Factors Reducing and Promoting the Effectiveness of Proline as an Osmoprotectant in *Escherichia coli* K12

By JOCELYN L. MILNER, DENISE J. McCLELLAN AND
JANET M. WOOD*

Department of Chemistry and Biochemistry, University of Guelph, Guelph,
Ontario N1G 2W1, Canada

(Received 12 January 1987; revised 5 March 1987)

Proline accumulation in *Escherichia coli* is mediated by three proline porters. Proline catabolism is effected by proline porter I (PPI) and proline/ Δ^1 -pyrroline carboxylate dehydrogenase. Proline did not accumulate cytoplasmically when *E. coli* was subjected to osmotic stress in minimal salts medium. Although PPI is induced when proline is provided as carbon or nitrogen source, its activity decreased following growth of the bacteria in minimal salts medium of high osmotic strength. Proline dehydrogenase was induced by proline in low or high osmotic strength media. Proline porter II (PPII) was both activated and induced in osmotically stressed bacteria, though the dependencies of the two responses on medium osmolarity differed. Osmotic downshift during the transport measurement decreased the uptake of proline, serine and glutamine by bacteria cultured in media of high osmotic strength. Thus, while osmotic upshift caused specific activation of PPII, osmotic downshift caused a non-specific reduction in amino acid uptake. Glycine betaine inhibited the uptake of [^{14}C]proline via PPII and PPIII but not via PPI. The dependence of that inhibition on glycine betaine concentration was similar when PPII was uninduced, induced or activated by osmotic stress, or induced by amino acid limited growth. Thus PPII and PPIII, not PPI, contribute to the mechanism of osmoprotection by proline and glycine betaine. The tendency for exogenous proline to accumulate in the cytoplasm of bacteria exposed to osmotic stress would, however, be countered by increased proline catabolism.

INTRODUCTION

The accumulation of inorganic solutes such as K^+ and organic solutes such as polyols, amino acids and *N*-methylated amino acid derivatives is frequently correlated with osmotic stress tolerance (Yancey *et al.*, 1982). Recently the enzymic and regulatory mechanisms mediating stress-induced accumulation of these osmoprotective compounds, often called compatible solutes, have been examined in the enteric bacteria *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae* (Le Rudulier *et al.*, 1984*a, b*). Although not halophilic, those organisms can grow in media supplemented with up to 0.7 M-NaCl (Brown, 1976). Proline, proline betaine, glycine betaine and the glycine betaine precursor choline, stimulate bacterial growth and nitrogen fixation when added to media of elevated osmotic strength (Csonka, 1982; Le Rudulier *et al.*, 1984*b*), and proline overproduction also enhances osmotolerance (Csonka, 1981; Le Rudulier & Bouillard, 1983).

Measures (1975) showed that large amounts of proline and glutamate were accumulated when an *E. coli* strain was cultivated in a rich medium supplemented with NaCl. The proline pool of a proline prototrophic *E. coli* K12 strain increased only 2-fold from 8.8 to 20 nmol (mg protein) $^{-1}$ when 0.55 M-NaCl was added during growth in a defined salts medium (Jakowec *et al.*, 1985);

Abbreviation: AC, L-azetidine-2-carboxylate; PPI, PPII, PPIII, protine porters I, II and III.

Csonka (1981) detected little change in the proline pool of *S. typhimurium* during growth in minimal medium plus 0.65 M-NaCl.

Cytoplasmic solutes are likely to provide osmoprotection in two ways. They may provide osmotic balance, preventing dehydration of the cytoplasm in response to high concentrations of solutes that remain extracellular (Measures, 1975). In addition, they may protect cellular macromolecules from denaturation by permeant osmolytes or by ions such as K^+ that are accumulated to provide osmotic balance (Somero, 1986; Sutherland *et al.*, 1986). Arakawa & Timasheff (1985) have demonstrated that proteins are preferentially hydrated in aqueous solutions that include certain organic solutes. That thermodynamic effect, which stabilizes the native, highly folded conformations of the proteins examined, is characteristic of polyols, amino acids and amino acid derivatives that are known to be osmoprotective. Proline and glycine betaine are equally effective stabilizers by that criterion. Efforts have been made to demonstrate specific enzyme activation by compatible solutes *in vitro* (Bowls & Somero, 1979). Pollard & Wyn Jones (1979), examining the inhibition of barley leaf malate dehydrogenase by NaCl, showed that the protective effects of *N*-methylated glycine derivatives were directly proportional to their degree of methylation. Glycine betaine, which yielded the greatest stimulation of enzyme activity among the solutes tested, was slightly more protective than proline. However, as in most other studies, neither compound restored full enzymic activity.

On the basis of the cited studies we expected that proline and glycine betaine would be similarly osmoprotective when accumulated to the same cytoplasmic concentration. In some cases, however, glycine betaine and proline betaine have shown significantly greater osmoprotective capacity than proline (Cairney *et al.*, 1985*b*; Le Rudulier & Bouillard, 1983; Le Rudulier *et al.*, 1984*a*). We have assessed the influence of the proline catabolic enzymes proline porter I (PPI) and proline/ Δ^1 -pyrroline carboxylate dehydrogenase (EC 1.5.99.8) on proline accumulation in response to osmotic stress in *E. coli*. We have also compared the specificities of the known proline transport systems, proline porters I, II and III, for proline and glycine betaine. Our studies suggest that the greater osmoprotective ability of glycine betaine than of proline reflects a corresponding difference in the capacity of the bacteria for their accumulation.

