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SHORT COMMUNICATION

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# The osmoprotectant proline betaine is a major substrate for the binding-protein-dependent transport system ProU of *Escherichia coli* K-12

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**Abstract** The ProP and ProU transport systems of *Escherichia coli* mediate the uptake of several osmoprotectants including glycine betaine. Here we report that both ProP and ProU are involved in the transport of the potent osmoprotectant proline betaine. A set of isogenic *E. coli* strains carrying deletions in either the *proP* or *proU* loci was constructed. The growth properties of these mutants in high osmolarity minimal media containing 1 mM proline betaine demonstrated that the osmoprotective effect of this compound was dependent on either an intact ProP or ProU uptake system. Proline betaine competes with glycine betaine for binding to the *proU*-encoded periplasmic substrate binding protein (ProX) and we estimate a $K_D$ of 5.2 μM for proline betaine binding. This value is similar to the binding constant of the ProX protein determined previously for the binding of glycine betaine ($K_D$ of 1.4 μM). Our results thus demonstrate that the binding-protein-dependent ProU transport system of *E. coli* mediates the efficient uptake of the osmoprotectants glycine betaine and proline betaine.

**Key words** Adaptation to high osmolarity
Osmoprotectants · Substrate binding protein · ProP
ProU

## Introduction

An integral part of the adaptation reaction of *Escherichia coli* to a high osmolarity habitat is the osmotically modulated uptake of osmoprotective compounds from the environment (for overviews see Csonka and Hanson 1991; Lucht and Bremer 1994). Two transport systems, ProP and ProU, play a central role in scavenging organic osmoprotectants from the extracellular milieu. The *proP*-encoded transporter consists of a single polypeptide embedded in the cytoplasmic membrane (Culham et al. 1993), whereas the *proU* operon (*proVWX*) encodes a multicomponent, binding-protein-dependent transport system (May et al. 1986; Gowrishankar 1989). Both the ProP permease and the ProU system were originally identified through the uptake of the osmoprotective solute proline under high osmolarity growth conditions (Csonka 1983). Subsequent studies showed that both ProP and ProU were also responsible for the osmotically controlled uptake of the potent osmoprotectant glycine betaine (*N*, *N*, *N*-trimethyl glycine; Perroud and Le Rudulier 1985; May et al. 1986; Cairney et al. 1985a, b). In addition, both ProP and ProU function in the accumulation of the osmoprotectants taurine and ectoine (McLaggan and Epstein 1991; Jebbar et al. 1992) and ProU also serves as a low-affinity uptake system for choline, the precursor for glycine betaine synthesis (Lamark et al. 1992). Thus, ProP and ProU exhibit a broad substrate specificity for various osmoprotectants and serve a key function in the cellular reaction against osmotic stress (Lucht and Bremer 1994). Despite the involvement of ProU in the uptake of glycine betaine, proline, and ectoine under high osmolarity growth conditions, only glycine betaine is efficiently recognized by the *proU*-encoded periplasmic glycine betaine-binding protein (ProX; $K_D$=1.4 μM) (May et al. 1986; Barron et al. 1987; Jebbar et al. 1992).

Proline betaine (*N*, *N*-dimethyl-L-proline) can serve as an osmoprotectant in *E. coli* with approximately the same effectiveness as glycine betaine and both substances are accumulated to the same intracellular steady-state level in osmotically stressed cells (Chambers and Kunin 1987; Chambers et al. 1987; Larsen et al. 1987). However, the systems mediating proline betaine transport are unknown. We have therefore investigated the route of proline betaine uptake in *E. coli* K-12.

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## Construction of a set of strains carrying deletions in the *proP* and *proU* loci

