BBA 3949

ON THE DISTINCTION BETWEEN PEPTIDASE ACTIVITY AND PEPTIDE TRANSPORT

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(Received September 21st, 1962)

SUMMARY

1. Mutants of a glycine-requiring strain of *Escherichia coli* have been isolated and used to show that active transport of peptides, and peptidase activity, are separable functions of the bacterial cell.

2. One mutant, lacking the transport system for glycylglycine but possessing the peptidase, grew well in media containing high levels of glycylglycine but failed to grow at low levels.

3. Another mutant, lacking the peptidase but possessing the transport system, was unable to grow on glycylglycine supplied at high or low levels. In this mutant, intracellular glycylglycine reached as much as one hundred times the extracellular level.

4. Studies of competition between peptides show that glycylglycine is carried by a transport system with broad specificity.

INTRODUCTION

A number of systems has been found in bacterial cells for the transport of small molecules, including sugars[1-4], amino acids[2,5-10] and cations[11-13]. Their specificity has been delineated by several methods, chiefly studies of competition between compounds for uptake[5-7,14,15], and studies of mutant strains which have lost the ability for transport[9,16].

The analysis of peptide transport by similar methods has been complicated by the presence of very active intracellular peptidases[17]. These peptidases could account for the observed rapid accumulation of radioactive counts from media containing ^{14}C-labeled peptides, as well as the rapidity with which auxotrophic strains utilize peptides for growth[8,23].

A mutant strain of *Escherichia coli* W has been isolated which lacks glycylglycine dipeptidase, but which retains the ability to concentrate glycylglycine from an extracellular source. In addition, a mutant has been found which cannot concentrate glycylglycine, but contains glycylglycine dipeptidase activity. We have used these mutants to demonstrate unambiguously the active transport of glycylglycine, and to distinguish between this transport system and peptidase activity.

METHODS

[1,4-$^{14}C_2$]Glycylglycine was obtained from Calbiochem, and other peptides and amino acids from Mann Research Corp. For measurement of peptidase, by the method of MITZ AND SCHLUETER[18], cells were grown and harvested as described below, resuspended in medium A (ref. 21), and treated for 15 min in a MSC sonic oscillator. After centrifugation at 20000 × *g* for 15 min to remove cell debris, the supernatant fluid was used for assay.

To extract from cells the pool of small molecules, cells were collected by centrifugation, and the washed pellets were suspended in 5 volumes of 40% ethanol[19]. The resulting mixture was poured into an equal volume of chloroform, the aqueous layer was evaporated to dryness at 0° under reduced pressure, and the residue was taken up in water for subsequent chromatography.

Glycylglycine was separated from other components of the extract by descending chromatography in 80% *n*-propanol for 36–66 h (ref. 20). Radioactivity was located by scanning the strips in an Atomic Accessories strip scanner, or by counting portions of the chromatograms in a Packard liquid scintillation counter. Intracellular [^{14}C] glycylglycine was identified by elution from chromatograms, followed by conversion to DNP-glycylglycine and acid hydrolysis[20], which yielded two radioactive products identified chromatographically as DNP-glycine and glycine.

For all experiments, except as noted in the text, cells were grown in minimal medium A (ref. 21) containing 0.2% glucose supplemented with 1 mg/ml of glycine, and were harvested during exponential growth. Uptake of radioactive compound was measured in cells resuspended at a density of 400 Klett units (420 mμ blue filter). Each millilitre of this suspension contained about 0.0025 ml of intracellular water.

To measure the uptake of [^{14}C]glycylglycine, the cells were washed twice in medium A, and incubated at 24° in medium A containing 0.2% glucose and specified amounts of radioactive substrate, usually 40 mM. Compounds tested for ability to inhibit uptake of [^{14}C]glycylglycine were added as specified in the text, simultaneously with the [^{14}C]glycylglycine. Samples of the bacterial suspension were taken after 1 min of incubation, by which time the rapid accumulation of counts was nearly complete. The cells were collected on Millipore filters (0.45 μ pore size) and were briefly washed at 24° in medium A containing 0.2% glucose. The radioactivity retained on the filter was determined with a Nuclear-Chicago thin window counter. In some cases accumulation of counts was observed to proceed for as long as 5 min, although the rate had decreased greatly after the first minute of incubation. Incorporation of counts into 5% trichloroacetic acid-insoluble pools was found to be extremely low, only 1–2% of the total counts, during 1 min of incubation.