METHODS

Materials. L-[^{14}C]Proline was purchased from ICN Radiochemicals (Irvine, Calif., USA) or New England Nuclear. The radiochemical purity of each batch was examined by thin layer chromatography followed by autoradiography (Wood & Zadworny, 1979). The solvent systems used were phenol/water (3:1, w/v) and butanol/acetic acid/water (5:4:1, by vol.). All other reagents were from previously described sources and culture media were prepared as previously described (Wood & Zadworny, 1980) except for Proline Bioassay Medium (Takara Kohsan Co. Ltd Tokyo, Japan). The culture media used were LB broth (Miller, 1972) and the MOPS medium described by Neidhardt *et al.* (1974). MOPS medium contained 9.5 mM- NH_4Cl as nitrogen source and was additionally supplemented with 50 μg L-tryptophan ml^{-1} and 1 μg thiamin hydrochloride ml^{-1} to meet auxotrophic requirements unless otherwise indicated. D-Glucose (2 mg ml^{-1}) was provided in solid media and D-fructose (2 mg ml^{-1}) was provided in liquid media as carbon source.

Strains. The bacterial strains used in this study, all derivatives of *E. coli* K12, are listed in Table 1. Genetic manipulations were done as described by Miller (1972). Bacterial phenotypes were determined as described previously (Wood, 1981; Grothe *et al.*, 1986; Miller, 1972).

Transport measurements. Bacterial cultures were prepared for transport measurements as previously described (Grothe *et al.*, 1986). The level of osmotic stress present during growth was maintained during cell harvesting, resuspension and the transport assay by supplementing the solutions with the appropriate level of osmolyte as indicated in Results. Amino acid uptake was measured using a filtration assay which was initiated by the addition of radioactive substrate, as follows: L-[^{14}C]proline, 20 μM , 25 $\mu Ci \mu mol^{-1}$ (925 kBq μmol^{-1}), or 200 μM , 10 $\mu Ci \mu mol^{-1}$ (370 kBq μmol^{-1}) as indicated; L-[^{14}C]serine 20 μM , 25 $\mu Ci \mu mol^{-1}$ (925 kBq μmol^{-1}); and L-[^{14}C]glutamine, 20 μM , 25 $\mu Ci \mu mol^{-1}$ (925 kBq μmol^{-1}). Samples were withdrawn and filtered on Millipore filters (type HA; 0.45 μm pore size) at 15, 30 and 45 s following initiation of the assay. Each filter was washed once with unsupplemented MOPS medium (Grothe *et al.*, 1986) with osmolyte added as indicated. Filters were dried and radioactivity was measured as described previously (Wood & Zadworny, 1979). All assays were done in triplicate.

Table 1. *Escherichia coli* K12 strains

Genetic nomenclature is that of Bachmann (1983). The *proU* locus is defined in the text. Δ , Chromosomal deletion; ::, transposon insertion.

Strain	Genotype	Source or derivation
CSH4	F ⁻ <i>trp lacZ rpsL thi</i>	Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA
JT31	CSH4 <i>putA1</i> :: Tn5	Wood (1981)
JT34	CSH4 <i>putP3</i> :: Tn5	Wood (1981)
RM2	CSH4 Δ (<i>putPA</i>)101	Wood (1981)
WG146	CSH4 <i>pyr-76</i> :: Tn10	Stalmach <i>et al.</i> (1983)
WG170	CSH4 Δ (<i>putPA</i>)101 <i>proP219</i>	Stalmach <i>et al.</i> (1983)
WG203	CSH4 Δ (<i>putPA</i>)101 <i>proP219 proU205</i>	Grothe <i>et al.</i> (1986)
WG210	CSH4 Δ (<i>putPA</i>)101 <i>proU205</i>	Grothe <i>et al.</i> (1986)
WG225	CSH4 <i>proP219 proU205 pyr-76</i> :: Tn10	P1 transductant of WG203 from WG146
WG226	CSH4 <i>proP219 proU205</i>	P1 transductant of WG225 from WG203
WG227	CSH4 <i>putA1</i> :: Tn5 <i>proP219 proU205</i>	P1 transductant of WG203 from JT31
WG228	CSH4 <i>putP3</i> :: Tn5 <i>proP219 proU205</i>	P1 transductant of WG203 from JT34
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>)U169 <i>rpsL150 relA1 deoC1 ptsF25 rbsR</i> <i>flbB5301 thiA</i>	Casadaban (1976)
EF047	MC4100 Δ (<i>putPA</i>)101 <i>proP</i> Δ (<i>proU</i>)1	May <i>et al.</i> (1986)
WG2300	CSH4 <i>recA srl-300</i> :: Tn10	Wood & Zadworny (1980)
WG256	EF047 <i>srl-300</i> :: Tn10	P1 transductant of EF047 from WG2300
WG258	CSH4 Δ (<i>putPA</i>)101 <i>proP219</i> Δ (<i>proU</i>)1 <i>srl-300</i> :: Tn10	P1 transductant of WG170 from WG256

and all experiments were done at least twice. Each set of replicate assays was used to determine the rate of amino acid uptake over the 15 to 45 s interval.

The presence of a radioactive contaminant in some batches of L-[¹⁴C]proline, which was detectable by autoradiography after chromatography in the butanol/acetic acid/water but not the phenol/water system, correlated with an uptake component resistant to inhibition by unlabelled proline. That uptake component was particularly apparent in bacteria grown under conditions of osmotic stress (0.3 M-NaCl) (data not shown). The results of the chromatographic analysis and the absence of such an uptake component were used routinely as criteria for adequate radiochemical purity of the proline used.

