Amino Acids (2001) 21: 161–174

Amino
Acids

Substrate recognition by proline permease in *Salmonella*

M.-K. Liao[1] and **S. Maloy**[2]

[1]Department of Biology, Furman University, Greenville, South Carolina, U.S.A.
[2]Department of Microbiology, University of Illinois, Urbana, Illinois, U.S.A.

Accepted December 1, 2000

Summary. Proline transport is required for catabolism of proline as a carbon, nitrogen, and energy source, and for accumulation of proline during adaptation to osmotic stress. These physiological processes are widespread in nature, and play essential roles in the virulence of both prokaryotic and eukaryotic pathogens. In enteric bacteria, the major proline permease is encoded by the *putP* gene. To identify the structural features required for substrate recognition by PutP, we assayed the transport and toxicity of a variety of natural and synthetic derivatives of proline. The results indicate that the substrate binding site of proline permease consists of a hydrophobic pocket that accommodates C3, C4, and C5 of the pyrrolidine ring. Both 4- and 5-membered rings fit into the substrate binding pocket, but 6-membered rings are excluded. Analogs with substituents on the C4 position are also excluded. In addition, the binding site includes a hydrophilic region that recognizes the imino and carbonyl groups. A free carboxyl group is not required. Taken together, these results may be used to design new synthetic inhibitors of proline transport that can effectively block proline uptake by microbial pathogens.

Keywords: Amino acids – Proline permease substrate specificity

Introduction

Solute transport is an essential and often rate-limiting step in a metabolic pathway. In *Salmonella* and *Escherichia coli*, approximately 40% of all transport is catalyzed by ion/solute co-transport systems (permeases) (Wilson, 1978). The binding of the substrate and the counter-ion to their respective binding sites in the permease presumably leads to a conformational change that results in the release of substrate into the cytoplasm. Hence, permeases must display a high degree of specificity in substrate recognition in order to discriminate between different molecules presented to the cell (Saier, 2000). Four general approaches have been taken to gain insight on the structure and function of the substrate binding sites in permeases: (i) labeling with inhibitors such as N-ethylmaleimide that bind tightly to a permease and

prevent transport (Frillingos and Kaback, 1996; Venkatesan and Kaback, 1998; Sahin-Toth et al., 2000); (ii) isolation of mutants that alter the substrate specificity of the permease (Dila and Maloy, 1986; Kawakami et al., 1988; Myers and Maloy, 1988; Myers et al., 1991; Franco and Wilson, 1996); (iii) site-directed mutagenesis to alter amino acid residues believed to be in or near the substrate binding site (Viitmen et al., 1985; Kaback, 1987; Quick et al., 1996; Quick and Jung, 1997; King and Li, 1998; Quick and Jung, 1998; Quick et al., 1999); and (iv) transport assays using substrate homologues to determine the structural motifs required for recognition and binding by the substrate (Tristram and Neale, 1968; Rowland and Tristram, 1975; Olsen and Brooker, 1989). Although these approaches are indirect, direct approaches are generally infeasible due to the hydrophobicity of membrane proteins and the difficulty of obtaining suitable crystals of many permeases (Kaback and Wu, 1997).

Proline transport is a useful model system for understanding how an ion-driven permease functions. The major route of proline uptake in *Salmonella* and *E. coli* is via the *putP* gene product, proline permease (Wood and Zadworny, 1979). Proline permease is an integral membrane protein with a molecular weight of about 25 Kd that catalyzes active transport of proline by sodium-proline symport (Cairney et al., 1984; Chen et al., 1985). The *Km* for proline uptake by this system is 2 μM (Myers et al., 1991). Two betaine transport systems can also transport proline (Anderson, Menzel et al., 1980; Stalmach et al., 1983; Grothe et al., 1986; Higgins et al., 1987; Milner et al., 1988; Wood, 1988; Csonka and Hanson, 1991): a low-affinity permease encoded by the *proP* gene with a *Km* for proline uptake of 300 μM, and a permease encoded by the *proU* gene that is induced by high osmolarity. However, the PutP permease is the primary proline transport system when proline is used as a sole nitrogen or carbon source (Ratzkin and Roth, 1978; Liao et al., 1997).

Direct approaches have been taken to study the active site of proline permease. Isolation and genetic characterization of mutants that alter the substrate specificity of proline permease localized amino acid residues that constitute the proline and sodium binding sites (Dila and Maloy, 1986; Myers and Maloy, 1988). In this study, we used an indirect biochemical approach to infer the structure of the substrate-binding site. Assaying the binding specificities of proline analogs allows the identification of structural features that are required for recognition by proline permease (Tristram and Neale, 1968; Rowland and Tristram, 1975; Hitz et al., 1986; Marmorstein and Sigler, 1989; Olsen and Brooker, 1989). Numerous natural and synthetic derivatives of proline are available. The kinetics of inhibition of proline transport by several proline analogs were determined in *E. coli* over two decades ago (Tristram and Neale, 1968; Rowland and Tristram, 1975). However, the results can not be directly applied to proline permease for several reasons: (i) the study was done before multiple proline transport systems were identified so it was not possible to distinguish which transport system was being studied, (ii) expression of the *putP* gene was not induced, (iii) the concentration of proline used was less than 1/10 of the *Km*, and (iv) the time points taken did not measure the initial velocity of transport (Myers et al., 1991).

