Peptide Transport in *Candida albicans*

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The tripeptide L-methionyl-L-methionyl-L-[methyl- ^{14}C]methionine was taken up into *Candida albicans* by a saturable system with a pH optimum of 3.5, a temperature optimum of 37 °C and an apparent K_m of 3.3×10^{-5} M. Metabolic inhibitors such as sodium azide and dinitrophenol completely prevented uptake. Neither methionine nor dimethionine effectively competed with trimethionine uptake. (Leu) $_3$, Gly-Met-Gly, acetyl-(Met) $_3$, D-Met-L-Met-L-Met and Met-Met-Ile effectively competed with (Met) $_3$ uptake, whereas (Lys) $_3$, L-Met-L-Met-D-Met, D-Met-D-Met-D-Met, (Met) $_3$ methyl ester and (Ala) $_3$ did not. Trimethionine was rapidly hydrolysed by a peptidase after entry into the cell.

INTRODUCTION

Studies in this laboratory have attempted to characterize the structural specificity of the peptide transport system in *Candida albicans* (Lichliter *et al.*, 1976). The aim of the project is to use the information gained from transport studies to design peptide carriers which would bring toxic agents specifically inside this pathogenic yeast and kill it. In our preliminary investigations, the growth response of a methionine/lysine auxotroph, strain WD 18-4, was used as a measure of peptide uptake. Since the growth curve was somewhat variable, and the generation time for this yeast was rather long, we found it difficult to obtain reliable data on the ability of different peptides to compete for the peptide uptake system. Furthermore, the results of growth experiments could not give information on the number of peptide transport systems present in *C. albicans* WD 18-4 or on the relative affinities of different peptides or peptide derivatives for the peptide transport systems in this strain. A transport assay using a radioactive peptide substrate is a direct approach to gain further information on the peptide transport system in the pathogenic yeast. In this communication, we report results on the uptake of ^{14}C -labelled trimethionine by *C. albicans* WD 18-4.

METHODS

Chemicals. Most of the peptides were purchased from Bachem Chemical Co., Marina Del Ray, Calif., U.S.A. Trilysine was purchased from Miles-Yeda, Rehovot, Israel. Radioactive trimethionine (L-methionyl-L-methionyl-L-[methyl- ^{14}C]methionine) was synthesized as reported previously (Becker & Naider, 1977). All other chemicals were reagent grade or the purest commercially available.

Organism. *Candida albicans* WD 18-4, a methionine/lysine double auxotroph, was used in all experiments. It was kindly supplied by Dr Alvin Sarachek, Wichita State University, U.S.A. The yeast was maintained on slants of YEPD agar containing (% w/v): yeast extract, 1; Bacto-peptone (Difco), 2; glucose, 2; agar (Difco), 2. The organism was transferred monthly to a fresh agar slant, incubated for 24 h at 37 °C and stored at 4 °C.

Growth conditions. The mineral growth medium used for all experiments was Vogel's N-medium (Vogel & Bonner, 1965) modified by the removal of NH_4NO_3 , unless stated otherwise, and by the addition of ($\mu\text{g ml}^{-1}$): inositol, 36; pantothenic acid, 2; pyridoxine.HCl, 4; thiamin.HCl, 4; biotin, 0.0005; methionine, 10; lysine, 10. The medium was autoclaved for 10 min at 121 °C. Sterile solutions of glucose (final concn 2%, w/v) as carbon and energy source and isoleucine (6.56 mg ml^{-1}) as nitrogen source were added aseptically. For growth, cells were taken from a YEPD agar slant and inoculated to give 10^6 cells ml^{-1} in 25 ml fresh medium contained in a 125 ml flask. The culture was aerated by stirring with a magnetic stir-bar and grown for 18 to 24 h at 37 °C. A portion (7 ml) of this culture were inoculated into 93 ml fresh medium (giving 10^6 cells ml^{-1}) in a 500 ml flask fitted with a sidearm and stirred vigorously at 37 °C. Growth was determined turbidimetrically at 400 to 420 nm (blue filter) using a Klett-Summerson photoelectric colorimeter. One Klett unit corresponded to 5×10^4 cells ml^{-1} . Under these growth conditions, the cells remained in the yeast phase.

