The Use of Cysteinyl Peptides to Effect Portage Transport of Sulphydryl-containing Compounds in *Escherichia coli* *

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We describe a method by which sulphydryl compounds may be transported into *Escherichia coli* as the mixed disulfides with a cysteine residue of a di- or tripeptide. Transport occurs through the di- or oligopeptide transport systems, and it is suggested that subsequent release of the sulphydryl compound occurs as a result of a disulfide exchange reaction with components of the sulphydryl-rich cytoplasm. The free sulphydryl compounds used here (2-mercaptopyridine and 4-[N-(2-mercaptoethyl)]aminopyridine-2,6-dicarboxylic acid) show weak growth-inhibitory properties in their own right, but sulfdide linkage to a cysteinyl peptide results in a considerable enhancement (up to 2 orders of magnitude). This is the first example of the use of the peptide transport systems of *E. coli* to effect portage transport of a poorly permeant molecule by using attachment to the side chain of one of the amino acid residues of a peptide; all previous examples have involved the incorporation of amino acid analogues into the peptide backbone. The synthesis of cysteinyl peptides containing disulfide-linked 2-mercaptopyridine is described. Displacement of the 2-mercaptopyridine by sulphydryl compounds of interest proceeds rapidly and quantitatively in aqueous alkaline solution to provide the required peptide disulfides.

The di- and oligopeptide transport systems of *Escherichia coli* have been studied extensively with respect to the molecular features of their substrates which are pertinent to their transport. Both systems require a primary or secondary NH, ular features of their substrates which are pertinent to their transport. Both systems require a primary or secondary NH, whereas the dipeptide system will allow carboxyl-substituted peptides to be transported, although such peptides have considerably reduced affinities compared to their unsubstituted counterparts (Mostillo, 1977a, 1977b). In contrast to these exacting requirements with respect to the peptide backbone, both systems show little side chain specificity. For this reason it was recognized early in the study of oligopeptide transport that, by incorporation of a normally impermeant or poorly permeant molecule into a peptide backbone (in such a way as not to alter the features of the peptide necessary for its transport), its entry into the cell could be effected or enhanced (Fickel and Gilvarg, 1973; Ames et al., 1973). Numerous examples of synthetic and naturally occurring peptides which elaborate this principle now exist. As a general description for this type of assisted entry the term "portage transport" has been suggested as more appropriate than other terms which have been used in the past (Gilvarg, 1981).

Most examples of portage transport by the di- and oligopeptide transport systems involve amino acid analogues which inhibit various intracellular enzyme activities. In their own right, these compounds usually exhibit poor antimicrobial activities, presumably because their structures differ sufficiently from those recognized by the amino acid transport systems to prevent efficient uptake. However, when incorporated into peptides, uptake is followed by rapid intracellular liberation of the inhibitory compound as a result of peptidase activity. Therefore, such peptides are often potent antimicrobial agents. Peptide transport systems appear to be widespread in both prokaryotic and eukaryotic microorganisms, and the idea of using portage transport with peptide vehicles as a means of developing new antimicrobial agents has received considerable interest recently.

There are, however, no examples in the literature of portage transport involving a peptide in which one of the amino acids has had attached to its side chain a potentially inhibitory compound. It is, of course, a prerequisite in the design of such a peptide that the bond linking the compound to the amino acid side chain be capable of rupture within the cell, either by enzymic means or through chemical reaction with a component of the cell pool. It seems likely that an amide bond would be unsuitable for this purpose, since it was shown in a lysine auxotroph of *E. coli* that, although a peptide containing ε-acyethylsine was transported, the lysine residue did not become available for use (Gilvarg and Katchalski, 1965). A consideration of the side chains of the naturally occurring amino acids suggested to us that the use of a disulfide linkage to a cysteine residue of a peptide might best meet our criteria for an investigation of portage transport by this method. It is known that many bacteria and fungi contain a high intracellular concentration of sulphydryl-containing compounds, principally glutathione (Roberts et al., 1963; Jocelyn, 1972). This pool of sulphydryl compounds could serve to release a sulphydryl compound from a mixed disulfide via (noneyzymic) disulfide exchange, a facile reaction at physiological pH. We have developed a method for the synthesis of such peptides by the use of (2-mercaptopyridine)-cysteine disulfide. The 2-mercaptopyridine moiety is an excellent leaving group, and participates extremely readily in exchange reactions with

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FIG. 1. Structures of Cys-2MP and MEPDA.

other sulfhydryl-containing compounds at alkaline pH. The applications of 2-mercaptopyridine disulfides in such reactions have been reviewed (Brocklehurst, 1979, 1982). After synthesis of peptides containing Cys-2MP

Fig. 2. Comparison of 2MP, AlaCys-2MP, and AlaAlaCys-2MP as growth inhibitors of E. coli strains in agar diffusion tests. Zones of inhibition produced by 2MP (■), AlaCys-2MP (O), and AlaAlaCys-2MP (×) on seeded agar plates after overnight incubation were determined as described under “Experimental Procedures,” using strains CB64recA/F’123 (A), CB64TORrecA/F’123 (B), and CB64TORrecA/F’123TOR (C).