RESULTS

Isolation and growth properties of mutants

The parent strain, from which mutants were derived, was a glycine-requiring strain, and could also grow very well on supplements of the peptide glycylglycine when present in the medium at low levels. The mutants, unlike the parent strain, were unable to grow in this medium. This group of mutants contained at least two types, one lacking glycylglycine dipeptidase, and another lacking the glycylglycine

transport system. Mutants lacking the peptidase would not be expected to grow at any level of glycylglycine supplement, but those lacking the transport system could be recognized by their ability to grow when glycylglycine was provided in the medium at very high levels (500 μg/ml).

TABLE I

GROWTH PROPERTIES OF MUTANTS ON SOLID MEDIUM

Strain No.	Genotype for glycylglycine peptidase and transport	Supplement in medium (μg/ml)			
		50 Glycine	1000 Glycine	50 Glycylglycine	500 Glycylglycine
W5	$GG^+Tr_{gg}^+$	+	+	+	+
W833	$GG^+Tr_{gg}^+$	o	+	+	+
W856	$GG^+Tr_{gg}^-$	o	+	o	+
W864	$GG^-Tr_{gg}^+$	o	+	o	o

To isolate these mutants, a modification of the penicillin selection method was used[24] in which the supplement in the medium during penicillin treatment was glycylglycine at 50 μg/ml, and the supplement during regrowth of the surviving population was glycine at 1000 μg/ml. The growth properties of the parent strain W833, and two derived mutants, W856 and W864, are shown in Table I, which records observations made qualitatively after incubation of plates for 24 h at 37°. In addition, Table I shows the growth properties of strain W5, a glycine auxotroph obtained from Dr. B. D. DAVIS, from which W833 had been derived.

Strains W5 and W833 both have the transport system for glycylglycine, but differ in that W833 lacks the transport system for glycine[16]. The use of strain W833, in preference to W5, as the parent strain from which other mutants were obtained, lessened the possibility of "feeding" of mutants on the small amount of glycine which might contaminate the medium during the period of treatment of the population with penicillin. Most of the experiments reported in this paper were carried out with strains W833, W856 and W864, although the studies on competition of peptides for entry were largely done on strain W5.

Symbols for the genotypes, designating the presence or absence of peptidase activity and of the glycylglycine transport system, have been tentatively assigned to the mutants in Table I. For convenience, these mutants are referred to in the text by these symbols, although the evidence which supports these designations is presented in later sections of the RESULTS.

Peptidase activity of cell extracts

Sonic extracts of W5 ($GG^+Tr_{gg}^+$) and W833 ($GG^+Tr_{gg}^+$) hydrolyzed glycylglycine, glycyl-L-leucine, L-leucylglycine, and L-leucyl-L-leucine at similar rates. In contrast, cell extracts from these strains were inactive toward glycyl-D-leucine or D-leucylglycine. The mutant strain W864 ($GG^-Tr_{gg}^+$) was incapable of utilizing glycylglycine for growth and lacked glycylglycine dipeptidase, but retained hydrolytic activity toward other peptides containing L-amino acids (Table II).

Identification of intracellular glycylglycine

After incubation in a medium containing 5 μg/ml of [^{14}C]glycylglycine for 1 min,

cells of $GG^+Tr_{gg}^+$ and $GG^-Tr_{gg}^+$ were extracted as described under METHODS. The radioactive material in $GG^-Tr_{gg}^+$ chromatographed with glycylglycine. In contrast, extracts of $GG^+Tr_{gg}^+$ contained only a trace of radioactive material chromatographing with glycylglycine, and most of the remaining activity appeared in the glutathione and glycine fractions.

