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Portage of various compounds into bacteria by attachment to glycine residues in peptides

(peptide transport/portage transport/oligopeptide permease/antimicrobial agents/bacterial transport)

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ABSTRACT **Synthetic di- and oligopeptides are described that contain nucleophilic moieties attached to the α carbon of a glycine residue. These peptides are accepted by the peptide transport systems of *Escherichia coli* (and other microorganisms) and are capable of being hydrolyzed by intracellular peptidases. After liberation of its amino group the α-substituted glycine is chemically unstable (although it is stable in peptide form) and decomposes, releasing the nucleophilic moiety. Thus, the combined result of peptide transport and peptidase action is the intracellular release of the nucleophile. Peptides containing glycine residues α-substituted with thiophenol, aniline, or phenol are used as models for this type of peptide-assisted entry and their metabolism by *E. coli* is described. Peptides of this type have broad applicability to the study of microbial physiology and the development of an additional class of antimicrobial agents.**

Transport systems for peptides have been found in a wide variety of microorganisms (1). *Escherichia coli* has been studied most extensively in this regard and has been shown to possess three distinct systems, one for dipeptides, another for oligopeptides, and a third for tripeptides composed of amino acids with hydrophobic side chains. Extensive data have been accumulated over the last 20 years on the range of factors that determine the recognition of peptides by these transport systems. Because of the diversity of side-chain combinations that may be found in peptides composed of the 20 naturally occurring amino acids, the peptide transport systems of *E. coli* (and other microorganisms) appear to have developed so as to possess little demonstrable side-chain specificity.

The early recognition of this fact in the study of peptide transport in *E. coli* led to the demonstration (2, 3) that normally impermeant amino acid analogues (which are not recognized by the more stringent amino acid transport systems) could be transported into the cell when incorporated into the backbone of a peptide. After transport these amino acid analogues are released by the intense peptidase activity normally found in the cytoplasm. Several descriptive terms for this type of assisted entry have been used in the past, and the term "portage transport" has been suggested as the most appropriate of these (4). Numerous natural and synthetic examples of this phenomenon now exist (reviewed in ref. 4), mostly involving growth-inhibitory amino acid analogues. Considerable interest has been shown in utilizing this phenomenon as a means of developing chemotherapeutic agents, exemplified by the description of the wide-spectrum antibiotic alaphosphin (5) (L-alanyl-L-1-aminoethylphosphonic acid).

In efforts aimed at broadening the scope of portage transport to molecules other than amino acid analogues, we have recently (6, 7) described a method that allows the transport of sulfhydryl-containing compounds by their attachment through a disulfide bond to the cysteine residue of a di- or tripeptide. After transport, the sulfhydryl compounds are released by disulfide exchange reactions in the sulfhydryl-rich cytoplasm. Those findings validated the feasibility of using side-chain attachment to effect portage transport through the peptide transport systems of *E. coli*, but the method is limited to sulfhydryl compounds. In this paper, we describe a synthetic approach that again employs linkage to the side chains of the amino acid residues in a peptide, but it can be used in a more general way to achieve portage transport of compounds containing nucleophilic substituents. The compound is released, after transport, by intracellular peptidases.

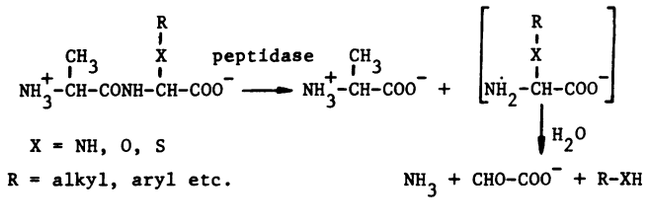

FIG. 1. Structure of α-glycine-substituted peptides and their mode of breakdown after peptidase cleavage.