Transport studies. Cells were grown for 6 to 8 h (40 to 70 Klett units; 2×10^6 to 3.5×10^6 cells ml^{-1}), and they were then harvested by filtering through a 0.65 μm pore-size membrane filter, washed twice with cold (0 to 4 °C) distilled water, resuspended so that a 1:10 dilution contained 1.4 mg dry wt ml^{-1} (30 Klett units) and kept cold until assayed. When assayed for uptake of peptides, the cells were incubated at 37 °C for 10 min and added to an equal volume of reaction mixture containing 2% (w/v) glucose, 0.03 M-citric acid/ KH_2PO_4 buffer (pH 3.5) and radioactive trimethionine. At intervals, 0.5 ml samples of the reaction mixture were applied to 0.45 μm pore-size filters and washed twice with 2.5 ml distilled water. The filters were placed in 5 ml Bray's solution (Bray, 1960) and counted in a Searle Isocap/300 6868 Liquid Scintillation System. There was no peptide adsorption to the cell surface or sticking to the filter since at 0 °C the counts were at background levels. The uptake results, calculated on the basis of 80% counting efficiency and the known specific activity of the peptide (1.0 $\mu\text{Ci } \mu\text{mol}^{-1}$), are expressed as nmol peptide taken up (mg dry wt cells) $^{-1}$.

Thin-layer chromatography. Extracts from cells incubated with the radioactive peptide were spotted on an Eastman Chromagram Sheet (13254 cellulose with fluorescent indicator, no. 6065) and developed in a solvent system containing propan-2-ol/butan-2-one/1 M-HCl (6:15:25, by vol.). A portion of the chromatogram sheet was sprayed with ninhydrin to detect the markers. For detection of radioactive compounds, the cellulose was scraped from the remaining chromatogram area, placed in Bray's solution and counted.

RESULTS

Characteristics of uptake of trimethionine

The uptake of radioactive trimethionine was linear for approximately 5 min (Fig. 1) using cell concentrations up to 1.4 mg dry wt ml^{-1} with the rate of uptake being directly proportional to the number of cells. At cell densities > 1.4 mg dry wt ml^{-1} the concentration of peptide became rate-limiting. In most experiments, therefore, cell concentrations of 0.7 mg dry wt ml^{-1} were used in the transport assays. The initial rate of uptake for 3×10^{-5} M-trimethionine was 1.0 nmol (mg dry wt cells) $^{-1} \text{ min}^{-1}$ (Fig. 1). In other experiments at this trimethionine concentration, we observed a variation in the initial rate from 0.25 to 1.0 nmol (mg dry wt cells) $^{-1} \text{ min}^{-1}$. Previous studies of *Saccharomyces cerevisiae* (Becker & Naider, 1977; Grenson *et al.*, 1966) and *Neurospora crassa* (Wolfenbarger & Marzluf, 1974) have also shown variable initial rates for the uptake of peptides and amino acids. Thus, in all experiments where the effect of pH, temperature, competitors or energy inhibitors was examined, the control rate of trimethionine uptake was also determined.

The source of nitrogen in the growth medium influenced the uptake of trimethionine by this yeast (Fig. 2). Cells grown on NH_4NO_3 or NH_4Cl (not shown) as the nitrogen source had a lower transport activity than cells grown with tryptophan or isoleucine. Cells grown with isoleucine as the nitrogen source gave the highest transport activity. The amino acids were poor sources of nitrogen with isoleucine giving the slowest growth rate and lowest cell yield. NH_4NO_3 was apparently a good source of nitrogen as the cells had a fast rate of growth and reached a high final concentration. Similar apparent repression of uptake by nitrogen has been shown for peptide (Becker & Naider, 1977) and amino acid (Grenson *et al.*, 1966; Roon *et al.*, 1975) uptake in *S. cerevisiae*. However, as different nitrogen sources produce different growth rates, uptake rates might be controlled by the growth rate *per se*.

Fig. 1

Fig. 2

Fig. 1. Uptake of radioactive trimethionine into *Candida albicans* WD 18-4. Cells at the mid-exponential stage of growth were harvested, washed and resuspended to 1.4 mg dry wt ml⁻¹ in distilled water. The suspension was added to an equal volume of a reaction mixture containing 2% (w/v) glucose, 0.03 M-citric acid/KH₂PO₄ buffer (pH 3.5), and radioactive trimethionine (6.06×10^{-5} M, 1 μ Ci μ mol⁻¹). The final volume was 4 ml. At the times indicated, samples of the suspension were removed, filtered and counted in a liquid scintillation counter.

