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# Regulation of Envelope Protein Composition during Adaptation to Osmotic Stress in *Escherichia coli*

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**Adaptation to osmotic stress alters the amounts of several specific proteins in the *Escherichia coli* K-12 envelope. The most striking feature of the response to elevated osmolarity was the strong induction of a periplasmic protein with an $M_r$ of 31,000. This protein was absent in mutants with λ *plac* Mu insertions in an osmotically inducible locus mapping near 58 min. The insertions are likely to be in *proU*, a locus encoding a transport activity for the osmoprotectants glycine betaine and proline. Factors affecting the extent of *proU* induction were identified by direct examination of periplasmic proteins on sodium dodecyl sulfate gels and by measuring β-galactosidase activity from *proU-lac* fusions. Expression was stimulated by increasing additions of salt or sucrose to minimal medium, up to a maximum at 0.5 M NaCl. Exogenous glycine betaine acted as an osmoregulatory signal; its addition to the high-osmolarity medium substantially repressed the expression of the 31,000-dalton periplasmic protein and the *proU-lac*$^+$ fusions. Elevated osmolarity also caused the appearance of a second periplasmic protein ($M_r$ = 16,000), and severe reduction in the amounts of two others. In the outer membrane, the well-characterized repression of OmpF by high osmolarity was observed and was reversed by glycine betaine. Additional changes in membrane composition were also responsive to glycine betaine regulation.**

Extremes of medium osmolarity are generally deleterious to cell growth. However, *Escherichia coli* and other enteric bacteria are capable of adapting to a wide range of external osmotic pressures (16, 17, 30). The mechanism of adjustment to elevated osmolarity and restoration of turgor pressure is not fully understood but involves the accumulation of ions and metabolites through synthesis or uptake from the environment. $K^+$ is the major osmoactive ion in *E. coli* (9). In minimal medium, potassium transport is stimulated almost immediately by osmotic shock, restoring turgor pressure within minutes (9, 20, 26). Cells shocked by addition of as much as 0.2 M NaCl can resume growth at normal rates. Higher concentrations of external osmolytes cause growth rates to slow, implying that the mechanism of cellular accommodation to elevated osmotic strength is incomplete or that it impairs the efficiency of metabolic processes.

An exogenous supply of osmoprotectant compounds such as proline, choline, or glycine betaine enhances the ability of the cell to grow at elevated osmolarity (7, 10, 19, 27). Transport of proline and glycine betaine is stimulated by increasing medium osmotic pressure, resulting in intracellular accumulation of these osmolytes (2, 3, 8, 10, 24). Presumably these compounds act as compatible solutes to increase the internal osmotic pressure without perturbing vital protein functions (6, 17).

Some aspects of the osmotic adaptation mechanism have been studied at the molecular level. Synthesis of the products of the *kdp* locus, specifying a high-affinity potassium transport system in *E. coli*, is transiently stimulated by elevated external osmolarity (15). Increased osmolarity induces sustained expression of the *proU* locus, originally identified as an osmoregulated transport system for proline (7, 8, 10). Recent studies of *Salmonella typhimurium* indicate that the *proU* transport system has a greater affinity for glycine betaine (2). Transport of glycine betaine via the *proU* system is regulated by osmotic pressure at two levels: transcription and transport activity (2, 8). Expression of the OmpF and OmpC porins is osmoregulated (12, 18), as is alkaline phosphatase (29), synthesis of periplasmic membrane-derived oligosaccharides (13), and putrescine transport (22). Induction of three unknown proteins by high osmolarity has been observed in *E. coli* by two-dimensional gel electrophoresis of total cellular protein (5). Although the adaptive value of increasing transport of osmoprotectant solutes is clear, the functional significance of the other changes is unknown.

In this paper, characterized osmotically induced protein alterations within the *E. coli* envelope that are modulated by the osmoprotectant glycine betaine. The most striking adaptation to elevated osmolarity was the appearance of a new periplasmic protein with a mass of 31 kilodaltons (32 kDa). This protein was completely absent in mutants carrying a λ *plac* Mu insertion in an osmoregulated gene that is probably *proU*. The addition of glycine betaine to high-osmolarity medium rescued cells from growth inhibition and acted as a feedback signal to suppress osmotically induced alterations in protein composition and gene expression.

