Regulation of compatible solute accumulation in *Salmonella typhimurium*: evidence for a glycine betaine efflux system

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(Received 20 May 1991; revised 25 July 1991; accepted 19 August 1991)

The regulation of glycine betaine accumulation has been investigated in *Salmonella typhimurium*. The size of the glycine betaine pool in the cells is determined by the external osmotic pressure and is largely independent of the external glycine betaine concentration. Analysis of the activity of the ProP and ProU transport systems suggests that other systems must be active in the regulation of the glycine betaine pool. Addition of *p*-chloromercuribenzoate (PCMB) or *p*-chloromercuribenzenesulphonate (PCMBs) to cells that have accumulated glycine betaine provokes rapid loss of glycine betaine. The route of glycine betaine efflux under the influence of PCMB is independent of either the ProP or ProU transport systems. Rapid loss of the accumulated pool of glycine betaine in the presence of PCMB is specific to glycine betaine and proline; accumulated pools of serine and lysine are not significantly affected by the –SH reagent. A specific glycine betaine/proline efflux system is postulated on the basis of these data and its role in the regulation of glycine betaine and proline accumulation is discussed.

Introduction

Bacteria require the presence of an outwardly directed turgor pressure in order to grow. When the osmotic pressure of the growth medium is increased, the maintenance of a relatively constant turgor pressure is initially achieved through increases in cytoplasmic salt concentrations (usually potassium glutamate) (reviewed by Epstein, 1986; Booth *et al.*, 1988; Csonka, 1989). The ability of cells to grow under conditions of elevated osmolarity is determined both by the salt-tolerance of their enzymes and by their ability to accumulate compatible solutes in place of salts (Imhoff, 1986). Compatible solutes are usually neutral molecules that can be accumulated by cells to molar concentrations without damaging enzyme structure. The accumulation of compatible solutes in cells is usually accompanied by the reduction of the cytoplasmic salt concentration and in addition compatible solutes will protect enzymes bathed in high salt concentrations (Pollard & Wyn-Jones, 1979; Riou & Le Rudulier, 1990). The ability of cells to accumulate high intracellular concentrations of compatible solutes poses significant problems, both in terms of

membrane permeability and in the regulation of solute accumulation.

We have proposed that the regulated accumulation of solutes in response to environmental stress requires the functioning of controlled transport systems (Booth *et al.*, 1988; Booth, 1988). Such transport systems should increase their activity under conditions of stress and their activity should decline once the stress is alleviated. The best examples of this type of activity have emerged from the study of K⁺ transport in *Escherichia coli* (Rhoads & Epstein, 1978; Meury *et al.*, 1985). The activity of K⁺-uptake systems is under negative-feedback control from high turgor pressure (and possibly from high internal K⁺ concentrations). Conversely, K⁺-efflux systems are activated under conditions of high turgor leading to controlled loss of K⁺ (Bakker *et al.*, 1987; Dinnbier *et al.*, 1988). The controlled activity of the uptake and efflux limbs of the K⁺ cycle enables the cell to vary the cytoplasmic K⁺ concentration according to the external osmotic pressure and the availability of compatible solutes (Epstein, 1986; Dinnbier *et al.*, 1988).

In the enteric bacteria glycine betaine transport is effected through the ProP and ProU transport systems (Cairney *et al.*, 1985*a, b*) which show both regulation of their activity and of their synthesis by the osmotic pressure of the growth medium. The ProP system of *Salmonella typhimurium* is a proton-driven transport system that is constitutively expressed and the level of its

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Abbreviations: PCMB, *p*-chloromercuribenzoate; PCMBs, *p*-chloromercuribenzenesulphonate; NEM, *N*-ethylmaleimide; CDNB, 1-chloro-2,4-dinitrobenzene.

synthesis is modulated only 2–3-fold by osmotic pressure (Cairney *et al.*, 1985a). The ProP system is active in membrane vesicles and activation of the system by osmotic pressure has been demonstrated in this sub-cellular system (Milner *et al.*, 1988). The ProU system, a high-affinity scavenging system for glycine betaine, is only expressed at significant levels in the period after the initial adaptation to osmotic stress (Csonka, 1982; Cairney *et al.*, 1985b). Induction of *proU* expression is substantially reduced if ProP is active and glycine betaine is present in the medium (Cairney *et al.*, 1985b). Thus the ProP system represents the major route for glycine betaine accumulation during the initial phase of the adaptation of the cell to osmotic shock (Cairney *et al.*, 1985a).