Fig. 2. Effect of the nitrogen source in the growth medium on the uptake of trimethionine. Yeasts were grown in Vogel's medium with either isoleucine (6.56 mg ml⁻¹), tryptophan (5.11 mg ml⁻¹) or NH₄NO₃ (2.0 mg ml⁻¹) as the sole source of nitrogen. Cells were harvested at the mid-exponential stage of growth, washed, resuspended to 1.4 mg dry wt ml⁻¹ in distilled water and added to an equal volume of reaction mixture as described in Fig. 1. Nitrogen sources: ■, isoleucine; ▲, tryptophan; ●, NH₄NO₃.

The transport of trimethionine was pH and temperature dependent. Optimum pH values for uptake were between 3.5 and 4.5 with initial rates of uptake at pH 3.0 and 5.0 about one-half the optimal rates. The temperature optimum was 37 °C with no uptake observed at 0 or 45 °C.

The initial rate of uptake of radioactive trimethionine was determined at various concentrations of radioactive peptide. The apparent K_m , calculated from a double-reciprocal plot of this data, was 3.3×10^{-5} M. The results suggest that the transport system has a limited capacity for carrying the peptide (i.e. is a saturable system). It is possible that the K_m determined could represent a combination of transport and intracellular hydrolysis of the peptide.

Competition for transport between trimethionine and structurally related compounds

Methionine did not compete with trimethionine uptake when present in 20-fold molar excess (Table 1) and dimethionine, in 5- and 20-fold molar excesses, competed only slightly, suggesting that the dipeptide is probably not taken up by the oligopeptide transport system which recognizes trimethionine. (Leu)₃ and Met-Met-Ile inhibited (Met)₃ uptake by almost 90% when present in 20- and 10-fold molar excess, respectively, whereas Met-Ala-Met, (Lys)₃, (Ala)₃ and Gly-Met-Gly showed less inhibition. Of the various stereoisomers of trimethionine, D-Met-L-Met-L-Met effectively competed with radioactive L-Met-L-Met-L-Met uptake whereas D-Met-D-Met-D-Met and L-Met-L-Met-D-Met did not. Acetyl-L-Met-L-Met-L-Met competed strongly with L-Met-L-Met-L-Met uptake while L-Met-L-Met-L-Met methyl ester and L-Met-L-Met-L-Met amide did not. These results indicate that the uptake system not only distinguishes between amino acids, dipeptides and oligopeptides but also between oligopeptides of different sequence, stereochemistry and chain ends.

Table 1. *Competition for transport between trimethionine and structurally related compounds*

Cells suspended at 1.4 mg dry wt ml⁻¹ in distilled water were added to an equal volume of reaction mixture containing 2.0% (w/v) glucose, 0.03 M-citric acid/KH₂PO₄ buffer (pH 3.5), radioactive trimethionine (6.06×10^{-5} M, 1.0 μ Ci μ mol⁻¹) and methionine or peptide at the concentration indicated. The final volume was 4 ml. Initial rates of trimethionine transport were determined. All amino acid residues were of the L configuration unless indicated otherwise.

Competitor	Molar excess relative to trimethionine (-fold)	Inhibition of trimethionine transport (%)
Met	5	0
	20	3
Met-Met	5	22
	20	16
Met-Ala-Met	5	48
	20	55
Leu-Leu-Leu	5	72
	20	87
Lys-Lys-Lys	5	24
	10	15
Ala-Ala-Ala	5	9
	10	38
Gly-Met-Gly	5	35
	10	64
Met-Met-Ile	5	67
	10	85
Met-Met-D-Met	5	9
D-Met-Met-Met	5	80
D-Met-D-Met-D-Met	5	12
Met-Met-Met methyl ester	10	0
Met-Met-Met amide	5	10
	10	5
	20	10
Acetyl-Met-Met-Met	10	80

Table 2. *Effect of metabolic inhibitors on the uptake of trimethionine*

Cells were incubated at 37 °C for 10 min with the various inhibitors at the concentration indicated. They were then added directly to an equal volume of reaction mixture containing 2.0% (w/v) glucose, 0.03 M-citric acid/KH₂PO₄ buffer (pH 3.5) and radioactive trimethionine (6.06×10^{-5} M, 1.0 μ Ci μ mol⁻¹). The final volume was 4 ml. Initial rates of trimethionine uptake were determined.

