Monitoring Enzyme Synthesis as a Means of Studying Peptide Transport and Utilization in *Escherichia coli*

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SUMMARY

A new method has been developed for measuring peptide transport in amino-acid auxotrophs of *Escherichia coli* by following induction of β -galactosidase. Appearance of the enzyme was determined after addition of inducer and peptides to amino-acid starved bacteria. For a given number of lysine equivalents, the rate and the extent of enzyme synthesis were the same for lysine and lysyl peptides; similar results were found for glycine and glycyI peptides. Saturation constants for peptide transport were determined from the exogenous peptide concentration that gave half maximal rates of enzyme synthesis. The saturation constants, studies with mutants defective in peptide transport, and detection of competition between peptides for uptake, all endorsed earlier conclusions from growth tests about the structural specificities for peptide transport. The new method is quicker, more sensitive and more informative than growth tests.

INTRODUCTION

Peptide transport in *Escherichia coli* occurs by permeases which are specific for di- or oligopeptides. After uptake, peptides are hydrolysed by intracellular peptidases and then incorporated into protein. Because of the difficulty in obtaining radioactive peptides, the present picture of peptide transport and utilization has been obtained mainly from studies of the growth responses of amino-acid auxotrophs to peptides, and of the effects of inhibitory peptides on the growth of wild-type strains (e.g. Barak & Gilvarg, 1975*a*; Payne, 1975, 1976; Payne & Gilvarg, 1971; Simmonds, 1972; Sussman & Gilvarg, 1971). There are several limitations to these methods: they require fairly large quantities of peptide and long incubations (e.g. 8 h), and measurement of growth by following turbidity is relatively insensitive. The present paper describes a method which is faster, and more sensitive and informative. Amino-acid auxotrophs are used, but instead of measuring growth, the peptide-dependent synthesis of β -galactosidase (EC. 3.2.1.23) is measured.

METHODS

Materials. Lysine peptides were synthesized as described previously (Payne, 1968). Amino acids and glycyI peptides were obtained from BDH. Isopropyl- β -D-thiogalactopyranoside and *o*-nitrophenyl- β -D-galactopyranoside were purchased from Sigma.

Growth of bacteria and β -galactosidase assay. All strains were derived from *Escherichia coli* W ATCC9637. The lysine auxotroph (M-26-26), the glycine/serine auxotroph (M-123) and their respective oligopeptide permease-deficient mutants M-26-26.TOR and M-123.TOR

have been described previously (Payne, 1968, 1974). Cultures (75 to 100 ml) were shaken at 160 strokes min^{-1} in a water bath (Grant Instruments, Cambridge) at 37 °C; each flask (250 ml) was fitted with a side-arm so that growth could be measured at 660 nm in a Bausch & Lomb Spectronic 20. The minimal medium A of Davis & Mingioli (1950) was used but, to prevent catabolite repression, citrate was omitted and sodium lactate (0.6 %, w/v; prepared by neutralizing lactic acid with NaOH) was used as carbon source. Growth was limited by lysine (0.05 mM) or glycine (0.5 mM) to an absorbance of about 0.5 at 660 nm (about 5×10^8 bacteria ml^{-1}). Incubation of the bacteria was continued for 1 to 2 h after cessation of growth in order to ensure adequate depletion of the appropriate amino acid. The bacteria were collected by centrifuging at about 8000 g for 10 min, and then resuspended to about 2×10^8 bacteria ml^{-1} in fresh minimal medium (approx. 100 ml) equilibrated at 37 °C and containing 1 mM-isopropyl- β -D-thiogalactopyranoside as inducer. This suspension was equilibrated with shaking under the conditions described above for 30 to 40 min. Portions (8.0 ml) of the suspension were then added to aqueous solutions (2.0 ml; 37 °C) of appropriate amino acids or peptides in tubes (2×15 cm) and incubated at 37 °C in a water bath with shaking (200 strokes min^{-1}). Samples (0.5 ml) were removed periodically into test tubes (1×10 cm) containing toluene (50 μl), mixed on a vortex mixer for 30 s and then incubated for 20 min at 37 °C in a water bath without shaking. One ml of 5 mM-*o*-nitrophenyl- β -D-galactopyranoside, pre-equilibrated at 37 °C, was added and the solutions were incubated in a water bath at 37 °C without shaking for 20 to 60 min as convenient; enzyme action was then stopped by adding 0.2 ml 1 mM- Na_2CO_3 . The extent of β -galactosidase synthesis is proportional to the amount of *o*-nitrophenol released; this was measured at 420 nm in a Gilford model 2000 spectrophotometer using 1 cm path-length cuvettes. Under these conditions an E_{420} of 1 is equivalent to 0.23 μmol *o*-nitrophenol formed per ml of incubation mixture.

