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Transport of Sulfanilic Acid via Microbial Dipeptide Transport System

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Sulfanilic acid (4-aminobenzenesulfonic acid) alone is normally not permeant in bacteria but can be readily delivered via the microbial dipeptide transport system. A dipeptidyl derivative of this compound, L-phenylalanyl-L-2-sulfanilylglycine (PSG), prepared by attachment of its primary amino group to the phenylalanyl α-glycine moiety, appeared to have a Km of 0.125 mM and a Vmax of 1.9 nmoles/ml/min (A_{660}, 1.0) in *Escherichia coli*. From competitive spectrophotometric analysis, it was found that the type of amino acids in both of the N- and C-terminals affected the kinetic power of dipeptides. The growth inhibitory effect of PSG was over 7 times more potent than that of the sulfanilic acid against *E. coli*, suggesting that this potential inhibition was presumably due to the increased hydrophobic nature of the sulfanilyl dipeptide.

Microbial uptake for impermeable compounds can be facilitated when they are delivered via certain peptide transport systems (17). Most of these, both natural and synthetic, are attached to peptidyl α-termini then readily liberated to be active after hydrolysis by intracellular peptidases (1, 4, 18). In alternative method (12), certain nucleophilic molecules can be loaded onto any peptidyl α-glycine residues internally so that the hydrolytic release of the glycine amino group allows them to be expelled. These compounds may contain a nucleophilic atom, i.e., S, O, N, to link with α-carbon of a glycine residue (10).

There are numerous papers dealing with peptide transport systems in microorganisms (15). Among these, extensive studies have been carried out with *E. coli*. It is now generally accepted that this bacterium contains di-, tri- and oligopeptide transport systems with their own specificities for the nature of peptide side chains (16). Unnatural amino acid side chains were reported to be less tolerable by the dipeptide transport system than by the oligopeptide transport system in *E. coli*. For example, the antibacterial activity of sulfanilic acid, a competitive inhibitor of dihydropteroic acid synthase involved in folic acid biosynthesis, could be increased over 200 times as the constituent of a tripeptide. On the contrary, by dipeptidyl delivery of this compound the growth inhibitory effect against this organism was rather decreased (5). This was presumably caused by the decreased rate of intracellular accumulation of sulfanilic acid because of difficulty in the transport.

It was therefore of interest to know whether the dipeptide transporter allows more permeability to the dipeptide by increasing its hydrophobicity. In this paper, we describe the portage kinetics of a number of microbial dipeptide transport systems and discuss rational ways of delivering impermeable molecules via these systems.

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Key words: sulfanilic acid, microbial dipeptide transport system, dipeptide prodrug

MATERIALS AND METHODS

Chemicals

L-Phenylalanyl-L-2-sulfanilylglycine (PSG) and L-phenylalanyl-L-3-thia-phenylalanine (PSP) were prepared by substituting sulfanilic acid and thiophenol to the acetoxy moieties of the corresponding protected 2-acetoxyglycyl dipeptides, respectively, followed by deprotections. Detailed procedures will be described in a separate report (6). 2,4,6,-Trinitrobenzenesulfonic acid (TNBS), 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), dipeptides, and sulfanilic acid were purchased from Sigma Chem. Co., USA. Thiophenol was obtained from Junsei Chem. Co., Japan. Unless otherwise indicated, all chemicals were commercial preparations of analytical reagent grade.

Microorganisms and Culture Condition

Escherichia coli No.20 (11) and *Staphylococcus aureus* No.19 (2) are stock strains in our laboratory. *Pseudomonas aeruginosa* 1630 and *Candida albicans*

1940 were purchased from KCTC, Korea. Strains were inoculated into culture tubes (1.8×20 cm) with 5 ml of nutrient medium containing 0.5% polypeptone and 0.3% yeast extract, pH 7.0, followed by cultivation at 30°C overnight in a water bath shaker (90 rpm). Antimicrobial activity was determind by measuring the growth inhibitory zone of disc on agar plates of the Lascelles' basal medium (13) containing additionally 0.1% NaCl and 1% glucose, pH 6.8.