1 Portions of this paper (including “Experimental Procedures,” Table I, and Fig. 4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-3055, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

charged at physiological pH, we wanted to test the effect on their transport of being attached to both neutral and positively charged peptides. The following synths were prepared as described under “Experimental Procedures”: AlaCys-2MP, AlaAlaCys-2MP, and OrnAlaCys-2MP.

Preliminary screening showed 2MP itself to have antibacterial activity. When the synths were tested by disc assay
on seeded agar plates as sole cysteine source for CB64, all three peptides supported growth, but the zones of growth were complicated by the ability of these compounds to supply 2MP, a growth inhibitor, while simultaneously supplying cysteine. Growth immediately surrounding the disc was poorer than that further from it. By complementing the trp and cysB mutations of CB64 with the F'123 factor (known to carry a large segment of the E. coli chromosome from a position before gatu to beyond pyrF) it was possible to test these peptides as growth inhibitors alone.

Since the F'123 episome also carries the gene(s) for oligopeptide transport, it was possible to examine the effect of reducing the level of oligopeptide transport on the relative effectiveness of these peptides, by constructing strains having various combinations of chromosomal and episomal transport mutations. CB64recA/F'123, CB64TORrecA/F'123, and CB64TORKrecA/F'123TOR were isolated as described under "Experimental Procedures"; in this order, these strains show decreasing relative rates of transport of [14C]Gly, of 2:1:0. Fig. 2 shows inhibition zones produced in agar diffusion tests with these strains using 2MP, AlaCys-2MP, and AlaAlaCys-2MP. Although the seeded agar plates were prepared under rather standardized conditions, the response to these compounds was somewhat variable from one set of plates to another, the response to 2MP itself showing the most variability. Although exact quantitation of the relative inhibitory powers of these three compounds to the three strains was difficult, certain relationships were always observed, and Fig. 2 shows typical data. Against CB64recA/F'123, both peptides were significantly more inhibitory than 2MP itself, the average of four determinations showing the dipeptide to be 4 times, and the tripeptide 6 times, more inhibitory on a molar basis. Against CB64TORrecA/F'123, the tripeptide became somewhat less effective, the average of three determinations showing both it and the dipeptide to be 3 to 4 times more inhibitory than 2MP. The tripeptide showed no activity against CB64TORKrecA/F'123TOR, thus demonstrating that growth inhibition occurs subsequent to transport of the peptide. The dipeptide's effectiveness as growth inhibitor was considerably reduced in this latter strain compared to the other two; this manifested itself not only in a reduction in zone size, but also in increased haziness around the edges of the zones.

In all cases described here, the growth inhibition produced by AlaCys-2MP could be completely reversed by a 10- to 15-fold molar excess of diglycine; a 10-fold excess of triglycine partially reduced inhibition, but higher excesses produced no further effect. This result is in keeping with the observation that dipeptides can enter E. coli through both the dipeptide and oligopeptide transport systems (Payne, 1968, 1971a, 1971b; De Felice et al., 1973). Growth inhibition produced by AlaAlaCys-2MP could be completely reversed by a 10-fold molar excess of triglycine, but the same excess of diglycine was without effect. Neither di- nor triglycine affected growth inhibition produced by 2MP.

Inhibition of the Growth of E. coli by Peptides Containing Disulfide-linked 4-[N-(2-Mercaptoethyl)aminopyridine-2,6-dicarboxylic Acid]—4-[N-(2-Mercaptoethyl)aminopyridine-2,6-dicarboxylic acid (Fig. 1) was chosen as a test compound for the demonstration of portage transport. It shows only slight inhibitory properties toward E. coli. At physiological pH, it will exist predominantly in a negatively charged form, and so would be expected to diffuse poorly across the cell membrane. However, if actively transported into a cell, it seemed likely that its accumulation might produce a toxic effect (e.g. by chelation of essential metal ions, since pyridine-2,6-dicarboxylic acid is known to be a moderately strong chelating agent). Accordingly, this compound was reacted with each of the three peptide synthons as described under "Experimental Procedures," to produce the corresponding mixed disulfides.

