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Photoinactivation of Peptide Transport in *Saccharomyces cerevisiae*†

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ABSTRACT: Oligopeptides and dipeptides are transported into *Saccharomyces cerevisiae* by a carrier-mediated system. In the dark, leucyl-*p*-nitroanilide (Leu-*p*-NA) and leucyl-leucyl-4-azido-2-nitrophenylalanine [Leu-Leu-Phe-($4N_3$,$2NO_2$)] are competitive inhibitors of peptide transport by *S. cerevisiae* cells. The photolysis of yeast cells in the presence of Leu-*p*-NA or Leu-Leu-Phe($4N_3$,$2NO_2$) at 350 nm results in an irreversible inactivation of peptide transport. Protection against this inactivation is afforded by an excess of trimethionine, a transported peptide. Photolysis with Leu-*p*-NA or Leu-Leu-Phe($4N_3$,$2NO_2$) does not affect amino acid or sugar transport, and cell viability is maintained throughout the irradiation procedure. A 5-min irradiation of *S. cerevisiae* with 2.4 μM Leu-*p*-NA or 15 μM Leu-Leu-Phe($4N_3$,$2NO_2$) causes 50% inhibition of trimethionine uptake. *p*-Nitroaniline, a possible hydrolysis product generated from Leu-*p*-NA by cellular peptidase activity, has no effect on peptide transport. An exogenous energy source is not required for photoinactivation. The results suggest that a component(s) of the peptide transport system of *S. cerevisiae* is irreversibly modified by photolysis with Leu-*p*-NA or Leu-Leu-Phe-($4N_3$,$2NO_2$) and provide the first example of the use of amino acid *p*-nitroanilides as photoaffinity labels.

Peptide transport has been studied in a variety of cells including bacteria (Payne & Gilvarg, 1978; Payne, 1980), fungi (Becker & Naider, 1980; Wolfinbarger, 1980), plants (Higgins & Payne, 1980), and cells of mammalian origin (Matthews & Payne, 1980). We have studied the uptake of peptides into *Saccharomyces cerevisiae* (Becker & Naider, 1980). Our results show that peptides are transported intact across the cell membrane of *S. cerevisiae*. The peptide transport system has highest affinity for peptides containing hydrophobic amino acids and is capable of taking up peptides composed of a variety of amino acids. Growth of cells in the presence of ammonium ion reduces the transport of peptides (Becker & Naider, 1977), as well as the transport of other nitrogen-containing small molecules in yeast (Grenson et al., 1970). Finally, in contrast to *Escherichia coli* which has separate di- and oligopeptide transport systems (Payne, 1980), *S. cerevisiae* appears to have one peptide transport system for all peptides utilized (Becker & Naider, 1980).

In a previous study (Parker et al., 1980), we showed that at pH 7.0 intact cells of *S. cerevisiae* 139 hydrolyzed leucyl-*p*-nitroanilide (Leu-*p*-NA)[1] and other aminoacyl-*p*-nitroanilides by an activity similar to that of aminopeptidase II, a well-characterized external peptidase in yeast (Frey & Rohm, 1979). Furthermore, we showed that at pH 5.5 Leu-*p*-NA was a competitive inhibitor of trimethionine transport in *S. cerevisiae* but does not affect amino acid transport. Transport inhibition was not related to hydrolysis of Leu-*p*-NA by aminopeptidase because at pH 5.5, the optimum for trimethionine transport, there was no aminopeptidase II activity. These results also provided evidence that peptides were not hydrolyzed by the extracellular aminopeptidase II prior to transport; if trimethionine were cleaved extracellularly followed by the transport of methionine, Leu-*p*-NA would not competitively inhibit. Other studies support the conclusion that peptides are not hydrolyzed extracellularly before transport into yeast: a peptide transport mutant retains peptidase activity (Marder et al., 1978), both transported and nontransported peptides are hydrolyzed by cell extracts (Naider et al., 1974), and amino acids do not compete for peptide transport in yeast (Marder et al., 1977).