## MATERIALS AND METHODS

**Strains and growth conditions.** All strains used in this study were derived from *E. coli* strain K-12 and are shown in Table 1. Cultures were grown in minimal medium M63 plus 0.4% glycerol or MMA medium plus 0.2% glucose (21). To ensure adaptation to high-osmolarity medium, cultures were either maintained in exponential growth phase for six generations by dilution with fresh medium or were grown overnight as indicated. Medium osmolarity was increased by the gradual addition of either 5 M NaCl or granular sucrose to the desired concentrations. Growth was monitored spectropho-

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TABLE 1. Strain list

| Strain | Genotype[a] | Source (reference) |
|---|---|---|
| KL19 | Wild type | B. Bachmann |
| MC4100 | F⁻ Δ(*argF-lac*)*U169 araD139 rpsL150 relA1 ptsF25 deoC1 thiA flbB5301 rbsR* | M. Casadaban (4) |
| MH513 | MC4100 Φ(*ompF-lacZ*⁺)*16-23* [λp1 (209)] | Hall and Silhavy (11) |
| GM37 | MC4100 Φ(*proU-lacZ*)*2*(Hyb) (λ *plac* Mu15) | May et al.[b] |
| GM50 | MC4100 Φ(*proU-lacZ*⁺) *3* (λ *plac* Mu55) | May et al.[b] |
| GM86[c] | MC4100 *proU*⁺/Φ(*proU-lacZ*) *2*(Hyb) | This work |

[a] The symbol Φ indicates the presence of a *lacZ* fusion and the abbreviation Hyb indicates that the gene fusion encodes a hybrid protein. The symbols *lacZ*⁺ and *lacZ* denote *lacZ* genes with or without translational initiation signals, respectively.

[b] G. May, et al., submitted for publication.

[c] This strain is a *proU*⁺/Φ(*proU-lacZ*) *2*(Hyb) merodiploid because of the presence of a Lac⁺ λ specialized transducing phage (pGM1) derived from strain GM37.

tometrically at 578 nm or with a Klett-Summerson photoelectric colorimeter.

**Construction of strain GM86.** UV induction of the lysogen strain GM37 was performed as described before (28). Lac⁺ bacteriophage was detected as blue plaques on 5-bromo-4-chloro-3-indoyl-β-D-galactoside plates. One Lac⁺ phage, λpGM1, was lysogenized by homologous recombination into strain MC4100 by spotting phage on cells plated on Luria agar plates (21). After overnight incubation, cells were streaked from the turbid spot onto a Luria plate seeded with λ *cI h80*. Lambda immunity was detected by cross-streaking cells against λ *cI h80* and λ *vir*. One lysogen was purified by streaking onto a Luria plate with kanamycin (30 μg/ml), and the presence of the osmotically inducible *proU-lacZ* fusion was verified by testing the strain (GM86) on lactose MacConkey plates with and without 0.2 M NaCl.

**LacZ⁺ phage detection.** To detect LacZ⁺ phage, 0.1 ml of a 10-mg/ml solution of 5-bromo-4-chloro-3-indolyl-β-D-galactoside in *N,N*-dimethylformamide was added to Luria soft agar (28).

**Cell fractionation.** Cells were fractionated into periplasmic, membrane, and cytosol components by a modification of the method of Nossal and Hepple (23). Washed cell pellets were suspended in solution B (33 mM Tris, 0.5 M sucrose, 1 mM EDTA [pH 7.6]) and incubated for 10 min at 4°C. The cells were spun down and shocked by resuspension in 0.5 mM $MgCl_2$ at 4°C. After 10 min of incubation, the suspension was centrifuged to pellet the cells, and the supernatant shock fluid containing the soluble periplasmic components was removed. The shock fluid was concentrated by lyophilization. Cell pellets were resuspended in solution B and peptidoglycan digested with 0.04% lysozyme in 20 mM EDTA, pH 8.0, for 1 h at room temperature. The cells were completely lysed by brief sonication, and the membrane fraction containing both inner and outer membranes was collected by centrifugation in a 60 Ti rotor for 90 min at 30,000 rpm. Membrane pellets were suspended in 33 mM Tris–5 mM MgCl (pH 8.0) plus 25 μg each of DNase I and RNase per ml, incubated at room temperature for 30 min, and recentrifuged. Fractionated cell components were stored at −70°C.