When compared to limiting amounts of cysteine as growth support for CB64 on seeded agar plates, the AlaCys compound gave a barely detectable zone of growth. The AlaAlaCys- and OrnAlaCys-peptides in comparable equimolar amounts were efficient growth supporters, however, but as found previously with the peptide synthons, an inner zone of reduced growth was apparent. That the dipeptide was poorly transported was shown by displacing the mercapto compound from the peptide with dithiothreitol before applying the disc to the agar. A pronounced zone of growth was then obtained.

Fig. 3 shows the results of agar diffusion tests for inhibition of CB64recA/F'123 by MEPDA and its mixed disulfides with AlaCys, AlaAlaCys, and OrnAlaCys. Large amounts of MEPDA (2 to 5 μmol) produced small zones of inhibition which were not completely clear, whereas the peptides gave clear zones at considerably smaller molar quantities. AlaCys-

![Fig. 3. Comparison of MEPDA, AlaCys-MEPDA, AlaAlaCys-MEPDA, and OrnAlaCys-MEPDA as growth inhibitors of CB64recA/F'123 in agar diffusion tests. Zones of inhibition produced on seeded agar plates after overnight incubation were determined as described under "Experimental Procedures," using MEPDA (O), AlaCys-MEPDA (O), AlaAlaCys-MEPDA (x), and OrnAlaCys-MEPDA (■).]
MEPDA was the least effective growth inhibitor of these three peptides, being only some 10 times more inhibitory than MEPDA itself. Since it was also the poorest cysteine source for CB64 at subinhibitory amounts, it appears to be rather poorly transported. By comparing the amounts of each substance needed to produce a 12-15-mm zone it can be seen that AlaAlaCys-MEPDA is some 30 times more inhibitory than MEPDA, and OrnAlaCys-MEPDA some 100-150 times more inhibitory.

The highest levels of the two tripeptides shown in Fig. 3 were without effect on CB64TORrecA/F'123TOR. AlaCys-MEPDA was inhibitory toward this strain, and its action could be reversed by a 10-fold molar excess of diglycine.

DISCUSSION

The fact that AlaCys-S-Et was utilized by a cysteine auxotroph as a source of cysteine as efficiently as the amino acid itself provided a measure of the rapidity with which E. coli could release a compound from a disulfide linkage. However, it did not exclude the possibility that the disulfide bond cleavage occurred extracellularly. The first clear indication of intact transport of a peptide containing cysteine in a disulfide form was provided by the growth inhibition data of the peptides containing disulfide-linked 2-mercaptopyridine. Both AlaCys-2MP and AlaAlaCys-2MP were more effective growth inhibitors than 2-mercaptopyridine itself, presumably because of increased uptake of this compound through peptide transport followed by its release in the sulfhydryl-rich cytoplasm. That the mode of action of these compounds depends on transport as intact peptides was shown by the fact that their growth inhibitory properties were reversed by peptides, and that an oligopeptide transport-deficient mutant was unaffected by AlaAlaCys-2MP. Somewhat surprisingly, AlaCys-2MP was considerably less effective against this mutant than against the other two strains, suggesting that transport via the oligopeptide transport system is a substantial component of its overall entry. Since we do not find this result with peptides which carry more efficient inhibitors and are thus active at 50-fold lower molar amounts than those used here (data not shown), it is probable that we can observe this effect only because we have to use amounts of peptide which are saturating the transport systems.

The 2MP peptides were only moderately more growth inhibitory than 2MP itself, whereas the MEPDA-containing peptides were substantially more active than MEPDA as measured in agar diffusion assays. The lipophilic 2-MP is unlikely to build up to high intracellular concentrations following its release from the transported disulfide, due to its easy passage across the cytoplasmic membrane down its concentration gradient. The difference in the relative effectiveness of AlaAlaCys-MEPDA and OrnAlaCys-MEPDA may be due to the neutralization of one of the negative charges of MEPDA by the ornithine side chain amino group, or may simply reflect some preference of the transport system for ornithine at the NH_2 terminus of the peptide compared to alanine. AlaCys-MEPDA was substantially less inhibitory than either of the two corresponding tripeptides, and this observation suggested to us that the dipeptide transport system is more exacting in its substrate recognition requirements (of either, or both, size and charge) at the carboxyl-terminal side chain than is the oligopeptide transport system. We have verified this conclusion using new techniques which will be the subject of another paper.

