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Proline Uptake Through the Major Transport System of *Salmonella typhimurium* Is Coupled to Sodium Ions

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Strains of *Salmonella typhimurium* deficient in one or more of the proline transport systems have been constructed and used to study the mechanism of energy coupling to transport. Proline uptake through the major proline permease (PP-I, *putP*) is shown to be absolutely coupled to Na^+ ions and not to H^+ ions as has previously been assumed. Transport through the minor proline permease (PP-II, *proP*), however, is unaffected by the presence or absence of Na^+. The effect of Na^+ on the kinetics of proline uptake shows that external Na^+ increases the V_{max} for transport. It seems probable that proline transport through PP-I is also coupled to Na^+ ions in *Escherichia coli*.

The study of energy coupling to bacterial amino acid transport has been complicated by a multiplicity of transport systems with overlapping specificities. Proline transport was once considered to be exempt from these difficulties, but over the last few years it has emerged that there are also multiple systems for the entry of this amino acid into both *Escherichia coli* and *Salmonella typhimurium*. The proline transport systems of these two species are similar but have been more completely characterized in *S. typhimurium*. The major proline permease PP-I is encoded by the *putP* gene and is under negative control by the product of its neighboring gene, *putA* (15, 19, 28). Strains deficient in *putP* still exhibit a low level of proline transport via a second system, PP-II, which is abolished by mutations at the locus *proP* (16, 22). In addition, there is a recent report of a third proline permease in *S. typhimurium*, PP-III, specifically stimulated by elevated osmotic pressure and encoded by the gene *proU* (4).

Energy for bacterial active transport is provided by coupling transport to the free energy of phosphorylated compounds or of ion gradients (2). In the latter case transport is almost always coupled to proton influx. However, in *E. coli* two transport systems, those for melibiose (24–26) and glutamate (8, 10, 14), are coupled to Na^+ ions. Proline transport has generally been considered to be energized by a proton symport mechanism and, indeed, has often been used as a model system for studying H^+-linked amino acid transport. However, several studies over the last 10 years have indicated that lithium (11–13) and Na^+ (23) can stimulate proline uptake into *E. coli* cells. In this paper we examine critically the role of Na^+ ions in proline transport by the various systems of *S. typhimurium*. We demonstrate that the major proline permease (PP-I, encoded by the *putP* gene) is absolutely dependent upon a gradient of sodium ions and is energized by Na^+-proline symport and not by H^+-proline symport as has previously been assumed.

MATERIALS AND METHODS

Bacterial strains and media. The genotypes of all bacterial strains used in this study are described in Table 1. Bacteria were grown with aeration at 37°C in LB medium or on LB agar plates (17) unless otherwise stated. All strains containing a Mu d1 insertion were grown at 30°C. Antibiotics were used at the following concentrations: carbenicillin (ampicillin, Amp), 50 μg ml^{-1}; kanamycin sulfate (Kan), 50 μg ml^{-1}; tetracycline (Tet), 15 μg ml^{-1}. Minimal glucose plates were based on the E medium of Vogel and Bonner as described by Roth (20). Where required, biotin supplements were added at 10 μg ml^{-1}. Nitrogen- and carbon-free medium (7) and MacConkey plates (6) supplemented with 1% (wt/vol) of the appropriate sugar have been described. LC medium is LB to which $CaCl_2$ (2 mM), glucose (0.1%), and thymidine (0.001%) have been added. P1 salts contain 12 mM $CaCl_2$ and 30 mM $MgSO_4$.

Transductions. Transductions were carried out with a high-transducing derivative of phage P22 *int-4* (21) as described by Roth (20). When using *galE* strains as the donor or recipient for P22-mediated transduction, cells were grown in LB medium supplemented with 0.2% galactose and 0.2% glucose to permit efficient synthesis of lipopolysaccharide phage receptors.

