Peptide Uptake in *Saccharomyces cerevisiae*: Characteristics of Transport System Shared by Di- and Tripeptides

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(Received 14 December 1978)

Di- and tripeptides shared the same transport system in *Saccharomyces cerevisiae*. Typical rates of uptake were 1 to 5 nmol peptide min⁻¹ (mg dry wt)⁻¹; these rates were influenced by the composition and sequence of the peptide. Accumulation of intact sarcosine peptides was demonstrated. Rates of peptide uptake were essentially the same in the wild-type and in several amino acid permease-deficient mutants. These results were derived from application of two fluorescence methods that permit direct determination of peptide uptake.

INTRODUCTION

There is an extensive literature on the transport of small peptides by bacteria (Payne, 1976; Payne & Gilvarg, 1978). In contrast, there have been only a very few reports on the uptake of peptides by the yeast *Saccharomyces cerevisiae* (Becker *et al.*, 1973; Naider *et al.*, 1974; Marder *et al.*, 1977; Becker & Naider, 1977). In the last of these studies radioactive trimethionine was employed as a transport substrate, but in the remaining studies an indirect way of investigating peptide transport was used in which the growth responses of methionine, leucine or lysine auxotrophs were related to the compositions, sequences and concentrations of various peptides provided as sole sources of the required amino acids. Although only limited conclusions can be drawn from growth tests, these indirect assays of transport have been employed because of the great difficulty in obtaining an adequate variety of radioactively labelled peptides.

In this paper, we demonstrate that di- and tripeptides share the same uptake system in *S. cerevisiae* and compete with each other for transport; the relative rates of uptake of certain peptides were determined and some were shown to be accumulated intact. Evidence is presented for the distinction between amino acid and peptide uptake. These results have been made possible by application of new fluorescence procedures that permit direct measurement of the transport of any small peptide.

METHODS

Organisms. *Saccharomyces cerevisiae* haploid wild-type strain Σ 1278b (mating type α) was used. Mutants isogenic with strain Σ 1278b were of the following genotypes: 2512c, *gap* (general amino acid permease-deficient); MG276, *met-pl* (methionine permease-deficient); 5156d, *gap met-pl*; CR10, *gap arg-pl* (general amino acid and arginine permease-deficient). Mutants 2512c, MG276 and 5156d were isolated by Gits & Grenson (1967) and Grenson *et al.* (1970). Mutant CR10 (previously referred to as strain D) was isolated from 2512c by P. Earnshaw (Seaston *et al.*, 1973). All cultures were kindly supplied by Professor A. A. Eddy.

Media and growth conditions. Cultures were maintained on 1.5% (w/v) agar slopes containing glucose (2%, w/v), peptone (2%, w/v), yeast extract (1%, w/v) and a Yeast Nitrogen Base lacking amino acids (Difco 0919-15), in which (NH₄)₂SO₄ (0.5%, w/v) was the nitrogen source. Cultures were transferred from slopes to the same medium without agar, incubated overnight at 28 °C in a shaking water bath (120 to 140 strokes min⁻¹), and then transferred to a medium containing glucose (2%, w/v), proline (4 mg ml⁻¹) (as

nitrogen source) and a Yeast Nitrogen Base lacking amino acids and $(\text{NH}_4)_2\text{SO}_4$ (Difco 0335-15). Cultures for uptake studies were grown in this medium at 28 °C with shaking to an A_{660} (measured on a Bausch and Lomb Spectronic 20 spectrophotometer) of 0.2 to 0.7 (approx. 0.5×10^7 to 2×10^7 organisms ml^{-1} , equivalent to 0.2 to 0.7 mg dry wt ml^{-1}).

Organisms were harvested by membrane filtration (25 mm diam., 0.45 μm pore size; Oxoid), washed on the filter with 4 vol. citrate/phosphate buffer, pH 4.5, (20 mM with respect to phosphate) at room temperature, and resuspended in the same buffer to an A_{660} of between 2.0 and 5.0.

Peptide uptake studies. These were performed at 28 °C in a shaking water bath in the presence of glucose (0.8 %, w/v), citrate/phosphate buffer, pH 4.5, (10 mM with respect to phosphate), and yeast giving an A_{660} of 1.0 to 2.5, usually in a total volume of 1 ml. Suspensions were pre-incubated for 10 min before addition of peptide.

