Incorporation of $^{14}$C in the cyanobacterium
Synechococcus PCC 6301 following salt stress

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Abstract. Synechococcus PCC 6301 synthesized sucrose as a compatible solute following hyperosmotic shock induced by NaCl. Initial rates of photosynthetic $^{14}$C incorporation were reduced following salt shock. Photosynthetic rates were comparable in cells enriched for glycogen (by growth in NO$_3$-deficient medium) and cells grown in NO$_3$-sufficient medium in the absence of osmotic shock. Incorporation of $^{14}$C was predominantly into the NaOH fraction and the residual acidic fraction in cells grown in NO$_3$-sufficient medium, whereas incorporation was predominantly into the residual acidic fraction in cells grown in NO$_3$-deficient medium. Following salt stress, $^{14}$C incorporation was initially into the ethanol-soluble fraction and the majority of tracer was recovered in sucrose. Carbon-14 was detected in sucrose in cells which had been enriched for [a$^{14}$C]glycogen prior to salt stress, inferring that glycogen can act as a carbon source for sucrose synthesis following salt stress. Changes in the specific activity of sucrose are consistent with an initial synthesis of sucrose from glycogen followed by synthesis of sucrose using newly fixed carbon, in response to salt stress.

Key words: Cyanobacteria – Osmoregulation – Photosynthesis and salt stress – Salt stress – Sucrose synthesis and salt stress – Synechococcus

Introduction

The salinity-dependent biosynthesis of one or more low-molecular-mass organic compounds as intracellular solutes (osmolytes) is a feature shared by cyanobacteria from a diverse range of habitats (Reed et al. 1986). The accumulated solutes include the disaccharides sucrose and trehalose, the heteroside glucosylglycerol and the quaternary ammonium compounds glycine betaine and glutamate betaine (Reed and Stewart 1988). In general, a single strain-specific organic solute is synthesized as the principal intracellular osmolyte (Borowitzka et al. 1980), although certain cyanobacteria also accumulate osmotically significant quantities of a secondary organic solute (Warr et al. 1985a). Several studies, using diverse strains from culture collections, have suggested that there may be a relationship between the chemical nature of the principal organic osmolyte and the upper salinity limit for growth, with disaccharide accumulation in the least halotolerant isolates, heteroside accumulation in strains of intermediate halotolerance and betaine accumulation in the most halotolerant forms; this is in accordance with the relative compatibility of these osmolytes in vitro (Warr et al. 1988) and supports the hypothesis that the distribution of cyanobacteria in natural saline habitats may be affected by the category of organic solute accumulated in response to salt stress (Warr et al. 1987). However, a recent study using carbohydrate-accumulating isolates from a single marine site suggests that this proposal may be somewhat oversimplified, as there was no clear relationship between disaccharide and/or heteroside synthesis and the salinity tolerances of these cyanobacteria (Stal and Reed 1987). In contrast, the presence of glycine betaine or glutamate betaine in all strains from the most hypersaline habitats indicates that extreme halotolerance may be linked to the accumulation of such osmolytes (Reed et al. 1986).

The biosynthesis of the carbohydrate glucosylglycerol in the marine unicellular cyanobacterium Synechocystis N100 under osmotic stress has been studied by Mackay and Norton (1987), using $^{13}$C enrichment and nuclear-magnetic-resonance spectroscopy. A rapid increase in the level of this heteroside followed hyperosmotic shock of light-incubated cells, with most of the solute being synthesized from newly fixed carbon, rather than from carbon reserves (e.g. glycogen). Similar findings were reported for Synechococcus PCC 7002 in response to osmotic stress (Tel-Or et al. 1986). Pulse-chase experiments with Synechococcus PCC 7002 also showed that $^{13}$C-enriched glycogen, which had been accumulated by cells grown at low salinities under nitrogen-limitation,
could be used for the light-dependent biosynthesis of glucosyglycerol in response to hypersaline treatment. However, no quantitative data were obtained for the interconversion of low-molecular-mass carbohydrates and storage polysaccharides.

The interconversion of organic osmolytes and glycogen has been demonstrated in salt-stressed *Nodularia harveyana* using electron microscopy (Warr et al. 1984) and *Spirulina platensis* by chemical assay (Warr et al. 1985b). Synthesis of glucosyglycerol and sucrose in darkness by glycogen-enriched *Synechocystis PCC 6714* provides additional evidence that low-molecular-mass osmolytes can be synthesized from storage polysaccharides. Here, we report on the osmotic responses of salt-stressed *Synechococcus* PCC 6301 with particular reference to the role of newly fixed carbon and/or carbon reserves (presumably glycogen) in the biosynthesis of sucrose.