Isolation of Tn*5* insertions. Strain TT3416 contains an *E. coli* F factor with an inserted Tn*5*. A phage P22 lysate grown on TT3416 was used to infect a suitable recipient, selecting for Kan^r transductants. As the episome shares no homology with the *S. typhimurium* chromosome, Kan^r transductants can only arise by random transposition of the Tn*5* onto the recipient chromosome. An overnight culture of recipient cells (1.0 ml) was mixed with an equal volume of phage lysate and incubated with shaking at 37°C for 45 min. The cells were pelleted by centrifugation, suspended in 0.2 ml of LB medium, and plated on LB kanamycin plates containing 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid to prevent phage reinfection. About 2,000 Kan^r colonies were obtained per plate after 36 h of incubation. The Kan^r colonies from five separate plates were pooled and washed twice in E medium before applying further selection.

Isolation of Mu d1 insertions. The phage Mu d1(Ap^r *lac cts62*) (3) was used as a transposable element to deactivate specific genes. Because Mu is a coliphage and will not normally infect *S. typhimurium*, we used a Mu-P1 hybrid helper phage that confers P1 host range and thus is capable of infecting *galE* strains of *S. typhimurium* (5). A Mu d1 lysate was prepared by heat induction of JL3473, grown in LC medium plus 10 mM $MgSO_4$, as described by Csonka et al. (5). Recipient cells were grown in LC medium containing

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TABLE 1. Bacterial strains

S. typhimurium LT2 strain	Genotype	Source or construction
CH219	*galE503 bio-203*::Tn*10*	LT2 transduced to Tet^r Gal^- with TA3425 as donor
CH223	*galE503 bio-561*	Tet^s derivative of CH219
CH321	*galE503 bio-561 putP201*::Mu dl	Mu dl insertion into CH223
CH378	*galE503 bio-561 putP214*::Tn*5*	Tn*5* insertion into CH223
CH486	*galE503 bio-561 putP201*::Mu dl *proP1667*::Tn*5*	Tn*5* insertion in CH321, selected for AC^r in high salt
CH495	*galE503 bio-561 putP214*::Tn*5 proP1669*::Mu dl	Mu dl insertion in CH378, selected for Ac^r in high salt
CH496	*galE503 bio-561 proP1669*::Mu dl	CH495 transduced to *putP*$^+$
CH500	*galE503 bio-561 putP214*::Tn*5 proP1673*::Mu dl	Mu dl insertion in CH378, selected for DHP^r
CH571	*galE503 bio-561 proP1673*::Mu dl	CH500 transduced to *putP*$^+$
CH576	*galE503 bio-561 proP1673*::Mu dl *putA810*::Tn*10*	CH571 transduced to Tet^r with TT946 as donor
JL3473	*galE496 metA22 metE55 rpsL120 hsdL6 hsdSA29* Mu dl(Ap *lac*) Mu *cts* hP1 no. 1	C. L. Turnbough
LJ3	*melA301*	W. W. Kay
TA3425	Δ*hisF645 galE503 bio-203*::Tn*10*	G. F.-L. Ames
TT184	*proBA662*::Tn*10*	J. Roth
TT946	*putA810*::Tn*10*	J. Roth

10 mM $MgSO_4$. A collection of random Mu dl insertions in the *S. typhimurium* chromosome was prepared by mixing 0.1 ml of the Mu dl lysate (10^8 PFU) with 0.1 ml of P1 salts and 0.1 ml of an overnight culture of the appropriate *galE* recipient, incubating without shaking for 30 min at 30°C, and plating on LB ampicillin plates. Approximately, 10^4 Amp^r colonies were pooled and washed twice with E medium before further selection. Strains containing Mu dl insertions were tested genetically during each set of experiments to ensure the Mu dl had not transposed; in no case was instability observed.

Identification of genotypes and phenotypes. Proline auxotrophy was determined by radial streaking on MG plates around filter paper disks impregnated with proline (1 μmol). Gal^- or Mel^- strains were tested with MacConkey agar plates containing the appropriate carbon source. The Put^- phenotype was identified by the inability of strains to utilize 0.1% proline as the sole nitrogen source when grown on nitrogen- and carbon-free plates containing 0.1% succinate as the carbon source (18). *putA* mutations were tested by the method of Ratzkin and Roth (19). An overnight culture of cells was starved for 24 h at room temperature and then radially streaked around a disk of the dipeptide L-prolyl-L-valine (2 μmol) on a nitrogen- and carbon-free plate containing 0.1% succinate as the carbon source. The dipeptide is taken up via one of the peptide permeases and cleaved intracellularly into its constituent amino acids. In *putA*$^+$ strains the proline so generated can be catabolized and used as a nitrogen source; *putA* strains, however, are unable to utilize this substrate.