This study avoided the problem of multiple transport systems by: (i) eliminating the minor proline transporters, (ii) pregrowing the cells under conditions that induce expression of the *put* operon, (iii) assaying transport with proline concentration 5-fold higher than the *Km* of proline permease, and (iv) measuring the velocity of the permease activity during the first 15 seconds of the reaction. Using these conditions optimized for proline transport by PutP, we determined the *Ki* of the available proline analogs and determined the relative affinity for each analog. A molecular modeling program CAChe 3.5 (Computer Aided Chemistry, CAChe Scientific, Inc.) was used to visualize the three-dimensional structure of the proline analogs to predict important similarities and differences between the analogs that are substrates for proline permease and the analogs that are not substrates for proline permease.

Materials and methods

Bacterial strains

Strains used were derived from wild-type *Salmonella enterica* serovar Typhimurium LT2 (abbreviated *S*. Typhimurium). Strain MST183 has a *putP*::MudJ insertion mutation that disrupts the PutP permease (Myers et al., 1991). Strain MST700 has a *proP*::Tn10dCam insertion mutation that disrupts the ProP permease (Myers et al., 1991).

Media and growth conditions

For growth in rich medium, bacteria were routinely cultured in 8 g/l Nutrient Broth (Difco Laboratories, Detroit, MI.) with 0.5% NaCl. For growth in minimal medium, bacteria were cultured in NCE with indicated carbon sources and NH_4^+ as the nitrogen source (Berkowitz et al., 1968; Ratzkin and Roth, 1978). For transport assays, bacterial cells were grown in NCE medium containing 0.6% succinate as a carbon source and 0.2% proline to induce the *put* operon (NCE-succinate-proline). Cell growth was determined by measuring turbidity with a Klett-Summerson colorimeter with a green filter. Sensitivity to proline analogs was assayed by measuring the zone of inhibition after radial streaking (Roth, 1970). Appropriate amounts of each analog were added to a sterile filter disk placed in the center of an NCE-succinate plate (Table 1).

Transport assays

Transport of L-[^{14}C] proline (250 mCi/mmol or 265 mCi/mmol, ICN, Irvine, Ca.) was assayed as described by Myers et al. (1991). Cells were subcultured from NCE-succinate-proline medium into the same medium and grown to exponential phase (100 Klett Units). The cells were then placed on ice for 10 min and washed twice with an equal volume of ice-cold NCE medium by centrifugation at 3,000 × g for 10 min at 4°C. The washed cell pellet was suspended in a half volume of ice-cold starvation buffer (NCE, 0.5 mM glucose, 25 μg/ml chloramphenicol) and transferred to a 25 ml Erlenmeyer flask, where cells were starved for proline by shaking at 100 rpm for 15 min at room temperature. The transport assay was initiated by adding 0.2 ml of cell suspension to 0.2 ml of reaction mixture (NCE + 1 mM glucose + 50 μg/ml chloramphenicol + 10 μM of L-[^{14}C] proline) (Chen et al., 1985). Transport was stopped by adding 5 ml of stopping buffer (5 mM 2(*N*-morpholino)-ethanesulfonic acid, 5 mM Tris, 300 mM KCl, 2 mM $HgCl_2$, pH 7.0) (Lancaster and Hinkle,

Table 1. Growth inhibition by proline analogs

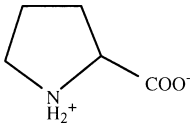
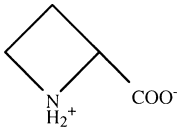
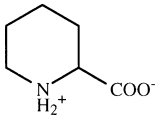
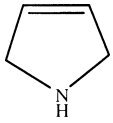
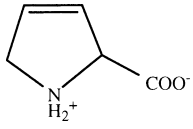
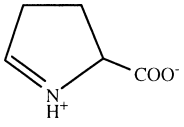
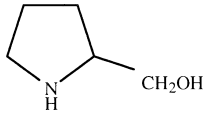
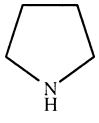
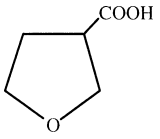
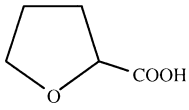
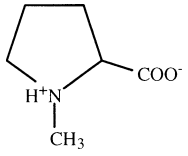
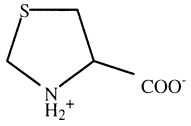
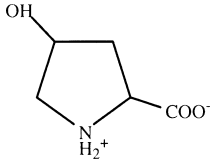
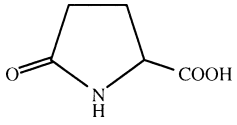
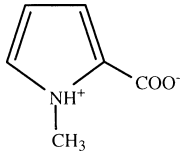
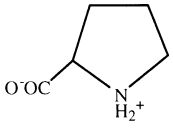
Compound	Chemical structures	Amount required for growth inhibition (μg)	Zone of growth inhibition[1] (mm)
L-Proline		>2,000	0
Group I: Modification of the ring structure			
azetidine-2-carboxyl acid		600	35
DL-pipecolinic acid		>2,000	0
Group II: Double bond in the ring			
3-pyrroline		>2,000	0
3,4-dehydroproline		100	25
pyrroline-5-carboxylic acid		>2,000	0
Group III: Modification of carboxyl group			
prolinol		>2,000	0
pyrrolidine		>2,000	0