Measurement of intracellular proline pools. Bacterial cultures were grown as described for transport measurements. A 10 ml sample of the culture was rapidly filtered on a Gelman filter (9 cm diameter, 0.45 μ m pore size). The cells were extracted on the filter with 10 ml ice-cold 70% (v/v) ethanol; the filtrate was then collected and dried under a stream of nitrogen at 60 °C. The residue was resuspended in 1.0 ml of water and sterilized by filtration. The proline content was determined by bioassay using *Pediococcus acidilactici* ATCC 8081 (*Leuconostoc citrovorum*). The inoculum was prepared by inoculating 6 ml of Proline Bioassay Medium and incubating for 16 h at 30 °C, centrifuging the culture, and washing and resuspending in 0.85% (w/v) NaCl to 10 times the original volume. The prepared inoculum was stored at -70 °C with sodium alginate (0.1% w/v) added as a cryoprotectant. Samples of the cell extract were added to 1.5 ml of double-strength Proline Bioassay Medium. Water was added and the tubes were inoculated with 75 μ l of prepared inoculum to a final volume of 3.0 ml. The mixture was incubated at 30 °C for 72 h. The optical density at 615 nm was compared with that produced by standard solutions of known proline concentrations.

Measurement of evolved ¹⁴CO₂. Bacterial cultures and the assay system were prepared as for transport measurements to a total volume of 1.0 ml in a 10 ml Erlenmeyer flask. The reaction was initiated by adding [¹⁴C]proline and stopped after 10 min with 6 M-HCl. Evolved CO₂ was captured in a 200 μ l phenylethylamine/methanol (1:1 v/v) trap. The intact trap was placed in a vial with scintillation fluid (Wood & Zadworny, 1979) and ¹⁴CO₂ was determined by scintillation counting.

Proline dehydrogenase activity. This was determined using toluenized bacteria and a modification of the *o*-aminobenzaldehyde assay described previously (Wood, 1981). The 2.5 ml enzyme assay mixture contained toluenized bacteria (1 to 1.5 mg protein), L-proline (80 mM) and *o*-aminobenzaldehyde (4 mM) in unsupplemented MOPS medium (Grothe *et al.*, 1986).

Protein determinations. Protein concentrations were determined using a micro modification of the Lowry assay for protein (Peterson, 1977). Bovine serum albumin was used as the standard.

RESULTS

Proline accumulation in response to osmotic stress

When *E. coli* strain CSH4 (*putA*⁺ *putP*⁺ *proP*⁺ *proU*⁺) was cultivated in minimal salts medium, the proline content increased from 3.5 ± 0.9 nmol (mg protein)⁻¹ in the absence of osmotic stress to 5.2 ± 1.7 and 8.7 ± 1.7 nmol (mg protein)⁻¹ when osmotic stress was imposed by adding NaCl and sucrose, respectively, at 0.3 M. When all known proline transport and catabolic functions were eliminated (*E. coli* strain WG203, *putA putP proP proU*) the corresponding contents were not significantly different: 4.3 ± 0.9 nmol (mg protein)⁻¹ with no stress; 6.1 ± 1.7 nmol (mg protein)⁻¹ when stress was imposed by 0.3 M-NaCl; and 7.0 ± 0.9 nmol (mg protein)⁻¹ when stress was imposed by 0.3 M-sucrose.

Csonka (1981) showed that glutamate and glutamine, not proline, accumulated when *S. typhimurium* was subjected to osmotic stress (0.65 M-NaCl) during growth in defined medium. The glutamate content was 290 nmol (mg protein)⁻¹, more than an order of magnitude higher than the reported proline contents. As glutamate is the biosynthetic precursor for proline, the small increase in proline content observed when osmotic stress is imposed in minimal salts medium may reflect osmotic stimulation of glutamate biosynthesis without modulation of the proline biosynthetic pathway. The low proline contents could also reflect a futile cycle of proline biosynthesis and catabolism. Thus the similarity in proline pools between strains CSH4 and WG203 could result from the absence of increased proline synthesis or from opposite and balanced effects of transport and catabolism on proline accumulation.

Influence of the proline catabolic enzymes on proline accumulation

PPI is a Na⁺/proline symport system with a high affinity and specificity for proline (Wood, 1981; Wood & Zadworny, 1979; Chen *et al.*, 1985). PPI and proline/ Δ^1 -pyrroline carboxylate dehydrogenase, encoded in genes *putP* and *putA* respectively, constitute a system for aerobic proline catabolism. The *putP* and *putA* loci are adjacent and divergently transcribed from a common regulatory region (Menzel & Roth, 1981; Wood, 1981). In addition to its enzymic function, the PutA protein serves as a repressor that regulates expression of the *put* genes in response to the inducer L-proline. Expression of *putP* is therefore constitutive in *putA* deletion and insertion mutants (Menzel & Roth, 1981; Wood, 1981). We have isolated *E. coli* strains defective at the *proP* and *proU* loci (Table 1) in order to examine the response of the proline catabolic enzyme system to osmotic stress.