The mechanisms by which the accumulation of glycine betaine is regulated are unknown at the present time. It has been shown that both the ProP and the ProU transport systems are activated by a sudden increase in the osmotic pressure of the medium (Cairney *et al.*, 1985a, b). ProU activity appears to take some minutes to respond to the change in osmotic pressure (Faatz *et al.*, 1988). The present study was initiated to gain an understanding of the mechanism of regulation of the ProP system and of the role of this system in the control over the accumulation of glycine betaine. The principal findings are that although the ProP and ProU transport systems are activated by osmotic stress, control over the glycine betaine pool appears to reside with another transport component. This putative component, a glycine betaine efflux system, is independent of ProP and ProU and is altered in its activity by the sulphydryl reagent *p*-chloromercuribenzoate (PCMB), and is responsive to changes in the osmotic pressure of the medium.

Methods

Bacterial strains. The strains used in this study were: *S. typhimurium* CH992 (*galE503 bio-561 putPA230 proU1697::Tn10*); *S. typhimurium* CH638 (*galE503 putPA230 proP1667::Tn5*); *S. typhimurium* CH710 (*galE503 putPA230 proP1667::Tn5 proU1697::Tn10*) (Cairney *et al.*, 1985a, b); and *E. coli* MJF335 [*F⁻ gal rha kdpABC gshA::Tn10(kan) kefB kefC::Tn10*].

Media for growth and transport studies. The medium LOM (Cairney *et al.*, 1985a) was used routinely; glucose (0.2%, w/v) was used as the carbon and energy source and growth media were supplemented with biotin (10 µg ml⁻¹). Medium was supplemented with NaCl or glucose to raise the osmotic pressure.

Growth protocols. Growth of cells was at 37 °C in Erlenmeyer flasks with a medium:flask volume ratio of approximately 0.2 to ensure good aeration. The flasks were incubated on a gyrotatory incubator at 250 r.p.m. Overnight cultures were subcultured into fresh medium to an approximate OD₆₅₀ of 0.05 (measured in LOM medium) and growth was monitored over the next 8–10 h. Cells for transport experiments were harvested in late-exponential phase.

Transport protocols. Transport experiments were carried out essentially as described previously (Cairney *et al.*, 1985a) using cells harvested and washed in LOM medium and stored on ice in the presence of chloramphenicol (12.5 µg ml⁻¹). All optical density measurements were made on cells incubated in LOM. Radioactive glycine betaine ([*N-methyl-¹⁴C*]glycine betaine; stocks 3 µCi µmol⁻¹ (111 kBq µmol⁻¹) from Amersham and 55 µCi µmol⁻¹ (2.035 MBq µmol⁻¹) from ICN) was added from stock solutions approximately 100-fold more concentrated than that required in the incubation. The specific activity of the glycine betaine was varied by mixing with unlabelled glycine betaine. Other radioactive amino acids used were serine [17.1 µCi µmol⁻¹ (633 kBq µmol⁻¹)], proline [20 µCi µmol⁻¹ (740 kBq µmol⁻¹)] and lysine [10 µCi µmol⁻¹ (370 kBq µmol⁻¹)]; these amino acids were purchased from Amersham.

Measurement of glycine betaine accumulation during growth. To determine glycine betaine accumulation during growth cultures were established as described above and incubated with glycine betaine (10–800 µM). Once exponential growth was established (OD₆₅₀ = 0.2) aliquots of culture (2.5 ml) were transferred to wide boiling-tubes containing 10–20 nCi (370–740 Bq) of radioactive glycine betaine and the incubation was continued. When growth in the control flask had reached OD₆₅₀ = 0.4 the tube was removed from the incubator and the following manipulations undertaken: the OD₆₅₀ of the culture in the tube was measured; three 0.5 ml samples were taken and filtered through glassfibre filters and washed with 3 ml of growth medium of the same osmotic strength but lacking glycine betaine – the filters were then dried and the radioactivity trapped in the cells determined by liquid scintillation counting; 50 µl samples were removed and transferred to filters and dried as standards for the determination of the glycine betaine specific activity.