**Protein gel electrophoresis.** Porin composition was analyzed by electrophoresis on 0.1% sodium dodecyl sulfate (SDS)–8 M urea–12.5% polyacrylamide gels for 12 h at 100 V (25). All other separations utilized 10% Laemmli gels (14). Gels were stained with Coomassie blue.

**β-Galactosidase assay.** Cell samples removed at appropriate times were collected by centrifugation and permeabilized for assay of β-galactosidase. Strains GM50 and GM37 were permeabilized with toluene and assayed as described by Miller (21). Strain MH513 was lysed by lysozyme-EDTA treatment and sonication, as described above, before the assay.

## RESULTS

**Porin osmoregulation is reversed by glycine betaine.** Extremely elevated medium osmolarity dramatically reduces both growth rate and expression of the *ompF* porin gene. Since glycine betaine substantially relieves the growth inhibition, we examined cell membranes to determine whether this osmoprotectant also influences osmoregulation of porin content. Cells were grown under conditions of osmotic stress with or without the osmoprotectant, and membrane protein composition was analyzed by urea-SDS-polyacrylamide gel electrophoresis. Cells of strain KL19 grown in M63-glycerol medium contain both OmpF and OmpC porins (Fig. 1, lane 1). After growth for 6 to 10 generations in medium supplemented with 0.5 M NaCl or 0.73 M sucrose, OmpF was virtually absent (Fig. 1, lanes 2 and 5). Cells grown in high-osmolarity medium supplemented with 1 mM glycine betaine (Fig. 1, lanes 4 and 6) expressed about the same amount of OmpF porin as those grown in low-osmolarity medium. Glycine betaine added to normal M63 medium did not alter growth rate or porin expression (Fig. 1, lane 3). The reversal of porin osmoregulation by glycine betaine was not simply a secondary consequence of stimulation of growth rate. Glycine betaine increased OmpF synthesis in cells subjected to 0.3 M NaCl, a condition that

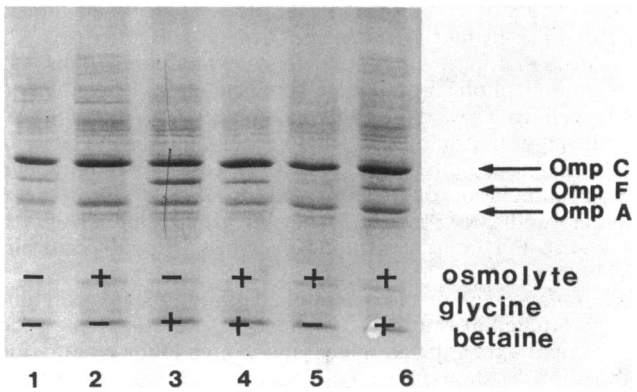


FIG. 1. Reversal of porin osmoregulation by glycine betaine. Cultures of wild type strain KL19 were grown for six generations in appropriate media and harvested, and membranes were examined by SDS-urea gel electrophoresis. Lanes: 1, M63; 2, M63 plus 0.5 M NaCl; 3, M63 plus 1 mM glycine betaine; 4, M63 plus 0.5 M NaCl plus 1 mM glycine betaine; 5, M63 plus 0.73 M sucrose; 6, M63 plus 0.73 M sucrose plus 1 mM glycine betaine. Glycerol was used as the carbon source in all cases.

had little effect on growth rate. The same pattern of regulation was also observed in strain MC4100 (data not shown).

Similar experiments with an *ompF-lac* operon fusion demonstrated that glycine betaine reversed OmpF osmoregulation by increasing the level of gene expression. Synthesis of β-galactosidase under the control of the *ompF* promoter was measured during growth under osmotic stress. Addition of 0.5 M NaCl to minimal medium reduced the rate of β-galactosidase synthesis to zero (Fig. 2). Supplementation of the high-osmolarity medium with 1 mM glycine betaine significantly restored β-glactosidase synthesis from Φ(*ompF-lac*).

**Other adaptations of envelope protein composition to elevated osmolarity.** Elevated osmolarity causes additional changes in *E. coli* envelope protein composition. Strain MC4100 was grown in minimal medium supplemented with osmotically equivalent amounts of NaCl or sucrose (0.5 and 0.73 M, respectively) and then was fractionated into membranes, periplasm, and cytosol. SDS-polyacrylamide gel electrophoresis of the membrane fraction from cells grown in sucrose revealed synthesis of small amounts of two new polypeptides with masses of 42 kDa and 20 kDa (Fig. 3). The same result was obtained with salt-supplemented medium (data not shown). Addition of the osmoprotectant glycine betaine to the high-osmolarity medium greatly reduced the extent of these changes.

