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PEPTIDE UTILIZATION IN *ESCHERICHIA COLI*: STUDIES WITH PEPTIDES CONTAINING β-ALANYL RESIDUES

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SUMMARY

A glycine auxotroph of *Escherichia coli* can utilize glycine oligopeptides as a source of its required amino acid. Glycylglycyl-β-alanine and β-alanylglycylglycine are both readily hydrolysed by intracellular peptidases, but only the former supports growth of the glycine auxotroph. Glycylglycyl-β-alanine is not nutritionally active towards a glycine mutant that is unable to transport oligopeptides. The nutritional responses to these β-alanine peptides are interpreted in terms of the structural requirements of the oligopeptide transport system, for which an α-peptide bond is required but the C-terminal α-carboxyl group is not essential. Dipeptides of β-alanine are generally poor sources of amino acids for auxotrophs of *E. coli*, although β-alanylhistidine (carnosine) is as effective as the free amino acid in supporting growth of a histidine auxotroph; this observation does not accord with the structural requirements established for dipeptide transport in general, and may indicate a separate uptake process. The results are related to the occurrence of β-alanyl peptides in the normal environment of enteric bacteria, and to the known ability of the intestine to transport carnosine.

INTRODUCTION

Many bacteria are able to take up peptides from their environment and to hydrolyse them with intracellular peptidases, and in this way the constituent amino acids are made nutritionally available[1]. The peptide uptake systems of *Escherichia coli* display strict specificities with respect to the structure of the peptides they are able to handle[1–4]. The specificities of the intracellular peptidases do not always parallel those of the transport systems. Thus, a peptide may be accumulated but may or may not be hydrolysed[3,5,6], or, alternatively, it may fail to enter even though intracellular peptidases exist for its cleavage[2,7,8]. The effect a particular peptide may have on a bacterium is therefore a reflection of the overall processes of uptake and cleavage, and these are governed by the nature of the organism and its environmental status[9,10]. This paper describes studies on the utilization by *E. coli* of dipeptides and oligopeptides containing β-alanine. The results, which further define this or-

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ganism's structural requirements for peptide utilization, are discussed in relation to the natural environment of enteric bacteria and to previous observations of intestinal absorption of β-alanyl peptides[11,12].

MATERIALS AND METHODS

Chemicals

Glycylglycyl-β-alanine and β-alanylglycylglycine were purchased from Cyclo Chemical Corp., through Baxter Laboratories, Thetford, Norfolk. All other peptides were obtained from Sigma (London) Chemical Co., London, S.W.6. All amino acid residues are of the L-configuration unless stated otherwise.

Micro-organisms

Escherichia coli W, strain M-123, a glycine–serine auxotroph, and the oligopeptide transport-deficient strain M-123.TOR[13] were grown in minimal medium A of Davis and Mingioli[14] supplemented with 0.5% glucose and 1 mM glycine. *E. coli* K12, strain EMG29, pro^-, his^-, $tryp^-$, lac^-, F^-, Str^R, was kindly provided by Dr W. Hayes. This strain was grown in minimal medium M-56 of Weismeyer and Cohn[15] supplemented with 0.5% glucose, and the required amino acids were supplied at 20 mg/l. Cultures (10 ml) were inoculated with about $2 \cdot 10^7$–$5 \cdot 10^7$ washed, exponential-phase organisms, and were incubated at 37 °C with shaking in 20-mm diameter tubes. Bacterial growth was followed by measuring the $A_{560\,nm}$ of the cultures with a Bausch and Lomb Spectronic 20.

Peptidase activities

Organisms were first made permeable by toluene treatment. Exponential phase organisms were collected by centrifugation, washed once with distilled water, and resuspended in water to give about $3 \cdot 10^9$ organisms/ml. Toluene (0.2 ml) was added to 3 ml of this suspension, which was mixed on a vortex mixer for 2 min; the suspension was then equilibrated at 37 °C for 15 min with frequent shaking. The assay for peptidase activity was started by the addition of 0.2 ml of toluene-treated organisms to the test solution (1 ml), which comprised 7.5 mM peptide, 20 mM potassium phosphate buffer (pH 7.6) and, if required, 1 mM Co^{2+} or Mn^{2+}. Samples (0.1 ml) were taken at intervals for assay by the copper–trinitrobenzene sulphonate procedure[16] described previously[17].