Analysis of the ProU transport system in cells grown at low osmolarity. In order to study the ProU transport system in cells that had been grown at low osmolarity we utilized the expression of the ProU system that occurs when the *proU* gene is cloned onto a multicopy plasmid (Stirling *et al.*, 1989). *S. typhimurium* CH710 was transformed with a partial *Sau*III A gene bank of *S. typhimurium* DNA cloned in pBR322 and ampicillin-resistant transformants that had recovered the ability to grow in the presence of 0.7 M-NaCl were isolated. Clones that expressed the ProU system were characterized as follows: their ability to restore osmoprotection by glycine betaine but not proline; their capacity for glycine betaine transport and the affinity of the encoded transport system (approximately 3–5 µM cf. 44 µM for ProP); their sensitivity to toxic proline analogues (Cairney *et al.*, 1985a, b); and by the presence of a 4.8 kb *Hind*III–*Bam*HI DNA fragment in the cloned region (Stirling *et al.*, 1989). By these criteria the positive clones carried the *proU* transport system. Such clones express the ProU transport system at low osmolarity, possibly due to the context of the plasmid vector. The clones were unstable, as we have reported previously (Stirling *et al.*, 1989), and thus the DNA was stored in the pure state and experiments were performed on fresh transformants.

Results

Steady state accumulation of glycine betaine

The steady state accumulation of glycine betaine was assessed in cells grown in media of different osmolarities and at a range of glycine betaine concentrations (Fig. 1a, b). The steady state accumulation of glycine betaine was essentially independent of the external glycine betaine concentration in the range 100–800 µM (Fig. 1a). In this

Fig. 1. Dependence of glycine betaine accumulation via ProP on osmotic pressure. Glycine betaine transport was measured as described in Methods. (a) The glycine betaine concentration in growing cells was measured during exponential growth in LOM medium in the presence of 0.5 M-NaCl and at the concentration of glycine betaine indicated (values indicate steady state external concentrations corrected for glycine betaine removed from the medium by the growing cells). The values shown are means of three measurements and the data are taken from parallel incubations at different concentrations of glycine betaine. (b) Glycine betaine accumulation was measured during mid-exponential phase in the presence of 200 μM-glycine betaine. Error bars indicate the variance between replicate experiments. No correction has been made for variations in cytoplasmic volume, but such changes are likely to accentuate further the effect of osmotic pressure on accumulation of glycine betaine (Larson *et al.*, 1987).

range the accumulation of glycine betaine showed a clear dependence upon the osmolarity of the growth medium (Fig. 1b: data are shown for 200 μM-glycine betaine; similar results were obtained at 100 μM and 400 μM). Thus, the accumulation of glycine betaine is regulated in response to the osmotic stress imposed on the cell. In this respect the system resembles the osmotically regulated K⁺-transport systems (Epstein, 1986).

Activation of the ProP system by osmotic pressure

We have previously shown that the ProP transport system is only active under conditions of osmotic stress (Cairney *et al.*, 1985a). It has been proposed that the system is activated by low turgor (Milner *et al.*, 1988). The activation of ProP was further investigated to determine the role of turgor pressure in the control of its activity. Glycine betaine transport via ProP increased 20-fold upon increasing the osmotic pressure of LOM medium by the addition of either NaCl or glucose to raise the osmotic pressure (Fig. 2). At high osmotic pressure

Fig. 2. Dependence of ProP activity on K⁺. Glycine betaine uptake was measured at an external concentration of 10 μM [specific activity 55 μCi μmol⁻¹ (2.035 MBq μmol⁻¹)]. Transport of glycine betaine was measured in the absence (○, □) and presence of K⁺ (10 mM, final concentration) (●, ■) in either LOM plus 1 M-glucose (○, ●) or LOM plus 0.7 M-NaCl (□, ■). Cells were incubated with K⁺ for 5 min prior to the addition of glycine betaine.