The most striking feature of the adaptive response to increased osmotic pressure was the change in protein content of the periplasm (Fig. 4). Growth in salt- or sucrose-supplemented medium caused induction of an abundant new 31-kDa protein. Another new 16-kDa protein also appeared, and two periplasmic proteins with masses of 41 and 24 kDa were strongly repressed (Fig. 4, lane 2). Again, the presence

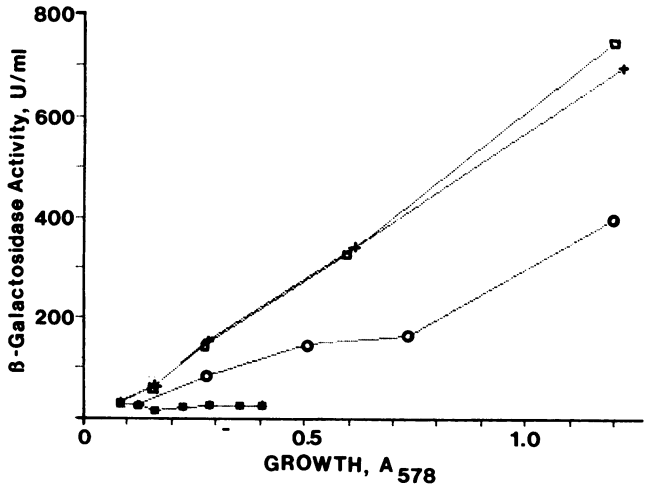


FIG. 2. Glycine betaine stimulation of Φ(*ompF-lacZ*$^+$) expression. A culture of strain MH513 Φ(*ompF-lacZ*$^+$) growing exponentially in M63-glycerol medium (□) was split four ways at time zero, and the growth medium was supplemented with either 0.5 M NaCl (■), 1 mM glycine betaine (+), or 0.5 M NaCl plus 1 mM glycine betaine (○). Cell growth was monitored as $A_{578}$ for 8 h. The figure shows the differential rate of synthesis of β-galactosidase activity from the *ompF-lac*$^+$ fusion. In the high-salt medium, cells grow slowly with a doubling time of 3.2 h, and *ompF* expression is completely repressed. Glycine betaine added to the high-salt medium reduces the generation time to 1.6 h and partially restores β-galactosidase synthesis. In low-osmolarity media (□, +), the doubling time was 1.4 h and was unaffected by glycine betaine addition.

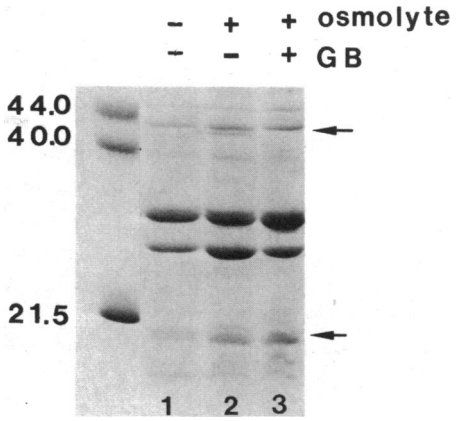


FIG. 3. Identification of other osmoregulated membrane proteins. Cells of strain MC4100 were grown as described in the legend to Fig. 1 and fractionated as described in the text. Membrane proteins were separated on a 10% polyacrylamide gel system (14). Proteins induced by elevated sucrose (indicated by arrows) had apparent masses of 42 and 20 kDa. The OmpF and OmpC porins are poorly resolved in this gel system. Markers are (from top of gel) ovalbumin (44.0 kDa), aldolase (40.0 kDa), and soybean trypsin inhibitor (21.5 kDa).

of 1 mM glycine betaine during growth in the high-osmolarity medium largely reversed all these changes (Fig. 4, lane 4). Comparison of the periplasmic proteins with those from the cytosols (Fig. 4, lanes 5 and 6) demonstrates that the salt-induced protein changes are unique to the periplasm and not a result of cytosolic contamination from cell lysis.