Electrophoresis

The products from the incubation of the various peptides with the disrupted bacteria were characterised by high-voltage electrophoresis, using a Shandon flat plate apparatus. Samples were run on Whatman No. 1 paper for 75 min, 55 V/cm at pH 2.1 in acetic acid (8%, v/v) and formic acid (2%, v/v). Papers were developed with cadmium–ninhydrin reagent[18].

RESULTS

Utilization of oligopeptides containing β-alanine

In studying these compounds, we sought to further characterise the structural features of an oligopeptide that are required for its bacterial utilization. In the

absence of extracellular peptidase, auxotrophic growth upon an oligopeptide requires its uptake and hydrolysis. Fig. 1 shows the growth response of the *E. coli* glycine auxotroph M-123 to glycylglycyl-β-alanine and to β-alanylglycylglycine. Significant growth was obtained with the former peptide but no growth was seen with the latter. In a control experiment, it was shown that at the concentrations used in Fig. 1, β-alanylglycylglycine did not inhibit growth of strain M-123 on media supplemented with glycine, or growth of a wild-type strain in minimal media. Fig. 2 shows the

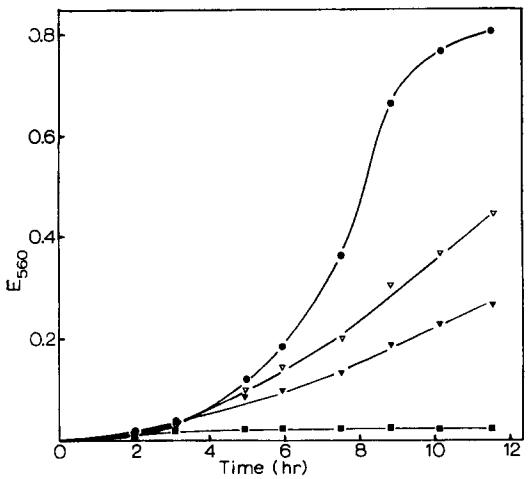

Fig. 1. Growth responses of the *E. coli* W glycine auxotroph M-123 to glycylglycyl-β-alanine and β-alanylglycylglycine. ●, +0.56 mM triglycine or +1 mM glycine; ▼, +0.56 mM glycyl-glycyl-β-alanine; ▽, +1.88 mM glycylglycyl-β-alanine; ■, +0.56–1.88 mM β-alanylglycyl-glycine, or +1 mM glycine+0.05 mM triornithine, or control *minus* glycine.

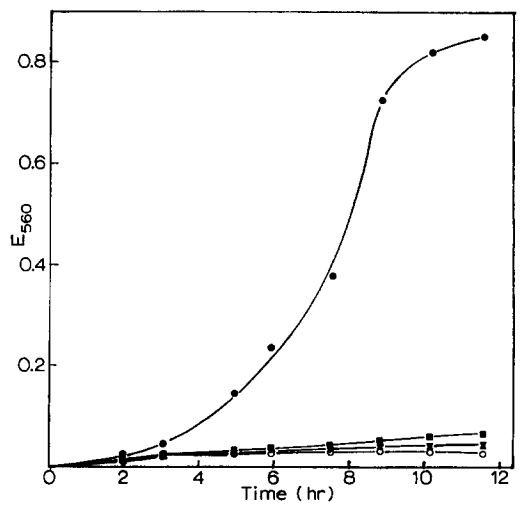

Fig. 2. Growth responses of the *E. coli* W oligopeptide transport-deficient, glycine auxotroph M-123.TOR to glycylglycyl-β-alanine and β-alanylglycylglycine. ●, +1 mM glycine, or +1 mM glycine +0.05 mM triornithine; ■, +0.56 mM triglycine; ▲, +0.56 mM glycylglycyl-β-alanine; ▽, +0,56 mM β-alanylglycylglycine; ○, control *minus* glycine.

