution (11 mg ml^{-1}). The change in A_{340} was continuously recorded at 30°C for 5 min (SP 800, Pye Unicam). Protein was determined by the Lowry method.

Photosynthesis and dark respiration. Photosynthetic and respiratory rates were measured using a Clark-type oxygen electrode (MV 85 F, Metra Radebeul). The measurement was made with undiluted

suspensions (5 ml) under conditions similar to those of the turbidostat (29°C , 20 W m^{-2}).

Cell number and mean diameter. Single and double cells, as well as larger cell aggregates, were counted on photographs taken using light microscopy. Biomass, cell number and mean diameter were determined with a particle size analyser using the Coulter principle (Laborscale, Medicor Budapest, Hungary). Cyanobacterial suspensions were diluted 500-fold with filtered NaCl solution (0.8%, w/v) and analysed at the following settings: aperture, $75 \mu\text{m}$; current, 100–400 μA ; lower discriminator, 50 mV; amplification, $\times 10^5$. The calculation of the mean diameter using the size distribution histogram (64 channels) was made as described (Heidenreich & Sackrow, 1978).

Chlorophyll *a*. The suspension (5 ml) was filtered through glass-fibre filters (SM 13400, Sartorius). The filters were dipped into 10 ml methanol for 1 h. Samples were centrifuged at $2000 g$ for 5 min. The absorbance of the supernatant was measured at 665 nm and Chl *a* concentration was calculated using a specific absorption coefficient of $79.24 \text{ l g}^{-1} \text{ cm}^{-1}$ (Lichtenthaler, 1987).

All turbidostat experiments were repeated at least twice using independent cultures. One representative experiment was used for the figures. All assays were done in duplicate and averaged.

Results

In the first series of experiments, two independent turbidostat cultures of *Synechocystis* PCC 6803 were set to equal conditions such that the major growth parameters of both cultures were synchronous (Table 1, Fig. 1, day –1 to 0). The first culture was upshocked by the addition of crystalline NaCl to the culture vessel (sudden shock), whereas the second one was fed with NaCl -containing medium from the reservoir vessel according to the growth rate (gradual shock). Fig. 1(a) shows the resulting courses of NaCl enrichment in each of the cultures.

The progress of salt acclimation was different during the first two days (Fig. 1). In the suddenly, but not in the gradually shocked culture, growth and photosynthesis declined during the first hours (Fig. 1b and 1c, respectively), with the greatest depression after about 10 h. Partial restoration of growth and photosynthesis coincided with the accumulation of glucosylglycerol, which started immediately after the shock and was quite similar in both cultures (Fig. 1d). After 1.5 to 2 d, growth and photosynthesis reached their maximal recovery. Although high levels of glucosylglycerol were accumulated and photosynthesis was almost fully active at this stage, the growth rate declined again and reached a minimum between the 6th and 8th day in both cultures. Thereafter a gradual recovery of growth led to salt-acclimated cells after more than two weeks.

To verify this uncommon acclimation course, the sudden shock was investigated with another glucosylglycerol-accumulating freshwater cyanobacterium (*Microcystis firma*) and in more detail in a second turbidostat series (Fig. 2). An immediate depression again occurred in growth and photosynthesis, and was also observed for

Fig. 1. Time courses of stress-relevant parameters after different hypertonic shocks (770 mM-NaCl) on a freshwater-based culture of *Synechocystis* PCC 6803. Two independent turbidostat cultures were set to equal conditions (main growth parameters are given in Table 1). After reaching a steady state of growth (days -1 to 0), the cultures were shocked either suddenly (○) or gradually (●) at day 0 (vertical line). (a) Course of NaCl enrichment in the medium; (b) course of growth as determined from the dilution rate of the turbidostat; (c) photosynthetic O_2 evolution per litre of suspension; (d) glucosylglycerol accumulation in the cells per litre of suspension.

respiration, Chl *a* content and the G6PDH activity. Glucosylglycerol synthesis started without a lag phase after the shock and without a proportional decrease in glycogen content. Glycogen is often considered a possible precursor of glucosylglycerol and sucrose (Warr *et al.*, 1985; Mackay & Norton, 1987; Reed & Stewart, 1988; Erdmann *et al.*, 1989; Kerby *et al.*, 1990) and interconversion of glycogen and glucosylglycerol has been shown to occur in the early period after illuminated salt shock in the marine cyanobacterium *Agmenellum quadruplicatum* (Tel-Or *et al.*, 1986).