**Insertions in the *proU* locus result in loss of the 31-kDa periplasmic protein.** Derivatives of the transposable λ *plac* Mu phage (1) were used to isolate *lacZ* gene and operon fusions in an osmotically inducible gene. These λ *plac* Mu insertions map to the 58-min region of the *E. coli* chromosome (May, Villarejo, and Bremer, manuscript submitted). Their chromosomal location and the osmoresponsive activation of *lac* expression strongly suggest that these insertions are in the *proU* locus (2, 7, 10). One operon fusion strain, GM50, and one protein fusion strain, GM37, were chosen for further study. The 31-kDa osmotically inducible periplasmic protein was absent in GM50 (Fig. 4); it was also missing in GM37 (data not shown). All other osmoinducible changes in periplasm and membranes seen in the parent strain MC4100 were observed in the mutants carrying λ *plac* Mu insertions. These data suggest that the *proU* locus includes the structural gene for the 31-kDa periplasmic protein or a regulatory gene involved in its expression.

**Regulation of *proU* expression.** The extent of induction of *proU* activity by osmotic pressure was studied by measuring β-galactosidase activity from the *proU-lacZ* operon fusion in GM50 as a function of the concentration of added NaCl (Fig. 5). β-Galactosidase activity was nearly zero in MMA medium and increased about 50-fold to full induction in medium supplemented with 0.5 M NaCl. The relationship between *proU-lacZ* expression and salt concentration was nearly linear between 0.1 and 0.45 M NaCl. A similar pattern of medium osmolarity dependence was observed for induction of the 31-kDa periplasmic protein, determined by examining gel patterns as a function of increasing salt concentrations (data not shown).

Since glycine betaine in the high-osmolarity medium reduced the amount of 31-kDa polypeptide in the periplasm (Fig. 4), we investigated the effect of the osmoprotectant on

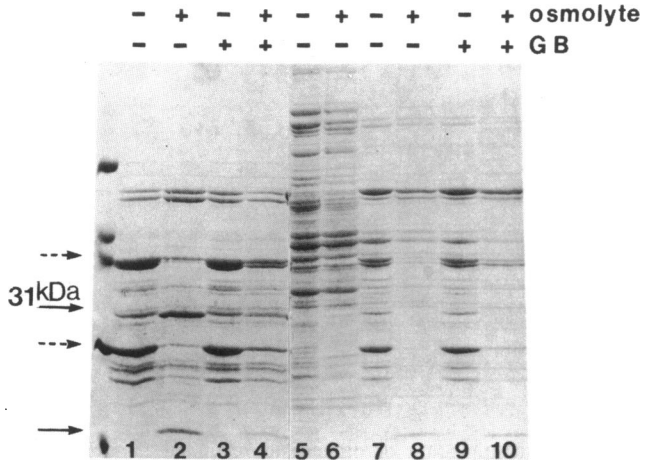


FIG. 4. Osmoregulation of periplasmic protein content. Cells of strains MC4100 and GM50 were grown for six generations in appropriate media, and their periplasmic contents were released and then examined by SDS-polyacrylamide gel electrophoresis. Lanes 1 to 4 contain periplasmic proteins from the parent strain MC4100, and lanes 7 to 10 contain periplasmic proteins from the *proU* mutant GM50. Lanes 1 and 7, M63; 2 and 8, M63 plus 0.5 M NaCl; 3 and 9, M63 plus 1 mM glycine betaine; 4 and 10, M63 plus 0.5 M NaCl plus 1 mM glycine betaine. Lanes 5 and 6 contain cytosols from cells whose periplasmic fractions are shown in lanes 1 and 2. All media contained 0.2% glycerol as the carbon source. Arrows indicate major osmotically induced changes in protein content; solid arrows show increases, and dashed arrows show decreases. Markers are (from top of gel) phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (44.0 kDa), aldolase (40.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

expression of a *proU-lacZ* fusion. Expression was initiated by addition of 0.3 M NaCl to a growing culture of strain GM37 (Fig. 6A). In the absence of glycine betaine, β-galactosidase activity was rapidly induced, reaching its maximum level in about 2 h (Fig. 6). When glycine betaine was present, β-galactosidase was initially synthesized at the same rate as the control but then terminated sharply after 30 min at about one-fourth of the fully induced level.