Mutations in *putP* and *proP* were identified by radially streaking on MG plates around filter disks impregnated with the appropriate toxic proline analog, L-azetidine-2-carboxylic acid (AC) or 3,4-dehydro-DL-proline (DHP) (4, 19). *putP*$^+$ *proP*$^+$ strains are sensitive to both AC (150 μg) and DHP (600 μg); *putP proP*$^+$ strains are AC^r, but DHP^s; *putP proP* derivatives are resistant to both analogs. Such tests could only be applied to proline prototrophs since the addition of proline to the media reverses the toxic effects of the analogs.

Transport assays. Cells for transport experiments were grown at 30°C in potassium- and sodium-free (KONO) medium. KONO medium contains 0.4 mM $MgSO_4$, 6 μM $(NH_4)SO_4 \cdot FeSO_4$, 20 mM $(NH_4)_2HPO_4$, 10 mM bistrispropane, and 120 mM choline chloride. The medium was titrated to pH 7 with concentrated HCl before sterilization. For bacterial growth, potassium chloride (1 mM) and glucose (0.2%) were added. For transport experiments glucose, KCl and NaCl were added as indicated. All solutions for transport experiments were made and stored in plastic ware. The contaminating sodium level was measured after every experiment by flame photometry (Corning 400) and was always less than 30 μM.

To measure transport activity, an overnight culture of cells was diluted 1:10 in KONO and grown at 30°C to an optical density at 600 nm of 0.8. The cells were harvested by centrifugation at 12,000 rpm for 5 min, washed twice in KONO, and finally suspended to 0.5 mg of cells ml^{-1} in KONO containing chloramphenicol (50 μg ml^{-1}). Glucose (1 mM), KCl (1 mM), and other compounds were added as indicated below. The cell suspensions were preincubated for 5 min at 30°C before adding [*U*-^{14}C]-proline (20 Ci $mmol^{-1}$; Amersham Corp.) to 10 μM (unless otherwise stated). Samples of 0.1 ml were removed at appropriate time intervals, passed through a glass fiber filter (Whatman; GFF), the filtered cells washed with 2.5 ml of preincubation buffer, and the accumulated proline determined by scintillation counting as previously described (23). Each data point was determined in duplicate, and each experiment was repeated with at least two independent cell preparations. In addition, although we generally only show data for one strain of each genotype, each experiment was repeated with at least one additional, independently derived strain of similar genotype. For experiments involving ^{14}C-labeled thiomethyl galactoside (TMG; 14 Ci mol^{-1}), or D-alanine (18.5 Ci mol^{-1}) these substrates were also added at 10 μM.

Sodium loading of cells. Cells were grown to an optical density at 600 nm of 0.8 in KONO, harvested by centrifugation, washed once in 20 ml of Tris-chloride (0.12 M, pH 8.0), and finally suspended to 10 mg of cells ml^{-1} in the same buffer. This cell suspension was incubated at 30°C for 2 min, EDTA was added to 1 mM, and the suspension was incubated for a further 10 min with gentle agitation. The cells were then diluted fivefold with 0.12 M Tris-chloride (pH 8.0) to dilute out the EDTA, washed twice with NaCl–HEPES (*N*-

2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–choline chloride buffer (containing [per liter] 50 mM NaCl, 10 mM HEPES [pH 7.5], and 100 mM choline chloride) and finally suspended in the same buffer to 0.5 mg ml^{-1} for the transport assays.

RESULTS

To ensure complete deactivation of the various proline transport genes and to facilitate strain constructions, the drug-resistant transposable elements Tn*5*, Tn*10*, and Mu dl (Ap^r *lac*) were used as mutagens. All strains used for transport studies were derived from CH223 and are therefore isogenic except for the introduced *pro* or *put* mutations.