(equivalent to LOM plus 0.7 M-NaCl) the transport system was progressively inhibited as has been noted for other transport systems (data not shown; Roth *et al.*, 1984). The maximum activity of the ProP system was dependent upon the presence of K⁺ in the incubation buffer (Fig. 2). Similar results were obtained when the osmoticum was glucose rather than NaCl (Fig. 2) and when proline, rather than glycine betaine, was used as the substrate of the ProP system (data not shown).

When the temporal pattern of the activation of the ProP system by K⁺ was investigated it was clear that K⁺ exerts its effect before substantial accumulation of the cation occurs (Fig. 3). Thus the glycine betaine transport rate achieved its maximum value within 10 s of addition of K⁺ to the cells. Once K⁺ accumulation had ceased there was a further slight stimulation (approximately 20% of the rate during K⁺ uptake) of glycine betaine accumulation (data not shown). From these data it appears unlikely that the K⁺-dependence of ProP reflects the generation of an activator, such as potassium glutamate, in the cytoplasm. Further, since the initial rate of glycine betaine transport is not diminished when turgor-regulated K⁺ uptake ceases it seems unlikely that the ProP system can be activated by reduced turgor pressure in the cell.

Is there feedback regulation of ProP and ProU activity?

We have previously noted that transport activities that are components of homeostatic systems are usually subjected to regulation of their activity (Booth *et al.*, 1988; Booth, 1988). Clearly the activation of ProP (and of ProU: Cairney *et al.*, 1985b; Faatz *et al.*, 1988) represents such control. However, regulation of the pool

Fig. 3. Restoration of turgor does not inhibit ProP. *S. typhimurium* CH992 cells were incubated in LOM in the presence of 0.15 M-NaCl and 10 mM-KCl and K^+ uptake measured (b) by flame photometry (Elmore *et al.*, 1990). At intervals the rate of glycine betaine uptake (a) was measured in parallel incubations by the addition of 10 μM -glycine betaine [specific activity 3 $\mu\text{Ci } \mu\text{mol}^{-1}$ (111 $\text{kBq } \mu\text{mol}^{-1}$)]. The initial rate, measured over the first 3 min of transport, is plotted against the time of addition of glycine betaine.

size of the compatible solute requires mechanisms both for the activation of the uptake system and for limiting accumulation. By analogy with the K^+ systems, feedback inhibition of the uptake system by the accumulated solute would represent the simplest mechanism for control. The possibility of a feedback loop was investigated by measuring the rate of glycine betaine uptake through the ProP and ProU transport systems once the steady state accumulation of glycine betaine had been achieved. Cells of *S. typhimurium* CH992 (ProP⁺ProU⁻) were incubated in the presence of 0.15 M-NaCl with radiolabelled glycine betaine and the initial rate of transport was determined. In parallel incubations cells were incubated with unlabelled glycine betaine (200 μM) for 30 min to allow the cells to accumulate glycine betaine to the steady state pool size. The rate of glycine betaine transport was then measured by pulsing the cells with radioactive glycine betaine and determining the rate of accumulation of radiolabelled solute (Fig. 4). Within experimental error the rate of initial transport and the steady state rate differed by a factor of two with the steady state rate being the higher value; similar glycine

Fig. 4. Comparison of initial and steady state rates of glycine betaine transport. Cells were incubated in LOM plus 0.15 M-NaCl in the presence of saturating glycine betaine concentrations (200 μM for ProP and 100 μM for ProU) and the initial rate of glycine betaine uptake was measured ($\square$). Parallel incubations in which cold glycine betaine was added at the start of the experiment were pulsed with 6 μCi (222 kBq) of radioactive glycine betaine [$55 \mu\text{Ci } \mu\text{mol}^{-1}$ (2.035 $\text{MBq } \mu\text{mol}^{-1}$)] after attainment of the steady state of glycine betaine accumulation (measured in the first incubation) and samples were taken to measure the inward rate of transport at the steady state ($\blacksquare$). (a) Transport via ProP (strain CH992); (b) transport via ProU (strain CH710 carrying the cloned *proU* gene).

betaine pools were established in the parallel incubations (data not shown). These incubations were conducted with non-growing cell suspensions but similar results were obtained in experiments with growing cells (data not shown).

