Osmotic Adjustment of Cyanobacteria: the Effects of NaCl, KCl, Sucrose and Glycine Betaine on Glutamine Synthetase Activity in a Marine and a Halotolerant Strain

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The effects of NaCl, KCl and the organic osmotica sucrose or glycine betaine on glutamine synthetase (GS) transferase activity in crude extracts of the marine *Nodularia harveyana* and the halotolerant *Synechocystis* sp. DUN52, respectively, were examined. NaCl at low concentrations (< 0.75 M) stimulated the enzyme (up to 30%) in the halotolerant strain but was inhibitory above 0.2 M in the marine strain. In both strains KCl (0.1–1.3 M) stimulated GS activity and was inhibitory only above 1.4 M. Sucrose, the major intracellular organic osmoticum in *N. harveyana*, had only a slight inhibitory effect until concentrations above 0.3 M; 2.0 M sucrose caused a 60% inhibition. The quaternary ammonium compound glycine betaine, the primary organic osmoticum in *Synechocystis* sp. DUN52, was not inhibitory at physiologically relevant concentrations up to 1.8–2.0 M and reduced NaCl inhibition by 10–30% at NaCl concentrations from 0.8 to 2.0 M in the halotolerant strain when used at 1.0 M. At NaCl concentrations above 0.2 M, 0.2 M KCl reduced the activity of GS by 6–15%. Sucrose and KCl showed similar degrees of protection against NaCl inhibition in *N. harveyana* when used at concentrations similar to those measured in seawater-grown cells of *N. harveyana*. Increasing concentrations of glycine betaine increased protection of GS activity against NaCl inhibition at NaCl concentrations above 0.5 M. These results suggest that the extent of salt tolerance of a particular strain of cyanobacterium may depend, in part at least, on the metabolic effects of compounds that are accumulated as internal osmotica in that strain.

INTRODUCTION

Low molecular weight carbohydrates including glucosylglycerol (Borowitzka *et al.*, 1980; Erdmann, 1983; Mackay *et al.*, 1983; Richardson *et al.*, 1983), sucrose (Blumwald & Tel-Or, 1982; Erdmann, 1983; Warr *et al.*, 1984), trehalose (Reed & Stewart, 1983; Reed *et al.*, 1984b) and the quaternary ammonium compound glycine betaine (Mohammad *et al.*, 1983) are known to function as major internal osmotica in a range of cyanobacteria under conditions of osmotic stress. For these compounds to be tolerated at high intracellular concentrations they must act as compatible solutes, i.e. they must allow enzymic function to continue at reduced water activity (Brown, 1978). The first described compatible solutes were the polyols which accumulate in sugar-tolerant yeasts (Brown & Simpson, 1972). Since then several other compounds have been shown to act as compatible solutes in a range of micro-organisms and higher plants: KCl (K^+) in halophilic bacteria (Aitken & Brown, 1972), glycerol in *Dunaliella* (Borowitzka & Brown, 1974), proline in *Triglochin maritima* (Stewart & Lee, 1974), sorbitol in *Plantago maritima* (Ahmad *et al.*, 1979) and glycine betaine in *Hordeum vulgare* (Pollard & Wyn Jones, 1979).

Abbreviation: GS, glutamine synthetase.

Here we report the effects of the inorganic solutes NaCl and KCl together with either sucrose or glycine betaine, respectively, on the activity of glutamine synthetase (GS), a key enzyme of nitrogen metabolism (Stewart, 1980), in *Nodularia harveyana*, a marine cyanobacterium in which sucrose serves as an internal osmoticum (Warr *et al.*, 1984) and in *Synechocystis* sp. DUN52, a halotolerant strain which accumulates glycine betaine as an internal osmoticum (Reed *et al.*, 1984a).