Transposition of the λ *plac* Mu phage into a gene disrupts its function and exerts a polar effect on the expression of other genes in the same operon located downstream from the point of insertion (1). The *proU* gene product or a regulatory gene possibly existing downstream in an operon could participate in the osmotic induction of the gene fusion. To investigate this, we isolated a specialized lambda-transducing phage carrying the entire *proU-lacZ* fusion after UV irradiation of strain GM37 and used this phage to construct a *proU*$^+$/*proU-lacZ* merodiploid. The kinetics of *proU-lacZ* induction in this strain (GM86) were essentially identical to those of the parent strain (Fig. 6B). Thus, we concluded that the ProU protein does not participate in the regulation of its structural gene.

*E. coli* apparently has the capability to transport both glycine betaine and proline when subjected to elevated osmotic strength (2, 10). However, exogenous proline was much less effective as an osmoregulatory solute than was glycine betaine. Proline supplementation of high-osmolarity medium did not relieve OmpF repression or significantly repress induction of the 31-kDa periplasmic protein (data not shown). The extent of *proU* regulation by proline was quantitated with the *proU-lacZ* fusion in experiments analogous to those in Fig. 6. Whereas 1 mM glycine betaine repressed *proU-lacZ* expression to 25% of the salt-induced activity, 1 mM proline allowed β-galactosidase synthesis at 80% of control levels.

## DISCUSSION

Osmoregulation involves several changes in protein composition of the *E. coli* envelope. The most striking aspect of the response to elevated osmolarity is the induction of large amounts of a periplasmic protein of 31-kDa. This protein is likely to be a product of the *proU* locus since it is absent in mutants with λ *plac* Mu insertions in *proU*, its level of induction parallels that of the *proU-lacZ* fusions in the presence of osmolytes, and both the protein and expression of the gene are repressible by glycine betaine. The high level of induction of this protein would account for the ready identification of *proU* as an osmoregulated locus in many laboratories, using gene fusion techniques. However, it is also possible that loss of the 31-kDa protein is due to a polar effect of the λ *plac* Mu insertion in *proU* on a distal gene is an operon or to loss of a positive regulatory function.

The function of *proU* has been the subject of considerable discussion. This locus was first identified as specifying an osmoregulated proline transport system in *S. typhimurium* (7, 8) and was recently shown to contribute to the ability of proline to act as an osmoprotectant in *E. coli* (10). Recent work with *S. typhimurium* suggests that *proU* encodes a high-affinity transport system for glycine betaine (2). If these observations extend to *E. coli*, the cells would take up both proline and glycine betaine when stressed by elevated osmolarity. The relative contributions of the two substances to osmoprotection would not only depend on their relative affinities for the transport system but also on their concentrations in the external environment.

While both proline and glycine betaine can act as osmoprotectants, they apparently differ in their roles in osmoregulation of protein synthesis. Glycine betaine had a dramatic feedback effect on osmoregulated events, largely reversing all the changes in protein composition associated

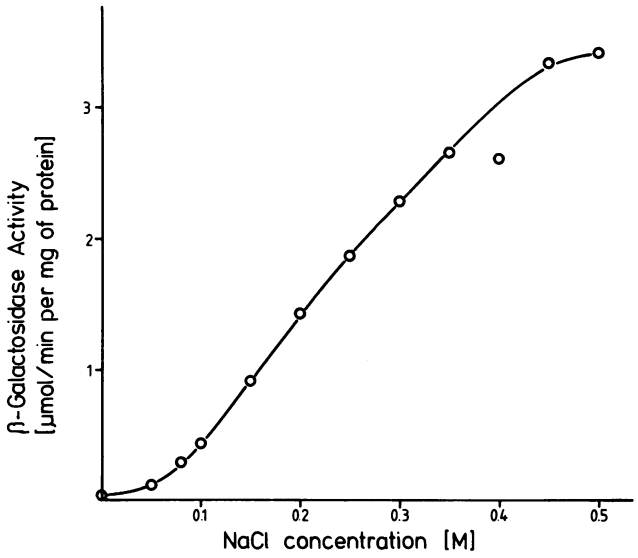


FIG. 5. Level of induction of Φ(*proU-lacZ*$^+$) as a function of osmolarity. Cultures of strain GM50 carrying a *proU-lacZ*$^+$ operon fusion were grown overnight in MMA-glucose medium supplemented with the indicated amount of NaCl, harvested, and assayed for steady-state levels of β-galactosidase.