The mode of action of these disulfide-substituted peptides may be affected by a number of factors. They are able to act as both growth supports (i.e. cysteine source) and growth inhibitors for the cysteine auxotroph CB64. Since one of the major thiol compounds in the cell pool, glutathione, is synthesized from cysteine, it might be expected that the intracellular concentration of glutathione, and hence the kinetics of the intracellular cleavage of the disulfide bond, would be affected in this strain. The introduction of the F'123 factor carrying the salu tonB trp cysB gyrF region of the E. coli chromosome into CB64recA allowed the inhibition of growth produced by these compounds to be demonstrated in the absence of any effects produced by cysteine auxotrophy.

Another factor in the consideration of the action of these compounds against different strains arises from the observation that thiols are excreted into the culture medium during the growth of E. coli (Roberts et al., 1963). A strain which excretes such compounds to a high concentration would obviously reduce the effectiveness of these peptides as growth inhibitors by extracellular reduction of the disulfide bond. The effect of this would be not only to lessen the concentration of the inhibitory peptide, but also to release as competitor for the transport system the free cysteiny1 peptide, or a new mixed disulfide of it, which might well have better affinity for the system than its parent mixed disulfide. This possible conversion has not been a problem in the present work, but is a factor to be considered in the use of these peptides with other microorganisms.

A further complication in the testing of these compounds is the growth inhibitory properties of cysteine itself to E. coli grown in liquid minimal medium (Roberts et al., 1963; Harris, 1981). This phenomenon is very dependent on the size of inoculum used, so that in liquid culture using 1-5% inocula of CB64recA/F'123, 0.5 mM cysteine inhibited growth completely for 18-24 h (data not shown). This behavior precluded the testing of the cysteinyl peptide mixed disulfides as growth inhibitors in liquid medium, as it was not possible to distinguish between inhibition due to the transported thiol compounds from that due to cysteine alone. The effect on growth of cysteine was not sufficiently severe to interfere in the disc assays for growth inhibition on seeded agar plates, however. Only partial zones of inhibition showing incomplete clearing, probably due to some slight slowing of growth, were apparent from cysteine or AlaCys-S-Et.

During the preparation of this manuscript, a report has appeared showing that mouse lymphoma cells in vitro can transport the mixed disulfide of cysteine and 2-mercaptoethanol through an amino acid transport system (Ishii et al., 1981). Using this mixed disulfide synthesized from [35S]cysteine, it was shown that rapid uptake of radioactivity occurred. Intracellular label was found only in free cysteine or glutathione synthesized from it, however, and no free disulfide was present. We suggest that a similarly rapid breakdown of the transported cysteinyl peptide mixed disulfides described here occurs in E. coli. The fact that at subinhibitory concentrations AlaCys-MEPDA is a very poor growth source for a cysteine auxotroph unless the disulfide bond is first broken by treatment with excess dithiothreitol supports the idea that cleavage of the disulfide bond normally occurs following transport across the cytoplasmic membrane.

The results described here provide the first example of portage transport of growth-inhibitory substances via the peptide transport systems of E. coli which depends upon their attachment to the side chains of an amino acid residue. All previous examples of portage transport have involved the incorporation of amino acid analogues into the peptide backbone (reviewed by Gilvarg, 1981). The use of peptide synthons containing Cys-2MP makes possible the synthesis of a range of peptides suitable for the comparison of various parameters.
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affecting transport by the di- and oligopeptide transport systems. It provides a method for the screening of thiols for growth-inhibitory properties in which the ability of the compounds to permeate the cell membrane(s) is no longer a factor to be considered. Such an approach may be capable of finding new inhibitors of intracellular processes in any organism which will effect portage transport in the way demonstrated for E. coli.

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SUGAR-ANAEROBIC BACTERIA 44

Prepared by the method of Felsen et al. (1950). A solution of 2.0 g (7.5 mmol) of S-guanidinoethyi2-ethyl disulfide in 30 ml of dimethylformamide was cooled to -10°C under a stream of nitrogen. To this solution was added, simultaneously from two separate addition funnels, a solution containing ethyl mercaptan (0.42 ml, 6.6 mmol) dissolved in 4.5 ml of dimethylformamide and a solution containing triethylamine (2.0. 0.25 ml) dissolved in 5.5 ml of dimethylformamide. The rate of triethylamine addition was slightly greater than the rate of addition of the mercaptan. After additions were completed, the reaction mixture was stirred for 1.5 h at -15°C and then adjusted to room temperature. The precipitated solid was collected, washed with 600 ml of ethyl ether and dried to give 29.5 g (72%) yield, 148–160°C (gas). A mixture of 2.0 g (7.5 mmol) of S-guanidinoethyl-2-ethyl disulfide, 30 ml of dimethylformamide, and 4.5 ml of dimethylformamide was stirred at -15°C under nitrogen. After 30 min the mixture was adjusted to room temperature and the precipitated solid was collected, washed with 600 ml of ethyl ether and dried to give 29.5 g (72%) yield, 148–160°C (gas).