Table 1. (continued)

Group IV: Modification of imino group			
(±)tetrahydro-3-furoic acid		>2,000	0
(±)tetrahydro-2-furoic acid		>2,000	0
N-methyl-L-proline		>2,000	0
Group V: Modification at C4 position			
thioproline		1,000	7
cis-4-hydroxy-L-proline		>2,000	0
Group VI: Modification at C5 position			
DL-2-pyrrolidone-5-carboxylic acid		>2,000	0
Other modifications			
1-methyl-2-pyrrole carboxylic acid		>2,000	0
D-proline		>2,000	0

Shown in this table are the predicted chemical structures of proline and proline analogs under the physiological conditions used in this study. They fell into six groups according to the structural differences between these analogs and proline. Also included are the growth inhibitions by different proline analogs. [1]Sensitivity to proline analogs was quantified by radial streaking bacterial culture MST700 (*putP*$^+$ *proP*$^-$) from a filter disc impregnated with 20 µl of the analogs at appropriate concentrations. The zone of growth inhibition extending from the edge of the disc was measured.

1977). Triplicate samples were taken at 0, 5, 10, and 15 sec. Within 8 min, the cells were filtered through Sartorius cellulose nitrate filters with 0.45 μM pore-size and washed once with 5 ml stopping buffer. The uptake of radioactive proline was quantified by adding 4 ml of BioSafe II scintillation cocktail to each air-dried filter and counting each sample in a 9800 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA.) using a full carbon window.

Proline analogs were purchased from Sigma Chemical Co. (St. Louis, MO.) Transport was assayed after addition of an analog at appropriate concentrations to reaction mixtures containing L-[^{14}C]proline.

The *Km* and *Vmax* values for proline permease were determined as described by Cornish-Bowden (Cornish-Bowden et al., 1978; Cornish-Bowden, 1979; Myers et al., 1990). The *Ki* values of the analogs were determined by re-plotting the initial velocity versus the concentration of the inhibitor. In such Dixon plots, the *Ki* equals the negative of the X intercept. Percent inhibition was determined by measuring the velocity of proline transport using 10 μM L-[^{14}C] proline in the presence and absence of the non-radioactive inhibitory analogs at indicated concentrations. All the proline transport assays included strain MST183 (*putP*::MudJ) as a negative control. Under the conditions used, the null mutation in the *putP* gene blocked all detectable proline transport.

Results

Kinetics of proline transport by LT2 (putP$^+$ proP$^+$) and MST700 (putP$^+$ proP$^-$) cells

When used as a sole carbon or nitrogen source, proline transport in *S.* Typhimurium is primarily mediated by PutP (Ratzkin and Roth, 1978). Proline uptake can also mediated by ProP, which is involved in glycine-betaine transport (Cairney et al., 1985). The *Km* for proline transport by the *proP* permease is over 100-fold higher than the *Km* of the *putP* permease (Cairney et al., 1985). Thus, it would not be expected to contribute greatly to proline transport activity at the concentration of proline used in our proline transport assay. However, to completely prevent proline transport activity by ProP, we used the strain MST700 which is *putP$^+$ proP* (Myers et al., 1991). The bacterial cells were grown in the presence of proline in order to induce the *put* operon. Proline transport in a *putP proP proU* mutant was indistinguishable from the transport observed in a *putP proP* mutant (data not shown), confirming that the *proU* system is not active at the osmolarity of the medium used. Exponential phase cells were harvested and the initial velocities for proline transport were assayed. The *Km* for proline transport in MST700 (*putP$^+$ proP$^-$*) was 1.8 μM and the *Vmax* was 47.9 nmol proline/min/mg protein.