METHODS

Experimental material. Axenic cultures of the marine cyanobacterium *Nodularia harveyana* SMBA 235 (see Warr *et al.*, 1984) were grown in BG-11₀ medium (Rippka *et al.*, 1979) supplemented with 0.5% (w/v) sea salt (Gerrard Biological Centre, West Sussex, UK) at 30 °C and at a photon flux density of 45 $\mu\text{E m}^{-2} \text{s}^{-1}$. Axenic cultures of the halotolerant cyanobacterium *Synechocystis* sp. DUN52, originally isolated from the hypersaline stromatolites of the Al-Khiran area of Kuwait (Mohammad *et al.*, 1983) were grown in BG-11 medium supplemented with 4.0% (w/v) sea salt under the same conditions as above.

Extraction and measurement of glutamine synthetase. The same procedure was used with both organisms. Cyanobacterial cells from exponential phase batch cultures were harvested by centrifugation at 2500 *g* for 20 min. The pellet was washed three times in 50 mM-Tris/HCl buffer, pH 7.5, then resuspended in the same buffer. The cells were disrupted by passage three times through a French pressure cell at 110 MPa. Debris was removed by centrifugation at 25000 *g* for 30 min and the supernatant was assayed for GS activity. Mn^{2+} -dependent GS transferase activity was measured using a modification of the method of Pamiljans *et al.* (1962). The basic reaction mixture contained, in a total volume of 2.0 ml: 0.1 ml extract, 15 mM-glutamine, 1.5 mM- MnCl_2 , 10 mM-potassium arsenate, 0.2 mM-ADP (sodium salt), 30 mM-hydroxylamine, 30 mM-NaOH, and various amounts of the solute under examination. Glutamine, ADP and all solute stock solutions were formulated in 50 mM-Tris/HCl buffer, pH 7.5. Samples were incubated for 15 min at 30 °C. The reaction was stopped by adding 2.0 ml of a solution containing 120 mM- FeCl_3 , 125 mM-trichloroacetic acid and 250 mM-HCl; the production of L-glutamic acid γ -monohydroxamate was then measured against a standard curve at 540 nm. In the case of samples containing glycine betaine, a series of standards containing different amounts of glycine betaine was prepared and incubated under the above conditions. The A_{540} of glycine betaine alone was subtracted from that in the presence of enzyme extract.

Protein and chlorophyll measurements. Protein was estimated by the Lowry method with bovine serum albumin as standard. Chlorophyll *a* was determined according to Mackinney (1941).

Measurement of intracellular Na^+ and K^+ concentrations. Cells were washed in ice-cold isotonic $\text{Ca}(\text{NO}_3)_2$ for 15 min, to remove Na^+ and K^+ from the cell wall matrix. The samples were then digested using 1.0 ml HNO_3 at 100 °C for 60 min and diluted to a known volume. Na^+ and K^+ concentrations were determined using an EEL model 100 flame photometer. Intracellular cation concentrations for *N. harveyana* were calculated using an intracellular volume/chlorophyll ratio as before (see Warr *et al.*, 1984); those for *Synechocystis* sp. DUN52 were calculated using intracellular volumes directly estimated on a Coulter counter, model ZB fitted with a C1000 Channelyzer (Coulter Electronics, Luton, UK) and linked to an Acorn microcomputer.

RESULTS

Effect of NaCl, KCl, sucrose and glycine betaine on glutamine synthetase activity. The addition of 0.1 M-NaCl resulted in a minor stimulation of GS activity in *N. harveyana* but NaCl concentrations in excess of 0.2 M were inhibitory; the degree of inhibition increased with increasing concentrations of NaCl (Fig. 1*a*). This contrasted sharply with the response of *Synechocystis* sp. DUN52 where NaCl stimulated enzyme activity at concentrations up to 0.75 M with maximum stimulation (30%) occurring at 0.3 M (Fig. 1*b*). Although NaCl concentrations above 0.75 M were inhibitory to GS in *Synechocystis* sp. DUN52 the enzyme from the halotolerant strain was more resistant to inhibition by NaCl than the enzyme from the marine *N. harveyana*, with 50% inhibition at 1.85 M-NaCl in *Synechocystis* sp. DUN52 compared to 50% inhibition at 1.3 M-NaCl in *N. harveyana*.

