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Topical Review

Proline Porters Effect the Utilization of Proline as Nutrient or Osmoprotectant for Bacteria

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Proline Transport in the Enteric Bacteria

The intestines of animal species, the habitat of *Escherichia coli* and *Salmonella typhimurium,* are environments low in oxygen which provide limited arrays of organic nutrients, frequently at low abundance. Since they are facultative aerobes the enteric bacteria serve as oxygen scavengers, helping to maintain the low oxygen concentration essential for the growth of the obligate anaerobes that predominate in that environment. Local environments may exist within the gut where oxygen concentration is relatively high or nutrient supply is transiently elevated. These organisms may gain a competitive advantage by retaining capabilities for the exploitation of such rare opportunities and for the survival of rare catastrophes. Koch (1976) suggests that the high affinity transport systems which catalyze the active accumulation of sugars and amino acids have been important determinants of their evolutionary success.

The multiple functions of the proline porters in *E. coli* and *S. typhimurium* reflect the physiological versatility of these organisms. Water-filled pores, the porins, mediate the diffusion of small molecules like proline across the outer membrane layer of the Gram-negative bacterial cell wall (Nikaido & Vaara, 1985; Milner, Vink & Wood, 1987*b;* Faatz, Middendorf & Bremer, 1988). Three proline porters aid these organisms in responding to a variety of environmental contingencies by transporting proline across the cytoplasmic membrane (Table 1). Proline porter I, which has a high affinity and rather strict specificity for proline, is dominant when proline is to be utilized as a source of carbon, nitrogen and energy during aerobic growth. Proline porters II and III, which have weaker proline affinities and broader substrate specificities, participate in bacterial stress responses. The rates of proline flux attainable via porters I and II are similar, so designation of those systems as major and minor proline permeases is appropriate only with reference to specific physiological contexts. Designation of the third system as a proline porter may be misleading (*see below*).

Interpretations of most early studies were based on the minimal but erroneous assumption that a single enzyme species mediated bacterial proline transport. The activities of the three porters can be resolved from one another experimentally through careful selection of culture conditions and of the proline concentrations used in transport assays. Recently genetic manipulation has been employed to ensure that each porter could be studied in isolation (e.g. Grothe et al., 1986; Csonka, 1982; Cairney, Higgins & Booth, 1984; Cairney, Booth & Higgins, 1985*a,b*). The activity of proline porter I clearly dominated most early measurements; the existence of proline porters II and III only occasionally complicated their interpretation.

Proline Porter I Is Induced to Promote Proline Catabolism

E. coli and *S. typhimurium* can utilize proline as sole nitrogen, carbon or energy supply by expressing genes *putP* and *putA* (between 22 and 23 units on the genetic maps of both organisms) (Maloy, 1987). The PutP protein is proline porter I, a Na^+/proline symporter with a high affinity for proline (K_M approximately 1 μM). The multifunctional PutA

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Table 1. The proline porters of *Escherichia coli* and *Salmonella typhimurium*

Proline porter	I	II	III
Genetic locus	*putP*	*proP*	*proU*
Map location	23	93	58
Substrate specificity	Proline	Proline Betaine	Betaine (Proline)
Coupling ion	Na^+	ND[a]	Unlikely
Function	Proline utilization	Stress responses	Stress response
Genetic regulation	Induced by proline	Induced by nutritional or hyperosmotic stress	Induced by hyperosmotic stress
Biochemical regulation		Activated by hyperosmotic stress	Activated by hyperosmotic stress

[a] ND = not determined.

protein has two enzymatic activities, respiratory chain-linked proline dehydrogenase (formerly called proline oxidase) and, in *S. typhimurium* at least, Δ^1-pyrroline carboxylate : NAD^+ oxidoreductase. The PutA protein also acts as a repressor mediating transcriptional regulation of both *put* genes in response to proline supply. The *S. typhimurium put* genes function in an *E. coli put* deletion strain to yield a proline utilization phenotype closely analogous to that of the wild type bacterium (T. Smith and J.M. Wood, *unpublished data*). Thus the proline utilization systems of these two organisms are highly homologous.

The cited proline utilization pathway is common to many organisms. In *Pseudomonas aeruginosa,* proline utilization is catalyzed by enzyme systems similar to those described above (Kay & Gronlund, 1969; Meile, Soldati & Leisinger, 1982). In *Saccharomyces cerevisiae* proline is oxidized by inducible, mitochondrial enzymes (Wang & Brandriss, 1987) and proline uptake is catalyzed by both a general amino acid permease and a proline-specific imino carrier (Horak, 1986; Jauniaux et al., 1987). Both are regulated in response to nitrogen supply. Although the enzymes effecting transport across the cytoplasmic, vacuolar and mitochondrial membranes have not been fully differentiated, a proline-inducible yeast mitochondrial proline uptake system comparable to proline porter I of the enteric bacteria may exist (Horak, 1986). High affinity, energy dependent proline uptake has also been demonstrated in *Agrobacterium tumefaciens* (Behki, 1968; Shonukan, 1985), *Bacillus subtilis* (Ordal et al., 1978), *Coxiella burnetii* (Hendrix & Mallavia, 1984), *Halobacterium halobium* (MacDonald, Green & Lanyi, 1977), *Methanococcus voltae* (Ekiel, Jarrell & Sprott, 1985), *Mycobacterium phlei* (Prasad, Kalra & Brodie, 1976) and *Rhodopseudomonas sphaeroides* (Kundu & Nicholas, 1986). The degree of homology between those systems and the proline porters of *E. coli* and *S. typhimurium* remains to be assessed.

Detailed genetic analyses defined the relative positions of the *put* genes and their regulatory sequences in *S. typhimurium* (Ratzkin & Roth, 1978; Menzel & Roth, 1981; Maloy & Roth, 1983; Hahn & Maloy, 1986). More limited studies on *E. coli* (Wood, 1981) were consistent with the view that *putP* and *putA* are adjacent and divergently transcribed from a central regulatory region. The nucleotide base sequences of the *put* intergenic regions in *E. coli* (Nakao, Yamato & Anraku, 1987*b*) and *S. typhimurium* (Hahn et al., 1988) have now been determined (Fig. 1). They are 419 and 423 base pairs in length, respectively, and show extensive but variable homology. The amino termini of genes *putP* and *putA* and the 116 base pairs adjacent to *putP* are exactly homologous; the homology in the regulatory region closer to *putA,* estimated over 60 base pair segments, varies between 67 and 95%.

On the basis of sequence analysis, expression of enzyme activity in vivo from plasmids containing fragments of the *put* region, gene fusion experiments and in vitro transcription, Nakao et al. (1987*b*) identified five potential *putP* promoters within 175 base pairs of the *putP* initiation codon and a single potential *putA* promoter beginning 75 base pairs upstream of the *putA* initiation codon in *E. coli.* On the other hand, the properties of *putPp, putAp* and operator mutants, expression of enzyme activity in vivo from plasmids containing fragments of the *put* region and sequence analysis led Maloy and his colleagues to suggest that transcription of *putP* and *putA* in *S. typhimurium* proceeds from overlapping, convergent promoters flanking a common *put* operator region near *putA* (Hahn & Maloy, 1986; Hahn et al., 1988). Transcription of the divergent structural genes would then occur across the common operator sequence. Surprisingly, the putative *put* operator sequence of *S. typhimurium* is not present in *E. coli;* indeed it is located in a DNA region of particularly low sequence homology. Resolution of the differences in interpretation illustrated in Fig. 1 will require further in vivo genetic analysis.

An open reading frame between *putP* and *putA* that begins 79 base pairs from the *putP* initiation codon encodes a putative 72 (*S. typhimurium*) or 111 (*E. coli*) amino acid peptide which would be translated from the same DNA strand as the PutA protein. No enzymatic activity was detected when gene *lacZ* was fused to the *E. coli* open reading frame (Nakao et al., 1987*b*).

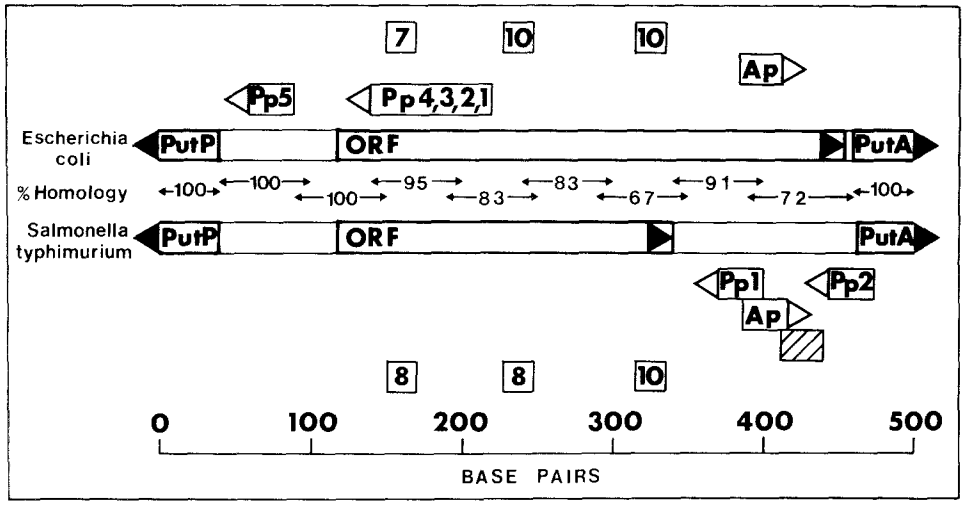

Fig. 1. The *put* intergenic region. The *put* intergenic regions of *Escherichia coli* and *Salmonella typhimurium* are compared. Sequences encoding the PutP and PutA proteins begin at opposite ends of each intergenic sequence and proceed divergently as indicated by the solid arrow heads. The apparently untranscribed open reading frame (111 amino acids in *E. coli* versus 72 amino acids in *S. typhimurium*) is indicated in a similar manner (*ORF* and solid arrow head). The percent homology between the two sequences was computed for overlapping 60 base-pair segments. The sequences encoding the amino termini of the PutP and PutA proteins were identical, as indicated. The positions of the putative promoter sequences, identified on the basis of the criteria cited in the text (Nakao et al., 1987*a* (*E. coli*); Hahn & Maloy, 1987, and Hahn et al., 1988 (*S. typhimurium*)), are labeled as *Pp* (*putP* promoter) or *Ap* (*putA* promoter), respectively. The box signifying the position of the *put* operator region proposed by Hahn et al. (1988) is hatched. The putative cAMP/catabolite activator protein binding sites are labeled with numbered boxes. The numbers indicate the degree of homology with the 14 base-pair concensus binding site observed at each position

Proline Catabolism is Genetically Regulated

Expression of the *put* genes is modulated in response to multiple signals. Proline Porter I shows substantial constitutive activity, whereas proline dehydrogenase is not detectable without induction. Early reports indicated that both *put* genes were subject to catabolite repression (Dendinger & Brill, 1970; Newell & Brill, 1972; Ratzkin & Roth, 1978; Wood & Zadworny, 1979; Wood, 1981). Recent experiments indicated quite stringent control of *putA* and little regulation of *putP* via that mechanism (Maloy & Roth, 1983; Grothe et al., 1986; Hahn & Maloy, 1986). The latter analyses may have been complicated by the influence of gene fusions and transposon insertions on the higher order structure of the *put* intergenic region, however.