Properties of proline analogs

Proline analogs used in this study fell into six groups according to the structural differences between these analogs and proline. The predicted chemical structures under the physiological conditions used are shown in Table 1 using molecular modeling program ChemDraw Std™ Version 4.0.1 (CambridgeSoft Corp.) Group I includes analogs with different ring structures: azetidine-2-carboxylic acid (AZT) with a 4-member ring, and DL-

pipecolinic acid with a 6-member ring. Group II includes analogs with 5-member rings having a single double bond: 3,4-dehydroproline (DHP) has a double bond between C3 and C4, pyrroline-5-carboxylic acid (P5C) has a double bond between C5 and N1, and 3-pyrroline has a double bond between C3 and C4 (3-pyrroline is similar to DHP but lacks the C2 carboxyl group). Group III includes analogs with an altered C2 carboxyl group: prolinol has CH_2OH, and pyrrolidine has no C2 carboxyl group. Group IV group includes analogs with an altered N1 imino group: N-methyl-L-proline has a methyl group attached to N1, (±) tetrahydro-2-furoic acid has a substituted oxygen at the N1 position and a carboxyl group attached at the C2 position, and (±) tetrahydro-3-furoic acid has a substituted oxygen at the N1 position and a carboxyl group attached at the C3 position. Group V includes analogs with alternations in the C4 position: thioproline (THP) has an S4 substitution, and cis-4-hydroxyl-L-proline has a hydroxyl group attached to the C4 position. Group VI includes a single analog with a change in the C5 position: DL-2-pyrrolidone-5-carboxylic acid has oxygen attached to the C5 position. In addition we tested the analogs 1-methyl-2-pyrrole, which has two double bonds in the closed ring structure and a methyl group attached to the imino group, and D-proline, the stereoisomer of L-proline.

Toxicity of proline analogs

When certain proline analogs are incorporated into proteins, the proteins are unstable (Rowland and Tristram, 1975). Hence, if transported into the cell, these analogs are toxic (Maloy, 1987). We initially assayed toxicity of each proline analog by measuring the zone of inhibition from a disk containing a known concentration of the analog (Roth, 1970; Dila and Maloy, 1986). Of the 16 analogs used in this study, only three showed toxicity toward MST700 (*putP*$^+$ *proP*$^-$): AZT, DHP, and THP (Table 1). The other analogs did not show significant inhibition of cell growth even at concentrations greater than 2 mg (Table 1). There are several potential explanations why these analogs are nontoxic: (i) proline permease may have a poor binding affinity for the analogs; (iii) the analogs may be transported but rapidly degraded or modified once inside the cell; (ii) the analogs may be transported but not efficiently recognized by prolyl-tRNA synthetase and thus not incorporated into proteins; or (iv) the analogs may be transported and incorporated into proteins, without disrupting protein structure and function. The first explanation can be distinguished from the other possibilities by assaying for the ability of the analogs to competitively inhibit proline transport. If the first explanation is correct, the analogs would be expected to be poor competitive inhibitors of proline transport. In contrast, the other explanations require that the analogs are readily transported.

Inhibition of proline transport by proline analogs

Radioactive proline analogs are not available, so their transport could not be measured directly. Therefore, the affinity of proline permease for proline

Table 2. Inhibition of proline transport by analogs

Compound	% Inhibition[1]	*Ki* (μM)[2]
L-Proline	100	2
3,4-Dehydroproline	95	9
Azetidine-2-carboxyl acid	77	125
Thioproline	50	190
Pyrrolidine	18.6	600
Prolinol	17.8	1,000

[1] Transport of 10μM [^{14}C] proline was assayed in the presence of 100μM of the competing analog. [2] Rate of transport of L-[U-^{14}C]proline was measured in the presence of varying concentrations of the analog. The [^{14}C]proline concentration ranged from 0 to 250μM, in 50μM increments. Transport assays were done as described in the Materials and methods and K_i values were determined from Dixon plots.

analogs was determined by assaying the inhibition of [^{14}C] proline uptake by the unlabelled analogs (Table 2). Initially the analogs were assayed at ten-fold higher concentrations than the concentration of [^{14}C] proline used. Experimental results showed that there were three strong competitive inhibitors of proline transport: DHP, an analog with a double bond in the ring structure, showed 95% inhibition; AZT, an analog with a different ring structure, showed 77% inhibition; and THP, an analog with alteration in the C5 position, showed 50% inhibition. Two analogs were weak competitive inhibitors of proline transport: pyrrolidine showed 18.6% inhibition and prolinol showed 17.8% inhibition. Both analogs have alterations in the C2 position.

The Ki for inhibition of proline transport by analogs

To determine the apparent affinity of proline permease for those analogs that competitively inhibit proline transport, we measured the *Ki* of each analog. The *Ki* for inhibition of radioactive proline transport by non-radioactive proline (1.3μM) was nearly equivalent to the *Km* for proline transport (1.8μM) in MST700 (Myers et al., 1991). Thus, we inferred that if an analog competitively inhibits proline transport, the *Ki* should approximate the *Km* for the analog. Therefore, we determined the *Ki* for each of the analogs that are potential competitive inhibitors (Table 2). The *Ki* for DHP was 9μM and the *Ki* for AZT was 125μM. These values agree with previously reported results (Myers et al., 1991). Thioproline (*Ki* = 190μM), pyrrolidine (*Ki* = 600μM), and prolinol (*Ki* = 1,000μM) were also competitive inhibitors of proline transport. As predicted from the assays described in Tables 1, the other analogs failed to show competitive inhibition at concentrations $\leq$1 mM.