A major goal of many transport studies is the isolation of components of the transport system. Chemical modification of transport proteins has been carried out for a variety of transport systems by affinity labeling techniques (Glover, 1977) to tag carriers. Photoaffinity reagents have been used to probe various transport systems because the highly reactive photolysis products generated in situ can react with virtually any amino acid side chain (Bayley & Knowles, 1977; Chowdhry & Westheimer, 1979). In the present study, incubation of cells with leucyl-*p*-nitroanilide or leucylleucyl-4-azido-2-nitrophenylalanine and irradiation with light of 350 nm led

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[1] Abbreviations: Leu-*p*-NA, leucyl-*p*-nitroanilide; Leu-Leu-Phe-($4N_3$,$2NO_2$), leucylleucyl-4-azido-2-nitrophenylalanine; Boc, *tert*-butyloxycarbonyl; OSu, hydroxysuccinimide ester.

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to the specific irreversible inactivation of the peptide transport system of *S. cerevisiae*.

Materials and Methods

Chemicals. Radioactive trimethionine was synthesized as reported previously (Becker & Naider, 1977). All of the chemicals were reagent grade or the purest commercially available, and all amino acids were in the L configuration. Leucylleucyl-4-nitrophenylalanine was the kind gift of Dr. Arieh Yaron, Weizmann Institute, Rehovot, Israel. 4-Azido-2-nitrophenylalanine was prepared from 4-aminophenylalanine (Bachem, Torrance, CA) as described (Staros & Knowles, 1978).

Synthesis of Leucylleucyl-4-azido-2-nitrophenylalanine. Boc-Leu-OSu (*tert*-butyloxycarbonyl-L-leucinehydroxysuccinimide ester, 6.93 g, 0.03 mol) in dioxane (75 mL) was added to a mixture of L-leucine (3.93 g, 0.03 mol) and sodium bicarbonate (5.04 g, 0.06 mol) in water (75 mL). The turbid mixture was stirred overnight at room temperature. Dioxane was removed by rotary evaporation and the remaining mixture acidified with 10% citric acid. On being stirred in the cold, a white product crystallized and was subsequently filtered and washed with distilled water followed by heptane. This product (6.5 g, 63%) was homogenous on thin layers of silica using two different solvent systems, had the anticipated NMR spectrum for Boc-Leu-Leu-OH, and was used without further purification. Dicyclohexylcarbodiimide (2.06 g, 0.01 mol) was added to Boc-Leu-Leu-OH (3.44 g, 0.01 mol) and hydroxysuccinimide (1.15 g, 0.01 mol) dissolved in dichloromethane (22 mL) at 5 °C. The resulting reaction mixture was stirred overnight at 5 °C and then treated with a few drops of acetic acid and filtered. The filtrate was concentrated to dryness and the residue redissolved in tetrahydrofuran and cooled in a refrigerator for 3 h. The resulting small precipitate of dicyclohexylurea was filtered and the process repeated until a urea-free solution was obtained. The solution was then diluted with ethyl acetate, and the organic phase was washed successively, with H_2O, a 10% citric acid solution, 5% $NaHCO_3$, and a saturated NaCl solution. After the organic phase was dried ($MgSO_4$), the solvent was removed, yielding an oil that crystallized (3.3 g, 75%) on trituration with petroleum ether: NMR ($CDCl_3$) δ 7.22 (d, 1 H, amide NH), 2.95 (s, 4 H, imide CH_2), 1.50 (s, 9 H, Boc CH_3), 0.98 (m, 12 H, CH_3). The product was homogeneous on silica thin layers (ethyl acetate and ethyl acetate:hexane, 4:1) and used without further purification. From this point on, all the reactions were carried out in the dark. Boc-Leu-Leu-OSu (176 mg, 0.40 mmol), 4-azido-2-nitrophenylalanine hydrochloride (116 mg, 0.40 mmol), and *N*-methylmorpholine (0.096 mL, 0.88 mmol) were mixed in 6 mL of dimethylformamide–H_2O (2:1). The turbid solution cleared after 2 h and was left at room temperature in the dark for 22 h. The solution was diluted with ethyl acetate and washed with 10% citric acid and then water. After the solution was dried ($MgSO_4$), the solvent was removed, yielding a solid product that was homogeneous on silica thin layers (CH_2Cl_2:acetic acid:water, 95:15:3); the product had an infrared spectrum (KBr) with a strong azide band at 2120 cm^{-1}. The compound was dissolved in 3 mL of trifluoroacetic acid–CH_2Cl_2 (1:1) and left for 30 min at room temperature. The solvent was removed under vacuum and the residue triturated with anhydrous ether, filtered, and washed with ether. The solid product as its trifluoroacetate salt (75 mg) was homogeneous and ninhydrin positive in two systems (2-propanol:H_2O, 2:1, and 1-butanol:acetic acid:H_2O, 4:1:1) and showed an infrared spectrum (KBr) with a strong azide band at 2122 cm^{-1}. Anal. Calcd for $C_{23}H_{32}N_7F_3O_9$: C, 46.70; H, 5.45. Found: C, 46.21; H, 5.87. In aqueous solution, the compound showed a UV spectrum with λ_{max} 250 (ϵ 1.7 × 10^4) and 334 nm (ϵ 1.5 × 10^3).