growth of strain M-123.TOR, an oligopeptide-transport mutant of the glycine auxotroph M-123; this mutant has been described previously, and shown to have lost the ability to transport oligopeptides while retaining the same facility as the original strain to utilize dipeptides and amino acids[1,13]. Thus, mutant M-123.TOR was unable to grow upon triglycine (Fig. 2), although strain M-123 could utilize it (Fig. 1); M-123.TOR was also resistant to the peptide triornithine (Fig. 2) although this peptide was toxic towards strain M-123 (Fig. 1). Fig. 2 shows that M-123.TOR was unable to use glycylglycyl-β-alanine nutritionally, suggesting that this tripeptide enters strain M-123 by the transport system absent in the mutant M-123.TOR. As with M-123, β-alanylglycylglycine failed to support growth of M-123.TOR.

Peptidase activity

β-Alanylglycylglycine may be devoid of nutritional effect towards M-123 for any of several reasons, for example, it may not enter the organism or it may be resistant to hydrolysis. To check the latter point we assayed for the presence of intracellular peptidases able to split these tripeptides of β-alanine. The results in Table I show that toluene-treated bacteria cause significant hydrolysis of both peptides. Furthermore, to confirm that free glycine was liberated during hydrolysis the products were examined electrophoretically.

TABLE I

HYDROLYSIS OF PEPTIDES OF β-ALANINE BY TOLUENE-TREATED *E. COLI* STRAIN M-123

Peptidase assays were carried out as described in Materials and Methods. The concentration of each peptide was 7.5 mM, and that of each cation was 1 mM. Activities are expressed as μmole amino acid released/min per mg bacterial protein. Results are calculated using the extinction coefficient for glycine, in those cases in which β-alanine is also released, this produces an overestimate of the extent of hydrolysis.

Peptide	*Supplement*	*Activity*
Gly–Gly–Gly	none	0.04
Gly–Gly–Gly	Co^{2+}	0.68
β-Ala–Gly–Gly	none	0.02
β-Ala–Gly–Gly	Co^{2+}	0.12
Gly–Gly–β-Ala	none	0.09
Gly–Gly–β-Ala	Co^{2+}	0.16
β-Ala–Gly	none	0.09
β-Ala–Gly	Co^{2+}	0.08
β-Ala–Gly	Mn^{2+}	0.05
Gly–β-Ala	none	0.09
Gly–β-Ala	Co^{2+}	0.06
Gly–β-Ala	Mn^{2+}	0.07

Electrophoresis of the products of peptidase action on tripeptides of β-alanine

Samples for electrophoresis were removed from incubation mixtures described in Table I; the results are given in Table II. Under these conditions triglycine was completely hydrolysed, and only glycine was detected. β-Alanylglycylglycine was about 50% hydrolysed, and glycine, β-alanine and uncleaved β-alanylglycylglycine

were detected. β-Alanylglycine was not detected in the incubation mixture, although it can easily be resolved from glycine, and distinguished from it by colour difference. The products observed are compatible with either amino-peptidase action followed by rapid diglycine cleavage (*E. coli* has been shown to contain an extremely active diglycine peptidase[17]), or with carboxy-peptidase action followed by rapid cleavage of β-alanylglycine. Oligopeptide cleavage is obviously the rate limiting step in the overall breakdown of tripeptide to amino acids, but it is not possible to decide between the two above alternative mechanisms. Peptidase-treated glycylglycyl-β-alanine yielded glycine, glycyl-β-alanine and uncleaved tripeptide, products that are compatible with amino-peptidase action. However, no free β-alanine could be detected, indicating that under these incubation conditions the carboxy-peptidases of *E. coli* are unable to liberate C-terminal β-alanyl residues, suggesting the enzyme(s) may have a requirement for a C-terminal α-carboxyl group. The absence of free β-alanine was surprising in view of the finding that free glycyl-β-alanine was split

TABLE II

ELECTROPHORETIC CHARACTERIZATION OF PEPTIDE HYDROLYSIS PRODUCTS

Samples were run at pH 2.1, and developed with cadmium–ninhydrin reagent as described in Materials and Methods. Mobilities are expressed relative to serine, measured from the origin. Samples were removed from Co^{2+}-supplemented incubation mixtures described in Table I.

