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TRANSPORT OF [^{14}C]Gly-Pro IN A PROLINE PEPTIDASE MUTANT OF *SALMONELLA TYPHIMURIUM*

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Summary

The transport of [^{14}C]Gly-Pro was examined using a mutant of *Salmonella typhimurium* (strain TN87) deficient in an X-Pro dipeptidase and an X-Pro-Y iminopeptidase. The dipeptide was taken up by one saturable transport system having a K_m of $5.3 \cdot 10^{-7}$ M and a V of 1.4 nmol/mg dry wt cell per min. The uptake of Gly-Pro was not inhibited by amino acids or tripeptides and the transport system exhibited a rather broad side chain specificity for dipeptides. Dipeptides containing hydrophobic residues were the most potent inhibitors of this dipeptide transport system exhibiting K_i values between 10^{-8} and 10^{-7} M. In contrast, dipeptides containing glycine residues were particularly weak inhibitors. Finally, Gly-Pro was found to be in the intact form inside the cell and was concentrated more than 1000-fold.

Introduction

The study of the transport of metabolites such as amino acids, sugars and ions by *Escherichia coli* and *Salmonella typhimurium* has received much attention [1—4]. Few detailed studies, however, have been carried out on the kinetics of dipeptide or oligopeptide transport. To some extent this is a consequence of the lack of availability of radioactively labelled peptides. In addition, the presence of high levels of intracellular peptidase activity makes the analysis of kinetic data subject to uncertainty. To date few examples exist where a dipeptide was found unequivocably to be taken up intact in *E. coli*: Kessel and Lubin [5] reported that glycylglycine was concentrated 100 times by a mutant of *E. coli* lacking the enzyme to hydrolyze this dipeptide; Meisler and Sim-

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monds [6] demonstrated that glycylleucine was taken up intact by *E. coli* and exhibited a toxic effect once inside the cell. Cowell [7] has studied the kinetics and energetics of glycylglycine uptake in *E. coli*. The strain he used in his investigation contained intracellular peptidases toward glycylglycine and the peptide was hydrolyzed once in the cell.

We have been working with a proline-requiring auxotroph of *S. typhimurium* (strain TN87) which lacks enzymes necessary for the hydrolysis of X-Pro dipeptides and X-Pro-Y oligopeptides. Preliminary studies on this strain showed that it contained a functional peptide uptake system and that Ala-Pro-[^{14}C]Gly was accumulated intact [8,9]. In this communication we report on the uptake of [^{14}C]Gly-Pro by strain TN87. Our results show that this peptide is concentrated more than 1000-fold in the intact form. Competition experiments give evidence that Gly-Pro enters *Salmonella* by a dipeptide transport system which is independent of amino acid and tripeptide transport. Furthermore, the system mediating Gly-Pro transport appears to have a very high affinity for dipeptides with hydrophobic side chains.

Methods

Bacterial strain and growth conditions

S. typhimurium TN87 was received from Dr. Charles Miller of Case Western Reserve University. This strain lacks an X-Pro dipeptidase and an X-Pro-Y iminopeptidase. Its genotype and growth characteristics on various peptides were described previously [8,9]. The organism was grown in Vogel-Bonner Medium E [10] modified by the deletion of citrate, adjustment to pH 7 with HCl, and the addition of glucose (0.5%) and proline (35 μg/ml). Strain TN87 was inoculated into 100 ml of minimal medium in a 250 ml flask and grown with shaking at 37°C until mid-log stage, a reading of 90 with a Klett-Summerson colorimeter (filter No. 42). This flask was then stored at 4°C and for uptake experiments 5 ml of these cells were used to inoculate 100 ml of fresh medium. The cells were again grown with shaking at 37°C to 90 Klett units. Cells which grew to 90 Klett units within 3.5—4.5 h showed excellent uptake characteristics and were used for kinetic determinations.

Uptake experiments

Cells grown to mid-log phase (90 Klett units) in 100 ml of growth medium at 37°C were spun down at 1000 × *g* for 10 min in a refrigerated centrifuge. The pellet was washed by centrifugation in 100 ml of fresh medium minus glucose and proline (medium A) and resuspended in 100 ml of medium A. Starvation for a carbon source was then carried out using a procedure similar to that described by Ames [11]. The cells, in medium A, were allowed to stand for at least 30 min at 25°C without aeration. After this equilibration time, portions of the cell suspensions were removed and used for uptake experiments. We observed that the initial rate of [^{14}C]Gly-Pro uptake diminished with the time of incubation at 25°C and the half-life of transport rate was approximately 100 min. In all cases the initial rates of uptake were corrected for the dimunition of transport rate during incubation. This phenomenon of decay was probably due to a loss of endogenous metabolic energy necessary to drive Gly-Pro trans-

port. In one experiment we found that the addition of glucose (final concentration 0.2%) to the starvation medium prevented the decay of Gly-Pro uptake. We are currently investigating in more detail the effect of glucose and other energy sources on Gly-Pro uptake by strain TN87.