In our illuminated cultures, glucosylglycerol was assumed to be synthesized via photosynthetic CO_2 assimilation (Fig. 2d). Although photosynthesis decreased to 20% of the non-shocked value in some experiments, the remaining C fixation rate was 10 times more than that needed for glucosylglycerol synthesis [assuming the C fixation rate at minimum was $12.59 \text{ mg l}^{-1} \text{ h}^{-1}$ and C demand for glucosylglycerol synthesis was

Fig. 2. Time course of salt acclimation after a sudden shock on a freshwater-based culture of *M. firma*. Cells were grown in a turbidostat until a steady state of growth was reached (see Table 1). Day -1 to 0, steady state; day 0, sudden NaCl addition to 770 mM; day 0 to 7, acclimation course; days 8 to 23, final recovery (compressed time scale). (a) Growth as determined from dilution rate; (b, c) light-dependent O_2 evolution and dark O_2 consumption, respectively, per litre of suspension; (d, e, f) contents of glucosylglycerol, glycogen and Chl *a*, respectively, per litre of suspension after separation and extraction of the cells; (g) specific G6PDH activity.

$1.30 \text{ mg l}^{-1} \text{ h}^{-1}$, when the accumulation rate of the first 10 h was averaged ($4.59 \text{ mg l}^{-1} \text{ h}^{-1}$)). Later, when the rate of glucosylglycerol synthesis diminished and growth became depressed (late depression), the excess of fixed carbon might be channelled into the glycogen-synthesizing pathway (Fig. 2e). This would explain the massive increase of glycogen and is consistent with the substantial increase in the number of glycogen granules reported previously (Schiewer & Jonas, 1977; Potts *et al.*, 1983; Lefort-Tran *et al.*, 1988). Glycogen accumulation seems to be a general feature for bacteria exposed to stress (Potts *et al.*, 1983; Shively *et al.*, 1988).

Photosynthesis, respiration, G6PDH activity, Chl *a* and growth increased in the same manner as glucosylglycerol content. When the maximum level of glucosyl-

Table 1. Comparison of major steady state growth parameters of the different turbidostat series

Values of the last 3 days before salt shock were averaged. Cultures were subjected to either sudden (s) or a gradual (g) shock.

Turbidostat series		$10^{-6} \times$ Cell number ml^{-1}	Doubling time (h^{-1})	Chl <i>a</i> content (mg l^{-1})	Photosynthetic rate [$\mu\text{mol O}_2 (\text{mg Chl } a)^{-1} \text{ h}^{-1}$]	Species
1 (Fig. 1)	s	74.2	10.0	ND	ND	<i>Synechocystis</i>
	g	69.7	9.1	ND	ND	PCC 6803
2 (Figs 2 and 3)	s	81.1	15.9	8.04	396.3	<i>Microcystis firma</i>
3 (Fig. 4)	s	112.6	15.3	7.17	426.5	<i>Synechocystis</i>
	g	121.6	17.3	7.20	414.8	PCC 6803

ND, Not determined.

glycerol was reached, all parameters reached levels similar to those of salt-acclimated cells (Fig. 2).

The recovery processes coincided closely with an increase in cell size and in the ratio of double to single cells. This transition was monitored microscopically as well as electronically, using a particle size analyser. Microscopic cell analysis revealed both cell enlargement and cell aggregation. Cell counts showed a decline in the number of single cells from about 50% (non-shocked culture) to 5% with a parallel rise of double cells from 50 to 75% (Fig. 3a). In the size histogram drawn by the particle analyser, these changes resulted in the splitting of the single peak, typical for cells grown in standard medium, into two. In Fig. 3(b), this splitting is represented by the branching of the diameter curve. At the zenith of the depression, single cells were nearly absent and almost all cells were present as unseparated cell aggregates. Cell division was apparently disturbed at this stage. A similar situation can arise from the effect of dark on cyanobacterial cell cycle events. Limitation of light stopped or slowed the cell cycle and arrested a certain fraction of the population in the doublet stage (Marino & Asato, 1986; Waterbury *et al.*, 1986; Armbrust *et al.*, 1989).

The late depression of growth could only partly be associated with an inhibition of cell metabolism (Fig. 2): respiration, glycogen synthesis and G6PDH remained active. G6PDH showed enhanced activity during the acclimation course and might cooperate with respiration, which is known to be integrated with the active exclusion of Na^+ ions (Molitor *et al.*, 1990).

Eighteen days after the sudden shock, the growth rate reached a value typical for salt-acclimated cells (about 0.03 h^{-1} in this experiment). Assuming this value was the growth rate of at least a few cells during the whole acclimation course, they would have undergone about 18 cell doublings.