A similar lack of feedback inhibition of the ProU system was observed using *S. typhimurium* CH710 (ProP⁻ProU⁺) carrying the cloned *proU* gene (Fig. 4b). The steady state rate of glycine betaine transport was identical to the initial rate of transport in parallel incubations.

These experiments with the ProP and the ProU system indicate that neither system exhibits significant feedback inhibition of the transport rate. Further, the undiminished rate of transport of glycine betaine once the steady state pool of betaine had been achieved would not be consistent with turgor regulation of the uptake system.

Is there a glycine betaine efflux system?

The above experiments suggest that the primary site of control of glycine betaine accumulation within the cell is

Fig. 5. Effect of PCMB on the accumulation of glycine betaine, proline, serine and lysine. *S. typhimurium* CH992 was incubated with (a) 100 μM -glycine betaine [20 $\mu\text{Ci } \mu\text{mol}^{-1}$ (740 kBq μmol^{-1})]; (b) 10 μM -proline [20 $\mu\text{Ci } \mu\text{mol}^{-1}$ (740 kBq μmol^{-1})]; (c) 10 μM -serine [17.1 $\mu\text{Ci } \mu\text{mol}^{-1}$ (633 kBq μmol^{-1})]; and (d) 10 μM -lysine [10 $\mu\text{Ci } \mu\text{mol}^{-1}$ (370 kBq μmol^{-1})]. Experiments (a) and (b) were conducted in LOM plus 0.15 M-NaCl and 10 mM-KCl and (c) and (d) were conducted in LOM plus 10 mM-KCl. Samples were removed at intervals and PCMB (0.5 mM, final concentration) was added (arrow) from a concentrated stock dissolved in 0.15 M-NaOH. $\square$, Control; $\blacksquare$, 0.5 mM-PCMB.

unlikely to be at the uptake systems, ProP and ProU. From the absence of feedback control over uptake we inferred the presence of a glycine betaine efflux system that could be the site of regulation of the glycine betaine pool. We investigated glycine betaine efflux using chemical inhibitors of the uptake system (West & Mitchell, 1973; Booth & Hamilton, 1980). Cells of *S. typhimurium* CH992 (ProP⁺ProU⁻) were incubated with a variety of -SH reagents to assess their effects on the activity of the ProP transport system. Pre-incubation of cells with *p*-chloromercuribenzoate (PCMB) and the related, but less permeant, -SH compound *p*-chloromercuribenzenesulphonate (PCMBS) resulted in inhibition of glycine betaine accumulation by the cells. Influx of glycine betaine was inhibited by more than 99% by pre-incubation with PCMB for 5 min. With PCMBS a longer pre-incubation period was required before inhibition became evident (see below). Pre-incubation with other -SH reagents, *N*-ethylmaleimide (NEM) and chlorodinitrobenzene (CDNB), inhibited the ProP transport system by 40% and 35%, respectively, and inhibition was not progressive.

PCMB-treatment of cells that had accumulated glycine betaine provoked the immediate release of the accumulated solute (Fig. 5a), and the onset of glycine betaine efflux was extremely rapid; with PCMBS there was a delay of some minutes before glycine betaine efflux was initiated (data not shown). Proline, which is also accumulated via ProP in response to osmotic pressure

(Cairney *et al.*, 1985a) also left the cells in response to treatment with PCMB (Fig. 5b). Essentially identical effects were obtained with cells of *S. typhimurium* CH710 (ProP⁻ProU⁻) carrying the cloned *proU* gene in which glycine betaine accumulation was via the ProU transport system (data not shown). As a control cells of *S. typhimurium* CH992 (ProP⁺ProU⁻) which had accumulated radiolabelled serine or lysine were treated with PCMB. No amino acid efflux was seen from these cell suspensions (Fig. 5c, d). Thus the effects of PCMB seen with glycine betaine and proline are specific to these solutes.