The disappearance of peptide from the incubation medium was determined on 0.3 ml samples taken at appropriate intervals using a 1 ml syringe and immediately freed from yeast by passing the suspension through a membrane filter (13 mm diam., 0.45 μm pore size; Oxoid) using a Swinnex (Millipore) filter holder. Filtrate was collected in a small vial. Uptake was usually followed for a period of 20 min.

Peptides were determined in cell extracts of organisms incubated as described, usually for 1 h. Cell suspension (1 ml) was harvested on a membrane filter under water vacuum, washed with 40 ml distilled water at room temperature, and finally extracted with 1 ml water in a stoppered tube for 15 min in a boiling water bath. The boiled suspension was passed through a membrane filter to remove insoluble debris and the filtrate was collected.

Assay of peptides. A dansylation method (Payne & Bell, 1979) was used to estimate peptides and amino acids in cell extracts, and also to follow the disappearance of peptides from the incubation media during uptake studies. Briefly, samples of the filtered incubation media and cell extracts (100 μl) were treated with dansyl chloride and the resulting dansyl peptides and (for cell extracts) dansyl amino acids were separated by two-dimensional chromatography on polyamide thin layers. Permanent records of the chromatograms were made by photographing under ultraviolet light. Diaminopimelic acid (5 μl of a 1 mM solution) was added as an internal standard to all samples before dansylation. The intensities of spots on the chromatograms were assessed visually, with reference to standard amounts of dansylated standards; within the approximate range 0.5 to 4 nmol per spot this procedure is accurate to ± 5 %. In certain cases, the intensities were quantified using a fluorescence spectrophotometer (Baird Atomic Fluoripoint) equipped with an automatic TLC scanner, peak areas being measured with a Beckman integrator.

The disappearance of peptides from the medium was also assayed using fluorescamine, the method being based on that of Perrett *et al.* (1975). Samples (50 μl) of the filtered incubation medium containing peptide (up to 10 nmol) were added to 2.5 ml of 0.1 M-sodium tetraborate (in deionized water, pH adjusted to 6.2 with HCl). Freshly prepared fluorescamine solution in acetone (0.5 ml, 0.15 mg ml^{-1}) was added while the peptide solution was being rapidly vortexed. Fluorescence was estimated using a Perkin-Elmer model 1000 or Baird Atomic Fluoripoint fluorescence spectrophotometer (excitation 390 nm; emission 485 nm). Fluorescence was stable for at least 30 min, but was routinely read after 2 min.

The relation between the amount of any particular peptide and its fluorescence was shown to be linear in the range used, though different peptides varied markedly in their fluorescence yield. Fluorescence values obtained for samples of the incubation medium alone were low compared with the values obtained for peptides, and at the pH used in the assay, interference by amino acids was also negligible (Perrett *et al.*, 1975).

Materials. Fluorescamine (Fluram) was a gift from Dr P. S. Ringrose, Roche Products. Glycyl-sarcosyl-sarcosine (Gly-Sar-Sar) and glycyl-sarcosyl-sarcosyl-sarcosine (Gly-Sar-Sar-Sar) were kindly provided by Dr S. Wilkinson, Wellcome Research Laboratories. All other peptides were from Sigma, and were of the all L-configuration. Peptone, 5-dimethylamino-1-naphthalenesulphonyl chloride (dansyl chloride) and polyamide sheets for chromatography (15 $\times$ 15 cm) were from BDH and yeast extract was from Oxoid. All other reagents were of analytical grade.

RESULTS

Uptake of physiological peptides

The uptake of 0.1 mM-Leu-Leu [(Leu)₂], Leu-Leu-Leu [(Leu)₃], Ala-Ala [(Ala)₂] and Ala-Ala-Ala [(Ala)₃] by strain $\Sigma 1278b$ (2 mg dry wt ml^{-1}) was followed to completion by the dansylation method. During uptake none of the constituent amino acids of the peptides was detected in the incubation medium (e.g. Fig. 1, A to C).

