The Osmotically Regulated *proU* Locus of *Salmonella typhimurium* Encodes a Periplasmic Betaine-binding Protein

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The *proU* locus of *Salmonella typhimurium* encodes an osmotically induced betaine transport system. We have identified a 31 kDa periplasmic protein, encoded by *proU*, whose synthesis is induced by osmotic stress. A specific betaine-binding activity with a K_D of about $1\ \mu\text{M}$ is also present in the periplasm of osmotically induced cells. This activity is absent in those *proU* mutants which lack the 31 kDa periplasmic protein. Thus, ProU is a periplasmic binding-protein-dependent transport system.

INTRODUCTION

Betaine plays an important role as an osmoprotectant in many plant, animal and bacterial species (Le Rudulier & Valentine, 1982; Le Rudulier *et al.*, 1984; Yancey *et al.*, 1982). In *Escherichia coli* and *Salmonella typhimurium*, betaine stimulates growth at high osmolarity, serving to restore turgor and to protect intracellular enzymes against the adverse effects of high cytoplasmic ionic strength (Cairney *et al.*, 1985*a, b*; Perroud & Le Rudulier, 1985). Two genetically distinct transport systems serve to mediate betaine uptake. The *proP* gene encodes a low-affinity betaine transport system which also has some affinity for proline (Cairney *et al.*, 1985*a*). The *proU* locus encodes a high-affinity betaine transport system with a K_t of $1.3\ \mu\text{M}$ (Cairney *et al.*, 1985*b*). Betaine uptake via the ProU transport system is regulated at two levels. Expression of the *proU* locus is induced by osmotic stress (Cairney *et al.*, 1985*b*; Dunlap & Csonka, 1985; Gowrishankar, 1985) in direct response to osmotically induced increases in intracellular K^+ concentrations (Sutherland *et al.*, 1986). Once expression of *proU* is fully induced, the activity of the ProU transport system is also regulated in response to osmotic stress (Cairney *et al.*, 1985*b*). Because of its importance for growth at high osmolarity, and because of these unusual regulatory properties, we have set out to characterize the ProU transport system.

Two principal classes of bacterial transport system can be distinguished by their organization and the mechanism of energy-coupling to transport (Berger & Heppel, 1974). Membrane-bound transport systems, typified by the lactose permease, are unicomponent and are energized by ion gradients. In contrast, binding-protein-dependent systems are multicomponent and are believed to be energized by the direct hydrolysis of ATP or a related nucleotide (Ames & Higgins, 1983; Higgins *et al.*, 1985; Ames, 1986). Binding-protein-dependent transport systems require the function of a specific substrate-binding protein located in the periplasm. These proteins exhibit high affinity for their specific substances which is reflected in the low K_t for transport when assayed in whole cells. In this paper we show that ProU is a binding-protein-dependent transport system and that the *proU* locus encodes a 31 kDa periplasmic betaine-binding protein.

METHODS

Bacteria. The two *S. typhimurium* strains used in this study were the wild-type LT2 (A) and its derivative CH946 [*proU*1702::Mu d1-8(*Ap^r*, *lac*)]. The construction of CH946 was described by Cairney *et al.* (1985*b*).

Pulse-labelling proteins. Cells were grown aerobically at 30 °C in M63 medium (Miller, 1972) containing 0.4% glucose as carbon source and each of the protein amino acids (except methionine) at 40 µg ml⁻¹. When appropriate, cells in mid-exponential growth were osmotically shocked by adding NaCl to a final concentration of 0.3 M and grown for a further 45 min before labelling. Samples (1 ml) of cells were pulsed for 1 min with [³⁵S]methionine [1.3 Ci mol⁻¹ (48.1 GBq mol⁻¹); 2 µCi (74 kBq); Amersham] followed by a 1 min 'chase' with 100 µg unlabelled methionine ml⁻¹. Total protein extracts of the labelled cells were prepared by rapidly sedimenting the cells by centrifugation, resuspending them in 200 µl of Laemmli sample buffer (Laemmli, 1970) and boiling for 5 min. Periplasmic fractions were isolated from the labelled cells by cold osmotic shock as described previously (Higgins & Hardie, 1983). Proteins were separated by SDS-PAGE (Laemmli, 1970; Ames, 1974) and detected by autoradiography after drying the gel.

