Proc. Nat. Acad. Sci. USA
Vol. 70, No. 2, pp. 456–458, February 1973

Illicit Transport: The Oligopeptide Permease

(histidinol phosphate transport/amino-acid analogue transport/*Salmonella*)

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Contributed by Bruce N. Ames, December 13, 1972

ABSTRACT **The oligopeptide permease of *Escherichia coli* has been characterized by Payne, Gilvarg, and their colleagues. We have confirmed its existence in *Salmonella typhimurium*, and have isolated a series of mutants lacking the permease. We use this transport system for smuggling a histidine biosynthetic intermediate, histidinol phosphate ester, into the bacteria as its glycylglycyl derivative, Gly-Gly-histidinol phosphate. Free histidinol phosphate ester is not transported into *Salmonella*. Several amino-acid analogues are shown to be much more inhibitory to *Salmonella* when presented to the bacteria in the form of tripeptides than as the free amino acids. The implications of this work for practical purposes are discussed. The synthesis of Gly-Gly-histidinol phosphate is described.**

The uptake and utilization of peptides in *Escherichia coli* has been studied by Gilvarg, Payne, Simmonds, and their colleagues (1–7). The uptake occurs through several systems for dipeptides and one system for oligopeptides of three or more residues (1, 2). Payne, Gilvarg, and their colleagues (1–6) have investigated extensively the properties of peptide uptake and concluded that the C-terminal carboxyl group is not necessary for the uptake of tripeptides and that the tripeptide permease system has a broad specificity.

Several years ago, in the course of other studies on the effect of amino-acid analogues, we observed (B. N. A., unpublished results) that analogues present in peptide form were much more inhibitory to *Salmonella typhimurium* than the free analogues, and that various mutants of the dipeptide and oligopeptide permeases could be isolated by use of these inhibitory peptides. We concluded that the *Salmonella* peptide transport was very similar to that reported for *E. coli*.

We have been interested in getting histidinol phosphate ester (HOLP), a histidine biosynthetic intermediate, into *S. typhimurium*, but were unsuccessful because there is no transport system for this intermediate. We have now solved this problem by synthesizing its peptide derivative, Gly-Gly-HOLP, which we show is transported by the oligopeptide permease.

MATERIALS AND METHODS

Bacterial Strains and Media. All strains were derived from *S. typhimurium* strain LT-2. Conditions for growth have been described (8). Mutants lacking the oligopeptide permease were isolated from plates containing inhibitory tripeptides (see legend to Table 1); following the standard rules of nomenclature, we name the gene for the oligopeptide permease *opp*.

Chemicals. All chemicals, except for Gly-Gly-HOLP, were commercially available. All amino acids, both free and in peptides, were the L isomer, except where noted. Gly-Gly-norvaline, Gly-Gly-norleucine, norleucyl-Gly-Gly, ethionyl-Ala, and L-histidinol phosphate were purchased from Cyclo Chemical Co. Gly-His-Gly and Lys-Lys-Lys were from Miles Laboratories. DL-norleucine, norvaline, and ethionine were from Calbiochem. Dicyclohexylcarbodiimide was from Schwarz/Mann and carbobenzoxy-Gly-Gly was from Fox Chemicals.

Synthesis of Gly-Gly-HOLP. A solution of 412 mg of dicyclohexylcarbodiimide (2 mmol) in dry pyridine (5 ml) was added to 530 mg of *N*-carbobenzoxy-Gly-Gly (2 mmol) and allowed to stand 1 min, at which time crystals started to appear. This mixture was then added to a solution of pyridine (10 ml), tri-*n*-butylamine (0.25 ml), water (5.6 ml), and HOLP·$2H_2O$ (129 mg; 0.5 mmol) in a 50-ml glass-stoppered Erlenmeyer flask. The mixture was magnetically stirred overnight at room temperature. Water (30 ml) was added and the mixture was filtered through a medium-porosity sintered glass funnel. The precipitate on the filter was washed with 70 ml of water and 100 ml of 1 N ammonia. The filtrate became cloudy on addition of the ammonia and was filtered through a fine sintered filter. This filtrate was extracted three times with 100-ml portions of ether. The water layer was brought to near dryness in a rotary evaporator and diluted to 25 ml with water. This was taken to dryness again, and further dried in a vacuum desiccator.

The above residue was decarbobenzoxylated, in the stoppered flask, by the addition of 5 ml of 30% HBr in glacial acetic acid containing 0.5 ml of dry anisole. The mixture was gently swirled and warmed by hand for 1 hr, a procedure that effected complete solution. The peptide was precipitated by the addition of 80 ml of anhydrous ether. The supernatant was decanted and the precipitate was transferred to a centrifuge tube and repeatedly washed with ether, centrifuging each time. The product was dried under reduced pressure over KOH and Drierite. It was then dissolved in water and brought to pH 8.1 with 10 N ammonia, to a final volume of 6.5 ml. A slight precipitate was removed by centrifugation.