The rates of (Leu)₂ and (Ala)₃ uptake at substrate concentrations between 0.025 and 0.2 mM were measured using the fluorescamine method. From the initial rates, assuming that

Fig. 1. Chromatograms of dansyl peptides and dansyl amino acids from incubation media and cell extracts.

A to C. Samples of medium during incubation with 0.1 mM-(Leu)₂: A, 0 min; B, 30 min; C, 60 min. D, Key to dansyl derivatives in A, B, C: (a) (Leu)₂, (b) position of Leu, (c) dansyl-ammonia, (d) diaminopimelic acid (standard), (e) dansyl-hydroxide, (f) origin.

E to G. Cell extracts prepared after 1 h incubation with: E, 5 mM-Gly-Sar; F, 5 mM-Gly-Sar plus 4 mM-(Leu)₂; G, no peptide. H. Key to dansyl derivatives in E, F, G: (a) Gly-Sar, (b) dansyl-ammonia, (c) Pro, (d) Val, (e) Ala, (f) Gly, (g) Glu, (h) Leu, (i) Lys, (j) Orn, (k) diaminopimelic acid (standard), (m) dansyl-hydroxide, (n) origin.

Table 1. *Mutual inhibition of (Leu)₂ and (Ala)₃ uptake*

Substrate was supplied at 0.1 mM; uptake was followed over 30 min by the dansylation method.

Initial concn of inhibitor (mM)	Percentage inhibition of	
	(Ala) ₃ uptake by (Leu) ₂	(Leu) ₂ uptake by (Ala) ₃
0.1	50	0
0.2	100	15
0.4	100	35
0.8	100	70

Michaelis-Menten kinetics apply, K_m values of the order of 10^{-5} M for (Leu)₂ and 10^{-4} M for (Ala)₃ were derived from a Lineweaver-Burk plot.

Competition between (Leu)₂ and (Ala)₃

The uptake of one peptide in the presence of one or more other peptides can readily be studied using the dansylation method, and this was used to study competition between different peptides. The peptide (Leu)₂ was a much better inhibitor of (Ala)₃ uptake than (Ala)₃ was of (Leu)₂ uptake (Table 1). This is consistent with the apparent affinities for uptake demonstrated above.

Uptake of sarcosine peptides

Saccharomyces cerevisiae has a generally high intracellular peptidase activity, making the detection of accumulation of unhydrolysed peptides in cell extracts difficult (Becker & Naider, 1977; T. M. Nisbet & J. W. Payne, unpublished results). However, in peptides containing sarcosine, the *N*-substituted peptide bond is very resistant to enzymic hydrolysis,

Fig. 2. Inhibition of Gly-Sar (a) and Gly-Sar-Sar (b) accumulation in *S. cerevisiae* strain $\Sigma 1278b$ by $(\text{Leu})_2$ (○) and $(\text{Ala})_3$ (●). Substrates were supplied at 5 mM. Accumulation of substrate during 1 h was estimated by analysis of cell extracts using the dansylation method.

although the peptides usually retain adequate affinity for peptide transport systems. These features have allowed the demonstration of the accumulation of unhydrolysed peptides in *Escherichia coli* (Payne, 1972; Payne & Bell, 1979), mammalian intestine (Addison *et al.*, 1972) and germinating barley embryos (Higgins & Payne, 1977).

Similarly, by dansylating cell extracts, it was also possible to demonstrate accumulation of unhydrolysed Gly-Sar, Gly-Sar-Sar and Gly-Sar-Sar-Sar in strain $\Sigma 1278b$. Accumulation was linear over the 1 h incubation period. Furthermore, after uptake of Gly-Sar no free sarcosine was detected in cell extracts, suggesting that the rate of Gly-Sar hydrolysis was slow (Fig. 1, E). Control experiments showed that free sarcosine was rapidly accumulated from the medium by *S. cerevisiae* and was not detectably metabolized during the 1 h incubation period.

The rate of Gly-Sar accumulation over the concentration range 2 to 30 mM was determined by examination of cell extracts by the dansylation procedure. Uptake occurred by a saturable system, and a K_m of the order of 10 mM was derived from a Lineweaver-Burk plot.