We showed previously that expression of proline dehydrogenase is apparently constitutive in *E. coli* strains with uncontrolled proline biosynthesis (Rancourt *et al.*, 1984). Induction of proline dehydrogenase might therefore be expected in response to elevation of cytoplasmic proline contents during aerobic osmotic stress. No induction of proline dehydrogenase was observed when strains CSH4 (*putA*⁺ *putP*⁺ *proP*⁺ *proU*⁺) and WG226 (*putA*⁺ *putP*⁺ *proP* *proU*) were cultivated in minimal salts medium supplemented with 0.3 M-sucrose (Table 2). Induction was observed, however, when proline was added to the same medium in the presence or absence of sucrose. Thus proline catabolism is not induced by osmotic stress, and induction of proline dehydrogenase by proline does occur during osmotic stress imposed by sucrose. Similar effects were observed when NaCl was used as an osmolyte (data not shown). These results suggest that the failure of proline to accumulate when *E. coli* is cultivated in minimal salts media of high osmotic strength without exogenous proline is not a consequence of futile cycling. Induction of *putA* gene expression did occur during osmotic stress, however, so proline supplied as an exogenous osmoprotectant would be subject to catabolism.

Like proline dehydrogenase, PPI was not induced in response to osmotic stress (Table 2). PPI activity decreased when osmotic stress was imposed on bacteria retaining or lacking the PutA protein (Table 2; Grothe *et al.*, 1986). The decrease in PPI activity was masked by induction of PPII and PPIII in strain CSH4; the proline uptake activity of strain WG210 (*putA putP proP*⁺ *proU*) was 5.2 (SD 0.2) nmol min⁻¹ (mg protein)⁻¹ when proline was provided at 20 μ M with 0.3 M-sucrose present during both growth and the transport assay. Limited induction of PPI by proline occurred in the *putA*⁺ strains CSH4 and WG226, as expected (Wood & Zadworny, 1979;

Table 2. Responses of the proline catabolic enzymes to osmotic stress

Bacteria were cultured in MOPS minimal medium supplemented with 245 μ M-tryptophan and 1 mM-proline (P) and/or 0.3 M-sucrose (S) as indicated. Proline transport was measured using 20 μ M-L-proline under conditions described in Methods. Proline dehydrogenase was measured as described in Methods. ND, Not detectable; NT, not tested.

Strain	Genotype				Addition to growth medium	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹] (SD)	
	<i>putA</i>	<i>putP</i>	<i>proP</i>	<i>proU</i>		Proline uptake	Proline dehydrogenase
CSH4	+	+	+	+	None	3.0 (0.9)	ND
					P	3.4 (1.4)	2.53 (0.07)
					S	7.1 (0.7)	ND
					P + S	5.0 (0.2)	1.49 (0.19)
WG226	+	+	-	-	None	3.0 (1.4)	ND
					P	3.0 (1.3)	2.05 (0.04)
					S	1.6 (1.6)	ND
					P + S	4.5 (1.2)	1.87 (0.06)
WG227	-	+	-	-	None	15.2 (1.2)	NT
					P	7.3 (1.2)	NT
					S	9.9 (1.2)	NT
					P + S	2.7 (0.5)	NT

Maloy & Roth, 1983). Surprisingly, 2- and 4-fold reductions in PPI activity were observed in strain WG227 (*putA*) when proline was added to growth media without and with 0.3 M-sucrose, respectively.

Transport is usually measured in *putA* strains so that catabolism of the substrate does not occur. To ensure that the low uptake activity of strains CSH4 and WG226 was not due to loss of the ¹⁴C-labelled substrate as ¹⁴CO₂, the amount of ¹⁴CO₂ evolved was determined under the conditions of the transport measurements described above. When strain CSH4 (*putA*⁺*putP*⁺*proP*⁺*proU*⁺) was grown with proline (0.2%, w/v) replacing NH₄Cl as nitrogen source to induce the proline catabolic enzymes, ¹⁴CO₂ equivalent to less than 1 nmol proline min⁻¹ (mg protein)⁻¹ was released. No evolved ¹⁴CO₂ could be detected when the culture of strain WG226 was prepared in minimal medium supplemented with sucrose and the evolved ¹⁴CO₂ was measured.

These results suggest that the proline catabolic activity of the PutA protein would negate the proline uptake capacity of PPI as an effector of proline accumulation when exogenous proline was provided and osmotic stress was imposed on *E. coli* strains retaining both functions.

Proline and glycine betaine as substrates for PPI, PPII and PPIII

PPII was originally identified as an uptake system with a high apparent *K_m* for proline, inactivated by mutations at *proP*, that was induced in bacteria whose growth rate was limited by amino acid supply (Anderson *et al.*, 1980; Menzel & Roth, 1980; Stalmach *et al.*, 1983). PPIII was identified as a function, eliminated by mutations at *proU*, that contributed to the osmoprotective effects of exogenous proline (Csonka, 1982). PPII and PPIII are now known to be osmoresponsive transport systems that mediate accumulation of both proline and glycine betaine in both *E. coli* (Perroud & Le Rudulier, 1985; Grothe *et al.*, 1986; Barron *et al.*, 1986; May *et al.*, 1986) and *S. typhimurium* (Cairney *et al.*, 1985*a, b*). Cairney *et al.* (1985*a, b*) suggested that PPII is a proline uptake system in the absence of osmotic stress and a glycine betaine transport system when osmotic stress is imposed, whereas PPIII is a glycine betaine transport system with very low proline uptake activity that functions only under osmotic stress.