The material contained three substances, as determined by high-voltage electrophoresis [4000 V, 30 min at pH 3.6 (pyridine–acetic acid–water 10:100:2890)]. The substances migrated toward the negative electrode: Gly-Gly (12 cm), HOLP (22 cm), and Gly-Gly-HOLP (18.5 cm). Visualization was by ninhydrin and by the Pauly spray for imidazoles (9). The first two compounds were identified by comparison with reference compounds.

The Beckman amino-acid analyzer was also useful in identifying the products. When the material was applied to the "short column" and eluted with the pH 4.25 buffer normally used as the second buffer on the "long column," Gly-

Abbreviation: HOLP, histidinol phosphate ester.

TABLE 1. *Growth inhibition by amino-acid analogues, free and in peptide form*

Compound	μmol	Strain Diameter (mm) of zone of inhibition *hisG46*	*hisG46 opp-7*	*hisG46 opp-8*	*hisG46 opp-10*	*hisG46 opp-16*
L-Norleucine	2	0	—	—	—	—
L-Norleucine	0.5	0	—	—	—	—
+ glycine	2					
L-Norvaline	2	0	—	—	—	—
L-Norvaline	0.5	0	—	—	—	—
+ glycine	2					
Norleucyl-Gly-Gly	0.8	16	0	0	0	0
Gly-Gly-norleucine	0.8	16	0	0	0	0
Gly-Gly-norvaline	1	17	0	0	0	0
Lys-Lys-Lys	2	20	0	0	0	0
L-Ethionine	2	0	0	0	0	0
L-Ethionyl-L-alanine	2	24	24	26	26	24

Bacteria (0.1 ml of a nutrient broth culture) and L-histidine (0.1 ml of a 0.1 M solution) were added to the soft agar layer of a minimal-medium petri plate (8). Filter paper discs (6 mm) containing the indicated amount of test substance were placed in the center. Incubation was at 37° overnight. A *dash* means "not assayed." A *zero* indicates no visible inhibition beyond the disc. Mutations *opp-7* and *opp-10* were isolated by resistance to Lys-Lys-Lys, *opp-10* by resistance to norleucyl-Gly-Gly, and *opp-16* by resistance to Gly-Gly-HOLP (see legend to Table 2).

Gly-HOLP eluted at 30 min, Gly-Gly at 14 min, and HOLP and ammonia emerged together at 56 min.

The material was preparatively fractionated (1.5 ml per column run) on a 1.5 × 160 cm column packed with AG-50-W-X8, 200–400 mesh resin (Bio-Rad) (10). The resin was washed on a sintered-glass funnel with: H_2O; 1 N NaOH; H_2O; 2 N HCl; H_2O; 2 N pyridine, and finally with a pH 3.1 buffer (557 ml of acetic acid–32.25 ml of pyridine in 2 liters) before the column was packed. The column was equilibrated and eluted at room temperature with the pH 3.1 buffer. The sample was adjusted to pH 3.0 with 6 N HCl before it was applied to the column. The flow rate was 98 ml/hr and 7-ml fractions were collected. Two aliquots from each of the fractions were spotted on separate sheets of paper. One sheet was sprayed with ninhydrin and the other with the Pauly reagent. Three separate peaks were obtained from the ion-exchange column fractionation: Gly-Gly (1722 ml), Gly-Gly-HOLP (2758 ml), and HOLP (3234 ml).

The pooled peak of Gly-Gly-HOLP was lyophilized and the residue was dissolved in water (1 ml). The yield from one column run of the peptide was 59.2 μmol of glycine (amino-acid analyzer analysis after 6 N HCl hydrolysis, 105°, 16 hr), and 32.5 μmol of phosphate (11), giving a yield of peptide based on the starting HOLP of 26%.

RESULTS

Table 1 shows that the analogues norleucine and norvaline are more inhibitory when present as components of peptides than as free amino acids. Oligopeptide permease mutants (*opp*) have been isolated by resistance to one of the various inhibitory tripeptides; each of them is cross-resistant to the other inhibitory tripeptides. These mutants are also unable to use Gly-His-Gly as a histidine source, while the wild-type strain can (Table 2). We conclude from these two observations that there is one oligopeptide permease functioning on various tripeptides. This conclusion is in agreement with the conclusion of Payne and Gilvarg (1), who had obtained a mutant for the oligopeptide permease (in *E. coli*) that is resistant to triornithine. The *opp* mutants and the wild type are still sensitive to a very inhibitory dipeptide analogue, ethionylalanine (Table 1), which is presumably transported through one of the dipeptide permeases.

TABLE 2. *Growth of histidine auxotrophs on histidine sources*

Compound	μmol	Strain Diameter (mm) of zone of growth *hisG46*	*hisB29*	*hisG46 opp-7*	*hisG46 opp-9*	*hisG46 opp-10*
L-Histidine	0.5	34	31	35	30	30
L-Histidinol	3	36	25	35	30	30
L-Histidinol phosphate	1.5	0	0	0	0	0
Gly-His-Gly	0.34	29	32	0	0	0
Gly-Gly-HOLP	0.05	19	0	0	0	0
	0.5*	29	0	—	—	—
	1.5*	39	0	—	—	—

The test was performed as described in Table 1, with the omission of L-histidine from the soft agar layer.