Competition against sarcosine peptides

The observation that sarcosine peptides were accumulated by *S. cerevisiae*, although suggestive, does not in itself indicate that accumulation occurs by the same transport system(s) as are used by physiological peptides. This point was investigated by competition studies. The peptide $(\text{Leu})_2$ was a better inhibitor than $(\text{Ala})_3$ of both Gly-Sar and Gly-Sar-Sar uptake, and both $(\text{Leu})_2$ and $(\text{Ala})_3$ inhibited Gly-Sar-Sar uptake more readily than Gly-Sar uptake (Fig. 2).

Uptake of peptides in amino acid permease-deficient strains

In all bacterial species examined to date peptide transport systems have been shown to be distinct from amino acid transport systems. In *S. cerevisiae*, the absence of competition between the two classes of substrate suggests that the same situation probably also exists (Marder *et al.*, 1977; Becker & Naider, 1977). However, this feature has not been investigated in transport-deficient mutants. The availability of amino acid permease-deficient strains made it possible to examine this aspect. The rate of transport of $(\text{Ala})_2$ was not diminished compared with that in the wild-type in any of the amino acid permease-deficient mutants examined (Table 2). Examination of the relative rates of uptake of peptides by strain $\Sigma 1278b$ and mutant 5156d (*gap met-p1*) showed that for the range of peptides tested, the specificity of the system is similar in both strains (Table 3).

Table 2. *Initial rates of uptake of (Ala)₂ by wild-type and amino acid permease-deficient mutants*

Uptake of peptide (initial concentration 0.1 mM) was followed for up to 20 min using the fluorescamine method. The number of determinations is given in parentheses.

Organism	Rate of uptake [nmol min ⁻¹ (mg dry wt) ⁻¹]	
	Range	Mean
Σ1278b	1.0–4.3 (17)	2.2
2512c	2.3–2.4 (3)	2.4
MG276	2.5–3.1 (3)	2.7
5156d	3.7–5.9 (4)	4.7
CR10	2.7–4.0 (3)	3.6

Table 3. *Relative initial rates of peptide uptake by S. cerevisiae strain Σ1278b and mutant 5156d*

Rates are expressed relative to (Ala)₂ uptake in strain Σ1278b [1.0 to 4.3 nmol min⁻¹ (mg dry wt)⁻¹] and mutant 5156d [3.8 to 4.3 nmol min⁻¹ (mg dry wt)⁻¹]. Uptake was followed for up to 20 min using the fluorescamine method. Results are the range for at least two measurements.

Peptide substrate	Rate of peptide uptake [% relative to (Ala) ₂ uptake]	
	Σ1278b	5156d
(Ala) ₂	100	100
(Ala) ₃	70–100	70–90
(Met) ₂	50–70	50–60
(Gly) ₂	< 10	< 10
(Leu) ₂	40–60	–
Ala-Met	100–120	100–120
Met-Ala	55–65	–
Ala-Lys	190–210	–
Lys-Ala	150–165	–

DISCUSSION

The results presented suggest that di- and tripeptides share the same transport system in *S. cerevisiae*. Inhibition by (Leu)₂ and (Ala)₃ of uptake of both di- and tripeptides containing sarcosine was demonstrated, and these two peptides were also mutually inhibitory. The inhibition of uptake was competitive, as it depended on the relative concentrations of inhibitor and substrate rather than the absolute inhibitor concentration (compare Table 1 and Fig. 2). Studies of the uptake of single peptides clearly indicated a higher affinity for (Leu)₂ than (Ala)₃ transport. This conclusion is corroborated by the relatively greater inhibitory effects of (Leu)₂ compared with (Ala)₃ on the transport of other peptides and supports the conclusion that the observed inhibition of uptake arose from competition at the transport level.