Larsen et al. (1987) have speculated that the high-level intracellular accumulation of proline betaine in an *E. coli* K-10 strain under high osmolarity growth conditions is mediated by the ProP and ProU transport systems. To assess the involvement of these transporters in proline betaine uptake, we constructed an isogenic set of *E. coli* K-12 strains with *proP* and *proU* deletions. For the construction of a defined *proU* deletion, we removed a *Hpa*I restriction fragment from the pBR322-derived *proU*$^+$ plasmid pOS25 [Ap$^r$] (Faatz et al. 1988; May et al. 1989) and replaced it with a DNA segment that encodes a spectinomycin resistance gene (Spc$^r$) from plasmid pHP45Ω (Prentki and Krisch 1984). The resulting deletion [Δ(*proU*)*608*] disrupts the entire *proU* operon and was integrated via homologous recombination into the chromosome (Silhavy et al. 1984). Loss of the *proU* material was verified by Southern hybridization experiments using DNA probes derived from plasmid pOS25. To isolate a *proP* deletion, we used a strain carrying a Tn*10* (Tet$^r$) insertion genetically tightly linked to the Φ(*proP-lacZ*)1 λp*lac*Mu55 [Kan$^r$] operon fusion. Tet$^s$ derivatives were selected (Maloy and Nunn 1981), and among these strains, mutants (LacZ$^-$ Kan$^s$) were found that had lost the Φ(*proP-lacZ*)1 fusion and the adjacent λp*lac*Mu55 prophage. The simultaneous appearance of the Tet$^s$ LacZ$^-$ Kan$^s$ phenotypes is readily understood as a Tn*10*-mediated deletion event. The *proP* mutation in one of the recovered mutant strains was designated Δ(*proP*)*2*. Starting with the Δ(*proU*)*608* [Spc$^r$] and the Δ(*proP*)*2* mutations, we then used phage P1*vir* and standard genetic procedures to construct a set of strains that differed only at the *proP* or *proU* loci: BK32 (Δ(*proP*)*2* *proU*$^+$), MKH17 (*proP*$^+$ Δ(*proU*)*608* [Spc$^r$]), and strain MKH13 (Δ(*proP*)*2* Δ(*proU*)*608* [Spc$^r$]). Each of these strains is a derivative of strain MC4100 (Casadaban 1976) and also carries the Δ(*putPA*)101 deletion to permit the rapid testing of the *proP* and *proU* mutant strains using the toxic proline analogues L-azetidine-2-carboxylic acid (AC) and 3,4-dehydro-DL-proline (DHP) (May et al. 1986).

## Uptake of proline betaine is mediated by the ProP and ProU transport systems

To investigate the osmoprotective properties of proline betaine and its route of uptake in *E. coli* K-12, we grew strains BK32 (ProP$^-$ ProU$^+$), MKH17 (ProP$^+$ ProU$^-$) and MKH13 (ProP$^-$ ProU$^-$) in minimal medium A (MMA) (May et al. 1986) with 0.2% glucose as the carbon source in the absence or presence of 0.8 M NaCl. There was practically no growth of any of these strains at high osmolarity in the absence of osmoprotectants (Fig. 1). The addition of either 1 mM proline betaine or glycine betaine to the high osmolarity growth medium rescued the growth of strains BK32 and MKH17 but not of strain MKH13 (Fig. 1). Thus, proline betaine uptake in osmotically stressed cells is mediated by both the ProP and ProU transporter systems. In agreement with previously reported data (Chambers et al. 1987; Larsen et al. 1987), glycine betaine and proline betaine exhibited similar osmoprotective activities and there was no significant difference between the growth patterns of strains MKH17 (ProP$^+$) and BK32 (ProU$^+$) (Fig. 1).

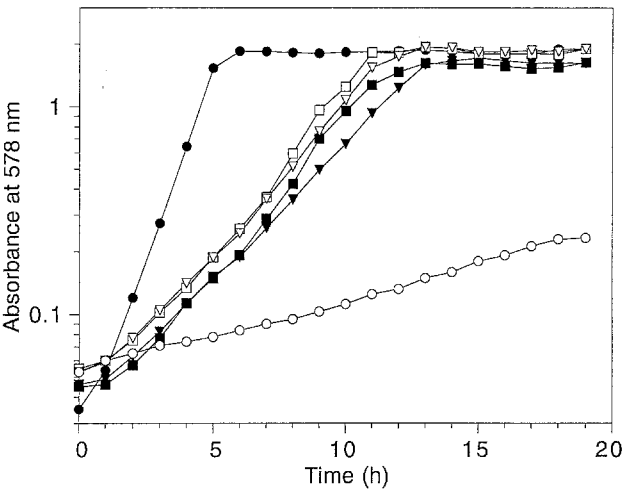


**Fig. 1** Influence of glycine betaine and proline betaine on the growth of *E. coli* in high osmolarity media. Strains BK32 (ProP$^-$ ProU$^+$), MKH17 (ProP$^+$ ProU$^-$) and MKH13 (ProP$^-$ ProU$^-$) were pregrown at 37°C overnight in glucose (0.2%) MMA (May et al. 1986) or glucose MMA with 0.3 M NaCl to preadapt the cells for growth at high osmolarity. The cells were then diluted 1:50 in 100 ml glucose MMA containing either no NaCl or 0.8 M NaCl and incubated in 500 ml flasks in a rotating water bath (220 rpm) at 37°C. Strain BK32 was grown in MMA (●), or high osmolarity MMA (○) or in high osmolarity MMA in the presence of glycine betaine (■) or proline betaine (▼). Growth curves for strain MKH17 in high osmolarity MMA in the presence of glycine betaine (□) and proline betaine (▽) are also shown. The growth kinetics of strain MKH13 in high osmolarity MMA in the presence of glycine betaine or proline betaine are identical to those of strain BK32 (○) grown in high osmolarity medium in the absence of osmoprotectants