S. typhimurium strain CH710 (ProP⁻ProU⁻) lacks the two major glycine betaine uptake systems and consequently cells of this strain do not exhibit rapid transport of glycine betaine. Cells incubated in LOM plus 0.5 M-NaCl in the presence of 100 μM -glycine betaine [specific activity 20 $\mu\text{Ci } \mu\text{mol}^{-1}$ (740 kBq μmol^{-1})] for 1 h exhibited no accumulation of radioactivity (data not shown). However, overnight growth in LOM plus 0.5 M-NaCl in the presence of 500 μM -glycine betaine did yield cells that were loaded with glycine betaine (see also Csonka, 1989). Addition of PCMB to such preloaded cells elicited rapid glycine betaine efflux (Fig. 6) which was complete within 15 min. PCMBS elicited a somewhat slower glycine betaine release and NEM and CDNB were without effect (Fig. 6). In parallel experiments the half-times for glycine betaine efflux were 3.56 ± 0.2 min ($n = 3$) and 3.9 ± 0.6 min ($n = 4$),

Fig. 6. Glycine betaine efflux does not require either ProP or ProU. Cells of *S. typhimurium* CH710 (ProP⁻ProU⁻) were grown overnight at 37 °C in LOM plus 0.5 M-NaCl plus 10 mM-KCl and 0.5 mM-glycine betaine [final specific activity 4 μ Ci μ mol⁻¹ (148 kBq μ mol⁻¹)]. The cells were transferred to a small well-aerated reaction vessel and samples taken at timed intervals. $\circ$, Control; $\blacksquare$, 0.5 mM-PCMB; $\bullet$, 0.75 mM-PCMBs; $\square$, 0.5 mM-NEM; and $\triangle$, 0.5 mM-CDNB. -SH reagents were added at zero time.

respectively, for CH992 (ProP⁺ProU⁻) and CH710 (ProP⁻ProU⁻). The half-time for efflux was unaffected by the intracellular concentration of glycine betaine in the range 46–180 nmols glycine betaine per OD₆₅₀ unit of biomass. Given that the CH710 cells have mutations that inactivate both the ProP and the ProU uptake systems but exhibit similar rates of glycine betaine efflux we believe that the system activated by PCMB may be a separate efflux system required for the regulation of the glycine betaine pool.

Lack of involvement of glutathione in glycine betaine efflux

We have previously established that the reaction of -SH reagents with cytoplasmic glutathione can modulate the K⁺ pool (Elmore *et al.*, 1990). The role of glutathione in the PCMB-activation of glycine betaine efflux was investigated in *E. coli* strain MJF335 which lacks both glutathione biosynthesis [*gshA::Tn10(kan)*] and the Kef systems (*kefB kefC::Tn10*) that are required for glutathione-dependent K⁺ efflux. It can be seen that the absence of glutathione, and of the KefB and KefC systems, did not impair the activation of glycine betaine efflux by PCMB and PCMBs (Fig. 7). Thus the efflux of glycine betaine does not arise from modification of the glutathione pool or from the alteration of the K⁺ transport capacity of the cell.

Osmoregulation of glycine betaine efflux

To investigate the dependence of glycine betaine efflux on the osmotic pressure of the incubation medium, glycine-betaine-loaded cells of *S. typhimurium* CH710 (ProP⁻ProU⁻) were subjected to sudden lowering of the

Fig. 7. Inhibition of glycine betaine retention by PCMB is independent of glutathione. *E. coli* strain MJF335 [*gshA::Tn10(kan) kefB kefC::Tn10*] was incubated in LOM plus 0.15 M-NaCl, 10 mM-KCl and 10 μ M-glycine betaine [specific activity, 20 μ Ci μ mol⁻¹ (740 kBq μ mol⁻¹)]. After 5 min, PCMB (0.5 mM, final concentration) or PCMBs (0.5 mM) was added (arrow) and the incubation continued. $\square$, Control; $\bullet$, PCMB added; $\blacksquare$, PCMBs added.