The five nucleotide sequence:

$$5' - TGTGA - 3'$$

that is highly conserved within the 21 nucleotide concensus Catabolite Activator Protein (CAP) binding site (deCombrugghe, Busby & Buc, 1984; Ebright et al., 1984) occurs three times (and at identical locations) within the *put* intergenic regions from both organisms. The surrounding 21 base sequences vary in degree of homology with the concensus binding site by containing from 7 to 10 of the 14 highly conserved bases (Fig. 1). All are in the same orientation. Further studies will be required to determine whether these sites are indeed recognized by the catabolite activator protein and whether *putP* is subject to catabolite repression directly or as a secondary effect of *putA* regulation (Hahn & Maloy, 1986).

Funanage et al. (1978) sought evidence for glutamine synthetase-mediated nitrogen control of amino acid utilization systems in *S. typhimurium* by analogy with the established properties of *Klebsiella aerogenes* (Prival & Magasanik, 1971). They anticipated that elevated glutamine synthetase levels would be correlated with increased amino acid uptake activities and increased rates of growth on amino acids as nitrogen sources. Contrary to their expectation, reductions in both the rate of growth on proline as nitrogen source and the rate of proline uptake were observed. A twofold reduction in proline uptake rate was also observed if the wild type or mutant bacteria were grown on media containing only proline, rather than proline and ammonium, as nitrogen source. We observed no difference in proline uptake activity when an *E. coli* strain was cultured with proline or proline plus ammonia as nitrogen source (Wood & Zadworny, 1979). Amino acid

limited growth yielded a twofold reduction in proline porter I activity, however (Grothe et al., 1986). The earlier observations may have reflected an imbalance in amino acid pools resulting from elevated glutamine synthetase activity rather than the expected effect of nitrogen supply. Indeed Nakao et al. (1987*b*) found no evidence for a nitrogen control sequence in the *put* regulatory region of *E. coli*.

Expression of genes *putP* and *putA* is induced by proline (Dendinger & Brill, 1970; Newell & Brill, 1972; Morikawa, Suzuki & Anraku, 1974; Ratzkin, Grabnar & Roth, 1978; Wood & Zadworny, 1979; Wood, 1981). That the PutA protein of *S. typhimurium* acts as both the bifunctional proline/Δ^1-pyrroline-5-carboxylate dehydrogenase and as the repressor effecting transcriptional regulation of *putP* and *putA* was indicated by the complex array of *putA* mutant phenotypes observed by Menzel and Roth (1981) and by the gene fusion experiments of Maloy and Roth (1983). A regulatory function for the PutA protein of *E. coli* was also indicated by genetic studies (Wood, 1981). Several palindromic sequences are located between genes *putP* and *putA*. One such sequence, located 24 base pairs upstream from the *putA* initiation codon, overlaps the putative *putA* operator region identified through classical genetic analysis (Hahn & Maloy, 1986; Hahn et al., 1988).

Induction of *put* gene expression is proposed to follow a proline-dependent shift in localization of the PutA protein from the *put* operator to the cytoplasmic membrane sites at which it becomes catalytically active. Saturation of the latter sites would lead to accumulation of the soluble PutA protein and its reassociation with the *put* operator sequence (Menzel & Roth, 1981; Maloy & Roth, 1983; Maloy, 1987). Induction of multiple, plasmid-encoded *put* gene copies yields increased membrane-associated proline dehydrogenase activity in *E. coli* (Graham, Stephenson & Wood, 1984; Wood, 1987) but not *S. typhimurium* (Maloy, 1987). The increased activity observed in *E. coli* does not likely result from an excess of operator sites over repressor molecules since the plasmid copy number is expected to be approximately four and very high levels of PutA protein expression are observed (Wood & Zadworny, 1980; Wood, 1982). Thus, although the cited behavior of *S. typhimurium* is consistent with the view that saturation of cytoplasmic membrane binding sites is attained during *put* gene induction, that of *E. coli* is not.

The association between *E. coli* proline dehydrogenase (the PutA protein) and inverted cytoplasmic membrane vesicles has been reconstituted *in vitro* (Graham et al., 1984). A partially saturable response was observed when the reconstituted proline : O_2 oxidoreductase activity was measured as a function of soluble proline dehydrogenase concentration at constant membrane concentration. The levels of proline oxidative activity reconstituted were as much as 20-fold higher than that observed after induction with proline in vivo, again suggesting that saturation of membrane binding sites is not attained in vivo. Reconstitution experiments also showed that association of proline dehydrogenase with the cytoplasmic membrane was proline dependent and that the proline dependence might arise via a redox mechanism (Wood, 1987). Maloy and Roth (1983) suggested that proline might promote membrane association of the PutA protein through reduction of its endogenous flavin moiety.

The *putP* Sequence Has Yielded Structure Predictions and Facilitated PutP Protein Purification

The nucleotide sequence of the *putP* gene from *E. coli* has been determined and the PutP protein sequence deduced (Mogi et al., 1986; Nakao, Yamato & Anraku, 1987*a*). The PutP protein was predicted to consist of 502 amino acids (mol wt 54,343 daltons), 70% of which are nonpolar. Twelve membrane-spanning alpha-helices were predicted by hydropathy analysis. No highly homologous protein sequences were found when the IFIND program was used to search the Bionet data base for homology to the PutP protein in December, 1987. Similarly, little homology was detected when the same and more stringent procedures were used to compare the PutP sequence with those of bacterial melibiose (Yazyu et al., 1984), lactose (Buchel, Gronenborn & Muller-Hill, 1980), xylose (Maiden et al., 1987), arabinose (Maiden et al., 1987), maltose (Higgins et al., 1986), citrate (Maiden et al., 1987), ribose (Higgins et al., 1986), phosphate (Higgins et al., 1986) and oligopeptide (Higgins et al., 1986) transporters and with some eukaryotic transporters (Maiden et al., 1986) (A. Kurosky and R. Fritz, *personal communication*). As noted above, eukaryotic transporters homologous in function with the PutP protein might be expected to participate in mitochondrial utilization of proline and Δ^1-pyrroline-5-carboxylate. Since unexpected homologies between prokaryotic and eukaryotic transporter sequences have recently been detected (Higgins et al., 1986; Gerlach et al., 1986; Maiden et al., 1987), further homologies will be sought as new sequences become available.

Amplification of PutA protein expression was readily apparent when the *put* genes were first inserted into a multi-copy plasmid, but the PutP pro-

tein was much more difficult to detect (Wood et al., 1979; Motojima et al., 1979; Wood & Zadworny, 1980; Wood, 1982). Further amplification of gene *putP* permitted detection of the PutP protein, which migrates during gel electrophoresis as a diffuse band whose mobility depends anomalously on gel porosity. Its molecular weight was estimated as 60,000 daltons from the slope of a Ferguson plot, but its migration implied a mol wt near 35,000 daltons after SDS polyacrylamide gel electrophoresis (Hanada, Yamato & Anraku, 1985). Until the nucleic acid sequence is compared with the amino acid sequence of the mature protein, the differences between the predicted and observed protein molecular weights may be attributed to either post translational protein processing or anomalous electrophoretic behavior.

Proline transport activity was reconstituted when membrane protein extracts from *putP*$^+$, not *putP*$^-$, strains of *E. coli* were incorporated in liposomes (Amanuma et al., 1977*b*; Chen & Wilson, 1986). Hanada et al. (1987) replaced the carboxyl terminal serine residue of the PutP protein with the *lacZ* gene or with a collagen linker followed by *lacZ*. The fusion proteins retained full, membrane-associated proline transport and β-galactosidase activities and the latter activity was cryptic. Thus the carboxyl termini of the fusion proteins were cytoplasmic. The latter construction permitted purification of the PutP protein by chromatography of a dodecyl-maltoside extract on anti-β-galactosidase IgG-Sepharose, elution with collagenase and removal of the collagenase by anti-collagenase IgG Sepharose chromatography (Hanada et al., 1988). The purified protein yielded proline uptake activity kinetically similar to that of whole bacteria on reconstitution into liposomes. A high affinity, Na^+-dependent proline carrier from *Mycobacterium phlei* has also been purified and its activity reconstituted in vitro (Lee et al., 1979).

Proline Porter I is a Proline/Ion Symporter

Progress towards understanding the energetic basis for bacterial active transport accelerated when isolated cell-free membrane vesicles from *E. coli* were shown to accumulate proline and other substrates in a respiration-dependent manner (Kaback & Stadtman, 1966; Lombardi & Kaback, 1972). The proline and lactose transport systems then served as prototypes for studies of chemiosmotic coupling in bacterial active transport. In whole cells, a high affinity transport system effected proline accumulation powered by a transmembrane proton electrochemical potential gradient created through electron transport, ATP hydrolysis, or the proton-linked efflux of other solutes (Klein & Boyer, 1972; Singh & Bragg, 1976; Bentaboulet, Robin & Kepes, 1979). The membrane potential resulting from respiration or ionophore-mediated ion fluxes was also a sufficient energy supply in membrane vesicles (Kasahara & Anraku, 1974; Hirata, Altendorf & Harold, 1974; Ramos & Kaback, 1977).