Compound tested	Concn (mM)	Inhibition of trimethionine uptake (%)
NaN ₃	0.1	100
2,4-Dinitrophenol	0.1	100
KCN	10	60
	1	40
N-Ethylmaleimide	10	100
Dicyclohexylcarbodiimide	1	50
Cycloheximide	0.18	0

Fig. 3. Intracellular fate of transported methionine. Cells were incubated in reaction mixture containing radioactive trimethionine, and samples were taken at 1, 3 and 5 min. The cells were separated by filtration, washed twice with distilled water and then extracted with cold (4 °C) ethanol (70 %, v/v); a portion of the extract was developed by thin-layer chromatography. The chromatogram sheets were divided into 0.5 cm strip fractions, scraped into scintillation fluid and counted by liquid scintillation.

Effect of metabolic inhibitors

Compounds which inhibit various metabolic activities were potent inhibitors of trimethionine uptake (Table 2). 2,4-Dinitrophenol and NaN_3 at concentrations as low as 0.1 mM completely inhibited uptake, while KCN at 10 mM gave 60% inhibition. *N*-Ethylmaleimide, which reacts with thiol groups, and dicyclohexylcarbodiimide, an inhibitor of the Na^+, K^+ -ATPase, were both effective inhibitors. Cycloheximide, an inhibitor of protein synthesis, had no effect. These results suggest that transport is probably coupled to the provision of metabolic energy and an intact membrane system.

Fate of the transported trimethionine

To establish that active transport is occurring, it is essential to detect accumulation of the substrate against a chemical gradient. However, we could not demonstrate active transport of trimethionine because of the inability to show accumulation of the intact substrate due to rapid intracellular hydrolysis by cellular peptidase activity (Fig. 3). Yeast cells were incubated with the radioactive peptide for 1, 3 and 5 min; they were then extracted with cold ethanol and a portion of the extract was developed by thin-layer chromatography. All the radioactivity moved as a single peak and had the same mobility as the methionine marker. These results are consistent with the finding of rapid trimethionine cleavage in *S. cerevisiae* (Becker & Naider, 1977) and Gly-D,L-Leu-Tyr breakdown in *N. crassa* (Wolfenbarger & Marzluf, 1975).

DISCUSSION

The results of this study show that *C. albicans* WD 18-4 contains a saturable system which transports radioactive trimethionine into the cell. The transport system is temperature and pH dependent and affected by inhibitors of metabolic energy production. It thus exhibits most properties normally associated with carrier-mediated uptake. Unfortunately the rapid

degradation of trimethionine by intracellular peptidases (Fig. 3) did not permit us to determine whether uptake occurs against a concentration gradient.

The apparent K_m (3.3×10^{-5} M) for the uptake of trimethionine by strain WD 18-4 is comparable to the value of 7.7×10^{-5} M reported for its uptake by *Saccharomyces cerevisiae* (Becker & Naider, 1977) and a K_m of 3.4×10^{-5} M for Gly-Leu-Tyr uptake by *Neurospora crassa* (Wolfenbarger & Marzluf, 1975). At present, it is not known whether intracellular hydrolysis of the peptide contributes to the K_m . Our data show, however, that once inside the cell, trimethionine is rapidly cleaved to free methionine. The cells therefore appear to have significant intracellular peptidase activity and peptide hydrolysis is probably not the rate-limiting step.

The competition studies (Table 1) provide much information about the system mediating tripeptide uptake in *C. albicans* WD 18-4. Free methionine does not significantly compete with trimethionine uptake. This suggests that these metabolites enter the yeast through separate transport systems. Dimethionine competes only slightly, indicating either separate di- and tripeptide transport systems or that dimethionine has a very low affinity for the system mediating trimethionine transport. The fact that neither methionine nor dimethionine significantly inhibits trimethionine uptake also suggests that the latter is not hydrolysed before entering the cell. We cannot, however, preclude the possibility that transport and cleavage occur at the cell membrane, as proposed by Ugolev & DeLaey (1973), since the only extractable radioactivity was found to co-migrate with free methionine (Fig. 3).