Although the transport assays were more sensitive than the plate assays, the *Ki* value of each analog agrees well with the results from the zone of

inhibition assays. The relative affinity of the analogs was: DHP > AZT > THP > pyrrolidine > prolinol. The strongest competitive inhibitor, DHP, was the most toxic analog. The weaker competitive inhibitors AZT and THP showed less inhibition of cell growth. The two weakest competitive inhibitors pyrrolidine and prolinol did not significantly affect cell growth.

Discussion

The specificity of PutP provides insight into the structural requirements for substrate recognition. The structures of the analogs that are recognized or ignored by PutP suggest the following rules. (i) Only the L-isomer is recognized, indicating that proline permease is stereospecific. (ii) The 4-membered ring analog AZT is a competitive inhibitor but the 6-membered ring analog DL-pipecolinic acid is not, indicating that proline permease prefers a 4-member ring structure to a 6-member ring structure. Proline is the optimal substrate for proline permease indicating that a 5-member ring structure preferentially interacts with the substrate binding pocket of proline permease, and a smaller 4-member ring molecule fits into the substrate binding pocket of proline permease easier than a larger 6-member ring molecule. Apart from the obvious differences in sizes, the chair conformations of the 6-member ring are significantly different than the 4- and 5-member rings. (iii) DHP is a competitive inhibitor but pyrroline-5-carboxylic acid is not, indicating that the presence of a double bond between C5 and N1 position interferes with the substrate recognition while a double bond between C3 and C4 position is tolerated. (iv) DHP is a competitive inhibitor but 3-pyrroline is not, indicating that the C2 carboxyl group is involved in substrate recognition. (v) Prolinol and pyrrolidine are weak competitive inhibitors, suggesting that substitution of the C2 carboxyl group interferes with, but does not abolish substrate recognition. This result implies that the presence of the C2 carbonyl group is important for substrate recognition, but the charged carboxyl group is not essential. (vi) (±)Tetrahydro-2-furoic acid, (±)tetrahydro-3-furoic acid, and N-methyl-L-proline acid are not competitive inhibitors, suggesting that substitutions on the N1 position interfere with substrate recognition. These results imply that a free, charged imino group is required for substrate recognition. (vii) THP is a competitive inhibitor but cis-4-hydroxyl-L-proline is not, suggesting that the substitution of C4 to S4 does not significantly interfere with substrate recognition, but attaching a hydroxyl group to the C4 position abolishes substrate recognition. This supports the argument that the C3–C4 position plays a role in binding but it is not essential for substrate recognition. (viii) DL-2-pyrrolidone-5-carboxylic acid is not a competitive inhibitor, suggesting that the attachment of oxygen to the C5 position interferes with substrate recognition. The presence of the oxygen also changes the C5–N1 environment so that the imino group is no longer charged. This supports the argument that the C5-N1 environment and a free, charged imino group are essential for substrate recognition.