RESULTS

Ordinarily, an α-substituted glycine in which the α carbon is linked to a nitrogen, oxygen, or sulfur atom is unstable (see Fig. 1). However, various *N*-acylated α-substituted glycines of this type have been described in the literature (for instance, refs. 8 and 9). Acylation of the amino group provides stabilization of the molecule by delocalizing the nitrogen electrons into the peptide bond. Instead of using simple *N*-acylation to provide chemical stability to such α-substituted glycines, we have used a peptide bond to an amino acid, thus obtaining peptide mimetics of the structure shown in Fig. 1. Such peptides may be synthesized by the condensation of *N*-carbobenzoxy-L-alanine amide and glyoxylic acid hydrate to give the corresponding α-hydroxy derivative of alanylglycine, which is converted to its benzyl ester. After acetylation of the hydroxy function, the acetate may be displaced by a nucleophile of choice and after deblocking, the dipeptide LL and LD diastereomers are separated by high-performance liquid chromatography. Longer peptides may be prepared from these by standard synthetic methods.

If these peptides bearing a nucleophile are recognized as substrates by both the peptide uptake systems and the intracellular peptidases, the result of transport of such a peptide across the microbial cell membrane followed by intracellular peptidase actions would then be the portage transport of the nucleophile (R—XH as shown in Fig. 1). The following ex-

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periments utilize peptides of the structure shown in Fig. 1, in which R = phenyl (Ph), as a model system to investigate the feasibility of this approach to portage transport.

Growth Inhibition by α-Substituted Glycine Dipeptides. *E. coli* is relatively insensitive to thiophenol, aniline, and phenol. Hence, an immediate test of the portage transport of these phenyl derivatives would be an exaltation of their toxicity when they are administered in the form of α-substituted peptides. All three such peptides produced zones of inhibition on agar plates seeded with strains of *E. coli*. Fig. 2 shows the dependence of zone size on the amount of added peptide for CB64*recA*/F'123, a strain we have described previously (6). The order of toxicity of these peptides was consistently X = S > X = NH > X = O, but some strains, including CB64*recA*/F'123, were dramatically more sensitive to the sulfur analogue peptide than others.

A comparison of the growth-inhibitory properties of the α-substituted glycine dipeptides with thiophenol, aniline, and phenol shows the peptides to be far superior in this respect. Although 30 nmol of the dipeptides produce distinct zones of inhibition (Fig. 2), no zones were obtained with 12 μmol of aniline or 1 μmol of thiophenol, and it takes 5 μmol of phenol to produce a detectable zone. The peptides are therefore at least 150 times more effective [150 times in the case of Ala-Gly(OPh) and at least 300 times for the other two peptides] in this regard than the free phenyl compounds. This illustrates one of the advantages of portage transport of relatively hydrophobic substances in Gram-negative bacteria. Such compounds are much less active against Gram-negative than Gram-positive bacteria because they are excluded from the cell by the lipopolysaccharide and protein components of the outer membrane (10). Peptides, on the other hand, are able to gain entry to the cytoplasmic membrane through the hydrophilic protein channels of the outer membrane. A second advantage is that simple molecules such as phenol exert their antimicrobial action on a consequence of diffusion, whereas peptides are concentrated by active transport (11–13).