The increased activity of PPII and PPIII during osmotic stress was correlated with increased toxicity of the proline analogues L-azetidine-2-carboxylic acid (AC) and 3,4-dehydropyrolidine (DHP) (Csonka, 1982; Cairney *et al.*, 1985*a, b*; Gowrishankar, 1985; Grothe *et al.*, 1986). We used AC sensitivity in the radial streak test and a series of *E. coli* strains in which proline/ Δ^1 -

Table 3. *Effect of glycine betaine on proline uptake*

Bacteria were grown on MOPS medium supplemented with 245 μM -tryptophan. Both the growth medium and the assay medium were supplemented with 0.3 M-sucrose. Proline uptake was measured as described in Methods. Glycine betaine was supplied at the indicated concentration. PPI activity was measured in strain WG227 (*putA putP⁺ proP proU*) with proline supplied at 20 μM . PPII activity was measured in strain WG210 (*putA putP proP⁺ proU*), PPIII activity was measured in strain WG170 (*putA putP proP proU⁺*) and background activity was measured in strain WG203 (*putA putP proP proU*), all with proline supplied at 200 μM . NT, Not tested.

Glycine betaine concn (mM)	Proline uptake rate [nmol min ⁻¹ (mg protein) ⁻¹] (SD)			
	Background	PPI	PPII	PPIII
0	1.0 (0.5)	8.5 (0.4)	15.3 (0.7)	3.4 (0.6)
0.020	1.4 (0.1)	7.1 (1.0)	NT	3.1 (1.3)
0.10	NT	7.6 (0.1)	8.5 (1.7)	1.3 (0.7)
0.20	1.4 (0.3)	7.2 (0.1)	7.7 (1.4)	2.3 (1.3)
1.00	NT	NT	3.7 (0.2)	NT
2.00	1.7 (0.5)	7.7 (0.8)	3.3 (0.5)	1.0 (0.8)
5.00	NT	NT	2.6 (0.9)	NT

pyrroline carboxylate dehydrogenase was absent and the proline porters were isolated (Table 1) to assess the relative specificities of the porters for proline and glycine betaine. Proline and glycine betaine, incorporated at 1 mM in solid medium supplemented with 0.3 M-NaCl, were equally effective in protecting strains WG210 (PPII only) and WG170 (PPIII only) from AC toxicity. In contrast, proline but not glycine betaine reduced AC toxicity in strains WG227 (PPI only) and JT31 (all porters present).

We examined the effects of glycine betaine on the uptake of [¹⁴C]proline by the same series of strains (Table 3). Proline uptake via PPI was not inhibited by glycine betaine even when the concentration ratio of glycine betaine to proline was 100. Although glycine betaine uptake has not been measured in bacteria retaining only PPI, these data are consistent with the conclusion (Cairney *et al.*, 1985a) that glycine betaine is not a substrate for that enzyme.

PPII can be both activated and induced by osmotic stress. A dramatic activation of PPII occurred in seconds without protein synthesis when bacteria grown in a medium of optimum osmolarity (Neidhardt *et al.*, 1974) were shifted to a medium of higher osmotic strength (Grothe *et al.*, 1986). A 2- to 4-fold induction of the *proP* gene in response to osmotic stress has been observed with *lacZ* operon fusions in *E. coli* and *S. typhimurium* (Gowrishankar, 1986; Dunlap & Csonka, 1985; Cairney *et al.*, 1985a). The effects of varying osmotic strength differ for the activation and induction of PPII. Optimum activation of PPII was attained between 0.12 and 0.25 M-NaCl (0.25 and 0.5 M-sucrose); the uptake activity observed was greater than 40 nmol min⁻¹ (mg protein)⁻¹ at the optimum and less than 10 nmol min⁻¹ (mg protein)⁻¹ at 0.5 M-NaCl (Grothe *et al.*, 1986). When strain WG210 was grown in media supplemented with 0 to 0.4 M-NaCl, PPII activity approached a maximum [approximately 45 nmol min⁻¹ (mg protein)⁻¹], with activities close to that value observed in the presence of 0.2, 0.3 or 0.4 M-NaCl (Fig. 1). Thus, activation and induction of PPII depended differently on osmotic strength, and induction of PPII became clearly apparent at NaCl concentrations greater than 0.2 M. Proline uptake activity was eliminated in strains WG203(*proU205*) (Fig. 1) and WG258(Δ (*proU*)) (data not shown) which lack the three proline porters. Serine and glutamine uptake by strains WG210 and WG203 were not altered by the *proP* mutation or by the increasing NaCl concentration when those substrates replaced proline in the titration experiment of Fig. 1 (data not shown).

Cairney *et al.* (1985a) examined changes in proline and glycine betaine uptake via PPII in response to osmotic stress in *S. typhimurium*. The measured proline uptake rates were low since proline was provided at only 10 μM ; glycine betaine was provided at 30 μM . Solute uptake was measured in growth and assay media with and without NaCl as supplementary osmolyte, so that shifts both up and down in osmotic strength of the medium occurred during the transport assay.

Fig. 1. PPII activity is induced by osmotic stress. Strains WG210 (*proP*⁺*proU*) (■) and WG203 (*proP proU*) (□) were cultured on MOPS minimal medium supplemented with 245 μ M-tryptophan and with increasing concentrations of NaCl. Transport was measured with proline supplied at 200 μ M in assay medium which was supplemented with NaCl to the same concentration as the growth medium. Error bars represent SD. For strain WG203, the SD values fell within the areas covered by the graph symbols.

Table 4. *Effect of osmotic downshift on amino acid uptake*

Bacteria, which were all *putA*, were cultured in MOPS minimal medium supplemented with 245 μ M-tryptophan and 0.3 M-NaCl. Substrates were supplied at the following concentrations: proline, 200 μ M; serine and glutamine, 20 μ M. Transport was measured in assay medium supplemented with 0.3 M-NaCl (no downshift) or not supplemented (with downshift).