Construction of CH223. The *galE503* mutation required to render *S. typhimurium* PI sensitive was transferred to LT2 as follows. A P22 lysate of TA3425 (*bio203*::Tn*10 galE503*) was used to transduce LT2 to Tet^r. Because of the linkage between bio and *galE*, a proportion (about 10%) of the Tet^r cells also became Gal^-. One such derivative (CH219) was then made Tet^s by selection for fusaric acid resistance (1). This strain, CH223, was used as the parental strain from which all *pro* and *put* mutants were derived.

Isolation of Tn*5* insertions in *putP*. Wild-type *S. typhimurium* is sensitive to the toxic proline analog AC, whereas strains mutated for *putP* are resistant (19). A pool of random Tn*5* insertions in strain CH223 was plated on MG plates supplemented with biotin and AC (40 μg ml^{-1}). As AC resistance can be caused by a variety of different mutational events, AC^r colonies were screened for the Put^- phenotype by their inability to utilize 0.1% proline as the sole nitrogen source. The Tn*5* insertions were additionally mapped to the *put* region of the chromosome, at 22 min, by demonstrating cotransduction with a Tn*10* insertion in *putA* (strain TT946).

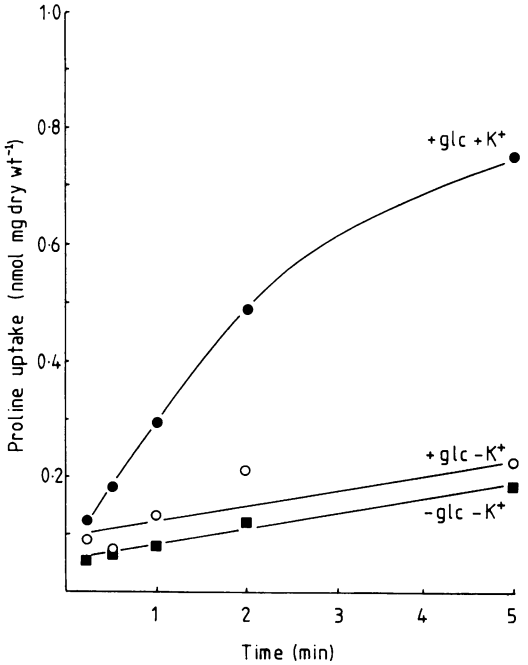

FIG. 1. Effect of K^+ on proline uptake in Na^+-loaded cells. Cells of CH496 (*putP*$^+$ *proP*) were preloaded with Na^+ and incubated with 10 μM [*U*-^{14}C]proline in the presence or absence of 1 mM glucose. KCl (1 mM) was included in certain incubations, as indicated, and proline uptake was assayed over a period of 5 min.

TABLE 2. Effect of K^+ on transport in Na^+-loaded cells[a]

Substrate	Strain	Transport rate (nmol mg^{-1} min^{-1})	
		Without K^+	With K^+
Proline	CH496 (*putP*$^+$ *proP*)	0.027	0.245
Proline	CH378 (*putP proP*$^+$)	0.122	0.129
TMG	CH378 (*putP proP*$^+$)	0.004	0.026
D-Alanine	CH378 (*putP proP*$^+$)	0.379	0.620

[a] Cells were preloaded with Na^+ as described in the text. Proline, TMG, or D-alanine uptake, each from a 10 μM solution, was assayed in the presence or absence of K^+ as indicated.

To confirm each strain carries only a single Tn*5* insertion, and that this is responsible for the $PutP^-$ phenotype, the linkage between Kan^r and AC^r was tested and shown to be 100%. (When the Tn*5*-containing strain was used as the donor, linkage was reduced to about 90% due to induction of transposition, which occurs upon introduction of the Tn*5* into repressor-free cells.)

Isolation of Mu dl insertions in *putP*. Mu dl insertions in *putP* were selected from a pool of independent Mu dl insertions in strain CH223. Selection for AC resistance and screening for the Put^- phenotype was carried out as described above. Transduction of putative *putP*::Mu dl insertions to Tet^r with a P22 lysate of TT946 (*putA810*::Tn*10*) showed 80 to 90% linkage between the Mu dl insert and *putA*. All Tet^r transductants that became AC^s also became Amp^s, confirming that only a single Mu dl insertion is present in each strain.