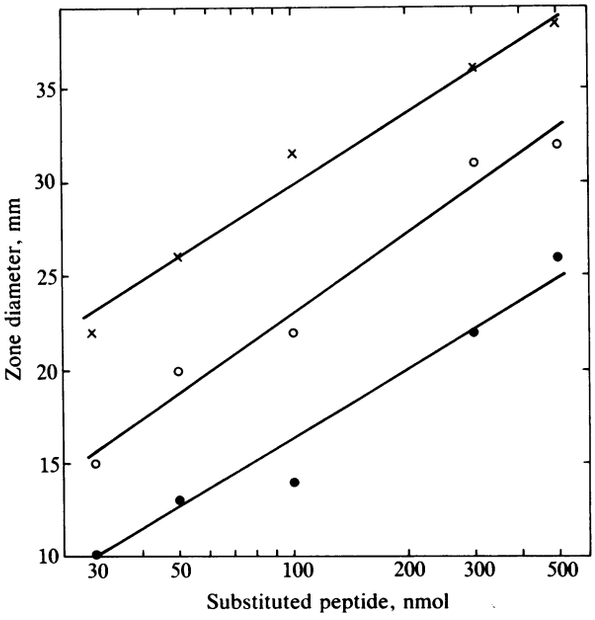

FIG. 2. Zones of inhibition produced by α-glycine-substituted peptides on *E. coli* seeded agar plates. A medium agar plates seeded with CB64*recA*/F'123 were prepared as described previously (6). Solutions of the three α-glycine-substituted dipeptides were added to filter paper discs (7-mm diameter) and the discs were transferred to the plates. After incubation at 37°C overnight the diameters of the zones of inhibition were measured. ×, Ala-Gly(SPh); ○, Ala-Gly(NHPh); ●, Ala-Gly(OPh).

Demonstration That α-Substituted Glycine Peptides Use Peptide Transport Systems. Oligopeptide transport-deficient mutants are easily obtained on the basis of their resistance to triornithine (14) (TOR mutants). We tested such strains with the tripeptide Ala-Gly(SPh)-Ala. Although the oligopeptide transport mutant CB64TOR*recA*/F'123TOR [described previously (6)] was still sensitive to the dipeptide, it was completely resistant to the tripeptide.

Further evidence that transport is necessary for the production of growth inhibition was obtained by the isolation of mutants resistant to the sulfur analogue dipeptide. Because dipeptides appear to be able to use the oligopeptide transport system as well as the dipeptide transport system (15), dipeptide transport mutants are usually isolated from strains deficient in oligopeptide transport. When approximately 10^8 cells of CB64TOR*recA*/F'123TOR were spread on an A medium (16) agar plate containing 1 mM Ala-Gly(SPh) and incubated (37°C) for 48 hr, several large colonies were apparent on the surface of a slow-growing lawn. As incubation proceeded the characteristic odor of thiophenol was apparent. After single-colony purification, a representative of the larger colony population was characterized as a dipeptide transport mutant by its cross-resistance not only to the oxygen and nitrogen analogue peptides but also to the dipeptide antibiotics alaphosphin (5) and bacilysin (17) and other dipeptides that inhibit the growth of *E. coli* K-12, such as Leu-Leu and Gly-Val. [Cell-free extracts prepared from parental and mutant cultures were comparable in their ability to hydrolyze Ala-Gly(SPh), and so resistance is not due to a change in peptidase activity.]

A further demonstration of the use of this method to effect portage transport of antimicrobial agents was obtained by preparing a dipeptide and a tripeptide containing a glycine residue α-substituted with the known antimetabolite 5-fluorouracil. The attachment was through N-1 and alanine was used for the remaining amino acid residues. These peptides were found to be effective growth inhibitors of *E. coli*, and testing the same transport-deficient mutants described above showed that their action depended upon accumulation via peptide transport systems. That growth inhibition was a result of intracellular liberation of 5-fluorouracil was shown by the fact that a mutant showing reduced sensitivity to 5-fluorouracil (presumably through a defect in uracil phosphoribosyltransferase activity) also showed reduced sensitivity to the peptides.

Metabolism of Ala-Gly(SPh) by Whole Cells and Cell-Free Extracts. Clearly, both the peptide transport systems and the intracellular peptidases have accepted these compounds as if they were normal peptides. The perfection of the mimicry can be assessed particularly well with the thiophenyl derivative, since the release of thiophenol is easily measured with Ellman's reagent [5,5'-dithiobis(2)-nitrobenzoic acid)]. This reagent reacts rapidly and quantitatively with sulfhydryl compounds, producing an increase in absorbance at 412 nm (ε = 13,600 M^{-1} cm^{-1}) (18).

When cell-free extracts were prepared from CB64TOR-*recA*/F'123TOR and added to Ala-Gly(SPh) in the presence of Ellman's reagent, a rapid liberation of sulfhydryl was observed. The concentration dependence of the initial rate of reaction is shown in Fig. 3. The reaction showed saturatable kinetics, from which a K_m of 850 μM could be calculated. The enzyme or enzymes responsible for the cleavage of this peptide were metal dependent (inhibited by EDTA) and sulfhydryl dependent (inhibited by prolonged incubation with Ellman's reagent), characteristics that have been found previously for *E. coli* peptidases.