RESULTS

In preliminary studies, bacteria were harvested in the exponential phase of growth, resuspended in fresh growth medium but without the required amino acid, and incubated for about 40 min at 37 °C to use up any residual amino acid before inducing β -galactosidase. However, this procedure gave variable, and occasionally unacceptably high, 'blank' or 'zero time' amounts of β -galactosidase. Subsequent use of amino-acid starved cells, as described in Methods, overcame this difficulty and still gave rapid initiation of enzyme synthesis.

Transport and utilization of lysyl and glycyl peptides

The ability of the lysine auxotroph M-26-26 to transport and to utilize di-, tri-, and tetra-lysine is shown by the capacity of these peptides to support β -galactosidase synthesis in bacteria starved of lysine (Fig. 1). In this experiment, enzyme synthesis was detected within 10 min of supplying each lysine source. Absorbances at zero time are mainly non-specific; they are caused by suspended bacteria and low basal activities of β -galactosidase. The same rate of enzyme synthesis was observed with lysine and with each of the lysine peptides, indicating that the uptake and the hydrolysis of these di- and oligopeptides [which occur by distinct permeases (Payne, 1968, 1975, 1976; Payne & Gilvarg, 1971) and by separate peptidases (Sussman & Gilvarg, 1970, 1971) respectively] are both extremely rapid processes and neither is rate limiting for protein synthesis. Additionally, the final plateaux of enzyme activity indicate that the same amounts of β -galactosidase are synthesized from given amounts of lysine, whether it is provided as the free amino acid or in a peptide. Analogous

Fig. 1

Fig. 2

Fig. 1. Transport and utilization of lysine and lysine peptides by *E. coli* lysine auxotroph M-26-26 monitored by β -galactosidase synthesis. Bacteria were used at $E_{660} = 0.14$ in the induction medium, and formation of *o*-nitrophenol was measured at 420 nm after 20 min enzyme action. The following additions were made at zero time: 15 μ M-lysine (▲); 7.5 μ M-dilysine (●); 5 μ M-trilysine (■); 3.75 μ M-tetralysine (○); 15 μ M-lysine plus 4 μ M-triornithine (Δ); control, no lysine source (□).

Fig. 2. Transport and utilization of glycine and glycy peptides by *E. coli* glycine auxotroph M-123 monitored by β -galactosidase synthesis. Bacteria were used at $E_{660} = 0.14$ in the induction medium, and formation of *o*-nitrophenol was measured at 420 nm after 30 min enzyme action. The following additions were made at zero time: 100 μ M-glycine (Δ); 50 μ M-diglycine (□); 33 μ M-triglycine (○); control, no glycine source (●).

curves were obtained using different concentrations of bacteria ($E_{660} = 0.05$ to 0.7); the rates of enzyme synthesis were proportional to the numbers of cells but the amount of β -galactosidase synthesized was determined solely by the amount of lysine added. The inhibitory effect of triornithine on protein synthesis (Gilvarg & Levin, 1972; Barak, Sarid & Katchalski, 1973) is also shown (Fig. 1) but the high concentration of *E. coli* apparently quickly hydrolysed the tripeptide and destroyed its inhibitory properties.

Use of the new method was further validated by results for the transport and utilization of glycine, di-, tri-, and tetraglycine by the glycine auxotroph M-123 (Fig. 2). Similar rates of enzyme synthesis occurred with each glycine source; tetraglycine gave the same results as diglycine but is omitted for clarity. Yields of β -galactosidase were the same for a given amount of free or peptide-bound glycine. However, the yield of β -galactosidase obtained with glycine was only about one-tenth that obtained with the same molarity of lysine, but rather than make the enzyme assay period unduly long, we chose to increase the concentrations of glycine used so that more β -galactosidase would be synthesized and the routine 20 to 30 min incubation time maintained.