For the assay of Gly-Pro transport, 4 ml of starved cells were added to 1 ml of reaction mixture that contained [^{14}C]Gly-Pro plus any desired inhibitors or competitors in a total of 1 ml of distilled water. The suspension of cells and transport substrate was vortexed and portions were removed for sampling at the desired intervals. Sampling was accomplished by filtering 0.5 ml portions through Millipore filters (0.45 μm). The filtered cells were washed 2 times with 2 ml of medium A (25°C), placed in 15 ml of Aqueous Counting Scintillant (ACS, Amersham/Searle) and counted by liquid scintillation. For determination of initial rates of uptake, samples were taken at 15, 30 and 45 s. In competition experiments both Gly-Pro and the competitor were present at a final concentration of 10^{-6} M for each compound unless otherwise noted. There was virtually no non-specific adsorption of Gly-Pro during uptake experiments as determined using boiled cells or cells incubated with [^{14}C]Gly-Pro at 4°C.

For pH and temperature profile determinations the procedure used was identical to the above with the following minor modifications. For the pH profile the 4 ml of starved cells were titrated with HCl or NaOH to the desired pH immediately prior to addition to the reaction medium. For the temperature profile the cells were starved at 25°C for 75 min and then transferred for 15 min to the uptake temperature in order to permit equilibration. Initial velocities in cpm were converted to nmol/min per mg dry wt. of cells by taking into account specific activity (12.7 Ci/mol), counting efficiency (72%), and the weight of cells sampled (0.172 mg dry wt./ml).

Fate of [^{14}C]Gly-Pro and intracellular concentration

S. typhimurium TN87 was allowed to incubate with 10^{-5} M [^{14}C]Gly-Pro for time intervals up to 40 min. At the desired time five 0.5 ml portions of the suspension were filtered through individual Millipore filters. One of these filters was used to determine the amount of radioactivity associated with the cells. This was used as a baseline for determining the efficiency of the extraction process. The other 4 filters were placed in 2 ml of 70% ethanol at 4°C for 10 min. The solution was then filtered through a 0.22 μm Millipore filter. Part of the filtrate was counted to determine the efficiency of extraction and part was electrophoresed at pH 3.5 at 3000 V for 3—4 h as previously described [9]. Glycine, proline and Gly-Pro were used as markers in the electrophoresis experiment. After electrophoresis the electrophoretogram was cut into strips and counted by liquid scintillation.

The intracellular concentration of Gly-Pro was calculated using the fact that at 90 Klett units there are $5 \cdot 10^8$ cells/ml of culture medium. This corresponds to a wet weight of $1.72 \cdot 10^{-9}$ mg per cell, and we assumed that 1 mg of cell (wet wt.) corresponds to one μl of cell volume [12]. Our calculation of the partition coefficient (ratio of the intracellular concentration of Gly-Pro to the extracellular concentration of Gly-Pro) contains a correction for depletion of Gly-Pro from the uptake medium. This depletion was calculated using the experimentally determined values for Gly-Pro contained inside the cells. Our

calculation showed that after 1 min the depletion of Gly-Pro was negligible (≈7%) whereas after 10 min the depletion of Gly-Pro was quite significant, reaching values greater than 50%.

Chemicals

All peptides and chemicals used in this study were of the highest purity available. Peptides used in the competition experiments were tested for homogeneity using high voltage electrophoresis. No peptide contained any ninhydrin positive impurity. [^{14}C]Gly-Pro was purchased from Amersham/Searle at a specific activity of 12.7 Ci/mol. The synthesis of Ac-Met-Met, Boc-Leu-Leu, and Leu-Leu-OBzl were carried out according to literature procedures [13,14].