The effect of KCl on GS activity in *N. harveyana* and *Synechocystis* sp. DUN52 is also shown in Fig. 1(*a,b*). In *N. harveyana* KCl was non-inhibitory at concentrations up to 1.4 M; there was a marked stimulatory effect at lower concentrations with maximum stimulation (up to 30%) between 0.2 and 0.5 M-KCl. Similarly in *Synechocystis* sp. DUN52, KCl was non-inhibitory at concentrations below 1.3 M, with maximum stimulation (48%) at 0.3 M-KCl. The internal K^+ concentrations of both strains are shown in Table 1; in both cases the measured *in vivo*

Fig. 1. Effect of NaCl (○), sucrose (●), KCl (□) and glycine betaine (■) on GS activity in (a) *Nodularia harveyana* and (b) *Synechocystis* sp. DUN52. Each point is the mean of two replicates (standard deviation < 2%). [The specific activities of GS were found to be between 523 and 602 nmol min⁻¹ (mg protein)⁻¹ in *Nodularia harveyana* and between 285 and 359 nmol min⁻¹ (mg protein)⁻¹ in *Synechocystis* sp. DUN52 throughout the present study.]

Table 1. Intracellular concentrations of NaCl, KCl, sucrose and glycine betaine in *N. harveyana* and *Synechocystis* sp. DUN52

Solute	Intracellular concentration (M)	
	<i>N. harveyana</i>	<i>Synechocystis</i> sp. DUN52
Na ⁺	0.024*	0.145*
K ⁺	0.142*	0.158*
Sucrose	0.270†	<0.003‡
Glycine betaine	<0.015†	0.782‡

* Values from cells used in this study.

† Values from Warr *et al.* (1984).

‡ Values from Reed *et al.* (1984a).

concentrations (on a total cell volume basis) were similar to those at which maximum stimulation of GS activity occurred *in vitro*. The fact that KCl caused only limited inhibition at the highest concentrations used shows that the inhibition by NaCl at lower concentrations is due to Na⁺ rather than to Cl⁻.

The effect of increasing sucrose concentration on GS activity in *N. harveyana* is shown in Fig. 1(a). Activity remained high (> 95%) at sucrose concentrations up to 0.3 M but declined rapidly at higher concentrations; 2.0 M sucrose caused a 60% inhibition. The concentration of sucrose in this marine organism, *N. harveyana*, is 0.27 M (Table 1), a concentration at which GS retained > 95% activity.

Figure 1(b) shows the effect of the quaternary ammonium compound glycine betaine on GS activity in *Synechocystis* sp. DUN52; at concentrations up to 1.8–2.0 M there was a slight stimulation (between 1.0 and 8.0%) of activity (*t* test: statistically significant at the 0.2% level). Glycine betaine is the major internal organic osmoticum in *Synechocystis* sp. DUN52 and is accumulated to approximately 0.8 M in seawater-grown cells (Table 1); it is clear that glycine betaine has no inhibitory effect at this concentration (Fig. 1b).

Effect of sucrose and KCl on glutamine synthetase activity of Nodularia harveyana in the presence of various amounts of NaCl. Figure 2(a) shows the effect of 0.2 M-sucrose and 0.2 M-KCl against NaCl inhibition of GS activity in *N. harveyana*. These concentrations were chosen because of their similarity to the physiological concentrations of sucrose and KCl in this organism (see Table 1). Above NaCl concentrations of 0.4 M there was little difference between the response curves for sucrose and KCl, both of which conferred limited protection (approximately 15%)

Fig. 2. (a) Effect of equimolar (0.2 M) sucrose (●) and KCl (□) on GS activity in *Nodularia harveyana* over a range of NaCl concentrations from 0 to 2.0 M. The effect of NaCl alone (○) is also shown. (b) Effect of 1.0 M-glycine betaine (■) and 0.2 M KCl (□) on GS activity in *Synechocystis* sp. DUN52 over a range of NaCl concentrations from 0 to 2.0 M. The effect of NaCl alone (○) is also shown. Each point is the mean of two replicates (standard deviation < 2%).