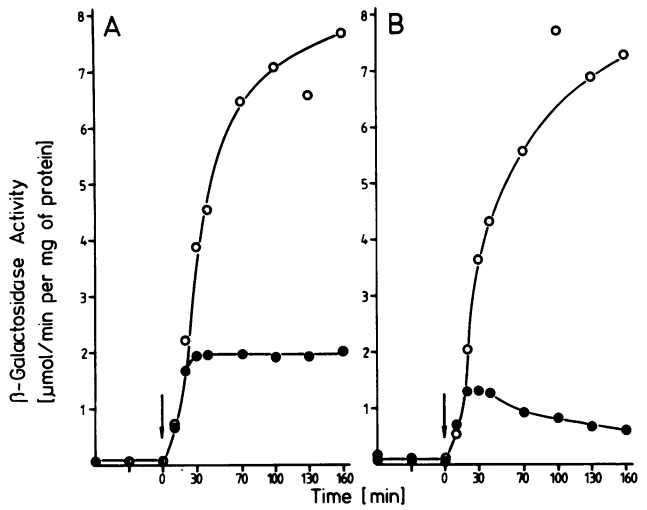


FIG. 6. Repression of Φ(*proU-lacZ*) expression by glycine betaine. A culture of the appropriate strain, actively growing in MMA-glucose, was divided in half and supplemented with 0.3 M NaCl (○) or with 0.3 M NaCl plus 1 mM glycine betaine (●). Aliquots were removed at indicated times and assayed for β-galactosidase activity and protein content. The strain used in panel A was GM37 (*proU*); the strain used in panel B was GM86 (*proU*$^+$/*proU-lacZ*).

with adaptation to elevated osmolarity. The extent of glycine betaine suppression of *proU* induction reported here is in agreement with its effectiveness in *S. typhimurium* (2). The kinetics of regulation (Fig. 6) suggest that intracellular accumulation of glycine betaine is required for activity. Proline, on the other hand, did not alter the extent of *proU-lacZ* induction in *E. coli* in a recent report (10). In our experiments, proline had little influence on osmoregulation of protein composition and *proU-lacZ* expression. Glycine betaine may be more effective as a regulator because it is accumulated to higher internal concentrations or because it is better recognized by some sensory mechanism.

The location of the 31-kDa osmoinducible polypeptide in the periplasm and its absence in *proU-lacZ* fusion strains leads to the attractive speculation that this protein is the binding protein component of a high-affinity glycine betaine transport system. This model is supported by preliminary experiments measuring binding of radiolabeled glycine betaine to periplasmic fractions and is also consistent with the stronger repression of *proU-lacZ* induction in a ProU$^+$ background (Fig. 6). However, cells lacking the periplasmic protein can still utilize glycine betaine as an osmoprotectant, presumably through a low-affinity transport system such as *proP* (3).

The modulation of porin synthesis was the first component of cellular osmoregulation to be well characterized. We showed that the osmoprotectant glycine betaine largely prevented the repression of *ompF* expression normally associated with elevated osmolarity. Since the amount of OmpC in the membranes did not vary greatly in these experiments, we did not have a sufficiently sensitive assay to detect possible glycine betaine effects on *ompC* expression. The extent of reversal of the OmpF phenotype by glycine betaine at very high osmolarities paralleled the extent of rescue from growth inhibition. Growth inhibition in minimal medium could be due to the deleterious effects of excessive concentrations of potassium and counterions or to the inability to develop sufficient turgor pressure through these mechanisms. Accumulation of the compatible solute glycine betaine would reduce the need for potassium and possibly increase the internal osmotic pressure. Thus, the signal for osmoregulation of *ompF* expression, and its reversal by glycine betaine, could be turgor pressure, intracellular potassium concentration, or the internal concentration of other osmotically active solutes.

The relationship of the other adjustments in envelope protein composition to alterations in cellular physiology is unknown. Membrane proteins induced by high osmolarity may be part of the *proU* transport system or may be involved in transport of other osmoprotectants. Several periplasmic and membrane proteins were reduced in amount under these conditions. Some might be enzymes involved in synthesis of membrane-derived oligosaccharides (13). Identification of all the metabolic and transport activities under osmotic control would contribute to understanding the mechanism of survival under osmotic stress.

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