Standards and peptide substrates	*Hydrolysis products*	*Mobilities*	*Colour*
Glycine	—	1.34	red
Serine	—	1.00	red
β-Alanine	—	1.66	violet
β-Ala–Gly	—	1.29	purple/brown
Gly–β-Ala	—	1.34	yellow
β-Ala–Gly–Gly	—	1.12	blue/grey
Gly–Gly–β-Ala	—	1.14	yellow
Gly–Gly	—	1.33	yellow
Gly–Gly–Gly	—	1.11	yellow
β-Ala–Gly–Gly	β-Ala–Gly–Gly	1.11	blue/grey
	Gly	1.33	red
	β-Ala	1.65	violet
Gly–Gly–β-Ala	Gly–Gly–β-Ala	1.11	yellow
	Gly–β-Ala	1.35	yellow
	Gly	1.33	red
Gly–Gly–Gly	Gly	1.33	red
β-Ala–Gly	Gly	1.33	red
	β-Ala	1.65	violet
	β-Ala–Gly	1.28	purple/brown
Gly–β-Ala	Gly	1.33	red
	β-Ala	1.64	violet
	Gly–β-Ala	1.34	yellow

under these incubation conditions, but it is possible that the presence of glycylglycyl-β-alanine may competitively inhibit cleavage of glycyl-β-alanine.

Demonstration of the ready release of free glycine from both glycylglycyl-β-alanine and β-alanylglycylglycine makes it unlikely that the nutritional failure of the latter peptide is attributable to lack of peptidase action, and it appears more likely that the peptide is taken up inadequately by the bacterium.

Utilization of dipeptides containing β-alanine

Fig. 3a shows that glycyl-β-alanine supports growth of strain M-123 but β-alanylglycine is nutritionally ineffective. A negligible growth response was also observed with β-alanylserine (not shown). The growth of M-123 upon the structural analogues glycylalanine (2 mM) and alanylglycine (2 mM) was similar to that upon diglycine, while the growth rate was even faster with the analogues glycylasparagine (2 mM) and glycylaspartic acid (2 mM). The growth rate on glycyl-β-alanine was reduced in strain M-123.TOR (Fig. 3b) suggesting that this peptide may use the oligopeptide transport system to enter the organism. In contrast, the growth response of M-123.TOR to glycylglycine (1 mM) (Fig. 3b), glycylalanine (2 mM), alanylglycine (2 mM), glycylasparagine (2 mM), and glycylaspartic acid (2 mM) all resembled that seen with strain M-123.

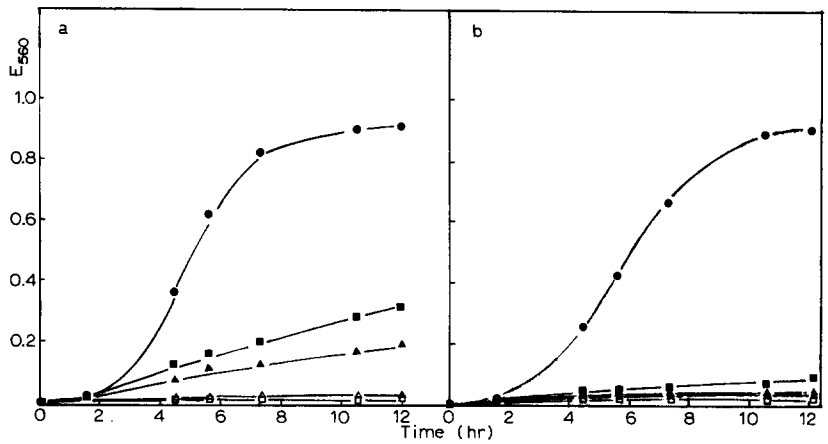

Fig. 3. Growth responses of the *E. coli* W glycine auxotroph M-123 (a), and the oligopeptide transport-deficient glycine auxotroph M-123.TOR (b), to dipeptides of β-alanine. ●, +1 mM diglycine; ▲, +1.85 mM glycyl-β-alanine; ■, +7.5 mM glycyl-β-alanine; △, +1.85–7.5 mM β-alanylglycine; □, control *minus* glycine.