The action of these inhibitory peptides could conceivably be a result of the liberated α-substituted glycine having a lifetime long enough for it to be recognized by cellular enzymes as a phenylalanine analogue. This seems unlikely, because

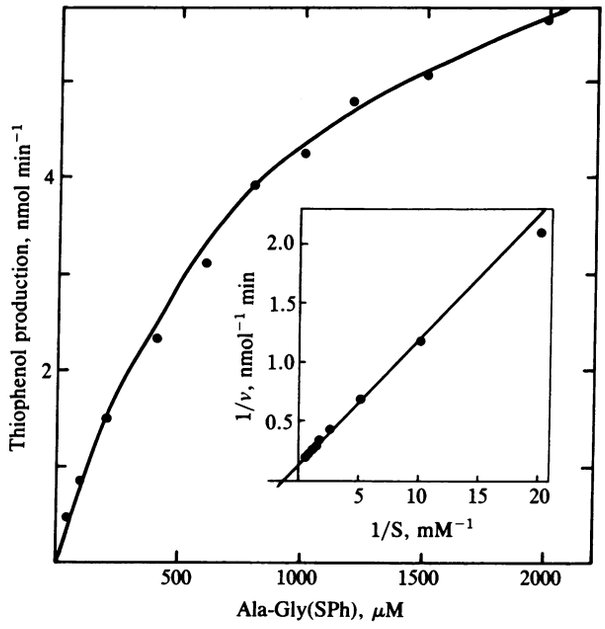

FIG. 3. Concentration dependence of the rate of thiophenol production from Ala-Gly(SPh) by extracts of *E. coli*. A culture of CB64TOR*recA*/F'123TOR in A medium (16) (200 ml) was grown to late logarithmic phase, centrifuged (4000 × *g*, 10 min) and washed twice with potassium phosphate buffer (200 ml, 20 mM, pH 7.6), and resuspended in this buffer (7.5 ml). After cooling on ice, the suspension was sonicated (10 min) with cooling in a Raytheon 10-kHz sonic oscillator. The sonicate was centrifuged (4°C, 12,000 × *g*, 15 min) and the supernatant was removed and kept on ice. The rates of production of thiophenol from various concentrations, S, of Ala-Gly(SPh) were measured in phosphate buffer (1 ml, 20 mM, pH 7.6) containing Ellman's reagent (0.5 mM) and cell extract (50 μl) by measuring the increase in absorbance at 412 nm at 1-min intervals for 15 min. From the linear increase in absorbance during the first 10 min, the rates of production of thiophenol, *v*, were calculated. Points are experimental; the solid curve is theoretical for K_m 850 μM, v_{max} 8.05 nmol min^{-1}. (*Inset*) Reciprocal plot of these data.

the addition of phenylalanine to the medium had no effect on the toxicity of these peptides to whole cells. In addition, with cell-free extracts with Ala-Gly(SPh), in the presence of Ellman's reagent, there was no detectable lag in the production of thiophenol.

We have shown previously (7) that Ellman's reagent can be used with suspensions of *E. coli* to follow the release of sulfhydryl compounds. When we applied this technique to whole cells in the presence of Ala-Gly(SPh) it was apparent that the concentration dependence of sulfhydryl production was very different from that observed with cell extracts, being independent of concentration down to 25 μM peptide and showing a small decrease in rate at 10 μM (the limit of detection with this method). It appears that with whole cells the rate of release of thiophenol is dependent upon the strong affinity of the dipeptide for its transport system and not upon the weak interaction with the peptidases. A comparison of the relative K_m values for transport of Ala-Gly(SPh) and Ala-Phe could be made by using Ala-Phe as a competitor for the release of thiophenol from Ala-Gly(SPh). Fig. 4 shows a Dixon (19) plot of data from such an experiment, the result of which shows Ala-Gly(SPh) to have a K_m of 5 μM and Ala-Phe a K_i of 15 μM. The α-substituted glycine dipeptide mimetic thus binds with somewhat enhanced affinity compared to the natural dipeptide. In the case of Ala-Gly(SPh), therefore, neither recognition by the dipeptide transport system nor the intracellular peptidases is a limiting factor in its metabolism by whole cells.