Fig. 3. Effect of 0.4 M (Δ), 0.8 M (▲), 1.0 M (▽) and 1.2 M (▼) glycine betaine over a range of NaCl concentrations on GS activity in *Synechocystis* sp. DUN52. The effect of NaCl alone (○) is also shown. Each point is the mean of two replicates (standard deviation < 2%).

against NaCl inhibition. At concentrations below 0.4 M-NaCl the curves diverge; KCl showed some stimulatory effect, as in Fig. 1 (a) while the sucrose curve converged to join that of NaCl at 0.1 M-NaCl.

Effect of glycine betaine and KCl on glutamine synthetase activity of Synechocystis sp. DUN52 in the presence of various amounts of NaCl. The data for *Synechocystis* sp. DUN52 (Fig. 2b) show that the addition of 0.2 M-KCl was more inhibitory than NaCl alone at concentrations in excess of 0.2 M-NaCl. However, the addition of 1.0 M-glycine betaine, a concentration equivalent to the intracellular level (i.e. approx. 0.8 M, see Table 1), resulted in an increase in GS activity in the presence of NaCl at concentrations above 0.7 M of up to 30%; at lower NaCl concentrations there was no inhibition by NaCl (cf. Fig. 1 a,b) and thus no protection by glycine betaine.

Effect of various glycine betaine concentrations on NaCl inhibition of glutamine synthetase activity in Synechocystis sp. DUN52. Since glycine betaine showed some protection of GS activity *in vitro* against NaCl inhibition at NaCl concentrations above 0.7 M (Fig. 2b) this phenomenon was further investigated. Figure 3 shows the effect of adding increasing concentrations of glycine betaine upon NaCl inhibition of GS activity in *Synechocystis* sp. DUN52. Lower concentrations of glycine betaine (0.4 and 0.8 M) showed similar responses to NaCl alone. However, samples containing 1.0 and 1.2 M-glycine betaine showed some relief of NaCl inhibition at NaCl concentrations above 0.5 M (1.0 M and 1.2 M-glycine betaine increased GS activity by 24% and

28%, respectively, at 2.0 M-NaCl). It should be noted that this, the greatest protective effect of glycine betaine concentrations tested, occurs at glycine betaine concentrations similar to those measured in cells of *Synechocystis* sp. DUN52 (Table 1; see also Reed *et al.*, 1984a).

DISCUSSION

The two cyanobacteria used in this study originated from different habitats and have been shown to accumulate different internal osmotica in response to salinity stress. *Nodularia harveyana*, a marine strain, accumulates sucrose to a concentration of 0.270 M when grown in seawater medium; *Synechocystis* sp. DUN52, a halotolerant species, accumulates glycine betaine in response to osmotic stress to concentrations varying from 0.782 M in full-strength seawater (3.5% sea salt) (Table 1) to 1.62 M in 14% sea salt (Reed *et al.*, 1984a).

The inorganic salts NaCl and KCl, and the relevant organic osmotic effector at concentrations similar to those at which these compounds occur intracellularly had little or no inhibitory effect on the *in vitro* GS activity of crude extracts of *N. harveyana* and *Synechocystis* sp. DUN52. Sucrose at 0.2–0.3 M in *N. harveyana* showed slight inhibition (5%) but the fact that 0.3 M-sucrose caused only limited inhibition of GS activity and that the degree of inhibition subsequently increased markedly with increasing sucrose concentration may partially explain why *N. harveyana* is frequently reported from brackish and hyposaline environments (Nordin & Stein, 1980) as opposed to hypersaline environments where the increased intracellular sucrose concentration required to balance the external osmotic potential would be more likely to severely inhibit enzyme activity within the cell (Fig. 1a). Sucrose is known to interfere with the assay of a variety of soluble and particulate enzymes, often by a reversible reduction of activity (Hinton *et al.*, 1969) and has been shown to inhibit the NADP-specific isocitrate dehydrogenase from *Saccharomyces rouxii* (Brown & Simpson, 1972). Such observations are consistent with the findings of the present study, i.e. increasing sucrose concentrations cause decreased GS activity.