The Identity of the Coupling Ion, Sodium, Proved Elusive

Although early experiments clearly implied that proline porter I catalyzed ion symport, efforts to establish the identity of the cotransported ion were complicated by the properties of proline porter I itself and by the often unrecognized coexistence of related transport activities. Our data now reveal that proline porter II, also likely an ion symport system, is activated by osmotic stress in both whole cells and membrane vesicles of *E. coli* (Milner, Grothe & Wood, 1988). Others have demonstrated that the activity of proline porter III is dependent on a periplasmic binding protein (Barron, Jung & Villarejo, 1987), implying that it does not couple proline uptake to an ion flux. Fluxes of organic solutes other than proline are linked to either proton or sodium ion movements, and additional ion fluxes are catalyzed by primary transport systems and by the Na^+/H^+ and K^+/H^+ antiporters.

Proline-linked Na^+ and Li^+ fluxes, but not H^+ fluxes, were demonstrated in *E. coli* cells retaining all three proline porters or porters I and III, but not in those lacking proline porter I (Tsuchiya et al., 1984; Chen et al., 1985). Furthermore, Na^+-linked proline uptake via proline porter I was supported by a membrane potential and/or a chemical potential gradient of Na^+ in the absence of a proton (electro)chemical potential gradient when a total membrane protein extract (Chen et al., 1985; Chen & Wilson, 1986) or the purified PutP protein (Hanada et al., 1988) was reconstituted in liposomes. These observations are consistent with repeated demonstrations that proline uptake is stimulated by Na^+ or Li^+ in *E. coli* and *S. typhimurium* (Kawasaki & Kayama, 1973; Kayama & Kawasaki, 1976; Kayama-Gonda & Kawasaki, 1979; Stewart & Booth, 1983; Cairney et al., 1984). They are not contradicted by the frequently reported correlations between the rate (Kaczorowski, Cheung & Walsh, 1977; Mogi & Anraku, 1984*a*) or extent (Ramos & Kaback, 1977; Mogi & Anraku, 1984*a*) of proline uptake via proline porter I and the proton electrochemical potential gradient since the latter serves as the driving force for the formation of a Na^+ electrochemical

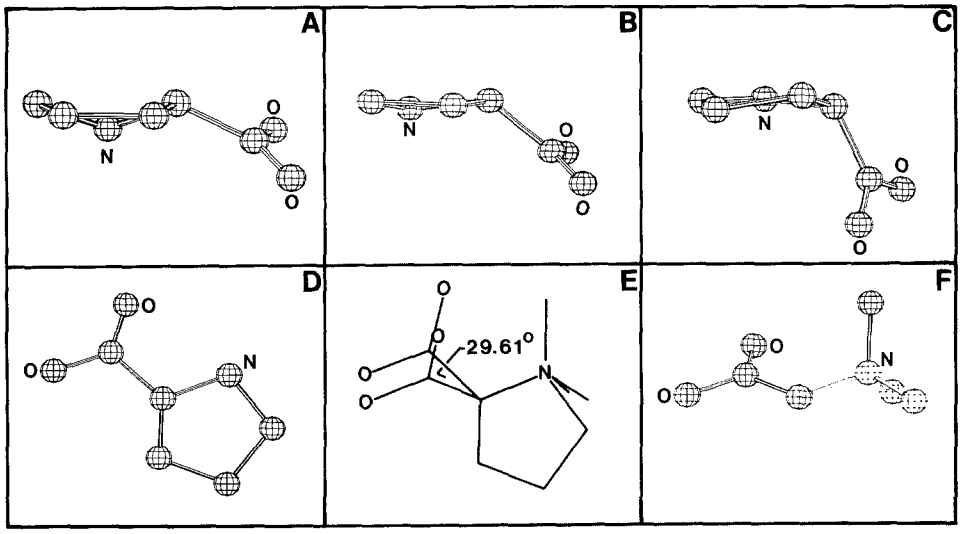

Fig. 2. Structures of proline and its analogues. Representations of the structures of proline and its analogues based on X-ray crystallographic data are provided as follows: *A, D* and *E,* L-proline (Swaminathan & Chacko, 1981); *B,* L-azetidine-2-carboxylate (Berman et al., 1969); *C,* 3,4-dehydro-L-proline (Benedetti et al., 1981); *E* and *F,* glycine betaine (Fischer, Templeton & Zalkin, 1970). For structures *A, B* and *C* the C(alpha)-carbonyl bonds are in the plane of the paper. For structures *D, E* and *F* the C(alpha)-N bonds are in the plane of the paper, and in structure *E* the C(alpha)-N bonds of L-proline and glycine betaine are superimposed

potential gradient which is catalyzed by the Na^+/H^+ antiporter.

The Na^+/proline symport mechanism was difficult to detect since *trans*-inhibition of Na^+/proline symport by Na^+ reduced the proline uptake activity observed when whole cells or membrane vesicles were incubated with Na^+ prior to initiation of a proline uptake assay (Stewart & Booth, 1983; Cairney et al., 1984) (*cf.* Lombardi & Kaback, 1972; Kasahara & Anraku, 1974; Mogi & Anraku, 1984*c*). A K^+ requirement for proline transport has now been attributed to a K^+/Na^+ exchange process which eliminates the Na^+ *trans*-inhibition (Strickland, Hamilton & Booth, 1980; Stewart & Booth, 1983; Cairney et al., 1984). In addition, the K_M of the porter for Na^+ is sufficiently low that the Na^+ requirement was probably met inadvertently in all experiments except those designed to specifically control Na^+ levels.

S. typhimurium grows well on L-proline as carbon source in media supplemented with NaCl but poorly when NaCl is replaced with LiCl. *S. typhimurium* mutants that retain Na^+/proline symport and show improved growth on proline plus lithium contain lesions which map at the 5′ and 3′ termini of the *putP* gene (R.S. Myers and S.R. Maloy, *personal communication*). Correlation of those mutations with the protein structure may define the cation binding site of proline porter I. Na^+-linked proline transport has also been detected in a number of organisms, including *Methanococcus voltae* (Ekiel et al., 1985), *Rhodopseudomonas sphaeroides* (Kundu & Nicholas, 1986), *Halobacterium halobium* (MacDonald et al., 1977) and *Mycobacterium phlei* (Prasad, Kalra & Brodie, 1975). That activity was retained in membrane vesicles prepared from *H. halobium* and *M. phlei.*

Proline Porter I Accepts a Limited Array of Organic Substrates

The selection of transport defective mutants using toxic proline analogues has been essential for the resolution of proline porters I, II and III (Wood, 1981; Csonka, 1982; Cairney et al., 1984, 1985*a,b*). The structure of L-proline (*A*) is compared with those of the toxic analogues L-azetidine-2-carboxylate (*B*) and 3,4-dehydro-L-proline (*C*) in Fig. 2. The substrate specificity of proline uptake has also been extensively analyzed by measuring the inhibition of radiolabeled proline uptake by unlabeled proline analogues (Tristram & Neale, 1968; Rowland & Tristram, 1975; DiGirolamo et al., 1984). A number of generalizations with respect to the substrate structure required for porter/substrate interaction emerge from those studies: (i) the carboxyl and secondary amine groups must be present and in appropriate relative disposition; (ii) the carbonyl portion of the carboxyl function is essential but the negative charge is not; proline esters and amides act as transport inhibitors; (iii) replacement or substitution of the pyrrolidone ring N is not tolerated; (iv) smaller but not larger ring sizes are recognized (*cf.* Rowland

& Tristram, 1971/72; Anderson, Menzel & Wood, 1980) and acyclic molecules are not inhibitors; (v) analogues with *cis*-substituents are more inhibitory than those with *trans*-substituents, and analogues with apolar substituents are more inhibitory than those with polar substituents at carbons 3 and 4 of the pyrrolidine ring.

These observations imply that the proline binding site of proline porter I includes a polar region with stringent specificity for the adjacent carbonyl and imino functions plus an apolar pocket with less stringent specificity.

S. typhimurium mutants with altered substrate specificity have been isolated and the mutations located in three discrete deletion intervals of the *putP* gene distinct from those containing mutations that alter cation binding (Dila & Maloy, 1986). They will soon be located within the protein sequence.

The Proline/Na^+ Stoichiometry Remains Uncertain

In spite of extensive experimentation, the proline/ion stoichiometry of proline porter I remains uncertain. The ratio of the transmembrane proline and proton electrochemical potential gradients maintained by cytoplasmic membrane vesicles from an *E. coli* ML strain increased from 1 at pH 5.5 to 2 at pH 8 (Ramos & Kaback, 1977), whereas for vesicles from an *E. coli* K-12 strain, Mogi and Anraku (1984*c*) observed a ratio close to 2 throughout the same pH range. In the former case the proton chemical potential decreased as the pH increased whereas in the latter no proton chemical potential gradient was detected within the pH range examined. For whole cells of *E. coli* K-12 maintained at pH between 6 and 7.5, a linear correlation was observed between the H^+ and Na^+ electrochemical potential gradients, though the latter exceeded the former by approximately 0.4 pU (Castle, Macnab & Shulman, 1986). Thus recognition that proline porter I is a Na^+, not a H^+, coupled system may not eliminate the apparent variability in its ion coupling stoichiometry based on these thermodynamic measurements. A direct comparison between the proline and Na^+ electrochemical potential gradients is clearly required.