Betaine-binding measurements. Large-scale preparations of periplasmic proteins for binding assays were obtained by cold osmotic shock from 1 litre of cells grown to saturation in LB medium (Hiles & Higgins, 1986). These preparations were dialysed extensively against 10 mM-Tris/HCl (pH 7.2), freeze dried and resuspended in Tris/HCl (pH 7.2) to a final protein concentration of 10–15 mg ml⁻¹. Betaine-binding assays were done by the filter-binding procedure (Dahl & Manson, 1985). Samples (20 µl, 200–300 µg protein) of periplasmic shock fluid were incubated for 2 min with the appropriate amount of [¹⁴C]betaine [0.1 µCi (3.7 kBq); 7.4 Ci mol⁻¹ (273.8 GBq); Amersham] after which time 500 µl of ice-cold saturated ammonium sulphate was added and the mix immediately passed through a 0.45 µm nitrocellulose filter (Schleicher and Schuell). The filters were washed three times with 3 ml of ice-cold saturated ammonium sulphate and the radioactivity retained by the wet filters measured by scintillation counting. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Identification of an osmotically induced periplasmic protein

The *proU* locus encodes a high-affinity betaine transport system which plays an important role in osmoregulation (Cairney *et al.*, 1985*b*). Expression of *proU* is osmotically induced. However, both genetic (Cairney *et al.*, 1985*b*) and biochemical (Clark & Parker, 1984) data indicate that very few other genes are similarly induced. In order to identify any protein(s) encoded by *proU*, cells of LT2 (*proU*⁺) and CH946 (*proU*) were pulse-labelled with [³⁵S]methionine either before or after osmotic shock. Fig. 1 shows a 12% (w/v) SDS-polyacrylamide gel of the labelled proteins in whole cell extracts. It is clear that the expression of very few proteins is altered significantly by osmotic shock. Only two proteins, labelled A and B in Fig. 1, were reproducibly found to be regulated by osmolarity. Neither of these proteins was absent from CH946 (Fig. 1) or from a variety of other independently isolated *proU* mutants (data not shown). Protein A has a molecular mass of 36 kDa and is only expressed in cells grown in media of low osmolarity. The use of appropriate mutants showed that this protein is not the osmotically regulated OmpF porin (Hall & Silhavy, 1981) (data not shown). Protein A is not periplasmic (see below) and its function remains to be determined. Protein B has a molecular mass of 21 kDa and its synthesis is induced by osmotic shock. This protein has the same molecular mass as the KdpC subunit of the Kdp potassium transport system which is known to be osmotically induced (Laimins *et al.*, 1981; Hesse *et al.*, 1984). However, as *kdp* mutants have not been isolated in *S. typhimurium*, it is not yet possible to determine rigorously whether or not protein B is a component of Kdp.

Because any periplasmic protein encoded by *proU* might not be detectable amongst total cellular proteins, periplasmic fractions from pulse-labelled cells were prepared and separated by SDS-PAGE (Fig. 2). An osmotically induced protein of 31 kDa, which is masked by other proteins in total cell extracts, can be identified in these periplasmic fractions. This protein is absent from the periplasm of CH946 (Fig. 2) and from a number of other independently derived *proU* mutants (data not shown). Thus, *proU* directs the synthesis of a 31 kDa periplasmic

Fig. 1

Fig. 1. Osmotically induced proteins. Cellular proteins were pulse-labelled with [35 S]methionine, either before (–) or after (+) osmotic shock, and separated on a 12% (w/v) SDS-polyacrylamide gel. Strain LT2 is wild-type; CH946 is a *proU* derivative. Proteins A and B are discussed in the text. The mobility of molecular mass markers is indicated.

Fig. 2

Fig. 2. Osmotically induced periplasmic proteins. Periplasmic proteins were isolated from cells pulse-labelled with [35 S]methionine before (–) or after (+) osmotic shock and separated on an 8% (w/v) SDS-polyacrylamide gel. Strain LT2 is the wild-type; CH946 is a *proU* derivative. The betaine-binding protein (BBP) is indicated, as is the mobility of molecular mass markers.

protein. Although it still remains a formal possibility that the *proU* locus encodes a regulatory component, rather than the 31 kDa protein itself, this seems highly unlikely in view of the fact that transcription of *proU* is so tightly regulated (regulatory genes are not normally subject to such stringent regulation) and because of the finding that all mutants defective in the ProU transport system map to this single locus (Cairney *et al.*, 1985*b*; Sutherland *et al.*, 1986).

Table 1. *Betaine-binding activities of periplasmic fractions*

Cells were grown in LB (low osmolarity) or LB containing 0.3 M-NaCl (high osmolarity). Binding assays were done in 10 mM-Tris/HCl (pH 7.2) with 0.3 M-NaCl or 0.44 M-sucrose added as indicated. Each measurement is the mean of three separate determinations carried out on independent preparations of periplasmic proteins. The range of values obtained was less than 10% of the average. Binding was assayed at saturating betaine concentrations (20 μ M).