## Purification of the *proU*-encoded glycine betaine-binding protein ProX

To study a possible interaction of proline betaine with the *proU*-encoded periplasmic glycine betaine-binding protein (ProX), we purified this substrate-binding protein and used it in competition experiments involving unlabelled proline betaine and radiolabelled [*methyl*-$^{14}$C] glycine betaine (7.1 mCi/mmol; Amersham Buchler, Braunschweig). Strain EF047(pOS40) carries a plasmid in which the *proX* structural gene is constitutively expressed from a *lacZ* promoter (May et al. 1989), resulting in high-level synthesis of the ProX protein. Periplasmic proteins were released by cold osmotic shock from cells of strain EF047(pOS40) grown in MMA, and initial purification of ProX by chromatography on a DEAE-

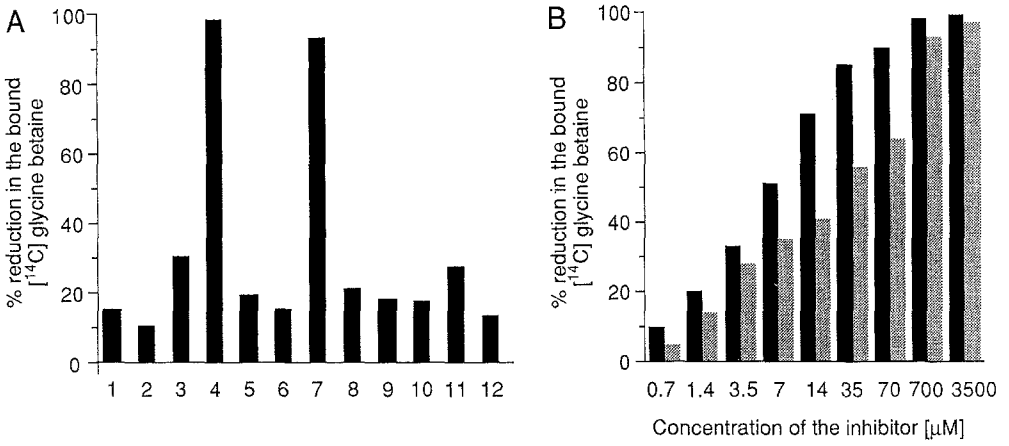


**Fig. 2A,B** The osmoprotectant proline betaine competes with glycine betaine for binding to the purified glycine betaine-binding protein. Competition between [*methyl*-$^{14}$C] glycine betaine and unlabelled compounds for binding by the purified ProX protein was assayed by the ammonium sulphate precipitation method (Richarme and Kepes 1983). **A** The concentration of [*methyl*-$^{14}$C] glycine betaine in each assay was 7 μM and the unlabelled competitors were present at a final substrate concentration of 700 μM; ProX was present at a concentration of 6 μM. The following compounds were used as potential competitors: (1) glycine, (2) N-methyl-glycine, (3) *N*, *N*-dimethyl-glycine, (4) *N*, *N*, *N*-trimethyl-glycine (glycine betaine), (5) L-proline, (6) *N*-methyl-proline, (7) *N*, *N*-dimethyl-proline (proline betaine), (8) γ-butyrobetaine, (9) crotonobetaine, (10) L-carnitine, (11) 3-dimethylsulphopropionate, (12) taurine. The solutions of the various competitors were adjusted to pH 7.3 prior to use in the competition assay. All compounds were purchased from Sigma Chemie (Deisenhofen, FRG), except for 3-dimethylsulphopropionate, which was obtained from Research Plus (USA). Proline betaine and crotonobetaine were kind gifts of A. Strøm and J. Brass, respectively. **B** Competition between [*methyl*-$^{14}$C] glycine betaine and either unlabelled glycine betaine (*solid bars*) or proline betaine (*stippled bars*) for binding by the purified ProX protein was assayed. The concentration of [*methyl*-$^{14}$C] glycine betaine in each assay was 7 μM and ProX was present at a concentration of 6 μM. The concentration of unlabelled glycine betaine or proline betaine in the binding assay was varied as indicated