Kinetic experiments employing a membrane protein extract reconstituted in liposomes implied a Na^+/proline coupling ratio of 1 (Chen & Wilson, 1986). Mogi and Anraku (1984*a*) analyzed the relationship between the maximal initial velocity for proline uptake via proline porter I and the membrane potential according to the model derived by Geck and Heinz (1976). They concluded that a porter/proline/2 ion complex with a net charge of plus two was translocated across the membrane. Alternate interpretations of their observations may also be valid, however (Sanders et al., 1984).

Kinetic Studies Provide Hints Regarding the Transport Mechanism

At constant coupling ion concentration and ionmotive force, ion symport reactions may be considered in terms of the reversible Michaelis-Menten relationship

$$v = \frac{V_o^f/K_M^0 - V^r i/K_M^I}{1 + o/K_M^0 + i/K_M^I} \quad (1)$$

where v is the net rate of organic solute uptake, V^f and V^r are the maximal velocities for organic solute influx and efflux, respectively, K_M^0 and K_M^I are the Michaelis constants for influx and efflux, respectively, and o and i are the extracellular and intracellular concentrations of the organic solute, respectively. A variety of kinetic mechanisms for solute-ion symport predict a hyperbolic relationship between initial rates of radiolabeled substrate influx and extracellular substrate concentration if internal substrate specific radioactivity remains negligible (Sanders et al., 1984). The existence of unlabeled intracellular substrate pools facilitates the estimation of initial rates of influx of radiolabeled substrate; as long as intracellular specific radioactivity is negligible, only influx is detected and use of the unidirectional Michaelis-Menten mechanism is justified. Deviations of measured transport rates from apparent adherence to the unidirectional Michaelis-Menten mechanism may arise through failure to satisfy that initial rate condition, through the coexistence of multiple transporters or of transport and passive transmembrane solute flux, and through transport mechanisms that genuinely fail to follow the simple Michaelis-Menten formalism.

Morikawa et al. (1974) first demonstrated that the initial rate of radiolabeled proline uptake did not show a simple hyperbolic dependence on extracellular proline concentration when a broad range of concentrations was tested. That deviation from Michaelis-Menten behavior was interpreted as indicating that proline uptake occurred via a single population of negatively cooperative transporters with multiple substrate binding sites. Subsequent work has revealed multiple proline porters with K_Ms for proline that differ by orders of magnitude (Wood & Zadworny, 1979; Anderson et al., 1980; Cairney et al., 1985*a*,*b*) and a passive proline flux pathway which becomes significant at high absolute proline concentrations and proline potential gradients (B.

Vink and J.M. Wood, *unpublished data*). The deviation from Michaelis-Menten behavior observed by Morikawa et al. was therefore most likely attributable to the coexistence of multiple routes for transmembrane proline flux.

Induction of the *put* genes by growth of *E. coli* on a variety of nitrogen and/or carbon sources, including proline, was correlated with a stepwise decrease in uptake K_M from 2 to 0.2 μM proline (Wood & Zadworny, 1979). That decrease was correlated with a stepwise increase in expression of both proline porter I and proline dehydrogenase. It was not a kinetic artifact due to proline catabolism or to changes in the relative contributions of multiple uptake pathways. Certain *putA* mutants are defective for proline uptake (Menzel & Roth, 1981) and some *putA* missense mutations are suppressed by mutations that map in *putP* (Maloy, 1987). These observations imply that the PutP and PutA proteins undergo a direct, functional interaction in vivo. Since proline uptake via proline porter I occurs in *putA* mutant bacteria and proline derived from glycyl- or leucyl-proline can serve as sole nitrogen source for *putP* mutants, that interaction must not be obligatory for the function of either enzyme.

K_Ms for energized proline uptake via PPI in the range 0.4 to 5 μM have been determined using whole cells (Tristram & Neale, 1968; Kawasaki & Kayama, 1973; Morikawa et al., 1974; Wood & Zadworny, 1979; Konings & Robillard, 1982; Stewart & Booth, 1983; Hanada et al., 1987) or membrane vesicles (Lombardi & Kaback, 1972; Kasahara & Anraku, 1974; Mogi & Anraku, 1984*a*) of *E. coli* K-10, K-12 or ML. In some cases, *putA*$^+$ bacteria grown under conditions that would induce *put* gene expression were employed. When a total membrane protein extract from *putA*$^-$ bacteria (Chen & Wilson, 1986) or the purified PutP protein (Hanada et al., 1988) was reconstituted in liposomes, the K_M for proline uptake was observed to be 0.5 or 3.6 μM, respectively.

The V_M, but not the K_M, for proline uptake varied with both fatty acid supplementation and transport assay temperature (range 3 to 41°C) for a fatty acid auxotrophic strain of *E. coli* (Eze & McElhaney, 1987). Nonlinear or biphasic linear Arrhenius plots were observed with low activation energies (10 to 14 kcal/mol) above and more variable, higher activation energies (40 to 80 kcal/mol) below the Arrhenius plot break point (13 to 20°C). That point varied with but did not coincide with the membrane phase transition midpoint. Whether these effects represent the temperature- and lipid-dependence of the energy supply or of the porter mechanism remains to be established.

In Eq. (1), coupling ion concentrations and the membrane potential are implicit to the values of V and K. The dependence of those values on coupling ion concentration and ionmotive force may be used to deduce details of the energy coupling mechanism (Sanders et al., 1984). Equation (1) can be related to the ionmotive force through the Haldane relationship, such that

$$\Delta\bar{\mu}_{ion} = -RT \ln K_{eq} \tag{2}$$

or

$$\Delta\bar{\mu}_{ion} = -RT \ln(V^f K_M^I / V^r K_M^0). \tag{3}$$

Thus the transporter will catalyze the approach of the solute concentration gradient to equilibrium with the ionmotive force. An equilibrium constant different from one, characteristic of an active transport process, will result if the mutual affinity of porter and organic solute differs at the two membrane surfaces and/or if the rate of translocation of solute and coupling ion inwards across the membrane differs from the rate of translocation outwards. V^f must then differ from V^r and/or K_M^I must differ from K_M^0 for an active transport process.

Proline porter I differs from other Na^+/solute symporters in that the K_Ms for both substrates are low and invariant; the coupling ion alters the maximal transport rate, not the affinity of the porter for proline. Surprisingly, the reported K_M values for Na^+ vary by an order of magnitude (350 μM in *S. typhimurium* (Cairney et al., 1984) and 37 μM (Chen et al., 1985) or 31 μM (Hanada et al., 1988) in *E. coli*). Further characterization of the *trans*-inhibition and V_{max} effects of Na^+ promises to yield new insights regarding the transport mechanism (Sanders et al., 1984).

In *E. coli* K-12, the K_M for proline uptake was observed to change from 1 to 250 μM in the presence and absence of a membrane potential, respectively (Mogi & Anraku, 1984*a*). The properties of bacteria lacking proline porter I were not determined, however, so the possibility that the latter value represents alternate influx pathways, not the activity of proline porter I, cannot be ruled out.

Ion-linked proline uptake can be inhibited at a variety of levels by thiol group-modifying reagents. Provision of an ionmotive force through ATP hydrolysis (Janick, Grunwald & Wood, 1977), phenazine methosulphate-mediated electron flow from ascorbate to oxygen via the respiratory chain (Kaback & Patel, 1978) or ionophore-mediated ion flux (Konings & Robillard, 1982) permitted transport inhibition due to the thiol group reactivity of a high affinity proline uptake system to be distinguished from the inhibition of respiration. Inhibition of proline porter I by maleimides was enhanced by proline or a proton ionophore in whole cells (Janick et al., 1977) but prevented by proline and accelerated by

the imposition of a protonmotive force in membrane vesicles of *E. coli* (Cohn, Kaczorowski & Kaback, 1981). Indeed, proline-sensitive reactivity with radiolabeled N-ethylmaleimide has been exploited to identify the PutP protein (Hanada et al., 1985). These observations imply a modulation of thiol group exposure during turnover of the porter that differs between whole cells and membrane vesicles.

The K_M for proline uptake by *E. coli* ML increased from 1 to 45 μM when membrane vesicles were treated with the membrane impermeant oxidizing agent, plumbagin (Konings & Robillard, 1982). Robillard and Konings (1982) proposed that the membrane potential and pH gradient modulate the redox poise of thiol and disulfide groups present at different depths within the porter protein. That modulation was proposed to cause the transmembrane differential in porter kinetic properties that is characteristic of active transport. Membrane permeant and impermeant oxidants, reductants and alkylating agents were used to demonstrate that redox-sensitive dithiols present at the inner and outer membrane surfaces of proline porter I participate in such redox modulation of proline transport (Poolman, Konings & Robillard, 1983). Again, assurance that the observed characteristics did not represent a hybrid of activities attributable to porters I and II and passive proline flux remains to be provided. The putative amino acid sequence of the PutP protein includes five cysteine residues (positions 12, 141, 281, 344 and 349 (Nakao et al., 1987*a*; Hahn et al., 1988). Modification of those residues through site-directed mutagenesis or in mutants with altered porter/substrate interactions will be useful in evaluating both their role in catalysis and the validity of the thiol-disulfide exchange model.

The rate of proline uptake (Mogi & Anraku, 1984*c*) and proline binding to cytoplasmic membrane preparations (Amanuma, Itoh & Anraku, 1977*a*; Mogi & Anraku, 1984*b*) are strongly dependent on extracellular (or extravesicular) pH in the range 5 to 7. Since protons are not cotransported with proline via proline porter I, that pH dependence must reflect the function of other enzymes or the dependence of other porter characteristics on functional group ionization. Since high concentrations of proline and/or NaCl were included in some measurements, those experiments may have reflected the summed binding activities of porters I and II.