In *N. harveyana*, physiological concentrations (0.2 M) of sucrose reduced NaCl inhibition by up to 15% at NaCl concentrations in excess of 0.5 M. An equivalent concentration of KCl relieved NaCl inhibition to a similar extent at NaCl concentrations above 0.5 M, while at lower NaCl concentrations 0.2 M KCl had a stimulatory effect. In general these results agree with the compatible solute hypothesis (Brown & Simpson, 1972; Borowitzka & Brown, 1974; Brown, 1976) that those compounds which are accumulated by cells as internal osmotica under conditions of osmotic stress have little or no detrimental effect on enzyme activity at physiologically relevant concentrations and may have a function in the protection of enzyme activities against inhibition by high NaCl concentrations (Pollard & Wyn Jones, 1979). From the results of this study this can be seen to be true for both K⁺ and sucrose at 0.2 M in *N. harveyana*.

In *Synechocystis* sp. DUN52, glycine betaine had no inhibitory effect on GS activity at concentrations up to and beyond those found in seawater-grown cells (Fig. 1b, Table 1). Glycine betaine at 1.0 M had a protective effect against NaCl inhibition of GS activity at NaCl concentrations in excess of 0.7 M (Fig. 2b). However, the effect of NaCl alone on the enzyme from the two different strains must also be considered, since the GS enzyme from the halotolerant *Synechocystis* sp. DUN52 appeared to be more resistant to NaCl inhibition than that from the marine *N. harveyana*. Thus *Synechocystis* sp. DUN52 showed decreased inhibition at higher NaCl concentrations and a stimulation by NaCl at concentrations below 0.7 M (Fig. 1b). KCl at 0.2 M further reduced the activity of GS from *Synechocystis* sp. DUN52 at concentrations of NaCl greater than 0.2 M. Glycine betaine functions here as a compatible solute in that it is not inhibitory to GS activity over a range of concentrations up to 1.8–2.0 M (Fig. 1b), whereas KCl, although stimulating GS activity at low concentrations, had an inhibitory effect at concentrations greater than 1.4 M. Furthermore, glycine betaine at physiologically relevant concentrations had a protective effect against NaCl inhibition of GS activity.

Glycine betaine is known to be involved in the adaptation of several micro-organisms to osmotic stress. Glycine betaine functions as an internal osmoticum in *Synechocystis* sp. DUN52 (Mohammad *et al.*, 1983) and in other halotolerant forms (Reed *et al.*, 1984a) and has been

reported from the halophilic bacterium *Ectothiorhodospira halochloris* (Galinski & Trüper, 1982). Glycine betaine has also been reported from several salt-tolerant higher plants including *Hordeum vulgare* (Pollard & Wyn Jones, 1979) and has been described in a range of species by Storey & Wyn Jones (1977). Bouillard & Le Rudulier (1983) have recently shown that externally supplied glycine betaine can be taken up by bacterial cells and can overcome the inhibition by NaCl of nitrogenase in *Klebsiella pneumoniae*. Glycine betaine has also been reported to affect membrane stability of a halotolerant bacterium (strain BA1; Shkedy-Vinkler & Avi-Dor, 1975) and has been shown to be compatible with several functions of plant cells including *in vitro* protein synthesis, polysome stability, chloroplast CO₂ fixation and coupled mitochondrial oxidative phosphorylation (see Wyn Jones & Gorham, 1983).

The results of the present study show that the compounds accumulated as internal organic solutes in response to osmotic stress in two strains of cyanobacteria are compatible with GS activity in those strains at physiological concentrations and, furthermore, confer a limited degree of protection against NaCl inhibition of this enzyme at high NaCl concentrations. The effect of increasing sucrose concentration on GS activity in *N. harveyana* is, however, markedly different from that of increasing glycine betaine concentrations on the enzyme from *Synechocystis* sp. DUN52 and if these results can be extrapolated to other enzymes and to other strains of cyanobacteria they may explain, in part, why those strains that accumulate glycine betaine do so to a higher internal concentration and can therefore tolerate more hypersaline conditions than those strains which accumulate sucrose as an internal osmoticum (cf. Reed *et al.*, 1984a; Warr *et al.*, 1984). It appears that glycine betaine may be tolerated at higher intracellular levels than sucrose due to its increased compatibility with enzyme activity.

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