Use of peptide permease mutants

In earlier growth studies, mutants defective in peptide transport have been used to establish structural specificities of peptide permeases of *E. coli* (Payne, 1968, 1975, 1976; Payne & Gilvarg, 1971; Barak & Gilvarg, 1975a). Results using our new method were similar to those obtained in earlier work. The oligopeptide transport mutant M-123.TOR transported and utilized glycine and diglycine for the synthesis of β -galactosidase (Fig. 3a) as did the parent strain (Fig. 2), but failed to utilize tri- and tetraglycine. Similarly, the oligopeptide permease mutant M-26-26.TOR of the lysine auxotroph transported and utilized dilysine (Fig. 3b) but not the oligopeptides trilysine or tetralysine (not shown in

Fig. 3. Transport and utilization of peptides by mutants of *E. coli* defective in the oligopeptide permease monitored by β -galactosidase synthesis. (a) Glycine auxotroph M-123.TOR was used at $E_{660} = 0.3$ in the induction medium, and formation of *o*-nitrophenol was measured at 420 nm after 20 min enzyme action. The following additions were made at zero time: 150 μM -glycine ($\circ$); 75 μM -diglycine ($\triangle$); 50 μM -triglycine ($\blacksquare$); 37.5 μM -tetraglycine ($\square$); control, no glycine source ($\bullet$). (b) Lysine auxotroph M-26-26.TOR was used at $E_{660} = 0.2$ in the induction medium, and formation of *o*-nitrophenol was measured at 420 nm after 20 min enzyme action. The following additions were made at zero time: 15 μM -lysine ($\square$); 7.5 μM -dilysine ($\circ$); 5 μM -trilysine ($\bullet$).

Fig. 4. Competition between dipeptides and oligopeptides for transport by *E. coli* glycine auxotroph M-123 monitored by β -galactosidase synthesis. Bacteria were used at $E_{660} = 0.3$ in the induction medium, and formation of *o*-nitrophenol was measured at 420 nm after 20 min enzyme action. The following additions were made at zero time: (a) 33 μM -triglycine ($\square$); 33 μM -triglycine plus 3.3 μM -trilysine ($\bullet$); 33 μM -triglycine plus 16.5 μM -trilysine ($\circ$). (b) 50 μM -Diglycine ($\blacksquare$); 50 μM -diglycine plus 2 μM -dilysine ($\circ$); 50 μM -diglycine plus 10 μM -dilysine ($\square$); 50 μM -diglycine plus 50 μM -dilysine ($\bullet$).

Fig. 3b). Both these mutants showed undiminished rates of enzyme synthesis in the presence of 4 μM -triornithine which inhibited enzyme synthesis in the parent strain (Fig. 1). Enzyme synthesis observed with some oligopeptides (Fig. 3) may arise from (i) contamination by lower homologues; (ii) protein turnover; (iii) cleavage by periplasmic amino-endopeptidase activity (Lazdunski, Basuttill & Lazdunski, 1975); (iv) uptake by diffusion and/or specialized transport systems (Payne, 1968; Cowell, 1974; Naider & Becker, 1975; Barak & Gilvarg, 1975b). The present procedure is a much more sensitive way of assessing possible contributions from (iii) and (iv) than the usual growth studies.

Fig. 5. Determination of saturation constants for transport of diglycine by *E. coli* glycine auxotroph M-123. Bacteria were used at $E_{860} = 0.1$ in the induction medium, and formation of *o*-nitrophenol was measured at 420 nm after 60 min enzyme action. The amount of β -galactosidase formed was determined after 30 min (●), 45 min (■), and 60 min (▲) biosynthesis.

Competition during peptide transport

Use of the new method was further endorsed by results on competition in agreement with earlier studies on effects on growth (Payne, 1968, 1975, 1976; Payne & Gilvarg, 1971; Barak & Gilvarg, 1975*a*). Thus, dilysine inhibited diglycine transport via the dipeptide permease (Fig. 4*b*), and trilycine competed with triglycine for uptake by the oligopeptide permease (Fig. 4*a*) making the supply of glycine rate limiting for enzyme synthesis in strain M-123. With the new approach, competition can be assessed within minutes, and peptides can be used at low concentrations.