Proline Accumulated via Proline Porter I Meets Multiple Physiological Demands

Proline serves as carbon source for the rapid aerobic growth of *E. coli,* but not for anaerobic growth with fumarate or nitrate as terminal electron acceptor (G. Baker and J.M. Wood, *unpublished data*). The chemotactic response of *E. coli* to proline gradients results from the respiratory stimulus provided by proline oxidation, not from the receptor-mediated sensory mechanism common to other amino acids and sugars (Clancy, Madill & Wood, 1981). Proline dehydrogenase induction is correlated with oxygen supply in *S. typhimurium* (Maloy & Roth, 1983) and the oxygen inducible gene fusion class *oxiB* is related to *putA* (Alibadi et al., 1986). It is thus likely that where oxygen concentration is low, the constitutive expression of *putP* allows traces of proline to be scavenged from the medium for use in protein synthesis. Where proline and oxygen are both available, proline transport becomes linked to the oxidative activities of the PutA protein so that proline can be utilized as an oxygen reductant and a carbon and/or nitrogen supply. The cytoplasmic proline concentration of wild type bacteria growing on carbon and nitrogen sources other than proline has been estimated as less than 1 mM (Piperno & Oxender, 1968; Csonka, 1981; Jakowec, Smith & Dandekar, 1985). Proline porter I is likely required to deliver proline efficiently to the protein synthetic and catabolic enzymes but not to maintain high intracellular proline levels.

Active Transport is Central to the Enterobacterial Osmotic Stress Response

Hyperosmotic shock threatens cellular survival. Unless protective mechanisms are invoked, exclusion of osmolytes leads to cytoplasmic dehydration; in consequence the environment of cytoplasmic macromolecules is altered and the turgor pressure maintained by walled cells is reduced. Some plant, microbial and animal cells tolerate variations in the osmolarity of their environment by modulating their cytoplasmic composition (Yancey et al., 1982; LeRudulier et al., 1984; Somero, 1986). They exclude potentially deleterious solutes like Na^+ and accumulate K^+ ions and/or organic solutes compatible with macromolecular function, thereby preventing dehydration of the cytoplasm. The compatible solutes include sugars, amino acids and their derivatives.

The enteric bacteria are moderately halotolerant, growing in media supplemented with up to 0.7 M NaCl (Measures, 1975; Brown, 1976). Growth rates are reduced in highly saline media through both hyperosmotic stress and specific ion toxicities. Recent genetic and biochemical experiments have dramatically enhanced our understanding of the molecular mechanisms underlying osmotic stress tolerance in *E. coli, S. typhimurium* and some related species. In *E. coli* and *S. typhimurium,* the

ubiquitous potassium ion is the primary osmoregulator. Cytoplasmic K^+ levels are directly proportional to medium osmotic strength (Epstein & Schultz, 1965; Sutherland et al., 1986), and bacteria unable to accumulate K^+ are unable to grow in media of elevated osmolarity (Gowrishankar, 1987). Glutamic acid and trehalose accumulate when the bacteria are cultivated in mineral media supplemented with NaCl. Proline, proline betaine, glycine betaine and γ-butyrobetaine may replace trehalose and glutamate as the accumulating osmolytes if they are available in the growth medium (Csonka, 1981; Larsen et al., 1987; T. Record, *personal communication*). Thus a variety of metabolites may be synthesized or acquired from the cellular environment to mount this crucial stress response.

Csonka (1979; 1981; 1982) first demonstrated that a wild type strain of *S. typhimurium* exposed to hyperosmotic stress accumulated proline by active transport, not *de novo* synthesis. A causal relationship between proline accumulation and osmotic stress tolerance was established when a genetic lesion deregulating proline biosynthesis was shown to cause both cytoplasmic proline accumulation and increased osmotolerance (Csonka, 1981). Exogenous proline provided osmoprotection to wild type bacteria but not to proline transport mutants, however (Csonka, 1982).

Proline porters II and III are now known to effect osmoprotection by both proline and glycine betaine. Although glycine betaine can be synthesized from choline, in the absence of that precursor the osmoprotective action of glycine betaine is fully transport-dependent (Landfald & Strom, 1986; Strom, Falkenberg & Landfald, 1986). Proline porter III is detectable only in bacteria exposed to hyperosmotic stress during growth, whereas proline porter II is constitutively expressed and it also undergoes limited induction. Although the activities of proline porters II and III are not readily distinguishable biochemically, they can be resolved by introducing appropriate genetic lesions or by manipulating the conditions of bacterial growth.

Proline Porters II and III Catalyze the Osmoprotective Accumulation of Proline and Glycine Betaine

Proline porter II was first identified as a function essential for the growth of *putP*$^-$, proline auxotrophic *S. typhimurium* strains in media containing proline at low (16 μM) but not high (8 mM) concentration. Mutants of *S. typhimurium* (Menzel & Roth, 1980) or *E. coli* (Stalmach, Grothe & Wood, 1983) defective at locus *proP* lacked proline porter II activity and required high exogenous proline for growth. The *proP* locus of *E. coli* is counterclockwise from *pheR*, between 92 and 93 chromosomal map units, and it is transcribed in a clockwise direction (Gowrishankar, 1986). The position of the *proP* gene in *S. typhimurium* is similar (Menzel & Roth, 1980). The ProP protein has not been identified, nor has direct evidence that it is PPII been provided.

Csonka (1982) first identified proline porter III as a function encoded in the *proU* locus of *S. typhimurium* that promoted the osmoprotective effects of exogenous proline. The *proU* locus, which is near 58 map units on the chromosome maps of *S. typhimurium* (Csonka, 1982) and *E. coli* (Gowrishankar, 1985), includes at least two cistrons, both of which are required for proline porter III activity (Gowrishankar, Jayashree & Rajkumari, 1986; Faatz et al., 1988).

The Activity of Proline Porter II is Elevated in Response to Nutritional or Hyperosmotic Stress

Although proline porter II is expressed constitutively, its activity is elevated approximately 10-fold in bacteria subjected to nutritional stress during growth; auxotrophic strains of *S. typhimurium* starved of proline, histidine or leucine ((Anderson et al., 1980) and tryptophan auxotrophs of *E. coli* undergoing tryptophan limited growth (Stalmach et al., 1983; Grothe et al., 1986) all showed elevated proline porter II activity. The glycine and glutamine uptake activities of the same bacteria were not altered by nutritional stress. Surprisingly, no increase in β-galactosidase activity was observed when an *S. typhimurium* strain containing a *proP* :: *lacZ* operon fusion was starved for proline, histidine or leucine (Dunlap & Csonka, 1985). Increased *lacZ* transcription may have been masked by increased proteolytic activity under those conditions, however.

Recently proline porter II was also shown to respond to hyperosmotic stress. β-galactosidase activity was elevated twofold when strains containing *proP* :: *lacZ* operon fusions were grown in media supplemented with NaCl (0.3 to 0.65 M) (Dunlap & Csonka, 1985; Cairney et al., 1985*a*; Gowrishankar, 1986). Similar effects were observed in merodiploid strains also containing a copy of the wild type *proP* gene. Addition of proline or glycine betaine at a concentration previously shown to effect osmoprotection (1 mM) did not alter the β-galactosidase activity of bacteria grown at optimal osmotic strength. Both compounds reduced the β-galactosidase activity expressed during growth under hyperosmotic stress (0.3 M NaCl).

The rate of proline uptake via proline porter II (but not glutamine, glycine or serine uptake) was elevated when the osmolarities of the growth and

transport assay media were increased by the addition of either NaCl or sucrose (Dunlap & Csonka, 1985; Grothe et al., 1986; Milner, McClellan & Wood, 1987*a*) (Fig. 3). Specific effects of osmotic downshock (growth at high osmolarity and assay at low osmolarity) on proline porter II could not be assessed since that treatment caused a nonspecific decrease in amino acid uptake activity (Milner et al., 1987*a*).

Activation of proline porter II (but not of glutamine or serine uptake) could be achieved in bacteria grown with or without nutritional stress if they were subjected to hyperosmotic shock with NaCl or sucrose when proline uptake was initiated (Grothe et al., 1986). Since the bacteria were treated with chloramphenicol, protein synthesis was not required for that activation. A 4.5-fold increase in activity was observed in nutritionally stressed bacteria when NaCl (0.125 to 0.25 M) or sucrose (0.25 to 0.5 M) was added to the transport assay medium.

The proline uptake activity of bacteria cultivated in media supplemented with 0.3 M NaCl was measured in media supplemented with NaCl at 0.3, 0.4 or 0.5 M (Fig. 3). The highest transport activity was observed when the growth and transport assay media were of the same composition. Osmotic upshock reduced the measured transport activities; the latter were comparable to those observed when bacteria grown under conditions of nutritional stress were exposed to the cited osmotic stress during the transport assay (Grothe et al., 1986). These observations suggest that the induction of proline porter II is balanced by enzyme inhibitory effects when NaCl is added to our growth and transport assay media at concentrations greater than 0.3 M.

A Tn*5* insertion mutation (*proQ220*::Tn*5*) located at 40.4 map units on the *E. coli* chromosome eliminated induction of proline porter II by nutritional stress and reduced the transport activity observed if osmotic stress was imposed during bacterial growth or during the transport assay (J.L. Milner and J.M. Wood, *submitted*). Small effects on glutamine and serine uptake were also observed. This insertion was isolated as conferring a phenotype identical to that of the *proP* mutation on bacteria grown at optimal osmolarity (Stalmach et al., 1983). Its properties lead us to suspect that *proQ* is a regulatory locus, but no genes related to proline metabolism or the nutritional or osmotic stress responses have previously been identified at that location.