Sephacel column (Pharmacia) followed the procedure described by Barron et al. (1987). The ProX protein was then further purified by FPLC chromatography. ProX-containing fractions from the DEAE-Sephacel column were concentrated with Aquacide (Calbio-Chem), dialyzed against a large volume of 16 mM TRIS-HCl (pH 8.3), and applied to a Mono-Q (Pharmacia) ion exchange column. The proteins were eluted from the column with an NaCl gradient (0–250 mM NaCl in 16 mM TRIS-HCl [pH 8.3]), and those fractions that contained pure ProX protein were identified by SDS-polyacrylamide gel electrophoresis. The purified ProX protein was extensively dialyzed against 10 mM TRIS-HCl (pH 7.3) and subsequently stored at –20°C.

## The ProX protein recognizes proline betaine with high affinity

The specificity of binding of ProX to various compounds was studied using the ammonium sulphate precipitation assay described by Richarme and Kepes (1983). Purified ProX protein (6 μM) was mixed with 7 μM [*methyl*-$^{14}$C] glycine betaine and a 100-fold excess of various unlabelled potential competitors in a final reaction volume of 100 μl. The binding reaction was allowed to proceed for 5 min at room temperature, and the ProX protein was then precipitated with a solution of ice-cold saturated ammonium sulphate. After 10 min in the cold the precipitated proteins were collected by filtration onto nitrocellulose filters (pore size 0.45 μm; Schleicher and Schuell) and the radioactivity retained on the filter was determined by scintillation counting. The binding of the [*methyl*-$^{14}$C] glycine betaine to the ProX protein was effectively reduced when either unlabelled glycine betaine or proline betaine was used as competitor (Fig. 2A). Thus, in addition to glycine betaine (May et al. 1986; Barron et al. 1987), proline betaine is also recognized as a substrate by ProX. In contrast, glycine, *N*-methyl-glycine, *N*, *N*-dimethyl-glycine, L-proline, *N*-methyl-proline, γ-butyrobetaine, crotonobetaine, L-carnitine, 3-dimethylsulphoniopropionate, and taurine reduced the binding of the radiolabelled glycine betaine by the ProX protein only slightly when present in 100-fold (Fig. 2A) or 500-fold (data not shown) excess. Thus, ProX does not exhibit significant affinity for any of these substances.

To estimate the binding constant of ProX for the binding of proline betaine, we measured the competition between unlabelled proline betaine and radiolabelled [*methyl*-$^{14}$C] glycine betaine (7 μM) over a wide range of inhibitor concentrations at a constant concentration of the ProX protein (6 μM) using the ammonium sulphate precipitation binding assay (Richarme and Kepes 1983). Even at low substrate concentration, both unlabelled glycine betaine and proline betaine competed efficiently with the radiolabelled [*methyl*-$^{14}$C] glycine betaine for binding to ProX (Fig. 2B). It is apparent from this competition assay that proline betaine is somewhat less efficiently bound by the ProX protein than is glycine betaine. As expected, 7 μM of unlabelled glycine betaine was

required to displace 50% of [*methyl*-$^{14}$C] glycine betaine from the binding protein (Fig. 2B). For proline betaine to reduce [*methyl*-$^{14}$C] glycine betaine binding by 50%, the relative proportion of proline betaine in the competition assay had to be 72% (this corresponds to 18 μM of proline betaine). Using the previously determined $K_D$ of 1.4 μM for the binding of glycine betaine to ProX (May et al. 1986) and assuming a single substrate-binding site, one can calculate a $K_D$ of 5.2 μM for the binding of proline betaine to the ProX protein.

## Conclusions

In contrast to a number of osmoprotectants that are also accumulated via the ProU transport system (e.g., proline, ectoine, taurine) (May et al. 1986; Barron et al. 1987; McLaggan and Epstein 1991; Jebbar et al. 1992), proline betaine is an excellent substrate for the *proU*-encoded periplasmic glycine betaine-binding protein ProX. Periplasmic glycine betaine-binding proteins have also been detected in the soil microorganisms *Rhizobium meliloti* and *Azospirillum brasiliense*. These proteins bind glycine betaine with affinities ($K_D$ between 2.5 and 3 μM) similar to that of the *E. coli* ProX protein, but they do not recognize proline betaine as their substrate (Le Rudulier et al. 1991; Riou et al. 1991). Our data clearly demonstrate that the *E. coli* ProX protein exhibits a dual substrate specificity and thus establish ProU as an efficient, binding-protein-dependent transport system for both glycine betaine and proline betaine.