In a third series of turbidostat experiments (Fig. 4) a

Fig. 3. Size and aggregation state of *M. firma* cells during the salt acclimation process shown in Fig. 2. (a) Composition of the population, as determined by microscopic cell counting (○, single cells; ●, double cells; ■, higher aggregates); (b) mean diameter of the cells estimated by particle size analyser; branching represents the transformation of single (lower curve) into aggregated (upper curve) cells.

culture of *Synechocystis* PCC 6803 was used which had been previously upshocked (to 600 mM) and then re-adjusted to freshwater conditions (each for 3 weeks, pretreated population). In contrast to the other two turbidostat experiments, a salt shock of 770 mM-NaCl had no dramatic consequences (Fig. 4). The acclimation period was only 3 d (4 doublings) for both suddenly and gradually shocked cultures, and productivity in the new steady state was only slightly reduced (growth rate by less than 10%, photosynthesis by about 20%). Only the suddenly shocked cells showed an immediate depression: growth and photosynthesis reached a minimum after 14 h and recovered within 3 d; the late depression was completely absent.

The more rapid acclimation of the pretreated population to 770 mM-NaCl was not the result of a more efficient accumulation of glucosylglycerol (Fig. 4d). Its accumulation started from nearly zero with the usual kinetics and to the usual internal concentration that counterbalanced approximately 25% of the external osmolality (medium of $1.4 \text{ osmol kg}^{-1}$; pretreated cells of $312 \pm 48 \text{ mM}$; non-pretreated cells of $357 \pm 137 \text{ mM}$;

Fig. 4. Time courses of stress-relevant parameters after different hypertonic shocks on a pre-treated culture (preceding NaCl acclimation and re-acclimation to standard medium) of *Synechocystis* PCC 6803 (for further details see Table 1 and Figs 1 and 2).

values are means of one and four experiments, respectively, with confidence intervals for 95% probability; calculation based on whole cell volume). The value of 25% is similar to reported data (>20 to <40%) of Reed & Stewart (1988) and Reed *et al.* (1985).

Compared to the sudden shock, the accumulation of glucosylglycerol was slightly retarded in gradually shocked cells (Figs. 1 and 4). It is unclear whether this difference was the result of either lower synthesis rates (caused by small NaCl gradients) or faster biovolume increases (noninhibited growth).

Discussion

Synechocystis PCC 6803 was subjected to severe salt shocks (770 mM) near the tolerance limit (about 1 M) to determine the importance of glucosylglycerol accumulation as a major subprocess of salt acclimation. Two different types of growth depression were observed. First, an immediate cessation which occurred in all sudden shocks and is well-known for both turbidostat and batch cultures (Blumwald & Tel-Or, 1984; Vonshak *et al.*, 1988; Sadka *et al.*, 1989). The depression

culminated at about 12 h, impaired photosynthesis, respiration and G6PDH activities, and was overcome in parallel with the accumulation of glucosylglycerol. This supported the view that compatible solutes possess protective functions (Schobert, 1980). No immediate depression was observed in gradually shocked cultures.

Second, a late depression was observed in all previously non-shocked cultures, but was absent in all NaCl-pretreated cultures. The growth depression culminated after about 7 d, and was characterized by most of the cells arresting at a two-cell stage. This type of depression was restricted to high-strength shocks; a shock of minor strength (600 instead of 770 mM-NaCl) did not induce this depression (data not shown). To overcome this late depression (after the 8th day), there must have been either selection of a few cells capable of growth in 770 mM-NaCl, giving rise to a new population, or adaptation by the entire cell population. The latter requires differential gene expression, and is assumed to occur in *Dunaliella* cells (Ginzburg *et al.*, 1990), and *Sorghum* seedlings (Amzallag *et al.*, 1990), both of which showed a similar late growth depression when exposed to high salt concentrations. Here, however, selection of salt-tolerant cells seems more probable, because the pretreated cultures hold the re-acclimation ability for a long time [at least 3 weeks, in contrast to 2.5 h for *Dunaliella* cells (Ginzburg *et al.*, 1990)].

The late depression appeared after glucosylglycerol had been accumulated in stress-relevant amounts. This led us to conclude firstly, that all cells must be able to synthesize glucosylglycerol when exposed to stress, and secondly, that glucosylglycerol is a necessary, but not totally sufficient, prerequisite of salt acclimation.

Part of the work was supported by a grant from the Fonds der Chemischen Industrie, FRG. The critical help of Dr D. N. Thomas and the technical assistance of Chem.-Ing. K. Sommerer and Mrs I. Dörr are gratefully acknowledged.

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