Proline Porter III Also Responds to Hyperosmotic Stress

Gene fusion experiments employing both *E. coli* and *S. typhimurium* readily detected both the *proU* locus and the *kdp* locus, the latter encoding an osmoresponsive K^+ uptake system, as among the most osmoresponsive genes in those organisms (Cairney et al., 1985*b*; Dunlap & Csonka, 1985; Gowrishankar, 1985; Barron et al., 1986; Gutierrez et al., 1987). Induction of *proU*::*lacZ* gene fusions in media supplemented with 0.3 M NaCl occurred after a lag of 10 to 20 min. Expression of β-galactosidase activity by such strains during steady-state growth showed a sigmoid dependence on medium NaCl supplementation: *proU* was not expressed in the absence of osmotic stress, a point of inflection occurred at approximately 0.2 M NaCl, and maximal induction, as much as 100-fold, was observed in media supplemented with more than 0.4 M NaCl. A variety of inorganic and organic osmolytes, provided to yield similar osmolarities, could induce *proU* expression. Only those compounds known to

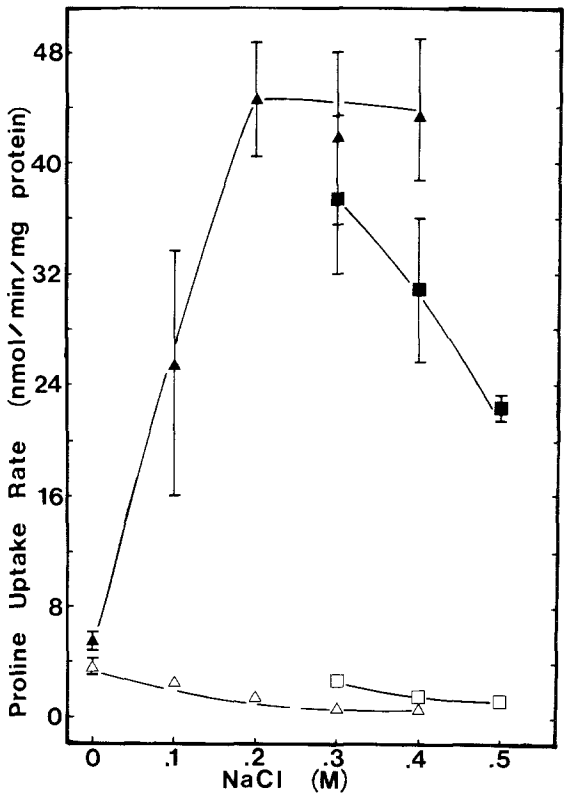

Fig. 3. The influence of hyperosmotic stress on proline porter II in *Escherichia coli* K-12. Rates of L-proline uptake by *E. coli* strains WG210, from which proline porters I and III and the proline oxidative enzymes are absent (filled symbols) and WG203, which is additionally defective in proline porter II (open symbols) are plotted versus the concentration of NaCl with which the bacterial growth and/or transport assay media were supplemented. Bacteria were either exposed to the indicated level of NaCl supplementation during both growth and the transport assay (triangles) or they were cultivated in media supplemented with 0.3 M NaCl and the transport assay media were supplemented with NaCl at the indicated concentration (squares)

Table 2. Kinetic properties of the osmoresponsive proline/betaine porters

Substrate	Organism	Parameter	Porter II	Porter III
Proline	*E. coli*	K_m	ND[a]	150[b]
		V	ND	10[b]
Proline	*S. typhimurium*	K_m	340[c]	ND
		V	32[c]	ND
Betaine	*E. coli*	K_m		35[d]
		V		42[d]
Betaine	*S. typhimurium*	K_m	44[e]	1.3[f]
		V	37[e]	12.5[f]

[a] Not determined.
[b] A *putP proP proU*$^+$ strain of *E. coli* K12 was subjected to osmotic stress (Barron et al., 1987).
[c] A *putP proP*$^-$ *proU*$^+$ strain of *S. typhimurium* was subjected to nutritional stress (Anderson et al., 1980).
[d] *E. coli* K10 was subjected to osmotic stress (Perroud & Le Rudulier, 1985).
[e] A *putP proP*$^+$ *proU* strain of *S. typhimurim* was subjected to osmotic stress (Cairney et al., 1985*a*).
[f] A *putP proP proU*$^+$ strain of *S. typhimurium* was subjected to osmotic stress (Cairney et al., 1985*b*).

rapidly penetrate the cell membrane through passive (e.g. ethanol) or facilitated (e.g. glycerol) diffusion failed to yield induction.

Neither provision of proline nor proline-limited growth of an auxotrophic strain altered expression of a *proU*::*lacZ* operon fusion in media of optimal osmotic strength (Dunlap & Csonka, 1985; Gowrishankar, 1985), but the β-galactosidase activity expressed by *proU*::*lacZ* strains in media of elevated osmolarity was reduced in the presence of proline or glycine betaine (1 mM) (Cairney et al., 1985*b*; May et al., 1986). The expression of β-galactosidase activity from the *proU* promoter during osmotic stress was altered when multiple copies of the intact *proU* locus were introduced but not in merodiploid strains containing a single copy of the intact *proU* locus (Barron et al., 1986; Gowrishankar, 1986).

Expression of *proU* was not altered by lesions at *ompR* or *envZ*, loci which have been shown to influence the expression of porin genes in response to osmotic strength (Cairney et al., 1985*b*; May et al., 1986). A variety of procedures have been applied to select mutants altered in the genetic regulation of *proU*. In addition to *cis*-dominant mutations closely linked to *proU* (Druger-Liotta et al., 1986), mutations that alter DNA supercoiling have been shown to influence *proU* expression (Higgins et al., 1988).

Proline porter III is not detectable in wild type bacteria cultivated in media of optimal osmolarity, but Faatz et al. (1988) demonstrated low constitutive activity of that system in bacteria containing multiple copies of the *proU* locus. That system permitted them to demonstrate activation of porter III in response to a hyperosmotic shift imposed by adding NaCl or sucrose. The dependence of that activation on NaCl concentration was similar to that previously reported for proline proter II (Grothe et al., 1986).

Proline Porters II and III Transport Both Proline and Glycine Betaine

Bacteria defective at *putP* (proline porter I) alone or at *putP* and *putA* (proline/Δ^1-pyrroline carboxylate dehydrogenase) are resistant to the toxic proline analogue azetidine-2-carboxylate (Fig. 2*B*) when cultured in media of optimal osmolarity. They regain sensitivity to that compound when proline porters II and III are induced in media of high osmotic strength (Csonka, 1982; Cairney et al., 1984; Grothe et al., 1986). Strains defective at *putP* are also resistant to a second toxic proline analogue, 3,4-dehydroproline (Fig. 2*C*), in media of optimal osmolarity. Since proline dehydrogenase detoxifies 3,4-dehydroproline, *putP putA* bacteria are sensitive to that compound (Wood, 1981). The sensitivity of *putP* or *putP putA* bacteria to 3,4-dehydroproline is also increased in media of elevated osmotic strength. Mutants lacking proline porters II and III can thus be isolated by selecting bacteria resistant to azetidine-2-carboxylate or 3,4-dehydroproline under appropriate conditions (Csonka, 1982; Stalmach et al., 1983; Cairney et al., 1984, 1985*a,b*; Grothe et al., 1986). That glycine betaine (Fig. 2*F*) protected bacteria retaining proline proters II and III, but not those retaining only proline porter I, from the toxicity of these proline analogues provided an initial clue to the broad substrate specificities of proline porters II and III (Cairney et al., 1985*a*; Grothe et al., 1986). Similar observations have been made in studies of *Serratia marcescens* (Sugiura & Kisumi, 1985*a,b*).

Early efforts to evaluate the biochemical characteristics of Proline Porter III were hampered by the low rates of proline uptake via that system detected with a variety of assay procedures (Csonka, 1982; Dunlap & Csonka, 1985; Grothe et al., 1986; Milner et al., 1987*a*). That difficulty was alleviated when Cairney et al. (1985*b*) demonstrated rapid uptake of glycine betaine via Proline Porter III in *S. typhimurium*. Several laboratories have since confirmed and amplified their observation that both proline and glycine betaine are substrates for both Proline Porters II and III by examining the transport activity of appropriately cultivated mutant bacteria (Table 2). The substrate preferences of the two

porters have not been clearly delineated since no laboratory has completed a direct and rigorous comparison of the kinetic parameters for proline and glycine betaine uptake within comparable bacterial strains. The available data suggest a much higher affinity of Proline Porter III for glycine betaine than for proline (Betaine/Proline Porter III may be a more appropriate designation). The conclusion that the substrate preference of Proline Porter II changes from proline to glycine betaine on imposition of osmotic stress (Cairney et al., 1985*a*) is questionable since it is based on experiments in which proline was provided as substrate at sub-saturating concentration and cells were exposed to a hypoosmotic shift during the transport assay. We observed similar effects of glycine betaine on uptake of radiolabeled proline via Proline Porter II if that system was uninduced or induced by nutritional or osmotic stress, unactivated or activated by hyperosmotic shock (Milner et al., 1987*a*). Approximately 50% inhibition of proline uptake was observed when glycine betaine and proline were supplied at the same concentration (200 μM). Thus we concluded that proline porter II has approximately equal affinities for proline and glycine betaine under all conditions examined. Peleg, Tietz and Friedberg (1980) examined the effects of hyperosmotic stress on proline transport in the moderately halophilic, halotolerant Gram-negative bacterium, Ba_1. They observed increased proline transport activity in bacteria exposed to hyperosmotic stress (up to 2 M NaCl) during or subsequent to growth. Less than twofold variations in the K_m for proline uptake were observed as V_{max} increased under hyperosmotic conditions. The data summarized in Table 2 imply that, with abundant substrate, flux via Porter II may be as much as 40-fold more rapid than that via Porter III.

Additional information regarding the substrate specificities of Porters II and III has been obtained by examining the inhibition of radiolabeled proline or glycine betaine uptake by unlabeled compounds. Proline uptake via proline porter II was strongly inhibited by L-azetidine-2-carboxylate and 3,4-dehydro-D-L-proline (50% inhibition of radiolabeled proline uptake by a twofold excess of inhibitor), very weakly inhibited by 4,5-dehydro-L-pipecolate and 4-hydroxy-L-proline and not inhibited by the other 19 commonly occurring amino acids (Anderson et al., 1980; Stalmach et al., 1983). Gowrishankar (1986) demonstrated osmoprotection of *E. coli* by 5-hydroxy-L-pipecolate in *proP*$^+$, but not *proP*$^-$, bacteria, implying a specific interaction of that compound with proline porter II. Dimethylthetin can substitute for glycine betaine as an osmoprotectant for *E. coli,* but mediation of that effect has not been attributed to a particular transport system(s) (Chambers et al., 1987).