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## References

Barron A, Jung JU, Villarejo M (1987) Purification and characterization of a glycine betaine binding protein from *Escherichia coli*. J Biol Chem 262: 11841–11846

Cairney J, Booth IR, Higgins CF (1985a) *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. J Bacteriol 164: 1218–1223

Cairney J, Booth IR, Higgins CF (1985b) Osmoregulation of gene expression in *Salmonella typhimurium*: *proU* encodes an osmotically induced betaine transport system. J Bacteriol 164: 1224–1232

Casadaban MJ (1976) Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J Mol Biol 104: 541–555

Chambers ST, Kunin CM (1987) Osmoprotective activity for *Escherichia coli* in mammalian inner medulla and urine. Correlation of glycine and proline betaines and sorbitol with response to osmotic loads. J Clin Invest 80: 1255–1260

Chambers ST, Kunin CM, Miller D, Hamada A (1987) Dimethylthethin can substitute for glycine betaine as an osmoprotectant molecule for *Escherichia coli*. J Bacteriol 169: 4845–4847

Csonka LN (1983) A third L-proline permease in *Salmonella typhimurium* which functions in media of elevated osmotic strength. J Bacteriol 151: 1433–1443

Csonka LN, Hanson AD (1991) Prokaryotic osmoregulation: genetics and physiology. Annu Rev Microbiol 45: 569–606

Culham DE, Lasby B, Marangoni AG, Milner JL, Steer BA, van Nues RW, Wood JM (1993) Isolation and sequencing of *Escherichia coli proP* reveals unusual structural features of the osmoregulatory proline/betaine transporter, ProP. J Mol Biol 229: 268–276

Faatz E, Middendorf A, Bremer E (1988) Cloned structural genes for the osmotically regulated binding protein-dependent glycine betaine transport system (ProU) of *Escherichia coli* K-12. Mol Microbiol 2: 265–279

Gowrishankar J (1989) Nucleotide sequence of the osmoregulatory *proU* operon of *Escherichia coli*. J Bacteriol 171: 1923–1931

Jebbar M, Talibart R, Gloux K, Bernard T, Blanco C (1992) Osmoprotection of *Escherichia coli* by ectoine: uptake and accumulation characteristics. J Bacteriol 174: 5027–5035

Lamark T, Stryrvold OB, Strøm AR (1992) Efflux of choline and glycine betaine from osmoregulating cells of *Escherichia coli*. FEMS Microbiol Lett 96: 149–154

Larsen PI, Sydnes LK, Landfald B, Strøm AR (1987) Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. Arch Microbiol 147: 1–7

Le Rudulier D, Gloux K, Riou N (1991) Identification of an osmotically induced periplasmic glycine betaine-binding protein from *Rhizobium meliloti*. Biochim Biophys Acta 1061: 197–205

Lucht JM, Bremer E (1994) Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system ProU. FEMS Microbiol Rev 14: 3–20

May G, Faatz E, Villarejo M, Bremer E (1986) Binding protein dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K12. Mol Gen Genet 205: 225–233

May G, Faatz E, Lucht JM, Haardt M, Bolliger M, Bremer E (1989) Characterization of the osmoregulated *Escherichia coli proU* promoter and identification of ProV as a membrane-associated protein. Mol Microbiol 3: 1521–1531

Maloy RM, Nunn WD (1981) Selection for loss of tetracycline resistance by *Escherichia coli*. J Bacteriol 145: 1110–1112

McLaggan D, Epstein W (1991) *Escherichia coli* accumulates the eukaryotic osmolyte taurine at high osmolarity. FEMS Microbiol Lett 81: 209–214

Perroud B, Le Rudulier D (1985) Glycine betaine transport in *Escherichia coli*: osmotic modulation. J Bacteriol 161: 393–401

Prentki P, Krisch HM (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. Gene 29: 303–313

Richarme G, Kepes A (1983) Study of binding protein-ligand interaction by ammonium sulfate-assisted adsorption on cellulose ester filters. Biochim Biophys Acta 742: 16–24

Riou N, Poggi, MC, Le Rudulier D (1991) Chracterization of an osmoregulated periplasmic glycine betaine-binding protein in *Azospirillum brasilense* sp7. Biochimie 73: 1187–1193

Silhavy TJ, Berman ML, Enquist LW (1984) Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York