Isolation of Mu dl insertions in *proP*. Mutations in *proP* cannot be selected directly, but must be selected in a *putP* background. Thus, a pool of random Mu dl insertions in strain CH378 (*putP214*::Tn*5*) was constructed, and insertions in *proP* were selected in one of two ways. (i) The first selection procedure was resistance to the toxic proline analog DHP. Although DHP is transported by PP-I, *putP*-deficient strains remain sensitive to 80 μg of DHP ml^{-1}. This is due to uptake via PP-II; an additional mutation in *proP* abolishes sensitivity (4; unpublished results). *proP* insertions were therefore selected in a *putP* background by spreading cells on MG plates supplemented with biotin and 80 μg of DHP ml^{-1}. (ii) The second selection procedure was resistance to AC at high salt concentrations. Although *putP* mutations confer resistance to 40 μg of AC ml^{-1}, sensitivity is restored by increasing the osmolarity of the medium to about 1.0 osM (e.g., by adding 0.3 M NaCl [4; unpublished data]). Mutations in *proP* confer resistance to 40 μg of AD ml^{-1} at high osmotic pressure. Although it has been reported that a third proline permease (PP-III, encoded by the *proU* gene) is specifically stimulated by high osmotic pressure (4), we have shown that in a *putP* background the major proline uptake system at high osmotic pressure is still PP-II (unpublished data). Uptake of AC through PP-III is insufficient to confer toxicity to AC at 40 μg ml^{-1}. Thus, Mu dl insertions in *proP* were obtained in a *putP* background by selection for resistance to 40 μg of AC ml^{-1} on MG plates containing 0.3 M NaCl.

To demonstrate that each derivative contained only a single Mu dl insertion and that this insertion was in *proP*, we had to be able to transduce these *putP proP* strains to $ProP^+$. This was achieved in a two-step process by transduction to proline auxotrophy with a P22 lysate of TT184 (*proBA662*::Tn*10*) and subsequently to $ProP^+$ by selecting for growth on 16 μM proline (16) with P22 grown on LJ3

(*melA*) as the donor. To avoid carry-over of proline from the phage preparations, P22 lysates were prepared on cells grown in E medium. Because transduction of a *putP proP proAB* strain to growth on 16 μM proline could be achieved by transduction to $putP^+$ or $proAB^+$ as well as to $proP^+$, transductions were carried out on MG plates containing both kanamycin and tetracycline to ensure that the insertion mutations in *proAB* and *putP* were retained. All $proP^+$ transductants simultaneously became Amp^s. Each of the Mu dl insertions was found to be about 20% cotransducible with *melA* (16), confirming that the insertions were indeed in *proP*. Strains CH495 and CH500 contain *proP*::Mu dl insertions selected by resistance to AC at high osmolarity and resistance to DHP, respectively.

Isolation of Tn*5* insertions in *proP*. Tn*5* insertions in *proP* were isolated from a pool of independent insertions in CH321 (*putP201*::Mu dl) by selection for AC^r at high osmolarity. The presence of a single copy of the transposon and its linkage to *mel* were determined as described above.

Construction of $putP^+$ *proP* derivatives. Since *proP* mutations could only be selected in a *putP* background, $putP^+$ *proP* strains had to be constructed by transducing a *putP proP* strain to $putP^+$. This was achieved by transduction to growth on 0.1% proline as the sole nitrogen source on N^-C^- plates containing 0.1% succinate as the carbon source. Colonies appearing after 3 days at 30°C were characterized as Put^+, AC^s and Kan^s (due to replacement of *putP*::Tn*5* with wild-type DNA), whereas the remaining Amp^r was due to the Mu dl insertion in *proP*. The Mu dl insertions were shown to have remained in *proP* and not to have transposed, by sequential transduction to *putP*::Tn*5* and $proP^+$; all such $proP^+$ derivatives became simultaneously Amp^s.