Previous evidence for a common peptide transport system in *S. cerevisiae* is indirect, being mainly based on growth response tests. Thus, since the dipeptide (Met)₂ was a poor inhibitor of labelled (Met)₃ uptake, Becker & Naider (1977) were initially led to speculate that separate di- and oligopeptide transport systems might occur in yeast, as had been found for *E. coli* (Payne, 1968). Subsequently, however, Marder *et al.* (1977) showed that a Lys⁻ Leu⁻ auxotroph of *S. cerevisiae* exhibited a prolonged lag in the growth response to (Leu)₃ on addition of (Met)₂ or (Ala)₂, and the growth responses to (Leu)₂, Lys-Gly and Lys-Leu were delayed by addition of (Met)₃. Thus it appeared probable that these growth lags arose from interactions between di- and tripeptides at the transport step. However, although growth will be limited when the rate of supply of the nutrient peptide falls below

that needed for optimal protein synthesis, it is difficult to see why competition should lead to varied lags rather than varied growth rates, and the simple competitive mechanism suggested by Marder *et al.* (1977) appears to us to be inadequate to explain the specificity in the observed growth inhibitions. Any inhibition must be a function of both the affinities for, and maximal rates of, uptake of the peptides and it is clear from the results presented here that both these parameters vary considerably between peptides. For example, (Ala)₂ was transported faster than (Met)₂ (each at 0.1 mM), but competition studies showed (Met)₂ had greater affinity for transport (results not shown). Apparently the $V_{\max}$ for transport of (Ala)₂ is greater than that for (Met)₂.

Changes in amino acid composition and sequence markedly influence the initial rates of uptake of peptides (Table 3). As growth tests yield only indirect results, with little information on uptake rates, these effects are best studied using the fluorescence methods presented here, the preparation of an adequate variety of radiolabelled peptides being impracticable. Results of this type are essential if optimal peptide structures are to form the basis of a rational design of peptide carriers for co-transporting antifungal compounds; this principle (Payne, 1976) has already been realized in the commercial development of antibacterial phosphonopeptides (Allen *et al.*, 1978) and has been discussed in relation to antifungal agents (Lichliter *et al.*, 1976).

No marked differences were found in the rates of peptide uptake by a wild-type strain and by a number of amino acid permease-deficient mutants (Tables 2 and 3). These results are consistent with earlier observations (Becker & Naider, 1977; Marder *et al.*, 1977) that amino acids and peptides do not compete for uptake and thus use separate transport systems. Using several peptide substrates, we also confirmed (unpublished results) the report (Becker & Naider, 1977) that organisms grown on proline as nitrogen source are able to transport peptides at about ten times the rate of ammonia-grown organisms; consequently we used proline-grown organisms throughout our studies. After incubation of these proline-grown organisms with a peptide, examination of their intracellular pools using the dansylation procedure frequently showed increased levels of the amino acid residues of the peptide (unpublished results). This observation leads to the speculation that these accumulated residues might bring about transinhibition of amino acid uptake, as described by Woodward & Cirillo (1977), a situation that should be clearly distinguished from that of direct peptide inhibition of amino acid uptake, i.e. competition.

In *E. coli*, which normally does not leak amino acids to a significant extent, peptide uptake is accompanied by rapid intracellular hydrolysis and selective exodus of those constituent amino acids of the peptide that become excessive in the pool (Payne & Bell, 1979). In *S. cerevisiae*, although intracellular peptide hydrolysis is also rapid, we observed no accompanying amino acid exodus. This difference may be because yeast has a comparatively greater capacity for accumulating amino acids (Indge *et al.*, 1977) and this capacity was not exceeded by the amount of added peptide; alternatively, some of the accumulated residues might be released as deaminated derivatives (Woodward & Cirillo, 1977) which probably would not be labelled by the fluorogenic reagents used here and would therefore be undetectable.

Note added in proof. After completion of this work, Marder *et al.* (1978) reported isolation of a mutant of *S. cerevisiae*, resistant to L-ethionyl-L-alanine, that was defective in transport of several di- and oligopeptides. Similarly, we have isolated a mutant of strain $\Sigma 1278b$, resistant to bacilysin, that is defective in the uptake of a wide range of both di- and tripeptides (Nisbet & Payne, 1979). These reports therefore confirm that all small peptides share a single transport system in this yeast.

This research was supported in part by a grant from the Science Research Council.

T.M.N. is in receipt of an SRC-CASE studentship in association with Roche Products Ltd. The contributions of C. F. Higgins and G. Bell to the development of the fluorescamine method are gratefully acknowledged. We thank A. Jamieson for photography and E. Ellis for help in preparing the manuscript.

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