* These higher concentrations of Gly-Gly-HOLP cause a zone of inhibition near the disc, with an outer zone of growth in the strains capable of utilizing it. A resistant mutant from the zone of inhibition is an *opp* mutant (*hisG46 opp-16*).

The mutant *hisG46* (12) requires histidine for growth because of a defective first enzyme of histidine biosynthesis; histidinol, a histidine biosynthetic intermediate, can replace histidine for growth (Table 2). However, histidinol phosphate, another biosynthetic intermediate, is not utilized (Table 2) despite the presence in the bacteria of the enzymes converting histidinol phosphate to histidinol (the *hisB* enzyme) and histidinol to histidine (the *hisD* enzyme). The histidinol phosphate is not transported into the cell, whereas histidinol is. Table 2 shows that mutant *hisG46* is able to utilize Gly-Gly-HOLP as a histidine source. Mutants defective in the oligopeptide transport system, the *opp* mutants, do not utilize Gly-Gly-HOLP. We conclude that Gly-Gly-HOLP enters the cell via the oligopeptide permease system *opp*. We also conclude that Gly-Gly-HOLP is split to histidinol phosphate by an internal peptidase. The internal histidinol phosphate can then be converted by the *hisB* and *hisD* enzymes to histidine. The possibility that phosphatases in the cell can split the phosphate from Gly-Gly-HOLP and that then the histidinol released by peptidase action is utilized an as intermediate is ruled out because a *hisB* mutant, which lacks the specific histidinol phosphate phosphatase, does not grow on the peptide. This is shown in Table 2: mutant *hisB29* grows well on Gly-His-Gly, and therefore has a normal *opp* system, but it does not grow on Gly-Gly-HOLP because it cannot convert internal HOLP to histidinol.

Mutants in all of the genes of the histidine operon have been tested. As expected, those blocked before histidinol phosphate behave as does *hisG46;* the *hisD* mutants, like *hisB29*, do not grow on Gly-Gly-HOLP, but do grow on Gly-His-Gly.

DISCUSSION

We call the entry of compounds into cells through transport systems that are designed for other substrates *illicit transport.* We cite several examples of this phenomenon.

(*i*) Many amino-acid permeases are very specific, and even closely related amino-acid analogues are excluded (13, 14). In contrast, the aromatic permease, which transports the three aromatic amino acids, probably for use as carbon or nitrogen sources (13–15), is much less specific. Various analogues of the aromatic amino acids, histidine and many of its analogues, and the antibiotic azaserine are among the compounds that can be transported into the bacteria through this system (13, 14).

(*ii*) The dipeptide permeases are able to recognize various different dipeptides (1–7); therefore, analogues in the form of peptides, such as ethionylalanine, can be transported very efficiently into cells, while ethionine cannot be transported very efficiently through specific methionine permeases (Table 1).

(*iii*) The oligopeptide permease appears to be a single system designed to bring the wide variety of tripeptides and tetrapeptides into cells and, thus, it does not recognize the individual amino acids in the peptides, but only the terminal amino end and the peptide bonds (1–7). One can make use of these facts to get a normally nontransportable biosynthetic intermediate (e.g., histidinal phosphate) into cells, or to get various amino-acid analogues into cells (Tables 1 and 2).

(*iv*) The effectiveness of an antibiotic often depends not only on its ability to inhibit an enzyme, but also on its ability to enter bacteria through some preexisting transport system. The cases of albomycin and phosphonomycin can be used as examples of such illicit transport. Albomycin is an antibiotic that has an unnatural pyrimidine attached to a ferrichrome (a natural iron chelator taken up by bacteria as an iron source), and that enters through the ferrichrome uptake system (16). The antibiotic phosphonomycin enters cells through the glycerol phosphate and the glucose-6-phosphate transport systems (F. Kahan, cited in ref. 17), and then inhibits an enzyme that is involved in cell-wall synthesis (17).

Clearly, the further description of the various transport systems in bacteria and in humans should help in the utilization of illicit transport for practical purposes.

NOTE ADDED IN PROOF

We have learned from Fickel and Gilvarg that they have a paper in press in *Nature New Biol.* on an analogous study using homoserine phosphate in peptide form.

This work was supported by USPHS Grants AM 12092 (B. N. A.), AM 12121 (G. F. A.), and A1 10412 (J. D. Y.). We are indebted to Robert Karp for some of the early growth tests. The Space Sciences group acknowledges the encouragement and financial support of Thomas H. Jukes (NASA Grant NGR-05-003-460), as well as the extremely helpful advice of Danute Nitecki in the synthesis of Gly-Gly-HOLP.

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