TABLE II

HYDROLYSIS OF PEPTIDES BY MUTANT STRAINS

Units refer to μmoles of peptide split/g of wet cells/h. Sonic extracts of cells were prepared as described in METHODS. The accuracy of repeated assays on a single sample was ± 10%. System: 0.1 ml of *E. coli* extract (from 3 mg of cells), 10 μmoles of substrate, 0.05 ml of 0.01 M cobalt chloride, and phosphate buffer (pH 8.0) in a total volume of 3.0 ml at 37°. Absorbancy decrease at 225 mμ was followed over a 90-min period.

Strain No.	*Genotype*	*Substrate*		
		Glycylglycine	*L-Leucylglycine*	*L-Leucyl-L-leucine*
W833	$GG^+Tr_{gg}^+$	750	500	1500
W856	$GG^+Tr_{gg}^-$	710	450	1450
W864	$GG^-Tr_{gg}^+$	< 5	300	1400

From a duplicate chromatogram of extracts of strain GG^-TrN^+ the radioactive region presumed to contain glycylglycine was eluted with water, and this substance was identified as glycylglycine by conversion to the dinitrophenyl derivative, acid hydrolysis, and chromatographic separation, as described under METHODS.

Uptake of [^{14}C]glycylglycine

Observations of growth properties of $GG^+Tr_{gg}^+$, $GG^+Tr_{gg}^-$ and $GG^-Tr_{gg}^+$ cells suggested that significant differences in uptake of [^{14}C]glycylglycine would be expected. Fig. 2 shows the observed accumulation of counts by strains $GG^+Tr_{gg}^-$ and $GG^-Tr_{gg}^+$ from media containing specified levels of radioactive glycylglycine, with samples taken after 1 min of incubation. Control samples show that very little radioactivity was incorporated during this brief interval into material insoluble in 5% trichloroacetic acid.

The data in Fig. 2 are consistent with the proposal that $GG^-Tr_{gg}^+$ has a transport system for concentrative uptake of glycylglycine from the medium, but $GG^+Tr_{gg}^-$ has not. Other experiments showed that $GG^+Tr_{gg}^+$ accumulated counts from a medium containing low levels of [^{14}C]glycylglycine.

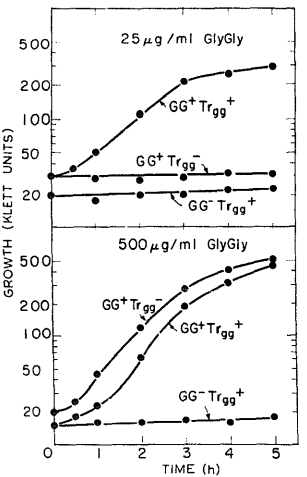
The kinetics of uptake of [^{14}C]glycylglycine by strains $GG^+Tr_{gg}^+$, $GG^+Tr_{gg}^-$ and $GG^-Tr_{gg}^+$ are shown in Fig. 3. The period of rapid uptake of counts was nearly complete in 1–2 min, and after 5 min very little change occurred.

Inhibition of [^{14}C]glycylglycine uptake by other compounds

The effect of other peptides and amino acids on accumulation of glycylglycine is shown in Table III. These values were obtained from strain W5, and most have been duplicated with the $GG^-Tr_{gg}^+$ strain. Cultures were incubated with [^{14}C]glycylglycine at an extracellular level of 5 μg/ml (40 mM) with inhibitors present at 75 mM. The affinity constants were derived as described by PAINE AND HEINZ[14]. The values obtained relate inhibition of uptake of radioactive glycylglycine by a test compound

to "self inhibition" by a similar concentration of non-radioactive glycylglycine, assuming a common transport site.

Other experiments have shown that peptides which inhibited glycylglycine



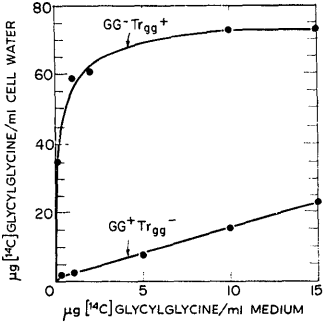


Fig. 2. Cells were grown as described in METHODS, then resuspended in medium A at 24° containing specified levels of [14C]glycylglycine. Intracellular radioactivity was determined from samples collected at 1 min by filtration, and is expressed for all strains as μg/ml glycylglycine, although other experiments show that strains GG+Trgg+ and GG+Trgg− contain in fact only trace amounts of glycylglycine because of dipeptidase activity.