Preparation of Intact Cells

Late logarithmic cells were harvested by centrifugation at 10,000 rpm for 10 min and washed typically by resuspending into 50 mM phosphate buffer (PB), pH 7.0. Cells were then suspended into 50 mM PB, pH 7.0 to give 1.0 O.D. units at 660 nm. Cell suspensions were either directly used for transport experiment or could be stored at -80°C before use without significant loss of transport functions.

Portage Transport of Dipeptides

Cell suspensions (0.9 ml; A_{660}, 1.0) were placed in test tubes (1×10 cm) on ice and then initial O.D. was measured. Transport was initiated by the addition of 0.1 ml of 10 mM dipeptides at 30°C. Aliquots of 0.2 ml were collected at 2-min time-intervals and centrifuged. The amounts of extracellular primary amino group in the supernatant were determined by using the TNBS method as described previously (ε_{420}=10,000/M/cm) (7).

Determination of Kinetic Constants of Dipeptide Transports

One mililiter of the cell suspension containing 0.1 mM PSP and 0.1 mM DTNB or, if necessary, with 0.1 mM dipeptide was placed in 1-cm light-path cuvette on ice. And the thiophenol production was monitored spectrophotometrically (ε_{412}=16,000/M/cm) (3). From the progress curves obtained by time course production of thiophenol with or without a dipeptide, the time lag could be measured. Since the delayed time to achieve same extent of thiophenol yielded by PSP alone was exclusively caused by the presence of competing dipeptide, the portage kinetic constants of dipeptides could be determined by competitive spectrophotometry (8).

RESULTS AND DISCUSSION

Microbial Transports of Sulfanilic Acid and PSG

Cellular uptake rates of primary amine compounds (14) can be easily determined by monitoring progressive reductions in the extracellular concentration of yellow pycryl adducts formed in the presence of TNBS. Diazotization (11) in particular was employed to measure the amount of sulfanilic acid. To discriminate delivery systems between sulfanilic acid and PSG in microbial cells, uptake rates of these compounds were measured at different pHs. The transport of sulfanilic acid was generally not affected by glucose but with the only exception of *Pseudomonas aeruginosa* appeared to be rather dependent upon the medium pH (Table 1). However, the uptake of PSG was apparently accelerated by the addition of glucose in most strains examined. Also, the optimal uptake pH of sulfanilic acid was observed at 6.0 whereas that of PSG was at 7.0, indicating that these compounds would be delivered in different ways. It is of interest that the *Candida albicans*, a well known pathogenic eukaryote, showed similar properties as bacteria.

Portage Kinetics of Microbial Dipeptide Transport Systems

Although the data presented in Table 1 were not sufficient to postulate the inducing antimicrobial activity of the PSG, we assumed the potential of this dipeptide in relation to the successful application for pathogenic cells. In order to elucidate the properties of functional barriers in those microbial transport systems for dipeptides that contain unnatural side chains, an attempt to determine the portage kinetics of microbial dipeptide transport systems was performed. In the previous paper (9), it was suggested that competitive spectrophotometry is a suitable methodology for this purpose. Therefore, a number of dipeptides including PSG were examined to evaluate their kinetic constants using PSP as a detector peptide (8). As an example, the analysis of Phe-Phe transport kinetics in *Escherichia coli* is shown in Fig. 1. Two progress curves were obtained by plotting the product concentration as a function of time in the presence or absence of Phe-Phe. It gave different time lags (Δt) by

Table 1. Determination of the cellular uptake rate of sulfanilic acid and PSG.