Fig. 8. Glycine betaine and serine efflux in response to osmotic downshock. Cells of *S. typhimurium* CH710 (ProP⁻ProU⁻) were preloaded with glycine betaine (a) [specific activity, 4 μ Ci μ mol⁻¹ (148 kBq μ mol⁻¹)] by growth overnight in LOM plus 0.5 M-NaCl, 0.5 mM-glycine betaine and 10 mM-KCl. Cells of CH710 were loaded with serine (b) [specific activity, 17.1 μ Ci μ mol⁻¹ (633 kBq μ mol⁻¹)] by incubation at 37 °C in the presence of chloramphenicol in LOM plus 0.5 M-NaCl for 1 h. [As a control for the different loading conditions cells of CH992 (ProP⁺ProU⁻) were loaded with glycine betaine using the same protocol as that used for serine; the loading protocol was found not to affect the results of the efflux experiment.] Washed cells were diluted into fresh warm medium at either the same osmolarity ($\square$) or with reduced osmolarity ($\blacksquare$, $\circ$, $\bullet$). $\square$, 0.5 M-NaCl; $\blacksquare$, 0.4 M-NaCl; $\circ$, 0.3 M-NaCl; $\bullet$, 0.2 M-NaCl.

osmotic pressure by mixing cell suspensions with media of lower osmolarity. Cells diluted into media of the same osmolarity (LOM plus 0.5 M-NaCl) lost glycine betaine slowly relative to cells that were simultaneously subjected to an osmotic downshock (Fig. 8). The osmotic shock accelerated the rate of glycine betaine efflux and the rate

was determined by the size of the downshock imposed on the cells. Similar data were obtained with *S. typhimurium* CH992 (ProP⁺ProU⁻) in which the ProP transport system was active (data not shown).

The loss of glycine betaine in response to osmotic downshock may not be a specific phenomenon since osmotic downshock in control incubations with serine-loaded cells provoked similar loss of the accumulated amino acid (Fig. 8).

Discussion

There has been substantial progress in the understanding of the events that take place in the enteric bacteria during adaptation to osmotic stress. In the main these advances have been in the understanding of the control over gene expression (Booth *et al.*, 1988; Csonka, 1989). However, cells possess constitutively expressed functions that can mediate osmo-adaptation in the absence of further gene expression (Booth *et al.*, 1988). Our understanding of these processes has received less attention and in consequence the control phenomena, which involve the regulation of the activity of enzymes and transport systems rather than genes, are less well understood. The mechanism by which compatible solute accumulation is regulated through control over enzyme and transport systems activity is one such poorly understood aspect of osmoregulation. It is to this question that the study described here was addressed.

The studies conducted here demonstrate that the accumulation of glycine betaine by *Salmonella typhimurium* is regulated by the osmotic pressure of the growth medium. It has previously been established that glycine betaine accumulation by *Escherichia coli* is regulated by the osmotic pressure (Perroud & Le Rudulier, 1985). The data presented here are consistent with control over glycine betaine accumulation residing in the regulation of both activation of the uptake systems and also control over the efflux of glycine betaine. The uptake systems are activated by the increase in the osmotic pressure of the environment although the precise mechanism of regulation of the two major glycine betaine uptake systems appears to be different. The activation of the ProP system occurs within 20 s of upshock in the presence of K⁺ but does not occur in the absence of K⁺ (Figs 2 and 3) whereas the ProU system requires several minutes to achieve maximum activity (Faatz *et al.*, 1988). The K⁺-dependence of the rate of glycine betaine uptake via the ProP system may indicate that this system is inhibited by low turgor. Cells subjected to a sudden osmotic upshock experience a loss of turgor which cannot be restored in the absence of K⁺. Cells that have been pre-adapted to high osmolarity in the presence of K⁺ show no inhibition

of transport activity at 0.5 M-NaCl. Thus loss of turgor may inhibit the ProP system.

The ProP system has been studied in membrane vesicles and in this semi-purified system has been shown to be activated by an increase in the osmotic pressure of the incubation medium (Milner *et al.*, 1988). These authors concluded that the system was activated by low turgor pressure. However, our data are not consistent with this conclusion. Thus, when the activity of the ProP system was studied in osmo-adapting cells and compared with the activity of the turgor-regulated TrkA system it was found that the ProP system was active when the activity of the TrkA system had subsided (Fig. 3; see also Meury *et al.*, 1985). Further, ProP activity was unaffected by achievement of steady state accumulation of glycine betaine. These measurements were conducted at moderate osmotic pressure (LOM plus 0.15 M-NaCl; approximately 0.29 Osm), conditions under which the restoration of turgor pressure could be expected to have occurred. These observations are not consistent with activation of the ProP system by low turgor pressure.