Fig. 1. Growth of cells at 37° in enriched medium containing specified amounts of glycylglycine. Cells were initially grown in medium A with 0.2% glucose + 1000 μg/ml of glycine, harvested during exponential growth, washed twice and transferred to the enriched medium. Enriched medium contained specified amounts of glycylglycine and 30 μg/ml each of 16 amino acids (glycine, serine, threonine and alanine were omitted), 30 μg/ml of adenine, and a vitamin mixture at levels used by EAGLE[22].

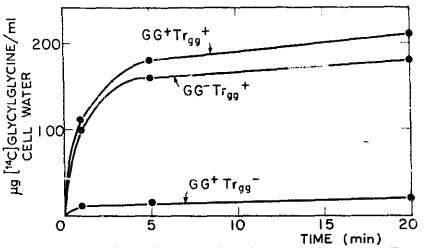


Fig. 3. Kinetics of uptake of radioactive glycylglycine at 24° by GG+Trgg+, GG+Trgg− and GG−Trgg+ cells. The conditions of the experiment were the same as for Fig. 2. The concentration of [14C]glycylglycine in the medium was 5 μg/ml. Measurements were corrected for the radioactivity which remained after washing duplicate samples with 5% cold trichloroacetic acid. In the case of GG+Trgg+, the trichloroacetic acid-insoluble material reached 30% of the total counts after 20 min. In the other strains, the correction was negligible.

uptake (*e.g.*, glycyl-L-methionine, histidylhistidine, glycyl-L-phenylalanine) did not inhibit uptake of [^{14}C]glycine or [^{14}C]proline by *E. coli* strains W5 or W833 ($GG^+Tr_{gg}^+$). Thus inhibition of glycylglycine uptake by other peptides is a specific effect, related to the specificity of the system responsible for glycylglycine transport.

TABLE III

THE EFFECT OF INHIBITORS ON ACCUMULATION OF [^{14}C]GLYCYLGLYCINE

Cells were incubated in 40 mM [^{14}C]glycylglycine + 75 mM of other compounds, as described in the text.

Compound	*Relative uptake**	*Affinity constant***
Glycyl-D-leucine 5-Aminovaleric acid	90%	0.07
L-Leucine, D- or L-serine Glycylglycylglycine	85%	0.1
Glycylglycine methyl ester D-Alanyl-D-alanine, D-leucylglycine Glycine, D-alanylglycine L-Lysine, L-glutamic acid	70%	0.25
L-Prolylglycine Glycyl-L-glutamic acid, L-alanyl-D-alanine L-Carnosine D-Alanyl-L-alanine	50%	0.60
[^{12}C]Glycylglycine	38%	1.0
Glycyl-L-lysine, histidylhistidine	30%	1.4
L-Leucylglycine, glycyl-L-alanine Glycyl-L-proline, glycyl-L-histidine Glycyl-L-serine, glycyl-L-threonine	25%	1.8
L-Alanylglycine, glycyl-L-leucine L-Alanyl-L-alanine	20%	2.5
Glycyl-L-methionine, L-leucyl-L-leucine Glycyl-L-phenylalanine, L-leucyl-L-alanine	15%	3.5

* Relative uptake $= \frac{\text{uptake of counts with inhibitor added}}{\text{uptake of counts without inhibitor}} \times 100$.

** Affinity constant relates inhibitory effect of [^{12}C]glycylglycine to inhibitory effect of other compounds, as described by PAINE AND HEINZ[14].

DISCUSSION

The experiments reported in this paper show that the transport of glycylglycine, and the activity of intracellular glycylglycine dipeptidase, are separable functions of the bacterial cell. Wild-type cells possess both functions, although the presence of the enzyme obscures the demonstration of the transport system. A tabular summary is presented of the properties of the parent strain, $GG^+Tr_{gg}^+$ and two derived mutants: $GG^+Tr_{gg}^-$ and $GG^-Tr_{gg}^+$ (Table IV).