**Material and methods**

**Organism and growth conditions.** An isolate of *Synechococcus* PCC 6301 was obtained in axenic culture from the collection of the Institut Pasteur (Paris, France). Cultures were maintained in BG11a (Rippka et al. 1979), a freshwater-based medium, until required for experimental purposes. Cells were grown in batch culture (21) bubbled with air, in BG11a, at a photon flux density of 50 μmol·m⁻²·s⁻¹ at room temperature to a chlorophyll a concentration of 3 μg·ml⁻¹. Prior to salt stress, cultures were divided and the cells resuspended in three different growth media which were incubated at room temperature on an orbital shaker at a photon flux density of 100 μmol·m⁻²·s⁻¹. Control cells were grown in nitrate-containing medium, BG11a, containing 10 mM NaHCO₃. To enrich cells with glycogen, they were grown in nitrate-free medium, BG11a, containing 10 mM NaHCO₃ or, to enrich with ¹⁴C-labelled glycogen, in BG11a containing 10 mM NaH¹⁴CO₃ (370 kBq·mmol⁻¹). In all treatments a further 100 g·l⁻¹ NaHCO₃ or NaH¹⁴CO₃ (370 kBq·mmol⁻¹) was added after 2 d. The pH of the medium was adjusted to 8.5 prior to the first addition of either NaHCO₃ or NaH¹⁴CO₃. After 5 d, cells were harvested by centrifugation, washed and resuspended in fresh medium in the absence of NaHCO₃ or NaH¹⁴CO₃. Salt stress was induced by the addition of NaCl to a final concentration of 250 mM, and NaH¹⁴CO₃ to a final concentration of 10 mM (370 kBq·mmol⁻¹) was added to cultures which had not been enriched for [¹⁴C]glycerol. 10 mM NaHCO₃ was added to [¹⁴C]glycogen-enriched cells. Growth conditions during salt stress were the same as those described for glycogen enrichment.

**Cell fractionation.** Aliquots of culture (35 ml) were removed from salt-stress and control treatments. Cells were harvested by centrifugation and washed in fresh medium, with or without NaCl depending on the treatment, to remove NaH¹⁴CO₃. Cell pellets were resuspended in 80% (v/v) ethanol (8 ml) containing 10 μmol glyceraldehyde as an internal standard. The ethanol extract was deproteinized using exhaustive centrifugation at 10000 × g for 10 min and the cell pellets were washed a further five times with 1 ml of 80% ethanol and the extracts combined. Residual pellets were then extracted with distilled water (2 ml) at 100°C for 30 min and after centrifugation the pellets were washed twice with water (1 ml). Residues were then extracted with 1 M NaOH (2 ml) at 100°C for 30 min followed by two further extractions with 1 M NaOH (1 ml). Finally, the remaining residues were extracted with 1 ml of 6 M HCl for 15 h at 50°C. Incorporation of [¹⁴C] into aliquots of the ethanol, hot water, NaOH and HCl fractions was determined by liquid scintillation counting using Ecoscint A scintillation fluid (BS and S (Scotland), Edinburgh, UK) and a Packard (Caversham, Berks., UK) Tri-carb 4000 liquid scintillation counter. Data for the aqueous and NaOH extracts have been combined. All data are the means of duplicate experiments.

Rates of photosynthetic [¹⁴C]CO₂ incorporation were determined by removing 100 μl of culture at appropriate time points and adding 10 μl of concentrated HCl. Following incubation for 24 h at room temperature, 5 ml of scintillant was added and liquid scintillation counting performed as described above.

**Separation of low-molecular-mass metabolites by high-performance liquid chromatography and determination of [¹⁴C] incorporation into sucrose.** The combined ethanol extracts were dried down on a hot-plate at 40°C, the residue from each sample was dissolved in 200 μl of Milli Q water (Millipore, UK) and centrifuged at 10000 × g for 5 min. Aliquots (10 μl) were injected onto Waters 10-cm-long, 8-mm-diameter Silica Pak cartridge (8SM HP41a), fitted in a Waters Radial Compression Unit (17 MPa), by a Waters Wisp Autosampler (Millipore, UK). Solutes were eluted at a flow rate of 3 ml·min⁻¹ and were detected using a refractometer with the mobile phase as a reference, as described previously (Kerby et al. 1989).

The [¹⁴C] incorporated into individual low-molecular-mass solutes was detected using a 9700 Series Radioactivity Monitor (Reeve Analytical, Glasgow, UK) fitted with a 200-μl heterogeneous flow cell in series prior to the refractometer. Efficiency of counting was calculated using known activities of [¹⁴C] glucose and [¹⁴C]fructose and was determined to be 72%.

The mobile phase consisted of acetonitrile:water:ΣSAM 1 reagent (77:21:2, by vol.). Milli Q water was used throughout, and the mobile phase was filtered through 0.22-μM Durapore filters (Millipore), bubbled with argon and stored under argon. The column was conditioned with ΣSAM 1 reagent (Waters) as described previously (Kerby et al. 1989).

A Waters Maxima 820 Chromatography Workstation was used to collect data and control a Waters 510 pump. Organic solutes were detected with a Waters differential refractometer (R401). Samples were injected onto the column using a Waters 712 Wisp Autosampler. The mobile phase was stored under argon using a Waters eluent-stabilization system.

**Chlorophyll concentration and cell size.** Chlorophyll a was extracted in methanol in the dark at 4°C. Absorbance was measured at 663 nm and the chlorophyll concentration calculated according to Mackinney (1941). Cell numbers and cell sizes were determined as described previously (Reed et al. 1987) using a ZB counting unit coupled to a C1000 channelizer sizing module (Coulter Electronics, Luton, Beds., UK).

**Chemicals.** The NaH¹⁴CO₃ was obtained from Amersham International (Amersham, Bucks., UK). All chemicals were of the highest grade available and were purchased from BDH (Poole, Dorset, UK), Millipore (UK), (Watford, Herts., UK) (for Waters products) or Rathburn Chemicals (Walkerburn, Borders Region, UK).

**Results**

Growth of *Synechococcus* PCC 6301 under nitrogen-limiting conditions results in the accumulation of glycogen (Smith 1982). Table 1 shows the data for cell number, size and chlorophyll a concentration for cells which were grown under nitrogen-limiting conditions