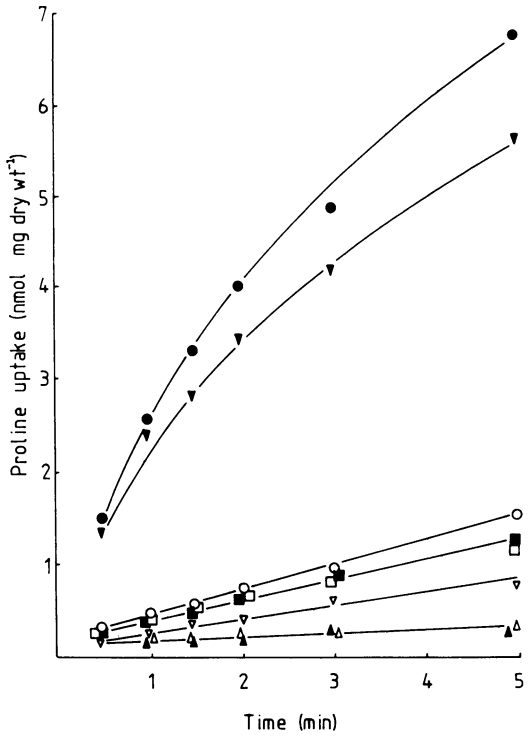

FIG. 2. Effect of Na^+ on proline uptake by strains deficient in PP-I or PP-II. Cells were grown in KONO in the presence (closed symbols) or absence (open symbols) of 5 mM NaCl. Proline uptake was measured as described in the text. The strains used were as follows: (●,○) $putP^+$ $proP^+$ (LT2, CH223); (▼, ▽) $putP^+$ *proP* (CH496, CH571); (■, □) *putP* $proP^+$ (CH321, CH322, CH378); (▲, △) *putP proP* (CH486, CH495, CH500).

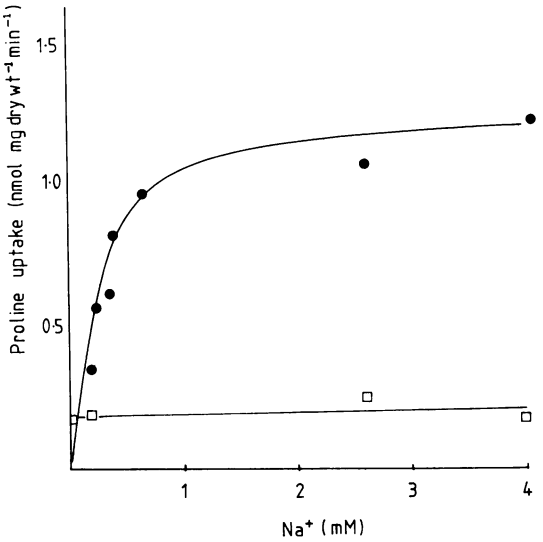

FIG. 3. Effects of various sodium concentrations on proline uptake through PP-I. Proline uptake by (●) CH571 ($putP^+$ *proP*) or (□) CH322 (*putP* $proP^+$) grown in KONO was measured in the presence of various concentrations of external sodium. Sodium concentrations were measured by flame photometry.

Construction of *putA* derivatives. The *putA* gene product represses the expression of *putP* (15). For certain experiments we required maximal expression of *putP*. *putA* mutation were therefore introduced into strains by transduction to Tet^r with a P22 lysate grown on TT946 (*putA*::Tn*10*). All Tet^r derivatives remained AC^s and were shown to be *putA* as described above.

Potassium stimulates proline uptake by Na^+-loaded cells. We have previously observed that in *E. coli* proline uptake by Na^+-loaded cells is stimulated by potassium (23). We initially wished to determine whether the same effect is observed in *S. typhimurium*. Cells of strain CH496 ($putP^+$ *proP*) were preloaded with sodium, and proline transport was assayed in the presence or absence of 1 mM KCl. Although glucose had no effect on proline uptake, the addition of external KCl stimulated proline transport sevenfold (Fig. 1). This effect is best explained by K^+-Na^+ exchange restoring the Na^+ gradient necessary for high rates of proline transport (23). If this explanation is correct, then the addition of potassium to Na^+-loaded cells should stimulate uptake by all Na^+-dependent transport systems, but not of H^+-dependent systems. We therefore measured the effects of such treatment on the uptake of TMG, which is transported by the Na^+-dependent melibiose system, and D-alanine, which is transported in a proton-dependent manner (2) (Table 2). Although the uptake of melibiose is stimulated six- to sevenfold by the addition of KCl, little effect is observed on D-alanine transport. Thus, the stimulation of transport by KCl in Na^+-loaded cells is specific for Na^+-linked systems. Table 2 also shows that whereas proline transport via PP-I is stimulated by external K^+, uptake via PP-II is unaffected.