Strain	Genotype		Substrate	Uptake rate [nmol min ⁻¹ (mg protein) ⁻¹] (SD)	
	<i>proP</i>	<i>proU</i>		No downshift	With downshift
WG210	+	-	Proline	19.5 (3.0)	2.7 (0.9)
			Serine	4.8 (0.6)	3.0 (0.8)
			Glutamine	1.3 (0.5)	0.2 (0.1)
WG203	-	-	Proline	2.2 (0.2)	2.3 (1.0)
			Serine	5.6 (0.9)	2.8 (<0.1)
			Glutamine	1.6 (0.1)	0.2 (<0.1)

They concluded that PPII specifically transports proline (not glycine betaine) at low osmolarity and glycine betaine (not proline) at high osmolarity. Further, the apparent K_m of PPII for glycine betaine at high osmolarity was reported to be almost 10-fold lower than that previously reported (Anderson *et al.*, 1980) for proline uptake via PPII at low osmolarity.

When strains WG210 (*proP*⁺*proU*) and WG203 (*proP proU*), cultured in medium supplemented with 0.3 M-NaCl, were exposed to osmotic downshift in the transport assay the proline uptake rate by strain WG210 was reduced to the background level represented by strain WG203 (Table 4). The rates of glutamine and serine uptake by these strains decreased by 90 and 38%, respectively, in response to the same osmotic downshift. Thus unlike osmotic upshift,

Table 5. *Specificity of PPII is not osmotic stress dependent*

Bacteria were grown on MOPS minimal medium supplemented with 245 μM -tryptophan except for amino acid limited growth (Trp Lim) where tryptophan was supplied at 24.5 μM . The growth and assay media were supplemented with 0.3 M-NaCl as indicated. The proline uptake rate is given as the fraction of that observed in the absence of glycine betaine. The absolute proline uptake rate [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$] in the absence of glycine betaine is given in parentheses. Uptake rates by strain WG210 (*putA putP proP⁺ proU*) were corrected for background proline uptake by strain WG203 (*putA putP proP proU*) in the same experiment. Proline was supplied at 200 μM . ND, Not detectable.

Agent of stress		Relative proline uptake rate (%) with glycine betaine at:		
Bacterial growth	Transport assay	0 mM	0.2 mM	2.0 mM
—	—	100 (2.3)	50	ND
—	0.3 M-NaCl	100 (13.9)	43	7
0.3 M-NaCl	0.3 M-NaCl	100 (27.6)	41	6
Trp Lim	—	100 (14.6)	35	10

which specifically activated proline transport, osmotic downshift resulted in a non-specific decrease in amino acid transport. The failure of bacteria cultured in medium of high osmotic strength to accumulate proline when shifted to medium of low osmotic strength (Cairney *et al.*, 1985a) cannot, therefore, be taken as an indicator of the specificity of PPII.

The uptake of [^{14}C]proline via PPII was inhibited by glycine betaine (Table 3). In this experiment, proline uptake via PPII was reduced to 50% of the uninhibited rate when glycine betaine and proline were present at equal concentrations (200 μM) and to 15% of the uninhibited rate when the concentration ratio of glycine betaine to proline was 25. The sensitivity of proline uptake via PPII to glycine betaine inhibition was also examined in unstressed bacteria, after osmotic activation of PPII and following amino acid limited growth (Table 5). In each case the degree of inhibition of proline uptake by glycine betaine depended similarly on glycine betaine concentration. Thus, we detected no change in the substrate specificity of PPII as its activity was modulated by either activation or induction. Our results imply similar affinities of that system for proline and glycine betaine, but more extensive kinetic analysis will be required to establish this point unequivocally.

As noted previously, the proline uptake activity correlated with genetic locus *proU* (PPIII) was much lower than that of PPI or PPII (Grothe *et al.*, 1986). Activity was undetectable when glycine betaine was present at low concentration (Table 3). This result is consistent with the view that PPIII is a glycine betaine porter with low proline uptake activity (Dunlap & Csonka, 1985; Cairney *et al.*, 1985b; May *et al.*, 1986).

DISCUSSION

Our results provide insight regarding the roles of proline and glycine betaine as osmoprotectants and the role of PPII in the osmotic stress response of *E. coli* K12. Proline pools were increased very little and the proline catabolic enzymes, PPI and proline dehydrogenase, were not induced when osmotic stress was imposed in the absence of exogenous proline. Conversely, both enzymes were induced when exogenous proline was provided in the presence or absence of osmotic stress to *putA⁺ putP⁺* bacteria (Table 2). These observations are consistent with that of Csonka (1981), who showed that the osmoprotective effects of proline in *S. typhimurium* were not altered by a deletion mutation eliminating both *putA* and *putP*. PPI would be expected to contribute to cytoplasmic proline accumulation whereas proline dehydrogenase would reduce proline pools.

The constitutive activity of PPI in *putA* bacteria was reduced after growth in the presence of proline with or without osmotic stress (Table 2). This surprising observation, which may reveal biochemical modulation of PPI activity, is also consistent with Csonka's observation that exogenous proline was equally osmoprotective in *putA* bacteria wild-type and defective in *putP*

(Csonka, 1981; Csonka, 1982). The lack of impact of PPI on osmoprotection by proline would be surprising if the high, constitutive uptake activity usually observed for PPI were retained in the presence of osmotic stress and exogenous proline.