In accord with the known stereospecific requirements of the peptide transport systems of *E. coli* (1) the LD stereoisomers of the α-substituted glycine dipeptides had no effect on the growth of *E. coli*, and furthermore no thiophenol was produced from the LD isomer of Ala-Gly(SPh) by cell-free extracts.

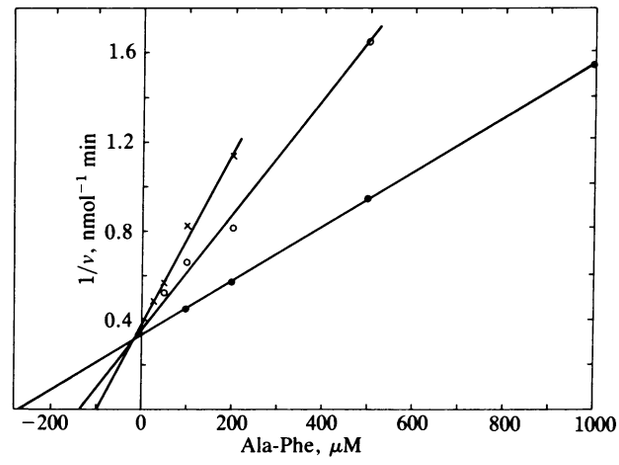

FIG. 4. Dixon (19) plot of the inhibition by Ala-Phe of thiophenol production from Ala-Gly(SPh) by *E. coli*. CB64TOR*recA*/F'123 was grown in A medium (16) to logarithmic phase (1.2 × 10^8 cells per ml), centrifuged (4000 × *g*, 10 min) and washed twice with 1 vol of buffer (50 mM potassium phosphate/0.5 mM magnesium chloride/25 mM ammonium chloride, pH 7.0), and resuspended in 2 vol of this buffer. To 1-ml aliquots of the suspension containing glucose (0.5% wt/vol) and Ellman's reagent (0.5 mM) were added Ala-Gly(SPh) and various concentrations of Ala-Phe. Absorbance at 412 nm was followed on a Gilford spectrophotometer with chart recorder relative to a blank of cell suspension with glucose and Ellman's reagent. The rate of increase of absorbance at a given concentration Ala-Gly(SPh) was linearly dependent upon cell density and independent of Ellman's reagent concentration up to 2 mM. From the linear increase in absorbance over a 12-min period, the rate of production of thiophenol, *v*, was calculated, and it was normalized to a cell density having an optical density of 1.0 at 660 nm. The reciprocal of the rate is plotted against Ala-Phe concentration for three Ala-Gly(SPh) concentrations: ×, 25 μM; ○, 50 μM; ●, 100 μM.

DISCUSSION

The demonstration of the feasibility of the α-substituted glycine peptide approach to portage transport using simple phenyl compounds provides a method that will allow the investigation of the effects of a wide variety of compounds on microbial physiology. By overcoming the permeability barrier of the outer envelope in Gram-negative bacteria to hydrophobic molecules, the use of α-substituted glycine peptides in the way described here provides opportunities for their investigation not previously available. Moreover, since the oligopeptide transport system of *E. coli* appears to be insensitive to the charge on the amino acid side chains (2), this method will also allow the accumulation of hydrophilic charged molecules that normally would not be expected to cross the cytoplasmic membrane. The ability of peptide transport to allow the accumulation of such a charged moiety has been demonstrated previously by using alaphosphin, when concentrations of up to 50 mM of L-1-aminoethylphosphonic acid were found in cell extracts (20). Peptide transport is widespread in the microbial world, and we have shown several α-substituted glycine peptides to be metabolized by a range of microorganisms, including yeast.