The behavior of strain $GG^+Tr_{gg}^-$ deserves further comment. In this strain, which

lacks glycylglycine transport, the very active peptidase should serve as a constant drain on the level of intracellular glycylglycine. The failure of W856 ($GG^+Tr_{gg}^-$) to grow rapidly on a low glycylglycine supplement suggests that the rate of uptake of peptide from low extracellular levels, in the absence of a specific transport process, is too slow to permit the dipeptidase to produce sufficient glycine for rapid protein synthesis.

TABLE IV

SUMMARY OF PROPERTIES OF BACTERIAL MUTANTS

Mutant	*Growth on 25–50 μg/ml of glycylglycine*	*Growth on 500 μg/ml of glycylglycine*	*Uptake of [^{14}C]glycylglycine from low levels*	*Presence of peptidase*	*Free cellular glycylglycine*	*Interpretation*
W833	+	+	+	+	o	Peptidase and transport present
W856	o	+	o	+	o	Peptidase only
W864	o	o	+	o	+	Transport only

If glycylglycine is provided at sufficiently high levels, rapid growth occurs, although the specific transport process is lacking. Presumably non-specific transport of the peptide occurs quickly enough to permit the maintenance of a high glycine pool. These observations are similar to findings with other strains lacking specific transport systems for amino acids required for growth[9,10].

We have designated the system studied here the "glycylglycine transport system", recognizing that its specificity extends over related peptides. The alternative name "peptide transport system" has the disadvantage of implying the existence of a unique system for peptide transport, when in fact future studies may show that there are several.

Competition studies show that the specificity of the glycylglycine transport system is quite broad, although individual amino acids are concentrated by systems of considerably greater specificity[5,6,9]. In general, peptides containing two L-amino acids, or glycine with one L-amino acid, have approximately equal affinities for this system. With longer aliphatic or aromatic side chains (glycyl-L-phenylalanine, glycyl-L-leucine) the affinity becomes more striking. In addition, free amino and carboxyl groups and the presence of a peptide bond are required. Some peptides containing D-amino acids (L-alanyl-D-alanine and D-alanyl-L-alanine) compete moderately well with glycylglycine for uptake. On the other hand, D-leucylglycine and glycyl-D-leucine are very poor competitors.

LEACH AND SNELL[17] have examined the specificity of the system responsible for uptake of glycyl-L-alanine and L-alanylglycine in *L. casei*. In general their findings are in agreement with ours, although the process responsible for peptide transport in *E. coli* is of somewhat lower specificity.

The broad specificity shown by peptides for uptake would suggest that the mutant unable to transport glycylglycine would also be unable to transport other peptides. The appropriate uptake studies have not been done, since peptides other than glycylglycine cannot be easily obtained in radioactive form. Growth data, however, show that the $GG^+Tr_{gg}^-$ mutant, unlike the $GG^+Tr_{gg}^+$ parent, fails to grow

well on low levels of glycyl-L-leucine or L-leucylglycine, but at higher levels grows rapidly. In the $GG^{+}Tr_{gg}^{-}$ strain, therefore, transport of at least two related peptides is lost.

Active transport of glycylglycine has been unequivocally demonstrated by our experiments. In the mutant strain lacking glycylglycine dipeptidase, glycylglycine was concentrated at least 100-fold, from a low level in the medium (Fig. 2), and the intracellular radioactive material was identified chemically as uncleaved glycylglycine. The intracellular level reached, however, under conditions which saturate the transport capacity of the cells, is modest (80–100 μg/ml of cell water) compared to the levels to which cells concentrate some amino acids and sugars.

It is not surprising that bacteria, which are normally found growing in heterogeneous mixtures of nutrients, should possess a transport system for peptides. Although broader in specificity than the corresponding systems for amino acid transport, the peptide transport process does share other features commonly observed in the growing list of transport systems.

ACKNOWLEDGEMENTS

This work was supported by U.S. Public Health Service Grant RG 6712, National Science Foundation Grant G 24163 and a U.S. Public Health Service post-doctoral fellowship, CF 10847, awarded to one of us (D.K.).

We are indebted to Dr. B. D. DAVIS for his criticism of the manuscript and to Mrs. J. S. MARVEL, Miss B. S. ORDICK and Mrs. H. M. HUNTER for laboratory assistance.

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