Proline transport through PP-I is sodium dependent. To demonstrate more directly the requirement for sodium ions during proline uptake, proline transport by the wild-type strain ($putP^+$ $proP^+$) was measured in the presence and absence of external sodium (Fig. 2). In the absence of Na^+

TABLE 3. Effect of Na^+ on kinetic parameters of proline uptake[a]

Sodium concn (mM)	K_m (μM)	V_{max} (nmol^{-1} mg^{-1} min^{-1})
0.1	3.6	0.7
10.0	3.4	4.6

[a] The initial proline uptake rates at both saturating (10.0 mM) and limiting (0.1 mM) concentrations of sodium were determined over a proline concentration range of 0.25 μM to 10 μM. The kinetic parameters were derived from Lineweaver-Burke plots of these data.

ions proline transport is very considerably reduced. Because of the multiple proline transport systems in *S. typhimurium*, we wished to determine which of these systems is involved in the sodium effect. Thus, proline uptake was measured for a variety of strains deficient in one or more of the proline uptake systems (Fig. 2). In strains deficient in PP-I (*putP proP*$^+$), proline transport is unaffected by sodium ions. In addition, in the absence of sodium ions proline transport by the wild-type strain is reduced to a level essentially identical to that of a strain deficient in PP-I (*put proP*$^+$). Thus, it is clear that it is the major proline permease, PP-I, that is sodium dependent; sodium has no effect on PP-II. Interestingly, in strains lacking both PP-I and PP-II (*putP proP*) proline transport is barely detectable. Thus, under the conditions used here, the putative third proline permease, PP-III (*proU*), is unimportant. It should also be noted that in strains deficient in PP-II, but proficient in PP-I (*putP*$^+$ *proP*), there is a small amount of residual uptake via PP-I in the absence of sodium ions. This is due to the small amount of contaminating sodium (15 to 30 μM) present in the "sodium-free" media.

Effect of various concentrations of external Na^+ on proline uptake. Figure 3 shows the effect of various concentrations of external sodium on proline transport. Proline transport via PP-I (strain CH571; *putP*$^+$ *proP*) increases with increasing sodium concentrations, saturating at millimolar levels of sodium. Such stimulation is not seen for PP-II (strain CH322, *putP proP*$^+$). From this data the K_t for sodium stimulation of proline transport via PP-I can be calculated to be 350 μM.

Sodium affects the V_{max} of proline transport. To examine the kinetic parameters of proline transport that are affected by Na^+, proline uptake over a range of substrate concentrations was assayed in the presence of both limiting and saturating concentrations of sodium ions. For these experiments strain CH576 (*putP*$^+$ *putA proP*) was used to ensure complete derepression of PP-I activity. The results of such experiments were used to calculate V_{max} and K_m for transport under each set of conditions (Table 3). Clearly, saturating levels of Na^+ cause a sevenfold stimulation in V_{max}, whereas the K_t is not significantly altered.

Sensitivity to toxic proline analogs is Na^+ dependent. If proline transport is Na^+ dependent, then the inhibitory effects of toxic proline analogs should also be affected by the presence or absence of sodium. An overnight culture of LT2 grown in KONO was diluted 1:20 in fresh medium with or without NaCl (5 mM). Cells were grown to an optical density at 600 nm of 0.4, at which point the indicated amounts of AC or DHP were added, and the subsequent growth rates were measured (Table 4). In the absence of Na^+ cells are insensitive to the concentrations of AC and DHP used. However, in the presence of Na^+ the toxicity of both analogs is restored.