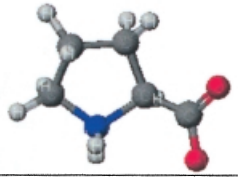
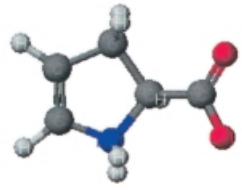
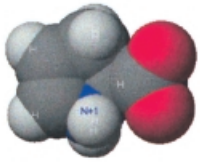
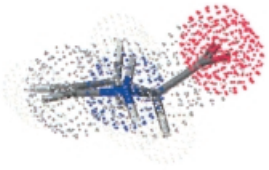
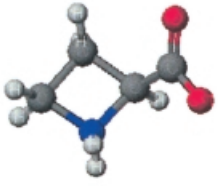
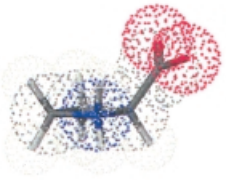
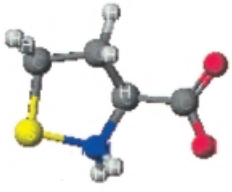
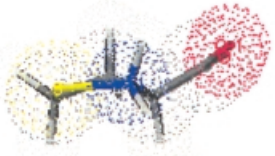
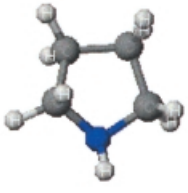
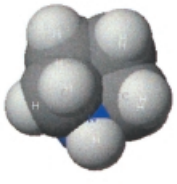
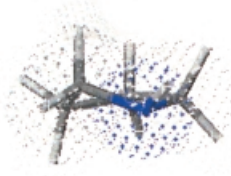
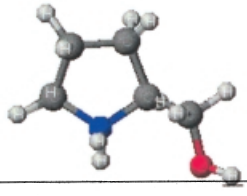
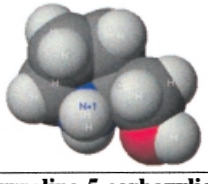
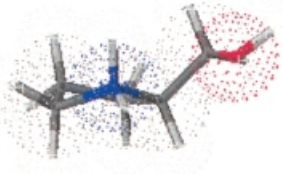
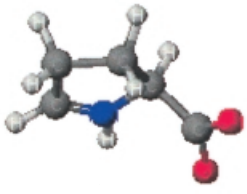
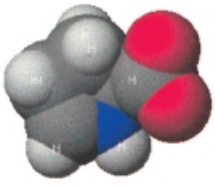
One of the simplest interpretations of these results is that the proline-binding site of proline permease consists of a hydrophobic pocket that accommodates C3, C4, and C5 of the pyrrolidine ring. DHP and THP maintain the structure of C3, C4, and C5 region, hence they are efficiently recognized by proline permease. In contrast, cis-4-hydroxyl-L-proline and DL-2-pyrrolidone-5-carboxylic acid have unacceptable alterations in the C4–C5 area. Cis-4-hydroxyl-L-proline has a hydroxyl group attached to the C4 position, which makes this molecule more polar than proline and alters the hydrophobicity of the C4–C5 area. DL-2-pyrrolidone-5-carboxylic acid has an oxygen molecule attached to the C5 position, which deprotonates the imino group and protonates the carboxyl group.

The results also suggest that the proline-binding site of proline permease includes a hydrophilic region that interacts with the secondary amine (N1 position) and the carbonyl group (C2 position). Substitutions or modifications at the N1 position completely abolish substrate recognition. Minor changes in the C2 position are tolerable but diminish substrate recognition.

The lowest energy, three-dimensional structures of proline, AZT, DHP, THP, prolinol, and pyrrolidine predicted by computer modeling are shown in Fig. 1. Comparison of the predicted three-dimensional structures provides additional insight into substrate recognition by proline permease. The structure of DHP is quite similar to proline, although the presence of a double bond in DHP makes the ring structure flatter but it does not change the overall structure dramatically. AZT is also very similar to proline, only smaller in size and with a somewhat flatter ring structure. A key point revealed by three-dimensional models is that the angle at which the carboxyl group intersects the pyrrolidine ring is important. This bond angle is much smaller in proline than in AZT, which may explain the preferential binding of proline by PutP. Likewise, the S4 group in THP changes the angle of the C2 carboxyl group to the pyrrolidine ring. The three-dimensional molecular models also help to explain why the intermediate in proline metabolism, P5C, is not recognized by PutP. P5C has a planar NH^+ group while proline has a tetrahedral NH_2^+ group. Thus, although the overall structure is similar, the essential spatial constraints of the N1 position are dramatically altered in P5C.

The results from this study agree with a previous study done in *E. coli* (Rowland and Tristram, 1975), indicating the basic principles of substrate recognition by proline permease are conserved among these two enteric bacteria. Proline permease is ubiquitous in both prokaryotes and eukaryotes. Recently, proline transport has been reported to promote colonization of the roots of plants by *Pseudomonas putida*, and infection of animals by

Fig. 1. The lowest energy, three-dimensional structures of proline, AZT, DHP, THP, prolinol, and pyrrolidine predicted by computer modeling. The structure of DHP is similar to proline. The ring structure of DHP is flatter. AZT is similar to proline, only smaller. The three-dimensional models revealed the angle at which the carboxyl group intersects the pyrrolidine ring is important. Both AZT and THP have altered angles. P5C has a planar NH^+ group while proline has a tetrahedral NH_2^+ group. The essential spatial constraints of the N1 position are altered in P5C

L-proline		
		
3,4-Dehydroproline		
		
Azetidine-2-carboxyl acid		
		
Thioproline		
		
Pyrrolodine		
		
Prolinol		
		
Pyrroline-5-carboxylic acid		
		

Staphylococcus aureus (Wengender and Miller, 1995; Coulter et al., 1998; Schwan et al., 1998; Bayer et al., 1999; Vílchez et al., 2000). Proline transport also plays an important role in eukaryotic systems. In tsetse flies, *Glossina morsitans*, proline is transported into the muscle mitochondria and provides energy for flight metabolism (Njagi et al., 1992). The antifungal agent cispentacin enters *Candida albicans* cells via proline permease (Jethwaney et al., 1997). The examinations of the structures of toxic proline analogs and the recognition requirements of proline permease will allow us to further identify the detailed structural features of the active site of proline permease. Understanding the diversity of substrate recognition and translocation mechanism of proline permeases in different species may facilitate the development of a proline analog that can effectively inhibit growth of microorganisms.