The inhibition of the initial uptake rates of trimethionine by various competitors could theoretically occur by inhibition at the transport level or at the level of peptide hydrolysis. As stated above, our experiments indicate a high level of *in vivo* peptidase activity and peptide hydrolysis does not seem to be rate-limiting for peptide transport. We have also observed that the hydrolysis of trimethionine is not affected by (Leu)₃ or (Lys)₃, which are, respectively, good and poor inhibitors of uptake (unpublished observations). Finally, both L-Met-L-Met-L-Met and L-Met-L-Met-L-Met methyl ester are hydrolysed at approximately the same rate by cell extracts of *C. albicans* WD 18-4, despite the fact that the free peptide is a good competitor of trimethionine uptake while the methyl ester does not compete. Based on all of these results, it appears reasonable to conclude that the inhibition in the uptake assays represents competition at the level of transport.

A number of peptide derivatives failed to compete with trimethionine uptake. Neither trimethionine amide nor trimethionine methyl ester prevent the entry of trimethionine, whereas acetyl-trimethionine is an effective competitor. These results are important for the design of peptide-drug conjugates. We also observed that the peptide transport system in *C. albicans* can tolerate D-residues. This ability is very specific, however, with D-Met-L-Met-L-Met causing an 80% inhibition of L-Met-L-Met-L-Met uptake under conditions where L-Met-L-Met-D-Met has virtually no effect. Since the design of a peptide-drug conjugate must include consideration of the hydrolysis of the conjugate by enzymes present in sera and the target micro-organism, a knowledge of the stereospecificity of the peptide transport system is of obvious importance.

In eukaryotic cells, little is known about the coupling of metabolic energy to the transport process while in *Escherichia coli* (Simoni & Postma, 1975) a great deal of knowledge has been attained. Metabolic energy inhibitors have been used extensively to determine whether or not metabolic energy is required for the transport process. We have observed that NaN₃ and 2,4-dinitrophenol completely inhibit uptake of trimethionine into *C. albicans* WD 18-4. Several other energy inhibitors were also effective, and the cells required glucose in the uptake medium. These findings are consistent with the uptake of trimethionine by *C. albicans* being coupled to energy provision.

Previous studies on peptide transport in eukaryotic micro-organisms have been primarily restricted to *N. crassa* and *S. cerevisiae*. A number of marked differences exist in the struc-

Fig. 4. Characteristics of a potential peptide carrier.

tural specificity for peptide uptake by these eukaryotes. *Neurospora crassa*, for example, cannot take in acylated peptides (Wolfenbarger & Marzluf, 1975). A methionine auxotroph of *S. cerevisiae*, however, grows on a number of acylated di- and tripeptides (Becker *et al.*, 1973) and a wild-type strain took up radioactively labelled acetyl-Met-Met-Met (Becker & Naider, 1977). In contrast, a leucine/lysine auxotroph of *S. cerevisiae* (strain Z1-2D) was not able to utilize acetyl-Leu-Leu-Leu or acetyl-Gly-Leu (Marder *et al.*, 1977). It appears that *S. cerevisiae* may contain separate transport systems for free and acylated peptides and this latter uptake mechanism may not be present in all strains. Acylated peptides are incorporated by *C. albicans* WD 18-4 and the results of the competition studies indicate that acetyl-trimethionine shares the transport system used by free tripeptides (Table 1). Furthermore, although peptide methyl esters were utilized by a methionine auxotroph of *S. cerevisiae*, they are not used by *C. albicans* (Lichliter *et al.*, 1976) and do not inhibit trimethionine transport (Table 1). Any attempts to use the peptide transport system to carry drugs into pathogenic eukaryotes will obviously be somewhat limited by these variations.

Despite this drawback, we believe that our results reveal certain features which should be included in the design of a peptide-carrier for toxic agent transport into *C. albicans* WD 18-4. These features are represented in Fig. 4 and include: (i) the exclusion of the C-terminus as an attachment point; (ii) the desirability of hydrophobic residues in the side-chain; (iii) the possible inclusion of D-residues to increase *in vivo* stability. This latter consideration obviates a different hydrolysis stereospecificity by peptidases in the serum and the pathogen. Using this model as a guide we are currently synthesizing conjugates of peptides and fluorinated pyrimidines. The effectiveness of these conjugates will then be subjected to biological examination.

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