DISCUSSION

The evidence presented here demonstrates that proline transport by the major proline permease of *S. typhimurium* occurs by sodium symport. Several lines of evidence lead us to this conclusion. (i) The addition of K^+ to cells preloaded with sodium stimulates proline transport. This effect is only observed for transport via PP-I and not via PP-II. To confirm that the K^+ stimulation of proline uptake is due to the formation of an Na^+ gradient by Na^+-K^+ exchange, we studied the effects of K^+ on TMG and D-alanine uptake. As anticipated, transport of TMG via the Na^+-dependent melibiose system was stimulated by external K^+, whereas the H^+-linked D-alanine transport system was not affected. (ii) The toxic effects of proline analogs transported by PP-I are reduced in the absence of sodium. (iii) Proline transport is severely reduced in sodium-free media. The use of strains defective in either PP-I (*putP*) or PP-II (*proP*) shows that this effect is entirely due to a reduction in transport via PP-I. Indeed, in the absence of sodium ions, proline uptake via PP-I is completely prevented; as sodium ions are restored, proline uptake increases until it reaches the maximum rate at about 1 mM Na^+. The presence or absence of Na^+ had no effect on proline transport via PP-II. Under the conditions used any transport via the putative third proline permease (PP-III, encoded by *proU*) was insignificant.

Although the data presented here refer only to *S. typhimurium*, it seems likely that proline transport in *E. coli* is similarly coupled. First, there is a great similarity between proline transport in the two organisms in many other respects. Second, proline transport in *E. coli* has been reported to be stimulated by Li^+ (11–13) and Na^+ (23), although in these instances a clear-cut role for the cation in symport was not established due to the multiplicity of proline transport systems. Only the present study of strains defective in one or more of the proline permeases allows an unequivocal demonstration of Na^+ coupling. Third, proline has recently been shown to stimulate lithium uptake in deenergized *E. coli* cells (27). In addition, using proline transport-defective mutations in *E. coli* we have recently shown a similar Na^+ requirement for transport via PP-I in this species (G. C. Rowlands and I. R. Booth, unpublished observations).

The data presented above show that Na^+ stimulates proline transport by increasing the V_{max}. Interestingly, Na^+ also effects an increase in the V_{max} for proline uptake by *Mycobacterium phlei* (9). This is in contrast with the stimulatory effects of Na^+ on the other Na^+-linked transport

TABLE 4. Effect of Na^+ on sensitivity to AC and DHP[a]

Analog	Without Na^+			With Na^+		
	Doubling time (min)		% Inhibition	Doubling time (min)		% Inhibition
	Without analog	With analog		Without analog	With analog	
AC (300 μM)	64	64	0	65	124	53
DHP (150 μM)	60	60	0	53	156	66

[a] The doubling time was calculated from the rate of change of optical density at 650 nm during exponential growth.

systems, those for melibiose and glutamate, where the effect is reported to be on K_t. It has previously been suggested that the *phs* mutation in *E. coli* may cause a specific defect in Na^+-linked transport systems possibly affecting a common Na^+-coupling subunit (29, 30). However, the *phs* mutation has no effect on proline uptake (29; unpublished results). At first sight this appears to contradict our evidence that proline uptake is linked to Na^+. However, it is now clear that the *phs* mutation is more complex than originally suspected. The mutation is quite pleiotropic; it affects transport systems other than those that are Na^+ linked and may affect the transcriptional-translational efficiency of several genes (unpublished results). Thus, there is no evidence for a common Na^+-coupling subunit or any reason to suppose that the mechanism of coupling to proline differs from that for melibiose and glutamate.

Proline uptake has often been used as a model system for characterizing proton-linked transport systems in both whole cells and vesicles. The present demonstration of Na^+-proline coupling does not negate most of the energetic studies of proline transport in vesicles, since the generation and maintenance of the Na^+ gradient is mediated by an H^+-linked system, the Na^+-H^+ antiport. It is, of course, also possible that there are strain differences. However, it would not be too surprising if linkage to sodium has been overlooked in vesicles in view of the multiple proline transport systems and the fact that the K_t for Na^+ is about 350 μM, well within the normal range of contamination from glassware. Nevertheless, it is clearly important to reassess certain results in the light of the present observations.

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