Strain	Osmolarity of growth medium	Addition to assay	Betaine bound [pmol (mg protein) ⁻¹]
LT2	Low	—	39
LT2	Low	NaCl (0.3 M)	28
LT2	High	—	486
LT2	High	NaCl (0.3 M)	511
LT2	High	Sucrose (0.44 M)	451
CH946	Low	—	11
CH946	High	—	32
CH946	High	NaCl (0.3 M)	21

Betaine-binding activity

The periplasmic components of transport systems function as the initial, high-affinity substrate-binding receptor (Ames & Higgins, 1983). Because *proU* mutants were both defective in transport and in the synthesis of a 31 kDa periplasmic protein, it seemed likely that the 31 kDa protein would be found to be a component of the transport system. To investigate this possibility, betaine-binding activity was assayed in periplasmic fractions isolated from LT2 or CH946 which had been grown at either low or at high osmolarity. Table 1 shows that periplasmic betaine-binding activity is absent from cells grown at low osmolarity but is present when wild-type cells are grown at high osmolarity. This is entirely consistent with the observation that *proU* expression and the ProU betaine transport system are only induced during growth at high osmolarity. However, betaine-binding activity was absent from the periplasmic fractions of CH946, even when grown at high osmolarity. This clearly demonstrates that ProU is a periplasmic binding-protein-dependent transport system and, in addition, strongly implies that the 31 kDa periplasmic protein is the betaine-binding component.

Further support for this view comes from measurement of the substrate-binding affinity of this protein for betaine. Betaine-binding activity at different substrate concentrations was assayed in periplasmic fractions isolated from LT2 grown at high osmolarity (Fig. 3). Although binding could not be accurately measured at betaine concentrations below 0.5 μ M, due to the low specific activity of the substrate, the K_D for betaine binding was found to be approximately 1.0 μ M. This is in very good agreement with the K_t for ProU-dependent betaine transport which has previously been measured as 1.3 μ M (Cairney *et al.*, 1985*b*).

Assuming that one molecule of protein binds one molecule of betaine, as is the case for other periplasmic binding proteins, it can be calculated from the data in Table 1 that the betaine-binding protein comprises 1.3% of the total periplasmic protein. While this figure might seem rather low when compared with the amount of the 31 kDa protein seen in pulse-labelling experiments (Fig. 2), it must be remembered that binding activity was measured in cells grown to steady-state in high osmolarity medium, rather than immediately after osmotic shock, which is when pulse-labelling was done and when *proU* expression is greatest relative to that of other proteins.

The demonstration that ProU is a binding-protein-dependent transport system has a number of important implications for the mechanisms, regulation and physiological function of this transport system. The finding is compatible with the high affinity of this transport system for its substrate (K_D 1.3 μ M) and with its ability to accumulate betaine against large concentration gradients (Cairney *et al.*, 1985*b*). These are features generally associated with binding-protein-dependent transport systems (Ames & Higgins, 1983). All other binding-protein-dependent transport systems have, in addition to the periplasmic substrate-binding component, several

Fig. 3. Betaine-binding activity in the periplasm. Betaine-binding by periplasmic fractions isolated from LT2 grown at high osmolarity (LB + 0.3 M-NaCl) was assayed at various betaine concentrations. Each point is a mean of five independent determinations. The data are expressed as the amount of betaine bound per assay mix (260 μ g protein).

(normally three) membrane-associated components (Ames & Higgins, 1983; Ames, 1986). It therefore seems likely that the *proU* locus is an operon consisting of several genes. If, as for other transport systems, the membrane components of ProU are very much less abundant than the periplasmic components, this would explain why they have not been detected as osmotically induced proteins.

In addition to transcriptional regulation of *proU* expression, we have previously shown that activity of the ProU transport system is modulated by the osmolarity of the medium. Even when the *proU* locus is fully induced, ProU-dependent betaine transport can only be detected when assayed in medium of high osmolarity. A possible role for the betaine-binding protein in this regulation was investigated by raising the osmolarity of the binding assay mix by the addition of 0.3 M-NaCl or 0.44 M-sucrose. Betaine-binding was found to be identical whether or not the assay was done at high osmolarity (Table 1). It is also interesting to note that the function of this binding protein is not inhibited by high ionic strength. These data show that the osmotic regulation of ProU activity is not simply a consequence of altered substrate binding by the periplasmic betaine-binding protein. Rather, osmotic regulation of transport activity must be achieved by modulation of the function of other, presumably membrane-bound, components of the transport system.

NB. Since submitting this paper we have learned that *E. coli* has a similar betaine-binding protein (E. Bremer, personal communication).

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