Perroud and LeRudulier (1985) examined the effects of unlabeled analogues on the uptake of radiolabeled glycine betaine in *E. coli* K-10. As noted above, those measurements probably detected the summed activities of proline porters II and III. They showed that: (i) tri-N-methyl molecules were more inhibitory than their di-N-methyl homologues; (ii) the carboxyl group was essential, and its close proximity to the N-methyl increased inhibition; (iii) although proline and its N-methyl derivatives were inhibitory, pipecolate betaine was not. The similar and incomplete inhibition of proline uptake observed when some analogues (e.g. β-alanine betaine, proline, monomethyl proline, pipecolate betaine) were provided at 20- or 40-fold excess over labeled glycine betaine may indicate that they competed effectively for uptake via only one glycine betaine porter. Since proline betaine caused 91% inhibition of glycine betaine uptake at the lowest concentration tested (20-fold excess), it is likely a co-substrate for both glycine betaine transporters.

Unambiguous analysis of the substrate specificities of proline porters I, II and III is now feasible. Proline porters I and III appear to behave as typical bacterial amino acid transporters (*see* Milner et al., 1987*b*) with high substrate affinities and narrow specificities for proline and glycine betaine, respectively. The similarities and differences between the structures of those compounds are illustrated in Fig. 2*D–F*). Proline porter II differs from the other systems in that no substrate transported by that enzyme with very high affinity has yet been identified. Proline betaine, glycine betaine and choline were recently shown to be the metabolites that permitted *E. coli* strains to grow in human urine, a highly hyperosmotic medium (Chambers & Kunin, 1987). As noted above, proline betaine is likely to serve as a substrate for both proline porters II and III. Perhaps it is, in fact, the preferred substrate for proline porter II.

The substrate specificity of the proline/sodium (IMINO) transporter from rabbit intestinal brush border membranes has recently been analyzed (Stevens & Wright, 1985). The specificity of that system is intermediate among those of the three bacterial transporters described above, showing similar affinities for proline and glycine betaine and a strong specificity for the spacing between the imino nitrogen and carboxyl functions shared by those molecules. It differs from all of them in accepting L-pipecolate, the six-membered cyclic analogue of proline. Interestingly, glycine betaine is among the osmotically active solutes that accumulate in rat and rabbit renal inner medulla under conditions of

antidiuresis (Bagnasco et al., 1986). It is tempting to speculate that homologous enzyme systems may mediate proline and betaine transport in both the enteric bacteria and some mammalian epithelia (*cf.* Stevens, Kaunitz & Wright, 1984).

Proline Porter II is Energized via Respiration and Active in Cytoplasmic Membrane Vesicles

Intracellular glycine betaine levels varying from 9 to 246 mM were observed when cells of *E. coli* K-10 exposed to various degrees of hyperosmotic stress (defined medium supplemented with 0 to 0.65 M NaCl) during growth were allowed to accumulate radiolabeled glycine betaine (provided at 250 μM) for 30 min at 37°C (Perroud & LeRudulier, 1985). Cytoplasmic glycine betaine and proline betaine concentrations of 0.2, 0.4 or 0.9 molal were observed radiochemically and by nuclear magnetic resonance spectroscopy in *E. coli* K-10 after growth in a mineral salts medium supplemented with 2 mM betaine and 0.2, 0.4 or 0.65 M NaCl, respectively (Larsen et al., 1987). Proline and glycine betaine attained intracellular concentrations of approximately 0.5 molal (detected by nuclear magnetic resonance spectroscopy) when they were provided at 1 mM to *E. coli* growing in a MOPS minimal medium supplemented with 0.5 M NaCl (T. Record, *personal communication*). No increase in the rate of proline synthesis and no synthesis of glycine betaine would be expected under any of these conditions (Csonka, 1981; Larsen et al., 1987; Csonka, 1988). Thus 500- to 1000-fold accumulation of proline and the betaines occurs in bacteria exposed to osmotic stress, but the relative contributions of the multiple proline and betaine porters to that accumulation have not been defined.

Proline entered a *putP proP proU* strain of *E. coli* with a permeability coefficient of approximately 0.2 nmol min^{-1} mg $protein^{-1}$ mM^{-1} (B. Vink and J.M. Wood, *unpublished data*). On that basis a proline influx rate of approximately 100 nmol min^{-1} mg $protein^{-1}$ would be required to maintain the proline pool at 0.5 molal in a nongrowing bacterium. The passive permeabilities of the cytoplasmic membrane for glycine betaine and proline betaine are not known, but they are likely to be similar to that for proline (Milner et al., 1978*b*). The maintenance of transmembrane compatible solute gradients in osmotically stressed bacteria is thus likely to require a substantial and continuous energy input. Increased respiratory rates during compatible solute uptake in response to osmotic stress have been reported in the halotolerant eubacterium, Ba_1 (Ken-Dror, Preger & Avi-Dor, 1986) and in *Paracoccus denitrificans* (Erecińska & Deutsch, 1985). No studies of respiratory activity in response to osmotic stress in *E. coli* and *S. typhimurium* have been published, to my knowledge.

Inhibition of proline porter II by arsenate, proton ionophores and respiratory chain inhibitors has been demonstrated repeatedly (Anderson et al., 1980; Dunlap & Csonka, 1985; Perroud & LeRudulier, 1985). Proline porter II was active in bacteria lacking the F_0F_1-ATPase if they were provided with either D-glucose or D-lactate as energy supply. That activity was eliminated by the proton ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) or by the cytochrome oxidase inhibitor potassium cyanide (Milner et al., 1988). Energy-dependent proline uptake attributable to proline porter II has been demonstrated in cytoplasmic membrane vesicles derived from *E. coli* (Milner et al., 1988). That activity was apparent only when a hyperosmotic shift, imposed by the addition of NaCl or sucrose, accompanied the transport measurement. The proline uptake activities of the membrane vesicles varied in proportion with those of the whole cells from which they were prepared. Energy-dependent serine uptake was reduced upon hyperosmotic shift, perhaps due to shrinkage of the vesicles. The elevation of uptake in response to a hyperosmotic shift was therefore a proline-specific phenomenon.

These experiments suggest that proline porter II is integral to the cytoplasmic membrane and that it is energized via respiration. They also explain both sporadic reports that proline uptake in whole cells or membrane vesicles of *E. coli* was stimulated by NaCl or other osmolytes (Kaback & Deuel, 1969; Morikawa et al., 1974; Van Heerikhuiizen, Boekhout & Witholt, 1977) and some of the complications encountered in attempting to deduce the nature of ion coupling to proline porter I (*see above*). Efforts to directly demonstrate proline-dependent ion transport catalyzed by proline porter II have not yet proven successful.

Glycine Betaine Transport via Proline Porter III is Mediated by a Periplasmic Glycine Betaine Binding Protein

A 31,000-kD periplasmic protein is amplified by osmotic stress and eliminated by insertions in the *proU* locus of *E. coli* (Barron et al., 1986) and *S. typhimurium* (Higgins et al., 1987*b*). Periplasmic protein preparations from bacteria grown at high, but not low, osmotic strength bound glycine betaine with high affinity; that binding was not influenced by the osmolarity of the binding assay medium (May et al., 1986; Barron et al., 1987; Higgins et al.,

1987*b*). A 31,000-D glycine betaine binding protein with the same properties was purified from *E. coli* (Barron et al., 1987). Since antibodies raised against the purified protein detected both the 31,000-D periplasmic protein induced by osmotic stress and a 150,000-D hybrid protein present in bacteria bearing a *proU*::*lacZ* fusion, the glycine binding protein is the *proU* gene product.

Although *proU* defects reduced both proline uptake activity and osmoprotection by exogenous proline, the purified ProU protein did not bind proline (the binding assay employed would have detected binding with a K_D as high as 200 μM). No proline-binding activity was detected among the periplasmic proteins of bacteria in which proline porter III was induced. Barron et al. (1987) suggest that weak, binding protein-independent proline uptake may occur via the integral membrane component(s) of the transporter encoded in the *proU* locus. A *putP proP proU* strain of *S. typhimurium* lacked osmoprotection by exogenous proline but retained osmoprotection by exogenous glycine betaine (LeRudulier & Bouillard, 1983). If a binding protein-deficient proline porter III were to retain more effective glycine betaine than proline uptake, that residual osmoprotection might still be attributable to proline porter III. Alternatively, a third glycine betaine uptake activity would be present but as yet undetected! We did not observe energy-dependent proline uptake when radiolabeled proline (200 μM) was provided to membrane vesicles from *putP proP* bacteria, but a transporter not energized via respiratory electron flow would not have been detected under our conditions (Milner et al., 1988).

Isolation of the glycine betaine binding protein implied that proline porter III is a member of the class of bacterial transport systems comprised of a periplasmic substrate-binding protein plus multiple (usually 3) proteins integral or peripheral to the cytoplasmic membrane (Ames, 1986). Proline porter III is therefore likely to include multiple protein components. The *proU* locus of *E. coli* has been cloned (Gowrishankar, 1985; May et al., 1986). Subcloning, Tn*1000* mutagenesis, interplasmid complementation in a deletion mutant and protein expression in minicells provided evidence that the *proU* locus is an operon encoding three proteins in addition to the glycine betaine binding protein (Gowrishankar et al., 1986; Faatz et al., 1988). Polyacrylamide gel electrophoresis has revealed other membrane proteins that appeared to be coregulated with the ProU protein, but they have not yet been correlated with genetic loci (Barron et al., 1986; Clark & Parker, 1984).

Periplasmic binding protein-dependent transporters are not powered by the protonmotive force; indirect evidence implies that they utilize the energy of ATP or a related compound (Ames, 1986). Neither the energy supply for proline porter III nor its capacity to maintain proline gradients has been determined. As noted above, when very high intracellular solute concentrations are maintained by synthesis or active transport, passive solute efflux is likely to become significant. Such leakage would prevent equilibration of the solute potential with the transport energy supply. Under those conditions, the intracellular solute concentration maintained would depend on the concentration of transporters in the cytoplasmic membrane. *E. coli* strains containing multiple copies of the *proU* locus tolerate media of higher osmolarity than their haploid parents when provided with glycine betaine as osmoprotectant (Gowrishankar et al., 1986). If that enhanced osmotolerance is due to maintenance of higher cytoplasmic glycine betaine concentrations, those data support the view that passive leakage is a significant factor limiting the cytoplasmic accumulation of osmoprotectants in response to osmotic stress.