Hydrolysis of dipeptides of β-alanine

The results in Table I show the ready cleavage of these dipeptides by toluene-treated bacteria. In fact, the extent of cleavage was greater with β-alanylglycine, even though it failed to support growth. The conclusions were endorsed by electrophoretic analysis of the incubation mixtures, which revealed more extensive cleavage of β-alanylglycine than of glycyl-β-alanine. It therefore appears that the nutritional failure of the former peptide is more likely to be through inability to enter the bacterium at an adequate rate than through failure to be hydrolysed sufficiently quickly.

Utilization of carnosine, homocarnosine and anserine

In view of the structural specificities of the peptide transport systems of *E. coli* (see Discussion) the nutritional failure of the above β-alanyl peptides is not surprising. On the other hand, there are a number of similarities between bacterial and intestinal transport of peptides[4,19], and yet the transport of carnosine (β-alanylhistidine) by the gut is well documented[11,12]. I therefore tested the growth response of the *E. coli* K12 multiple auxotroph EMG29 to various sources of histidine, including carnosine.

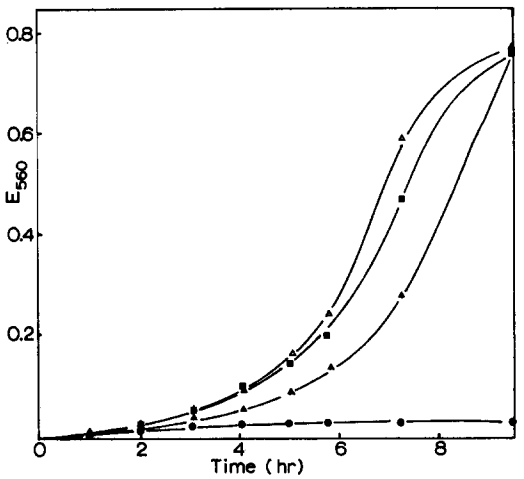

Fig. 4. Growth responses of the *E. coli* K12 histidine auxotroph EMG29 to various sources of histidine. △, +1.25 mM glycylhistidine; ▲, +1.25 mM alanylhistidine; ■, 0.375 mM β-alanylhistidine (carnosine) or 0.25 mM histidine; ●, control without histidine, or 0.375–3.1 mM β-alanyl-1-methylhistidine (anserine), or 0.375–3.1 mM γ-aminobutyrylhistidine (homocarnosine), or 1.25 mM 1-methylhistidine.

Fig. 4 shows that growth was rapid on histidine, alanylhistidine, glycylhistidine and on carnosine. No stimulation of growth over that of an unsupplemented control was seen with 1.25 mM 1-methylhistidine, 0.4–3.0 mM β-alanyl-1-methylhistidine (anserine), or with 0.4–3.1 mM γ-aminobutyrylhistidine (homocarnosine), and none of these was inhibitory.

DISCUSSION

E. coli possesses intracellular peptidase activity able to cleave a number of di- and tripeptides containing β-alanine residues. This fact has been demonstrated directly by assay of peptide cleavage using disrupted bacteria and by electrophoretic characterization of the split products; it has also been indicated indirectly for certain peptides by growth studies using amino acid auxotrophs. β-Alanyl peptidases have not been reported from bacteria although they are well characterised from mammalian sources[20–23]. It has not been established whether one or more peptidases are responsible for the observed cleavage but the activity seems to be distinct from certain other cation-activated peptidases from *E. coli*[17]. A strain of *Corynebacterium*, which required β-alanine for growth, used carnosine in place of β-alanine suggesting the

presence of carnosinase activity in this organism[24]. A β-aspartyl peptidase has been isolated from *E. coli*[25], but its specificity is unlikely to extend to peptides of β-alanine, and we have also found that a purified sample of the periplasmic asparaginase of *E. coli* fails to hydrolyse the peptides of β-alanine used here.