Strain		*Uptake rate (nmoles/ml/min)			
		pH 5.0	pH 6.0	pH 7.0	pH 7.0 (1% Glc)
Escherichia coli	sulfanilic acid	0.17	0.27	0.25	0.22
	PSG	0.40	0.40	0.55	0.63
Staphylococcus aureus	sulfanilic acid	0.19	0.22	0.11	0.11
	PSG	0.17	0.21	0.26	0.33
Pseudomonas aeruginosa	sulfanilic acid	0.17	0.17	0.17	0.17
	PSG	0.27	0.31	0.33	0.33
Candida albicans	sulfanilic acid	0.13	0.22	0.13	0.13
	PSG	0.33	0.30	0.33	0.42

*Uptake rates indicate mean values of the time course of decreased amounts of each compound in the medium for 10 min. The residual amounts of sulfanilic acid and PSG were determined as described in Materials and Methods.

change in the local concentration of PSP (S). According to the linear function of the competitive equation, a plot of t at [S] as a function of $[(So-y)/So]^n$ would give a straight line when the true n value ($[K_m$ PSP/V_{max} PSP] [V_{max} Phe-Phe/K_m Phe-Phe]) is computed. In Fig. 2 a straight line was achieved by computing 1 for n, indicated that the kinetic power (V_{max}/K_m) of the Phe-Phe would be identical to the PSP but different in each value of kinetic constants. This methodology is particularly useful when comparative data are needed to access a certain kinetic function. As shown in Table 2, individual tranporters apparently contain their own specificities for both N- and C-terminal amino acids. In fact, the presence of extra unnatural sulfone group in the dipeptide (PSG) caused all transporters to be reduced in their kinetic powers to some extent. The highest affinity for this compound was found in *Escherichia coli*, having the strongest kinetic power (K_m/V_{max}) among those tested. Interestingly, the kinetic power of Phe-Gly was 15 times higher than Ala-Gly in the case of *Escherichia coli*. It was therefore of interest to evaluate the *in vitro* anti-*Escherichia coli* activity of the PSG directly. In order to circumvent the possible existence of extra para-aminobenzoic acid in the medium, a defined medium (see Materials and Methods) and *Escherichia coli* were selected as a testing vehicles.

In vitro Antimicrobial Activity of PSG

From disc zone analysis, it was found that PSG was 7 times more potent than sulfanilic acid in antimicrobial activity (Fig. 3). On the contrary, L-alanyl-L-2-sulfanilylglycine (ASG) in the literature was paradoxically 4 times poorer than sulfanilic acid (5). This discrepancy may be the most closely interpreted in re-

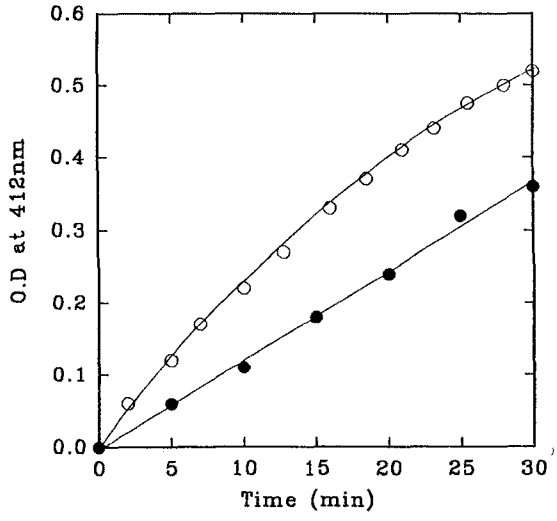

Fig. 1. Transport competition between PSP and Phe-Phe for intact cell system of *Escherichia coli*.
For the upper curve (○—○), the intact cells suspended in 50 mM phosphte buffer (pH 7.0) was consisted of 0.1 mM DTNB and 0.1 mM PSP. For the lower curve (●—●), the conditions were identical but, in addition, 0.1 mM Phe-Phe was present.