SUGAR-ANAEROBIC BACTERIA 45

Prepared by the method of Felsen et al. (1950). A solution of 2.0 g (7.5 mmol) of S-guanidinoethyl2-ethyl disulfide in 30 ml of dimethylformamide was cooled to -10°C under a stream of nitrogen. To this solution was added, simultaneously from two separate addition funnels, a solution containing ethyl mercaptan (0.42 ml, 6.6 mmol) dissolved in 4.5 ml of dimethylformamide and a solution containing triethylamine (2.0. 0.25 ml) dissolved in 5.5 ml of dimethylformamide. The rate of triethylamine addition was slightly greater than the rate of addition of the mercaptan. After additions were completed, the reaction mixture was stirred for 1.5 h at -15°C and then adjusted to room temperature. The precipitated solid was collected, washed with 600 ml of ethyl ether and dried to give 29.5 g (72%) yield, 148–160°C (gas). A mixture of 2.0 g (7.5 mmol) of S-guanidinoethyl-2-ethyl disulfide, 30 ml of dimethylformamide, and 4.5 ml of dimethylformamide was stirred at -15°C under nitrogen. After 30 min the mixture was adjusted to room temperature and the precipitated solid was collected, washed with 600 ml of ethyl ether and dried to give 29.5 g (72%) yield, 148–160°C (gas).

SUGAR-ANAEROBIC BACTERIA 46

Prepared by the method of Felsen et al. (1950). A solution of 2.0 g (7.5 mmol) of S-guanidinoethyl-2-ethyl disulfide in 30 ml of dimethylformamide was cooled to -10°C under a stream of nitrogen. To this solution was added, simultaneously from two separate addition funnels, a solution containing ethyl mercaptan (0.42 ml, 6.6 mmol) dissolved in 4.5 ml of dimethylformamide and a solution containing triethylamine (2.0. 0.25 ml) dissolved in 5.5 ml of dimethylformamide. The rate of triethylamine addition was slightly greater than the rate of addition of the mercaptan. After additions were completed, the reaction mixture was stirred for 1.5 h at -15°C and then adjusted to room temperature. The precipitated solid was collected, washed with 600 ml of ethyl ether and dried to give 29.5 g (72%) yield, 148–160°C (gas). A mixture of 2.0 g (7.5 mmol) of S-guanidinoethyl-2-ethyl disulfide, 30 ml of dimethylformamide, and 4.5 ml of dimethylformamide was stirred at -15°C under nitrogen. After 30 min the mixture was adjusted to room temperature and the precipitated solid was collected, washed with 600 ml of ethyl ether and dried to give 29.5 g (72%) yield, 148–160°C (gas).
3. Peptide containing disulfide-linked 4-[(2-mercaptoethyl)imino]pyrrolidine-2,6-dicarboxylic acid.

The displacement of the 2-mercaptoethylpyruvate entry to the peptide syntheses of this chapter were achieved in alkaline aqueous solution followed by fractionation of the reaction mixture on a column of Sephadex G-15 using acetone buffer as eluent. The absence of 300 nm of the fractions was monitored, and the fractions of the retentate peak corresponding to 2-mercaptoethylpyruvate were also used at 304 nm, allowing the extent of reaction to be quantitated from the increase absorption coefficient of 2-mercaptoethylpyruvate.

The synthesis of the mixed disulfide of AlaaCys and 4-[[(2-mercaptoethyl)imino]pyrrolidine-2,6-dicarboxylic acid was described as typical of such syntheses.

S-Sephadex 30 and 1-[[(2-mercaptoethyl)imino]pyrrolidine-2,6-dicarboxylic acid were dissolved in a solution of diisopropylamine hydrochloride solution and after 5 min at room temperature the solution was applied to a column of Sephadex G-10 previously equilibrated with acetone buffer and eluted with this buffer. 

Figure 4 shows the elution profile obtained. Peak 1 led to the preparation of the 4-[(2-mercaptoethyl)imino]pyrrolidine-2,6-dicarboxylic acid was used as a model for all the peptide syntheses of this chapter. The absence of 300 nm of the fractions was monitored, and the fractions of the retentate peak corresponding to 2-mercaptoethylpyruvate were also used at 304 nm, allowing the extent of reaction to be quantitated from the increase absorption coefficient of 2-mercaptoethylpyruvate.

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