The full potential of portage transport using α-substituted glycine peptides to provide chemotherapeutic agents is a subject for future research. Increasing use of enzyme inhibition screens is being made in the pharmaceutical industry to search for new antimicrobial agents. Synthetic studies aimed

at the definition of an inhibitor for a particular enzyme, perhaps based on substrate or transition state analogues, will often lead to small molecules that are relatively impermeant into microbial cells. The method of portage transport described here is thus an important adjunct to such an approach. The presence of multiple peptide transport systems with differing substrate requirements suggests that the simultaneous use of two different synthetic peptides carrying the same inhibitory agent would overcome potential problems of resistance due to loss of a transport system.

During the course of this work we have developed a simple spectrophotometric assay for peptide transport, based on the detection, by Ellman's reagent, of thiophenol liberated from α-substituted thiophenyl glycine peptides, which is applicable to a number of microorganisms and enables the relative affinities of peptides for the transport systems to be determined by competition experiments. The opportunity to synthesize a series of closely related peptides differing in a parameter of choice (e.g., size, charge, or hydrophobicity of the side chain) will greatly simplify the delineation of the substrate characteristics of peptide transport systems for a given microorganism.

In considering limitations of the procedures outlined in this article, from a chemical point of view one can foresee that the attachment in the way described here of molecules that can function as extremely good leaving groups might lead to problems of instability in aqueous solution, and indeed we have found L-alanyl-L-α-(*p*-nitrophenyloxy)glycine to have a half-life of 7 min at pH 6.8. Similarly, one would anticipate that easily protonated nitrogen atoms would not provide a stable linkage. On the other hand, no difficulty has been experienced in preparing pyrimidine, benzimidazole, and nucleoside peptide adducts, among others.

1. Payne, J. W. & Gilvarg, C. (1978) in *Bacterial Transport*, ed. Rosen, B. P. (Dekker, New York), pp. 325–383.
2. Fickel, T. E. & Gilvarg, C. (1973) *Nature (London) New Biol.* **241,** 161–163.
3. Ames, B. N., Ames, G. F., Young, J. D., Isuchiya, D. & Lecocq, J. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 456–458.
4. Gilvarg, C. (1981) in *The Future of Antibiotherapy and Antibiotic Research*, eds. Ninet, L., Bost, P. E., Bovanchaud, D. H. & Florent, J. (Academic, New York), pp. 351–361.
5. Allen, J. G., Atherton, F. R., Hall, M. J., Hassall, C. H., Holmes, S. W., Lambert, R. W., Nisbet, L. J. & Ringrose, P. S. (1978) *Nature (London)* **272,** 56–58.
6. Boehm, J. C., Kingsbury, W. D., Perry, D. & Gilvarg, C. (1983) *J. Biol. Chem.* **258,** 14850–14855.
7. Perry, D. & Gilvarg, C. (1983) *J. Biol. Chem.* **258,** 14856–14860.
8. Zoller, U. & Ben-Ishai, D. (1975) *Tetrahedron Lett.* **31,** 863–866.
9. Matthies, D. (1970) *Pharmazie* **25,** 522–524.
10. Nikaido, H. (1976) *Biochim. Biophys. Acta* **433,** 118–132.
11. Kessel, D. & Lubin, M. (1962) *Biochim. Biophys. Acta.* **71,** 656–663.
12. Jackson, M. B., Becker, J. M., Steinfeld, A. S. & Naider, F. (1976) *J. Biol. Chem.* **251,** 5300–5309.
13. Payne, J. W. & Bell, G. (1979) *J. Bacteriol.* **137,** 447–455.
14. Barak, A. & Gilvarg, C. (1974) *J. Biol. Chem.* **249,** 143–148.
15. Payne, J. W. & Gilvarg, C. (1968) *J. Biol. Chem.* **243,** 335–340.
16. Davis, B. D. & Mingioli, E. S. (1950) *J. Bacteriol.* **60,** 17–28.
17. Walker, J. E. & Abraham, E. P. (1970) *Biochem. J.* **118,** 563–570.
18. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77.
19. Dixon, M. (1953) *Biochem. J.* **55,** 170–171.
20. Atherton, F. R., Hall, M. J., Hassall, C. H., Lambert, R. W. & Ringrose, P. S. (1979) *Antimicrob. Agents. Chemother.* **15,** 677–683.