Our experiments (Tables 2, 3, 4 and 5; Fig. 1) and those of other laboratories, summarized above, suggested that the maintenance of osmoprotective proline gradients in *E. coli* is mediated primarily by PPII and antagonized by the proline catabolic enzymes. Proline oxidation requires respiratory electron flow and proline utilization is very limited when nitrate or fumarate is provided to anaerobic cultures of *E. coli* (L. G. Baker & J. M. Wood, unpublished data). Thus, catabolism of proline accumulated in response to osmotic stress may be less significant physiologically than is implied by experiments done under the commonly used aerobic growth and reaction conditions. Induction of the proline catabolic enzymes of *E. coli* by proline under osmotic stress is in contrast to the repression of proline catabolism reported to occur in some higher plants under those conditions (Wyn Jones & Storey, 1981).

Glycine betaine biosynthesis from exogenous choline, like choline and glycine betaine uptake, is stimulated by osmotic stress in *E. coli* (Landfald & Strom, 1986; Styrvold *et al.*, 1986; May *et al.*, 1986). The maintenance of osmoprotective glycine betaine gradients is effected by PPII and by PPIII, an osmotically activated uptake system now reported to catalyse glycine betaine uptake more effectively than proline uptake (Cairney *et al.*, 1985*a, b*; May *et al.*, 1986). Our results suggest that PPII has similar affinities for proline and glycine betaine (Tables 4 and 5), though Cairney *et al.* (1985*a*) concluded that PPII, like PPIII, had a higher affinity for glycine betaine than for proline in osmotically stressed bacteria. In either case, greater uptake capacity for glycine betaine (PPII plus PPIII) than for proline (primarily PPII) seems to be present in bacteria subjected to osmotic stress during growth. This difference and the induction of proline catabolism may be sufficient to explain the observed differences in osmoprotection by exogenous proline and glycine betaine (Cairney *et al.*, 1985*b*; Le Rudulier & Bouillard, 1983; Le Rudulier *et al.*, 1984*a*) without invoking different physicochemical effects of the two compatible solutes.

PPII is both activated and induced by osmotic stress (Grothe *et al.*, 1986; Fig. 1). Activation of PPII may provide a first line of defence against dehydration of the cytoplasm when proline or glycine betaine is available. In contrast, glycine betaine accumulation after induction of both PPII and PPIII may contribute to survival in the presence of acute and prolonged osmotic stress (Sutherland *et al.*, 1986).

This work was supported by an Operating Grant (J. M. W.) and a Postgraduate Scholarship (J. L. M.) from the Natural Sciences and Engineering Council of Canada. The authors are grateful to E. Bremer for strains MC4100 and EF047.

REFERENCES

- ANDERSON, R. R., MENZEL, R. & WOOD, J. M. (1980). Biochemistry and regulation of a second L-proline transport system in *Salmonella typhimurium*. *Journal of Bacteriology* **141**, 1071–1076.
- ARAKAWA, T. & TIMASHEFF, S. N. (1985). The stabilization of proteins by osmolytes. *Biophysical Journal* **47**, 411–414.
- BACHMANN, B. J. (1983). Linkage map of *Escherichia coli* K12. Edition 7. *Microbiological Reviews* **47**, 180–230.
- BARRON, A., MAY, G., BREMER, E. & VILLAREJO, M. (1986). Regulation of envelope protein composition during adaptation to osmotic stress in *Escherichia coli*. *Journal of Bacteriology* **167**, 433–438.
- BOWLUS, R. D. & SOMERO, G. N. (1979). Solute compatibility with enzyme function and structure: rationales for the selection of osmotic agents and end-products of anaerobic metabolism in marine invertebrates. *Journal of Experimental Zoology* **208**, 137–152.
- BROWN, A. D. (1976). Microbial water stress. *Bacteriological Reviews* **40**, 803–846.
- CAIRNEY, J., BOOTH, I. R. & HIGGINS, C. F. (1985*a*). *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. *Journal of Bacteriology* **164**, 1218–1223.
- CAIRNEY, J., BOOTH, I. R. & HIGGINS, C. F. (1985*b*). Osmoregulation of gene expression in *Salmonella typhimurium*: the *proU* gene encodes an osmotically-induced betaine transport system. *Journal of Bacteriology* **164**, 1224–1232.
- CASADABAN, M. J. (1976). Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *Journal of Molecular Biology* **104**, 541–555.
- CHEN, C. C., TSUCHIYA, T., YAMANE, Y., WOOD, J. M. & WILSON, T. H. (1985). Na⁺(Li⁺)-proline cotransport in *Escherichia coli*. *Journal of Membrane Biology* **84**, 157–164.