Results

Dependence of Gly-Pro uptake on time, pH and temperature

The uptake of [^{14}C]Gly-Pro by strain TN87 was linear for up to 2 min (Fig. 1). At longer times a significant decrease in the rate of uptake was observed. Even at times as long as 10 min, however, the steady state intracellular concentration of the peptide was not attained. As we will show later, the failure to saturate the cell with peptide is not due to its intracellular hydrolysis. Since the linear portion of the uptake curve occurred during the first 2 min, all initial velocities for kinetic measurements and competition experiments were determined within this time period. Details of the exact procedure used are given in Methods. The rate of uptake of Gly-Pro was affected by both pH and temperature. The maximum velocities of uptake occurred at pH 6 and at 37°C

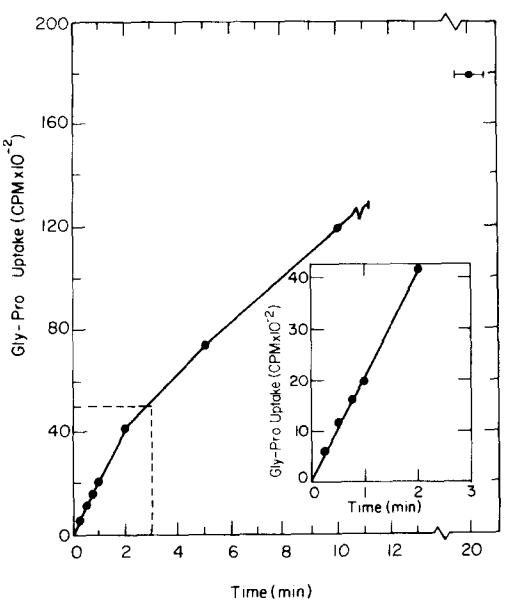

Fig. 1. Uptake of [^{14}C]Gly-Pro into *S. typhimurium* TN87. Cells from the mid-log phase of growth were starved for a carbon source and added directly to radioactive glycyl-proline ($1 \cdot 10^{-6}$ M, 12.7 Ci/mol in the final suspension). Uptake was determined at pH 7 and 25°C by removing portions at the time indicated, filtering and counting the radioactivity associated with the cells.

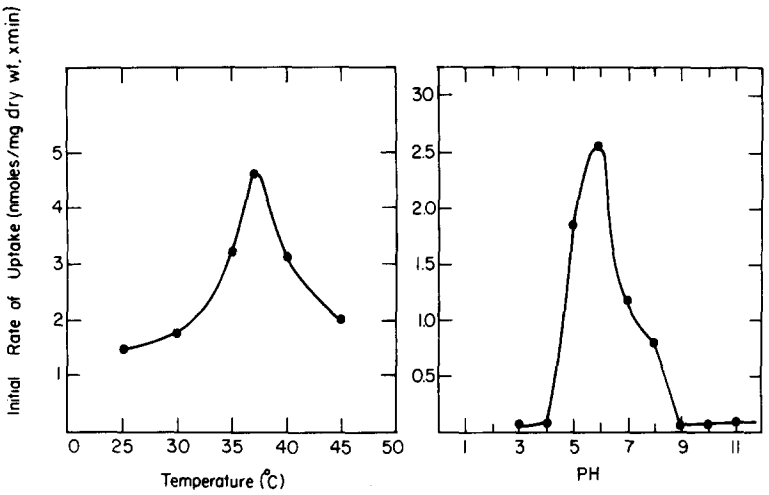

Fig. 2. Effect of pH and temperature on initial rate of uptake. Cells were suspended in buffers adjusted to the appropriate pH values as indicated (right-hand panel) and added to [^{14}C]Gly-Pro ($1 \cdot 10^{-6}$ M, 12.7 Ci/mol) at 25°C. For the results indicated in the left-hand panel, cells were suspended at pH 7 and incubated at the temperature indicated for 15 min and then added to [^{14}C]Gly-Pro ($1 \cdot 10^{-6}$ M, 12.7 Ci/mol). The initial rate of uptake was determined.

(Fig. 2). We decided to carry out all uptake studies at pH 7.0 and 25°C. These conditions are similar to those used for uptake of amino acids in *Salmonella* [12] and of glycylglycine in *E. coli* [7].