Acknowledgements

We thank Donald Cronkite (Biology Department, Hope College), William Mungall, and Nicole Bennett (Chemistry Department, Hope College) for useful comments, and Carrie Newell for assistance with molecular modeling. This work was supported by the NSF grant DCB901715 and the NIH grant GM34715 to SM.

References

Anderson RR, Menzel R, Wood, JM (1980) Biochemistry and regulation of a second L-proline transport system in *Salmonella typhimurium*. J Bacteriol 141: 1071–1076

Bayer AS, Coulter SN, Stover CK, Schwan WR (1999) Impact of the high-affinity proline permease gene (*putP*) on the virulence of *Staphylococcus aureus* in experimental endocarditis. Infect Immun 67: 740–744

Berkowitz D, Huston J, Whitfield H, Roth J, Ames B (1968) Procedure for identifying nonsense mutations. J Bacteriol 96: 215–220

Cairney J, Higgins CF, Booth IR (1984) Proline uptake through the major transport system of *Salmonella typhimurium* is coupled to sodium ions. J Bacteriol 160: 22–27

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

Chen C, Tsuchiya T, Yamane Y, Wood J, Wilson TH (1985) (Li^+)-proline cotransport in *Escherichia coli*. J Membr Biol 84: 157–164

Cornish-Bowden A (1979) Fundamentals of enzyme kinetics. Butterworths, Boston

Cornish-Bowden A, Porter W, Trager W (1978) Evaluation of distribution-free confidence limits for enzyme kinetic parameters. J Theor Biol 74: 163–175

Coulter SN, Schwan WR, Ng EYW, Langhorne MH, Ritchie HD, Westbrock-Wadman W, Hufnagle WO, Folger KR, Bayer AS, Stover CK (1998) *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. Mol Microbiol 30/2: 393–404

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

Dila D, Maloy S (1986) Proline transport in *Salmonella typhimurium*: *putP* permease mutants with altered substrate specificity. J Bacteriol 168: 590–594

Franco PJ, Wilson TH (1996) Alteration of Na(+)-coupled transport in site-directed mutants of the melibiose carrier of *Escherichia coli*. Biochim Biophys Acta 1282/2: 240–248

Frillingos S, Kaback HR (1996) Probing the conformation of the lactose permease of *Escherichia coli* by in situ site-directed sulfhydryl modification. Biochemistry 35/13: 3950–3956

Grothe S, Krogsrud R, McClellan D, Milner J, Wood J (1986) Proline transport and osmotic stress in *Escherichia coli* K-12. J Bacteriol 166: 253–259

Higgins C, Sutherland L, Cairney J, Booth I (1987) The osmotically regulated *proU* locus of *Salmonella typhimurium* encodes a periplasmic betaine-binding protein. J Gen Microbiol 133: 305–310

Hitz WD, Card PJ, Ripp KG (1986) Substrate recognition by a sucrose transporting protein. J Biol Chem 261/26: 11986–11991

Jethwaney D, Höfer M, Khaware RK (1997) Functional reconstitution of a purified proline permease from *Candida albicans*: interaction with the antifungal cispentacin. Microbiology 143: 397–404

Kaback HR (1987) Use of site-directed mutagenesis to study the mechanism of a membrane transport protein. Biochemistry 26: 2071–2076

Kaback HR, Wu J (1997) From membrane to molecule to the third amino acid from the left with a membrane transport protein. Q Rev Biophys 30/4: 333–364

Kawakami T, Akizawa Y, Ishikawa T, Shimamoto T, Tsuda M, Tsuchiya T (1988) Amino acid substitutions and alteration in cation specificity in the melibiose carrier of *Escherichia coli*. J Biol Chem 263/28: 14276–14280

King S, Li S (1998) Suppressor scanning at positions 177 and 236 in the *Escherichia coli* lactose/H^+ cotransporter and stereotypical effects of acidic substituents that suggest a favored orientation of transmembane segments relative to the lipid bilayer. J Bacteriol 180/10: 2756–2758

Lancaster J, Hinkle P (1977) Studies of the β-galactoside transport in inverted membrane vesicles of *Escherichia coli*. J Biol Chem 252: 7657–7661

Liao MK, Gort S, Maloy S (1997) A cryptic proline permease in *Salmonella* typhimurium. Microbiology 143: 2903–2911

Maloy S (1987) The proline utilization operon. *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. In: Neidhardt F, Ingraham J, Low K, et al (eds) American Society for Microbiology, Washington, D.C., pp 1513–1519

Marmorstein RQ, Sigler PB (1989) Stereochemical effects of L-tryptophan and its analogues on *trp* repressor's affinity for operator-DNA. J Biol Chem 264/16: 9149–9154