- CSONKA, L. N. (1981). Proline overproduction results in enhanced osmotolerance in *Salmonella typhimurium*. *Molecular and General Genetics* **182**, 82–86.
- CSONKA, L. N. (1982). A third L-proline permease in *Salmonella typhimurium* which functions in media of elevated osmotic strength. *Journal of Bacteriology* **151**, 1433–1443.
- DUNLAP, V. J. & CSONKA, L. N. (1985). Osmotic regulation of L-proline transport in *Salmonella typhimurium*. *Journal of Bacteriology* **163**, 296–304.
- GOWRISHANKAR, J. (1985). Identification of osmoprotective genes in *Escherichia coli*: evidence for participation of potassium and proline transport systems in osmoregulation. *Journal of Bacteriology* **164**, 434–445.
- GOWRISHANKAR, J. (1986). *proP* mediated proline transport plays a role in *Escherichia coli* osmoregulation. *Journal of Bacteriology* **166**, 331–333.
- GROTHE, S., KROGSrud, R. L., MCCLELLAN, D. J., MILNER, J. L. & WOOD, J. M. (1986). Proline transport and osmotic stress response in *Escherichia coli* K-12. *Journal of Bacteriology* **166**, 253–259.
- JAKOWEC, M. W., SMITH, L. T. & DANDEKAR, A. M. (1985). Recombinant plasmid conferring proline overproduction and osmotic tolerance. *Applied and Environmental Microbiology* **50**, 441–446.
- LANDFALD, B. & STROM, A. R. (1986). Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. *Journal of Bacteriology* **165**, 849–855.
- LE RUDULIER, D. & BOUILLARD, L. (1983). Glycine betaine, an osmotic effector in *Klebsiella pneumoniae* and other members of the *Enterobacteriaceae*. *Applied and Environmental Microbiology* **46**, 152–159.
- LE RUDULIER, D., BERNARD, T., GOAS, G. & HAMELIN, J. (1984a). Osmoregulation in *Klebsiella pneumoniae*: enhancement of anaerobic growth and nitrogen fixation under stress by proline betaine, γ -butyrobetaine, and other related compounds. *Canadian Journal of Microbiology* **30**, 299–305.
- LE RUDULIER, D., STROM, A. R., DANDEKAR, A. M., SMITH, L. T. & VALENTINE, R. C. (1984b). Molecular biology of osmoregulation. *Science* **224**, 1064–1068.
- MALLOY, S. R. & ROTH, J. R. (1983). Regulation of proline utilization in *Salmonella typhimurium*: characterization of *put::Mud(Ap, lac)* operon fusions. *Journal of Bacteriology* **154**, 561–568.
- MAY, G., FAATZ, E., VILLAREJO, M. & BREMER, E. (1986). Binding-protein-dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K-12. *Molecular and General Genetics* **205**, 225–233.
- MEASURES, J. C. (1975). The role of amino acids in osmoregulation of non-halophilic bacteria. *Nature, London* **257**, 398–400.
- MENZEL, R. & ROTH, J. R. (1980). Identification and mapping of a second proline permease in *Salmonella typhimurium*. *Journal of Bacteriology* **141**, 1064–1070.
- MENZEL, R. & ROTH, J. R. (1981). Regulation of the genes for proline utilization in *Salmonella typhimurium*: autogenous repression by the *putA* gene product. *Journal of Molecular Biology* **148**, 21–44.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- NEIDHARDT, F. C., BLOCH, P. L. & SMITH, D. F. (1974). Culture medium for enterobacteria. *Journal of Bacteriology* **119**, 736–747.
- PERROUD, B. & LERUDULIER, D. (1985). Glycine betaine transport in *Escherichia coli*: osmotic modulation. *Journal of Bacteriology* **161**, 393–401.
- PETERSON, G. L. (1977). A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Analytical Biochemistry* **83**, 346–356.
- POLLARD, A. & WYN JONES, R. G. (1979). Enzyme activities in concentrated solutions of glycine betaine and other solutes. *Planta* **144**, 291–298.
- RANCOURT, D. E., STEPHENSON, J. T., VICKELL, G. A. & WOOD, J. M. (1984). Proline excretion by *Escherichia coli* K12. *Biotechnology and Bioengineering* **26**, 74–80.
- SOMERO, G. N. (1986). Protons, osmolytes and fitness of internal milieu for protein function. *American Journal of Physiology* **251**, R197–R213.
- STALMACH, M. E., GROTHE, S. & WOOD, J. M. (1983). Two proline porters in *Escherichia coli* K12. *Journal of Bacteriology* **156**, 481–486.
- STYRVOLD, O. B., FALKENBERG, P., LANDFALD, B., ESHOO, M. W., BJORNSEN, T. & STROM, A. R. (1986). Selection, mapping, and characterization of osmoregulatory mutants of *Escherichia coli* blocked in the choline-glycine betaine pathway. *Journal of Bacteriology* **165**, 856–863.
- SUTHERLAND, L., CAIRNEY, J., ELMORE, M. J., BOOTH, I. R. & HIGGINS, C. F. (1986). Osmotic regulation of transcription: induction of the *proU* betaine transport gene is dependent on accumulation of intracellular potassium. *Journal of Bacteriology* **168**, 805–814.
- WOOD, J. M. (1981). Genetics of L-proline utilization in *Escherichia coli*. *Journal of Bacteriology* **146**, 895–901.
- WOOD, J. M. & ZADWORN, D. (1979). Characterization of an inducible porter required for L-proline catabolism by *Escherichia coli* K12. *Canadian Journal of Biochemistry* **57**, 1191–1199.
- WOOD, J. M. & ZADWORN, D. (1980). Amplification of the *put* genes and identification of the *put* gene products in *Escherichia coli* K12. *Canadian Journal of Biochemistry* **58**, 787–796.
- WYN JONES, R. G. & STOREY, R. (1981). Betaines. In *The Physiology and Biochemistry of Drought Resistance in Plants*, pp. 171–204. Edited by L. G. Paleg & D. Aspinall. Sydney, Australia: Academic Press.
- YANCEY, P. H., CLARK, M. E., HAND, S. C., BOWLUS, R. D. & SOMERO, G. N. (1982). Living with water stress: evolution of osmolyte systems. *Science* **217**, 1214–1222.