Fate of intracellular Gly-Pro

The ultimate fate of Gly-Pro after accumulation was determined by first allowing the cells to take up the peptide and then extracting the intracellular

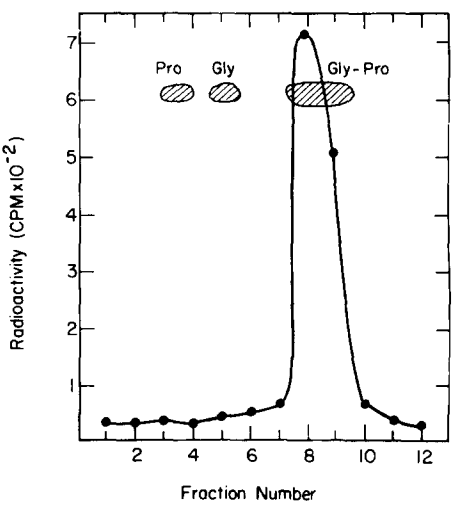

Fig. 3. Intracellular fate of [^{14}C]Gly-Pro. Cells incubated with radioactive Gly-Pro were removed from the incubation mixture by filtration at 45 s and extracted with cold ethanol. The cellular extract was electrophoresed and strips cut from the electrophoretogram and counted. The markers proline, glycine and glycyl-proline are indicated in the Figure.

radioactivity using cold 70% ethanol (see Methods). The extracted material was then electrophoresed. We observed that after a 45 s uptake all of the extractable radioactivity co-migrated with Gly-Pro (Fig. 3). In this experiment 97.3% of the intracellular radioactivity was extracted indicating no incorporation into macromolecular constituents. Identical results were observed after uptake for 10, 20 and 40 min. Thus, the peptide remains intact even after long time periods within the cell. Using the data presented in Fig. 1, we calculate that at 2 min the intracellular concentration of Gly-Pro is $0.6 \cdot 10^{-3}$ M. Similar calculations at 5 and 10 min indicate concentrations of $1.0 \cdot 10^{-3}$ and $1.7 \cdot 10^{-3}$ M, respectively. By allowing for the depletion of Gly-Pro from the uptake medium we were able to calculate partition coefficients (intracellular Gly-Pro concentration/extracellular Gly-Pro concentration) of 660, 1300 and 2600 at 2, 5 and 10 min, respectively.

Kinetics of uptake of Gly-Pro by Salmonella typhimurium

The kinetics of Gly-Pro transport were examined by measuring initial velocities in the presence of increasing concentrations of the dipeptide (Fig. 4). The double reciprocal plot of velocity versus substrate concentration was consistent with the presence of one saturable uptake system for the range of concentrations studied. The calculated K_m and V for the transport system at 25° C are $5.3 \cdot 10^{-7}$ M and 1.4 nmol/mg dry wt. cells per min. We have replicated this experiment three times and in all cases the K_m was between 4.5 and $5.5 \cdot 10^{-7}$ M. Although the kinetic data was always corrected for decay of uptake with time (see Methods) some variation in the V value, which seems to depend on the physiological state of the cell, was observed. In additional experiments we attempted to study Gly-Pro uptake at higher concentrations ($5 \cdot 10^{-6}$ M to 10^{-4} M), but high and variable background counts made it more difficult to get reliable data. The data which was obtained, however, were still consistent with one saturable transport system which had a K_m similar to that found using lower Gly-Pro concentrations. Thus, between $2 \cdot 10^{-7}$ M and 10^{-4} M only one saturable transport system is apparently involved in Gly-Pro uptake.

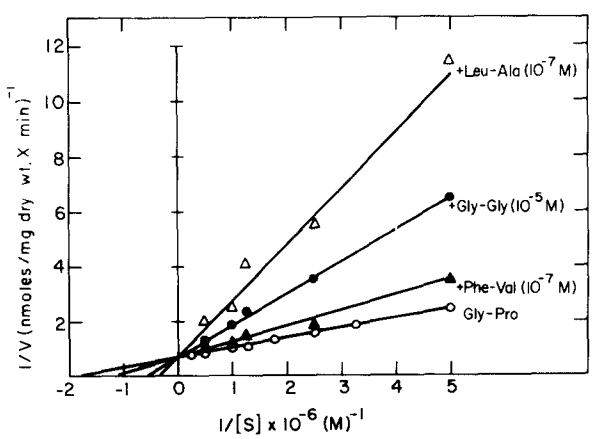

Fig. 4. Kinetics of [^{14}C]Gly-Pro transport. The uptake of radioactive dipeptide was determined without competitor (○), and in the presence of Phe-Val at 10^{-7} M (▲), Gly-Gly at 10^{-5} M (●) or Leu-Ala (△). K_i values were calculated from this plot using a K_m of $5.3 \cdot 10^{-7}$ M for [^{14}C]Gly-Pro uptake.