Milner JL, Grothe S, Wood J (1988) Proline porter II is activated by a hyperosmotic shift in both whole cells and membrane vesicles of *Escherichia coli* K12. J Biol Chem 263: 14900–14905

Myers R, Maloy S (1988) Mutations of *putP* that alter the lithium sensitivity of *Salmonella typhimurium*. Mol Microbiol 2: 749–755

Myers R, Olsen S, Maloy S (1990) Computer programs for the rapid determination of enzyme kinetics on MS-DOS compatible microcomputers. CABIOS 6: 51–53

Myers R, Townsend D, Maloy S (1991) Dissecting the molecular mechanism of ion-solute cotransport: substrate specificity mutations in the *putP* gene affect the kinetics of proline transport. J Membr Biol 121: 201–214

Njagi ENM, Olembo NK, Pearson DJ (1992) Proline transport by tsetse fly *Glossina morsitans* flight muscle mitochondria. Comp Biochem Physiol 102B/3: 579–584

Olsen SG, Brooker RJ (1989) Analysis of the structural specificity of the lactose permease toward sugars. J Biol Chem 264/27: 15982–15987

Quick M, Jung H (1997) Aspartate 55 in the Na^+/proline permease of *Escherichia coli* is essential for Na^+-coupled proline uptake. Biochemistry 36/15: 4631–4636

Quick M, Jung H (1998) A conserved aspartate residue, Asp187, is important for Na^+-dependent proline binding and transport by the Na^+/proline transporter of *Escherichia coli*. Biochemistry 37/39: 13800–13806

Quick M, Tebbe S, Jung H (1996) Ser57 in the Na^+/proline permease of *Escherichia coli* is critical for high-affinity proline uptake. FEBS 239/3: 732–736

Quick M, Stolting S, Jung H (1999) Role of conserved Arg40 and Arg117 in the Na^+/proline transporter of *Escherichia coli*. Biochemistry 38/41: 13523–13529
Ratzkin B, Roth J (1978) Cluster of genes controlling proline degradation in *Salmonella typhimurium*. J Bacteriol 133: 733–744
Roth J (1970) Genetic techniques in studies of bacterial metabolism. Methods Enzymol 17: 1–35
Rowland I, Tristram H (1975) Specificity of the *Escherichia coli* proline transport system. J Bacteriol 123/3: 871–877
Sahin-Toth M, Frillingos S, Lawrence MC, Kaback HR (2000) The sucrose permease of *Escherichia coli*: functional significance of cysteine residues and properties of a cysteine-less transporter. Biochemistry 39/20: 6164–6169
Saier MH Jr (2000) Families of transmembrane transporters selective for amino acids and their derivatives. Microbiology 146: 1775–1795
Schwan WR, Coulter SN, Ng EYW, Langhorne MH, Ritchie HD, Brody LL, Westbrock-Wadman S, Bayer AS, Stover CK (1998) Identification and characterization of the PutP proline permease that contributes to *in vivo* survival of *Staphylococcus aureus* in animal models. Infect Immun 66/2: 567–572
Stalmach M, Grothe S, Wood J (1983) Two proline porters in *Escherichia coli* K-12. J Bacteriol 156: 481–486
Tristram H, Neale S (1968) The activity and specificity of the proline permease in wild-type and analogue-resistant strains of *Escherichia coli*. J Gen Microbiol 50: 121–137
Venkatesan P, Kaback HR (1998) The substrate-binding site in the lactose permease of *Escherichia coli*. Proc Natl Acad Sci USA 95: 9802–9807
Viitmen P, Menick D, Sarka H, Trumble W, Kaback HR (1985) Site-directed mutagenesis of cysteine-148 in the *lac* permease of *Escherichia coli*: effect on transport, binding and sulfhydral inactivation. Biochemistry 24: 7628–7635
Vílchez S, Molina L, Ramos C, Ramos J (2000) Proline catabolism by *Pseudomons putida*: cloning, characterization, and expression of the *put* genes in the presence of root exudates. J Bacteriol 182/1: 91–99
Wengender PA, Miller KJ (1995) Identification of a PutP proline permease gene homolog from *Staphylococcus aureus* by expression cloning of the high-affinity proline transport system in *Escherichia coli*. Appl Environ Microbiol 61: 252–259
Wilson D (1978) Cellular transport mechanism. Ann Rev Biochem 47: 933–965
Wood JM (1988) Proline porters effect the utilization of proline as a nutrient or osmoprotectant for bacteria. J Membr Biol 106: 183–202
Wood JM, Zadworny D (1979) Characterization of an inducible porter required for L-proline catabolism by *Escherichia coli* K-12. Can J Biochem 57: 1191–1199

Authors' address: Dr. Min-Ken Liao, Department of Biology, Furman University, 3300 Poinsett Highway, Greenville, SC 29613-1118, U.S.A.,
E-mail: Min-ken.liao@furman.edu

Received November 1, 2000