We have not identified the regulatory signal for activation of the ProP system. However, there are several possibilities; firstly, the system could be activated by the accumulation of potassium glutamate in the cytoplasm. Although this would explain the dependence of the activity of the system on external K⁺ it would not readily explain the very rapid change in the activity of the system once K⁺ was provided. Further, glutamate would not be available in the vesicle system investigated previously (Milner *et al.*, 1989). Such a mechanism could operate for activation of ProU which takes several minutes to achieve maximum activity (Faatz *et al.*, 1988). Secondly, the ProP system could be responsive to changes in cytoplasmic pH consequent upon K⁺ uptake; the pH changes are very rapid but have been reported to be transient (Dinnbier *et al.*, 1988) and could not explain the sustained high activity of the ProP system. It is unlikely that such pH changes are seen in membrane vesicles. Finally, the system could be regulated directly by the external osmotic pressure, presumably by deformation of the structure of the protein. At the present time the latter seems the most probable explanation and would explain activation of ProP in membrane vesicles.

Evidence for a specific efflux system that could regulate glycine betaine accumulation was obtained. Although the accumulation of glycine betaine by the cells is regulated by the osmotic pressure the activity of the ProP uptake system is not subject to feedback at the steady state and, therefore, cannot alone regulate the size of the glycine betaine pool. The ProP system was found to be inhibited by PCMB yet rapid glycine betaine efflux is still observed. This was in sharp contrast to the effects of -SH reagents on serine and lysine transport (Fig. 5 and

unpublished data). Similarly, although PCMB inhibited the lactose permease the lactose pool was retained by the treated cells (Booth & Hamilton, 1980). Further, in mutants lacking both the ProP and the ProU uptake systems, which are incapable of rapid glycine betaine uptake, glycine betaine efflux can still be elicited by the addition of PCMB and was observed to occur at a rate similar to that found in cells possessing either ProP or ProU activity. These data point to the presence of a system that cannot participate in glycine betaine uptake but which, when modified by PCMB, results in rapid efflux of the accumulated solute. One interpretation of these data obtained with PCMB and PCMBs is that the efflux system possesses a reactive -SH group exposed at the inner face of the membrane (Driessen & Konings, 1990). Modification of this group leads to loss of control of the system and consequent glycine betaine efflux. This glycine betaine transport system might be analogous to the K⁺ efflux systems of the enteric bacteria which participate in osmoregulation of the K⁺ pool (Bakker *et al.*, 1987).

We were unable to show that the glycine betaine efflux system was uniquely responsive to increased cell turgor provoked by osmotic downshock. Thus both serine and glycine betaine efflux could be elicited by osmotic downshock (Fig. 8). However, early experiments to demonstrate activation of K⁺ efflux by high turgor created by downshock were similarly unsuccessful (Meury *et al.*, 1985). It has been reported that osmotic pressure controls the amount of proline that is excreted into the medium by mutants of *S. typhimurium* that are osmotolerant due to overproduction of proline (Csonka, 1988). At low osmolarity excretion is high but at higher osmolarity the proline is retained by the cells. We have shown that proline is likely to be a substrate for the glycine betaine efflux system (Fig. 4). The data presented by Csonka (1988) raise the interesting possibility that the efflux system for glycine betaine and proline is active at low osmolarity and that its activity is diminished at high osmotic pressure. The combination of activation of the uptake system and inhibition of the efflux system by high osmotic pressure would control the accumulation of the compatible solutes glycine betaine and proline. A fascinating parallel here is that the control over the trehalose pool, which is also modulated by osmotic pressure, appears to involve a specific efflux system (Styrvoid & Strom, 1991).

In conclusion, we have shown that the accumulation of glycine betaine by cells displays properties associated with stress-regulated transport (Booth *et al.*, 1988; Booth, 1988) in that the activity of the uptake system is regulated by osmotic stress and the level of accumulation of the transported solute is determined by the degree of osmotic stress. In contrast to K⁺ uptake systems there

appears to be no feedback regulation of the ProP and ProU activity and it is proposed that the magnitude of the glycine betaine pool is controlled by the efflux process characterized here.

The authors wish to acknowledge the support of the AFRC (grant FG1/527) during this study.

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