Although the simple demonstration of a specific peptidase activity in broken-cell extracts is no guarantee that the *in vivo* activity of the enzyme is sufficient for auxotrophic growth, the measured rate of hydrolysis of β-alanylglycylglycine (Table I) appears more than sufficient to meet auxotrophic requirements[9]. Therefore, the failure of this tripeptide to meet the nutritional requirement of an *E. coli* glycine auxotroph suggests that the tripeptide is not readily concentrated by *E. coli*. Such an observation reflects the specificity of the oligopeptide uptake system and indicates its requirement for an α-peptide bond contiguous with the N-terminal amino group. The importance of an α-linkage at the second (from the N-terminus) peptide bond was not studied here, but could be investigated using analogous peptides such as glycyl-β-alanyl-glycine. On the other hand, the ready utilization of glycylglycyl-β-alanine by the glycine auxotroph indicates that a β-substituent as the third residue is compatible with uptake. Furthermore, the failure of glycylglycyl-β-alanine to support growth of the oligopeptide transport-deficient mutant M-123.TOR, suggests that the uptake of this peptide is mediated by a specific oligopeptide transport system in the parental strain. Previous studies have indicated that the oligopeptide transport system lacks specificity towards the C-terminal α-carboxyl group, and indeed α-linked peptides devoid of this group continue to be transported[26]. The utilization of glycylglycyl-β-alanine therefore accords with these earlier findings, for it may be considered as glycyl-glycylaspartic acid devoid of a C-terminal α-carboxyl group.

In nature oligopeptides containing β-linkages are rare, and it is unlikely that they occur in the normal environment of *E. coli*. Notwithstanding the fact that they cannot enter by the α-oligopeptide system therefore, there is no pressure for the evolution of specific transport systems to handle them. In view of this, the presence in *E. coli* of β-oligopeptidase activity presumable reflects lack of specificity of α-peptidases.

The negligible growth observed with dipeptides containing β-alanine residues suggests that these compounds are also concentrated poorly by *E. coli*, for they were readily hydrolysed by broken organisms. Earlier studies[1] upon the structural requirements for dipeptide uptake by *E. coli* have indicated the importance of both the N-terminal α-amino group and C-terminal α-carboxyl group; a β-linkage necessarily destroys the fixed conformational relationship between these groups and with it presumably the ability to use the dipeptide transport system. However, the charge distribution on a peptide may also have a bearing on its uptake, and this would be influenced by the different pK_a values of α- and β-carboxyl groups. The limited ability of dipeptides to use the oligopeptide system has been described previously[1]; the results here suggest that glycyl-β-alanine (related to glycylaspartic acid) may use the oligopeptide system in an analogous manner to that reported for lysylcadaverine (related to lysyllysine)[26].

It has been shown that carnosine can be used by animals on a histidine-deficient diet[27], but the ready utilization of carnosine (as a source of histidine) by the histidine auxotroph of *E. coli* was surprising. Although the auxotrophic requirement for histidine is not high, carnosine would need to be concentrated by the bacterium.

for at the low concentrations used in the growth studies, diffusion alone is unlikely to meet the requirement. Because the structure of carnosine precludes it from entering *E. coli* by either the di- or oligopeptide systems it is possible that a separate uptake mechanism may exist. It is of interest to relate this conclusion to the natural occurrence of carnosine. Carnosine occurs widely in the skeletal muscle of animals and birds, and is ingested in significant amounts when meat or fish are consumed. Many studies indicate that intestinal peptide transport is an important nutritional process[28], and carnosine has been shown to be absorbed *in vivo*[11,12]. However, in man, protein digestion products are absorbed mainly in the virtually sterile upper two thirds of the small intestine, suggesting that competition for peptide uptake between enteric microorganisms and the intestine is unlikely to be significant; although in certain animals, *e.g.* ruminants, or the rat and rabbit, which eat faeces, competition for nutrients between bacteria and the small intestine may take place. Carnosinase has not been demonstrated amongst intestinal peptidases, and indeed, recent studies *in vitro* indicate that carnosine is readily transported but poorly hydrolysed by the intestine (Matthews, D. M., personal communication). It seems likely therefore, that carnosine will occur extensively in the usual environment of *E. coli*, and enteric organisms may therefore have evolved an uptake system specially for this nutrient; it should be recalled that *Corynebacteria*, which may occur as intestinal parasites can also utilize carnosine[24]. The existence of a transport mechanism in brain slices for actively transporting carnosine (but not homocarnosine) has also been reported[29].

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