Orchestration of the Osmotic Stress Response

An array of genes and enzymes related to osmotolerance in the enteric bacteria has now been enumerated. They include enzymes involved in both the transport (Epstein, 1986; this article) and the synthesis (Strom et al., 1986; Larsen et al., 1987) of several potentially osmoprotective substances. Future research will focus on the regulatory signals and responses required to invoke and coordinate the osmotic stress response at both the biochemical and the genetic level. The regulatory patterns deduced will reflect both the biochemical mechanisms by which cytoplasmic solutes afford osmoprotection and the availability of particular solutes or their precursors in each environment colonized by these organisms. For example, recent experiments have identified proline betaine and glycine betaine (whose accumulation is likely mediated by proline porters II and III (*see above*)) as the osmoprotective agents that allow growth of *E. coli* in human urine (Chambers & Kunin, 1987).

How Do Compatible Solutes Afford Osmoprotection?

Solutes accumulating in the cytoplasm clearly counteract the tendency for dehydration as extracellular osmolarity is elevated. Their relative effectiveness as osmoprotectants is thus expected to depend on the relative ease with which their cytoplasmic concentrations can be elevated through biosynthesis

and/or transport in the face of competing catabolism and passive transmembrane efflux. For example, glycine betaine is not catabolized by *E. coli* (LeRudulier & Bouillard, 1983) whereas the proline catabolic enzymes are induced by proline when bacteria are cultivated in aerobic media of low or high osmotic strength (Milner et al., 1987*a*; Csonka, 1988). Glycine betaine synthesis from choline is stimulated by osmotic stress only under aerobic conditions (Landfald & Strom, 1986). Thus, under aerobic conditions, choline and glycine betaine are likely to be more effective osmoprotectants for *E. coli* than proline, whereas under anaerobic conditions proline may be more effective and choline less effective. As noted above, multiple transporters are available to effect the accumulation of proline and glycine betaine. The relative cytoplasmic concentrations of proline, glycine betaine, or other solutes maintained by those systems under various conditions have not yet been assessed. Gutowski and Rosenberg (1976) showed that the relative activities of ion symporters and of transporters not energized via the protonmotive force differed under aerobic and anaerobic conditions. Thus the relative contributions of proline porter II, which is energized via respiration, and proline porter III, which is not likely to be energized in that manner, to the accumulation of osmoprotective solutes may be different under aerobic and anaerobic conditions. The passive membrane permeabilities of proline, glycine betaine and other compatible solutes have not been compared, but, as noted above, proline is likely to exit from *E. coli* at a rate comparable to that of active uptake when the cytoplasmic proline concentration is elevated to the levels required for osmoprotection. In addition to elevating the osmolarity of the cytoplasm, some compatible solutes may stabilize the higher order structures, and hence the catalytic activities, of macromolecules. Proteins are preferentially hydrated, and hence stabilized with respect to hydrophilic denaturants such as urea, in aqueous solutions that include certain organic osmolytes (Arakawa & Timasheff, 1985). Efforts to demonstrate specific enzyme activation by compatible solutes *in vitro* have met with limited success (Bowlus & Somero, 1979). Proline and glycine betaine have shown similar effectiveness as protein stabilizers using these criteria. The relative effectiveness of various compounds as osmoprotectants may thus be determined both by the mechanisms available for their accumulation and by the nature of their interactions with macromolecules.

As a ubiquitous solute, K^+ seems highly appropriate as a primary osmoregulator. However, high K^+ concentrations may lead to specific enzyme inhibition, competition for specific intracellular cation binding sites and unacceptable elevation of cytoplasmic ionic strength. Replacement of K^+ with organic osmolytes may thus meet the requirements of osmoregulation but avoid enzyme inhibition. Indeed, glycine betaine replaced K^+ as the predominant cytoplasmic osmolyte when *S. typhimurium* was exposed to increasingly extreme osmotic stress (media supplemented with 0 to 0.8 M NaCl and 1 mM glycine betaine) (Sutherland et al., 1986).

Glycine betaine and proline betaine support more rapid growth of enteric bacteria in aerobic media of inhibitory osmolarity than does proline (LeRudulier & Bouillard, 1983; Cairney et al., 1985*a*; Sutherland et al., 1986). Similarly, glycine betaine causes a larger reduction in the expression of *proU*::*lacZ* and *kdp*::*lacZ* gene fusions in response to osmotic stress than does proline (Cairney et al., 1985*a,b*). On this basis, glycine betaine has been considered intrinsically more effective as a compatible solute than proline. The difference in osmoprotective action between proline and glycine betaine may reflect differences between their interactions with cellular components and/or differences in the degree to which they accumulate under particular experimental conditions. The significance of those differences with respect to osmoregulation in natural settings remains to be determined.

Proline Porters II and III Will Serve as Foci for Future Studies of Osmosensory and Osmoregulatory Mechanisms

Both potassium transport via the TrkA and the Kdp transporters and proline or glycine betaine transport via proline porters II and III are activated in bacteria exposed to osmotic stress (Epstein, 1986; Faatz et al., 1988; Milner et al., 1988). Those responses occur with time constants on the order of one minute. Activation is sustained only in cells exposed to impermeant or excluded osmolytes like sucrose and Na^+; elevation of the extracellular osmotic strength with readily permeant solutes like glycerol elicits only transient activation. Those observations suggest that activation of both systems occurs in response to decreased turgor, not increased extracellular or intracellular osmolarity. The activation of K^+ uptake has been proposed to result directly from changes in transporter structure due to altered tension on the cytoplasmic membrane (Epstein, 1986). On the other hand, activation of K^+ or proline/betaine transport may result from primary changes in membrane potential and/or trans-membrane ion distribution. For example, Ken-Dror et al. (1986) proposed that osmoprotection of the halotolerant Gram-negative bacterium,

Ba_1, by glycine betaine arose through coupling between NADH-ubiquinone oxidoreductase-linked Na^+ extrusion and Na^+-linked betaine uptake. Since activation of proline porter II is observed in both whole cells and cytoplasmic membrane vesicles derived from *E. coli* (Milner et al., 1988), the biochemical mechanisms underlying its activation are now readily accessible to further study.

Potassium transport via the Kdp transporter, proline and betaine transport via proline porters II and III, choline transport and the synthesis of glycine betaine and other compatible solutes are all induced by osmotic stress in *E. coli* and *S. typhimurium* (reviewed above). The time courses of induction have not been fully analyzed. Jovanovich et al. (1988) reported differential rates of expression of *proU*::*lac*, *proP*::*lac*, *ompC*::*lac* and *ompF*::*lac* operon fusions when *E. coli* was cultivated in a rich medium. Interestingly, a dramatically increased rate of *proU* expression in response to osmotic stress (0.3 M NaCl) was maintained over many generations of growth, whereas induction of *proP* and *ompC* was both more limited and more transient. The accumulation of osmoregulatory solutes present in the culture medium may have influenced those responses. *proU*, but not *proP*, was identified as an osmoresponsive gene through gene fusion analysis. The above data serve as a reminder that the gene fusion technique may detect only those osmoresponsive functions whose induction is maintained in the steady state.

Only impermeant osmolytes induce and activate potassium (Epstein, 1986) and proline/betaine (Dunlap & Csonka, 1985; Milner et al., 1988) transport and induction ratios are reduced under conditions conducive to the accumulation of compatible solutes. In consequence, decreased turgor pressure, not absolute osmolarity, has been cited as the signal triggering those responses. Alternate proposals that induction of the *proU* locus occurs in response to cytoplasmic K^+ concentration (Sutherland et al., 1986; Gowrishankar, 1987) or cytoplasmic ionic strength (Higgins et al., 1987*a*) are based on correlations between steady-state K^+ levels and steady-state induction ratios for *proU*::*lac* operon fusions.

Many questions must be answered before the mechanics of osmoregulation can be understood. They include: (i) Does turgor decrease the elastic modulus (elasticity) of bacterial cytoplasmic membranes? (ii) What is the time course of turgor pressure (or membrane elastic modulus) variation in response to hyperosmotic stress? Are turgor and/or cell volume restored to pre-stress levels by osmoregulation? How does turgor depend on the provision of osmoprotectants and the function of osmoresponsive enzymes and genes? How can it be manipulated in vitro? (iii) What is the time course of changes in both membrane potential and transmembrane ion gradients in response to hyperosmotic stress (Castle et al., 1986)? How do they depend on the functions of both transporters and stretch- or voltage-activated ion channels (Martinac et al., 1987)? (iv) In what temporal sequence do the osmoresponsive transporters and genes respond to changes in turgor pressure, membrane potential and transmembrane ion distribution? (v) How do the relative contributions of the various osmoresponsive transporters to the maintenance of osmoprotective solute gradients vary as the cellular environment is altered? How are they relevant to the survival of organisms in natural environments?

Summary

Proline is utilized by all organisms as a protein constituent. It may also serve as a source of carbon, energy and nitrogen for growth or as an osmoprotectant. The molecular characteristics of the proline transport systems which mediate the multiple functions of proline in the Gram negative enteric bacteria, *Escherichia coli* and *Salmonella typhimurium*, are now becoming apparent. Recent research on those organisms has provided both protocols for the genetic and biochemical characterization of the enzymes mediating proline transport and molecular probes with which the degree of homology among the proline transport systems of archaebacteria, eubacteria and eukaryotes can be assessed. This review has provided a detailed summary of recent research on proline transport in *E. coli* and *S. typhimurium;* the properties of other organisms are cited primarily to illustrate the generality of those observations and to show where homologous proline transport systems might be expected to occur. The characteristics of proline transport in eukaryotic microorganisms have recently been reviewed (Horak, 1986).

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