Affinities of peptides for transport systems

Some measure of the relative affinities of peptides for their permeases can be obtained from their competitive abilities. However, a more direct measure of the affinity of a peptide for the transport system is obtained from measurement of the rate of enzyme synthesis as a function of peptide concentration. Results of an experiment using diglycine are shown as a double reciprocal plot (Fig. 5), with the intercept on the abscissa giving an affinity constant for transport of 6.3×10^{-5} M; i.e. the concentration that supports half maximal rate of β -galactosidase synthesis. In other experiments the following affinity constants were determined (ranges from four to six experiments): triglycine $4.0 \pm 0.8 \times 10^{-4}$ M; trilycine $3.9 \pm 0.4 \times 10^{-7}$ M; dilysine $3.4 \pm 0.5 \times 10^{-7}$ M. These values accord with the relative competitive abilities of the glycine and lysine peptides (see rest of text, and Payne, 1968). Providing intracellular hydrolysis was not rate limiting, the procedure could be used to determine affinity constants for the transport of any peptide.

DISCUSSION

The fact that glycyl and lysyl peptides were shown by the new method to be utilized for enzyme synthesis by the starved auxotrophs only a few minutes after addition (Figs 1 and 2) provides convincing evidence for the constitutive nature of the peptide permeases and also for the presence of active, constitutive peptidases (the bacteria had been grown without peptides in the medium) (Sussman & Gilvarg, 1971; Simmonds, 1970, 1972).

In determining permease affinity from the exogenous peptide concentration that makes peptide transport (and hence intracellular supply of required amino acid) become just limiting for enzyme synthesis in an auxotroph (Fig. 5), the approach is somewhat similar to that of Ames (1964) for finding 'limit concentration' values. An assumption of our approach is that uptake and not hydrolysis is the rate-limiting step. Pertinent to this assumption and to the method in general are the following calculations. At the cell concentration used in a typical assay ($E_{660} = 0.15$; 2×10^8 bacteria ml^{-1}), the dry weight of bacteria is about 0.05 mg ml^{-1} , of which about 50 % is protein. For, say, diglycine (Fig. 2), 50 nmol is completely utilized (and therefore necessarily hydrolysed) in 100 min. Thus, the minimum specific activity for 'diglycine peptidase(s)' *in vivo* under these experimental conditions is about $20 \text{ nmol peptide cleaved min}^{-1} (\text{mg protein})^{-1}$ and, similarly, for triglycine (Fig. 2) about $15 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$, and for di- and trilycine (Fig. 1) about $2 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$. These figures can be compared with the following values measured *in vitro* using crude extracts of several strains of *E. coli*: di- and triglycine, about 1 to $5 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ (Payne, 1972*b*; Simmonds, Szeto & Fletterick, 1976; Patterson, Gatmaitan & Hayman, 1975); di- and trilycine, about 2.25 and $0.25 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ respectively (Sussman & Gilvarg, 1970; Simmonds *et al.*, 1976). When comparing calculated activities *in vivo* with observed activities *in vitro* due attention must be given to the substrate concentration at which the peptidases are acting. For example, for the enzyme that cleaves diglycine, a K_m value *in vitro* of about 2 mM has been determined (Payne, 1972*a*; Hayman, Gatmaitan & Patterson, 1974). In the present method, diglycine was used at about 0.05 mM (Fig. 2), requiring it to be accumulated 40-fold over its concentration in the medium for the intracellular peptidase to work at half maximal velocity *in vivo*. This level of accumulation is not unusual and has been reported for diglycine (Kessel & Lubin, 1963).

We chose to monitor β -galactosidase synthesis because it is well characterized and easily assayed. Putnam & Koch (1975) recently described an improved assay. The sensitivity of the procedure could be increased by extending the time the enzyme acts on its substrate. Other inducible or derepressible enzymes could be used to monitor peptide transport and utilization, and with alkaline phosphatase we have obtained similar results to those described here. Cascieri & Mallette (1974) have described a related method in which wild-type bacteria were put into a state of 'physiological auxotrophy' by shifting from a medium containing methionine (to repress methionine biosynthetic enzymes) to one without methionine; synthesis of induced lysine decarboxylase in these cells was then shown to be dependent for a limited period on added methionine or methionine peptide. Their procedure was too involved for routine study of peptide transport. Furthermore, it cannot be used to assess peptide utilizability based on enzyme yield because physiological auxotrophy is too quickly lost. However, their approach offers a way of investigating peptidase activities *in vivo* under different physiological conditions from those present in the starved auxotrophs used here.

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