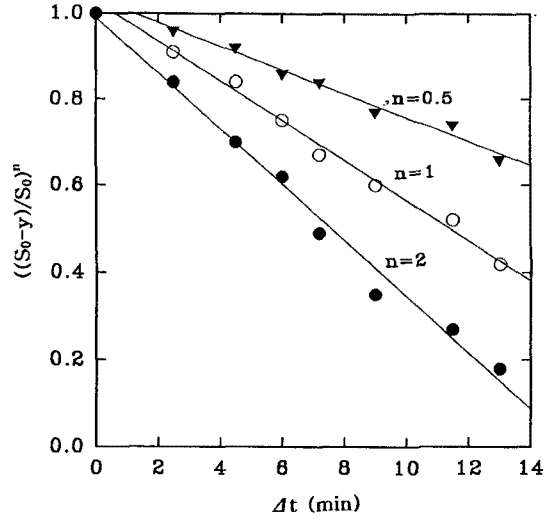

Fig. 2. Evaluation of the ratio of kinetic powers of PSP and Phe-Phe.
The additional time required to achieve the same extent of hydrolysis of PSP due to the presence of Phe-Phe (see Fig. 1) was plotted as a function of the fraction of the reaction completed raised to the exponent indicated of the curve. For details, refer to the text.

Table 2. The Kinetic constants of dipeptides by competitive spectrophotometry.

Compound	*Escherichia coli*		*Staphylococcus aureus*		*Pseudomonas aeruginosa*		*Candida albicans*	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
PSP	3.3	0.022	3.4	0.070	0.98	0.038	2.2	0.032
Phe-Phe	4.8	0.032	3.5	0.071	0.90	0.017	2.8	0.081
PSG	1.9	0.125	1.7	0.512	1.15	0.089	1.8	0.260
Phe-Gly	7.9	0.018	3.4	0.114	0.26	0.014	7.2	0.070
Ala-Gly	1.7	0.057	8.8	0.120	0.45	0.018	1.7	0.181

1 ml of the cell suspension dissoved in 50 mM phosphate buffer, pH 7.0 was adjusted to O.D. 0.5 at 660 nm and, placed in a 1-cm light-path cuvette and an initial spectrophotometric reading was taken. The reaction was then initiated by the addition of 10 μl of 10 mM PSP and the absorbance was read continuously at 412 nm (ε_{412}=10,000^{-1} cm^{-1}). The maximal rates were expressed as nmoles per mililiter per minute and the affinity constants, as mM. Same experiment was then carried out in the presence of dipeptide. The kinetic constants of dipeptides employed were calculated by the method shown in Fig. 2.

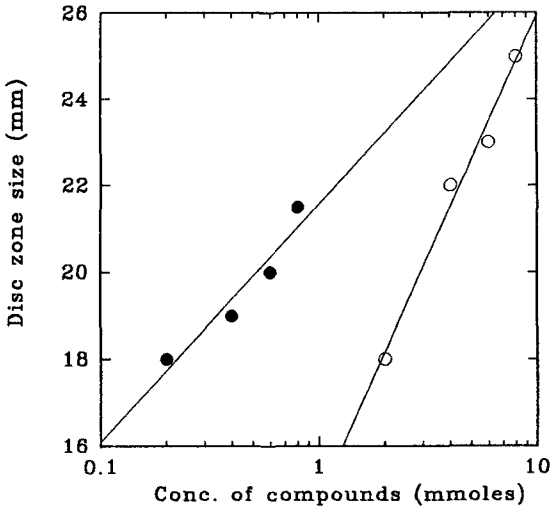

Fig. 3. Zones of inhibition produced by PSG and sulfanilic acid on *Escherichia coli* seed agar plate.
Disc holes were made on the agar plate and 25 μl each of solutions were added. After incubation at 30°C for 9 h the diameters of the zones of inhibition were measured. Open circles were for sulfanilin acid and closed circles, for PSG, respectively.

lation to alternative kinetic powers by changing N-terminal amino acid (X-Gly) as above, although we could not compare the kinetic powers of those two compounds simultaneously.

The potential induction of sulfanilic acid activity is conceivably due to the increased hydrophobic nature of this dipeptidyl prodrug. For this reason, it is realistic to assume that the unnatural amino acid side chain is generally more tolerated by microbial oligopeptide transport systems than by their dipeptide transport systems (16). In this paper, however, the proposed concept of impermeant amino acid delivery via microbial dipeptide transport systems by increasing their hydrophobic natures will provide promising design of dipeptidyl prodrugs. To do this, a comparative analysis of portage kinetics for individual compounds is required, i.e., by using competitive kinetics.

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