Competition by amino acids and peptides with [^{14}C]Gly-Pro uptake

The inhibition of Gly-Pro uptake by a variety of amino acids, dipeptides and tripeptides was examined. In most of these experiments both Gly-Pro and the competitor were present in the assay mixture at concentrations of 10^{-6} M. We found that there was some variability in the competition observed in replicate experiments. The data presented represent average values for at least two, and in most cases four, experiments. We also carried out a number of competition experiments using 10^{-5} M Gly-Pro and an equal concentration of the inhibitor. Conclusions from these experiments were totally consistent with results found at the lower substrate concentration.

The competition studies show that amino acids do not compete with Gly-Pro uptake. The ability of dipeptides to compete is dependent for the most part on the amino acid composition. Dipeptides composed primarily of hydrophobic amino acids such as Leu-Leu or Met-Met are excellent competitors, almost completely eliminating Gly-Pro uptake (Table I). In contrast Lys-Lys is a good competitor having an affinity almost equal to Gly-Pro itself whereas Glu-Glu is a poor competitor and Gly-Gly showed little competition under our assay conditions. Peptides containing proline exhibited inhibition intermediate between that of Gly-Gly and most hydrophobic dipeptides.

We examined a number of the dipeptides used in the competition experi-

TABLE I

COMPETITION FOR TRANSPORT OF Gly-Pro BY VARIOUS STRUCTURALLY RELATED COMPOUNDS

Cells were added to reaction mixtures containing [^{14}C]Gly-Pro and amino acids or peptides at equimolar concentrations (except where indicated for non-radioactive Gly-Pro at 10-fold). Initial rates of transport were determined as described in Methods.

Compound tested	Inhibition of Gly-Pro uptake * (%)	Compound tested	Inhibition of Gly-Pro uptake (%)
Glycine, proline, valine, leucine, serine, threonine, tyrosine, methionine, lysine, histidine	<10	Leu-Met, Leu-Ser	>90
		Leu-Val, Val-Leu	>90
		Leu-Ala, Ala-Leu	>90
		Leu-β-Ala, β-Ala-Leu	<10
Gly-Gly	<10	L-Leu-D-Leu, D-Leu-L-Leu	<10
Glu-Glu	20	Ac-Met-Met	<10
Lys-Lys	55	Ac-Leu-Gly	<10
Met-Met	>90	Boc-Leu-Leu	<10
Leu-Leu	>90	Leu-Leu-OBzl	<10
Phe-Val	70	Met-Met-Met	<10
Pro-Met, Met-Pro	50	Lys-Lys-Lys	<10
Pro-Val, Val-Pro	70	Ala-Ala-Ala	<10
Pro-Ala, Ala-Pro	60	Leu-Leu-Leu	<10
Gly-Pro	40		
Gly-Pro (10-fold)	90		
Pro-Gly	<10		
Gly-Leu	40		
Leu-Gly	20		

* Percent inhibition reflects the average of at least 2 independent experiments and is the ratio of the initial velocity of uptake in the presence of the competitor to the initial velocity of [^{14}C]Gly-Pro uptake without competitors.

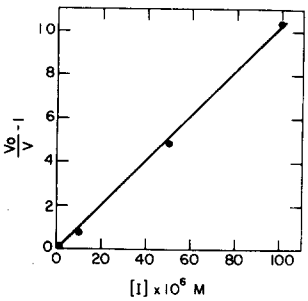

Fig. 5. Competition of [^{14}C]Gly-Pro uptake by Gly-Gly. The initial velocity of [^{14}]Gly-Pro at $1 \cdot 10^{-6}$ M (12.7 Ci/mol) was determined in the presence of various concentrations of Gly-Gly [I]. The data is plotted according to Eqn. 1 in the text.

ments to determine the nature of the inhibition of Gly-Pro uptake. Double reciprocal plots of the data obtained for Leu-Ala, Gly-Gly and Phe-Val were consistent with competitive inhibition kinetics (Fig. 4). Furthermore, we found that

$$\left(\frac{v_0}{v} - 1\right)\left(1 + \frac{S}{K_M}\right) = \frac{I}{K_i} \qquad (1)$$

where v_0 is uptake velocity in the absence of inhibitor, v is uptake velocity in the presence of inhibitor, S is Gly-Pro concentration and I is inhibitor concentration, $(v_0/v - 1)$ was proportional to Gly-Gly concentrations which varied from 10^{-6} to 10^{-4} M (Fig. 5). This gives further evidence for the competitive nature of the inhibition which we observed. Using data from Figs. 4 and 5 we calculated that the K_i for the effect of Gly-Gly, Leu-Ala and Phe-Val on Gly-Pro uptake was $3.6 \cdot 10^{-6}$ M, $1.8 \cdot 10^{-8}$ M and $1.6 \cdot 10^{-7}$ M, respectively.

Since Leu-Leu was a very good competitor we examined the ability of various Leu-X and X-Leu dipeptides to compete with Gly-Pro entry. For the most part almost all the leucine-containing dipeptides were strongly inhibitory to Gly-Pro uptake (Table I). For example, in comparing Leu-X dipeptides we observed greater than 90% inhibition of the initial rate of uptake when X was equal to Leu, Ser, Val, Ala or Met. In contrast, when X equaled β-alanine or Gly little inhibition of uptake was found. Furthermore both L-Leu-D-Leu and D-Leu-L-Leu were not competitors.

We also examined the competition of some dipeptide derivatives with Gly-Pro uptake. Neither Ac-Gly-Leu, Ac-Met-Met or Boc-Leu-Leu showed any competition. In addition, Leu-Leu-OBzl was also not competitive with Gly-Pro uptake. Finally, a number of tripeptides were found not to inibit Gly-Pro uptake when present at equimolar ratios. However, 10 : 1 excesses of $(Ala)_3$, $(Leu)_3$ and $(Met)_3$ slightly inhibited Gly-Pro uptake.

Discussion

In this communication we show that *S. typhimurium* TN87, a proline peptidase mutant, can transport [^{14}C]Gly-Pro via a saturable dipeptide transport system which exhibits a broad side-chain specificity. The usefulness of direct transport assays in this peptidaseless mutant was evident since we determined

that [^{14}C]Gly-Pro transport occurs against a significant concentration gradient; the partition coefficients obtained for Gly-Pro uptake by strain TN87 are comparable to those observed in the transport of sugars and amino acids by other bacteria. In addition, our data are compatible with previous findings [15,16] of separate amino acid, dipeptide, oligopeptide transport systems and with reports [15,16] on the structural specificity of the dipeptide transport system in *E. coli* based on indirect transport assays.

Most previous studies on dipeptide transport in bacteria have utilized the growth response of amino acid auxotrophs to peptides containing the essential amino acid [15,16]. Although much descriptive information has been compiled by this procedure, indirect growth assays cannot yield detailed information concerning the relative affinity of a transport system for various transport substrates. Our kinetic investigations give information concerning the types of molecular interactions which affect the affinity of the transport system for different dipeptides. It is obvious that the dipeptide transport system has a rather broad side-chain specificity. The much stronger inhibition by peptides containing leucine, methionine and valine as compared with peptides containing lysine, glutamic acid and glycine residues suggests that hydrophobic interactions might predominate at the binding site of the transport proteins(s) involved in Gly-Pro uptake. It is significant, we believe, that peptides containing glycine manifest particularly poor competition against Gly-Pro. Gly-Gly is a weak inhibitor having a K_i which is an order of magnitude higher than the K_m for the substrate and which is almost two orders of magnitude higher than K_i values found for two hydrophobic dipeptides; Leu-Ala and Phe-Val. Furthermore, of the Leu-X and X-Leu dipeptides which were examined, only Leu-Gly and Gly-Leu did not strongly inhibit the uptake of Gly-Pro by *S. typhimurium* TN87 (Table I). Similar results are found by comparing Pro-Gly with the other Pro-X dipeptides examined. We conclude, therefore, that the system responsible for Gly-Pro uptake in *S. typhimurium* TN87 shows a lower affinity for dipeptides containing glycine as one of the residues.

The apparent K_m for the system which takes in [^{14}C]Gly-Pro is $5.3 \cdot 10^{-7}$ M. Interestingly, the K_m of Gly-Pro uptake in a strain of *Salmonella* having a full complement of peptidases was almost identical (Becker, J.M. and Naider, F., unpublished results). Nevertheless, mutants devoid of specific peptidases are necessary in transport studies to establish the concentrative uptake of peptides by microorganisms, and to give experimental evidence that peptidases do not contribute to kinetic constants of peptide transport. In general, our kinetic analysis and the results of studies from other laboratories [7,17,18] demonstrate that many dipeptides are bound strongly by components, presumably proteins, of the transport system. This information suggests that dipeptides may serve as suitable starting materials to prepare affinity labelling reagents. Such reagents may specifically react with components of the dipeptide transport system and should be a practical tool to aid in their isolation.

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