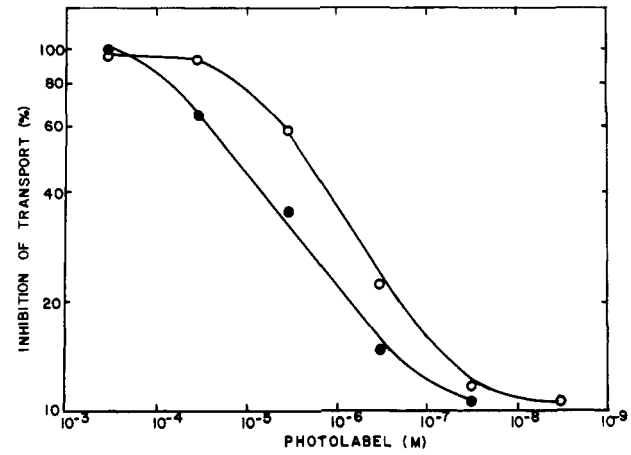

FIGURE 1: Inactivation of peptide transport at various photolabel concentrations. Yeast cells were photolyzed at 350 nm with Leu-*p*-NA (○) or Leu-Leu-Phe($4N_3$,$2NO_2$) (●) for 5 min at the concentrations indicated. The initial rate of transport of trimethionine was determined, and the percent inhibition was calculated and plotted.

Cells. Saccharomyces cerevisiae (ATCC 9896) was grown on Vogel's N medium or yeast nitrogen base without amino acids (Difco) as described previously (Becker & Naider, 1977). For transport assays, cells were harvested in the late logarithmic phase of growth as described previously (Becker & Naider, 1977) and resuspended to 1 mg dry weight/mL.

Treatment of Cells. Photolyses of cell suspensions were carried out in a quartz reaction vessel fitted with a cold finger. A pair of Rayonet 350-nm reactor bulbs (lot RPR-3500A, Southern New England Ultraviolet Co., Middletown, CT) was mounted laterally along the axis of the incubation chamber 4 cm from the sample. Water at 30 °C was continuously circulated through the cold finger so that the cell suspension was temperature controlled and received the light directly from the bulbs through the quartz glass. During photolysis, the cells were suspended in 0.05 M potassium phosphate adjusted to pH 5.5 with or without 55 mM glucose and were stirred by a Teflon-coated magnet. A portion of the cell suspension was removed at various times as indicated and immediately centrifuged at 3500 rpm for 10 min in a table-top centrifuge. The cell pellet was resuspended in distilled water, centrifuged again, and resuspended in distilled water. For transport assays, photolysed, washed cells were incubated at 30 °C for 5 min in a reaction mixture containing 55 mM glucose and 0.05 M potassium phosphate at pH 5.5, the optimal conditions from trimethionine uptake. Radioactive peptide, amino acid, or sugar was added, and at various times, portions of the cell suspension were filtered and counted as described previously (Becker & Naider, 1977).

Results

In a previous study (Parker et al., 1980), we demonstrated that in the dark leucyl-*p*-nitroanilide (Leu-*p*-NA) is a competitive inhibitor of trimethionine transport in *S. cerevisiae*. Upon irradiation of cells in the presence of Leu-*p*-NA, trimethionine uptake was severely lowered (Figure 1). The dose–response curve for a 5-min photolysis at 350 nm indicates that a low concentration (2.4 × 10^{-6} M) of Leu-*p*-NA caused a 50% loss of transport activity. Photolysis of cells under identical conditions in the absence of Leu-*p*-NA had a negligible effect (~10% inhibition). That inhibition of Leu-*p*-NA was irreversible was demonstrated by the fact that cells washed

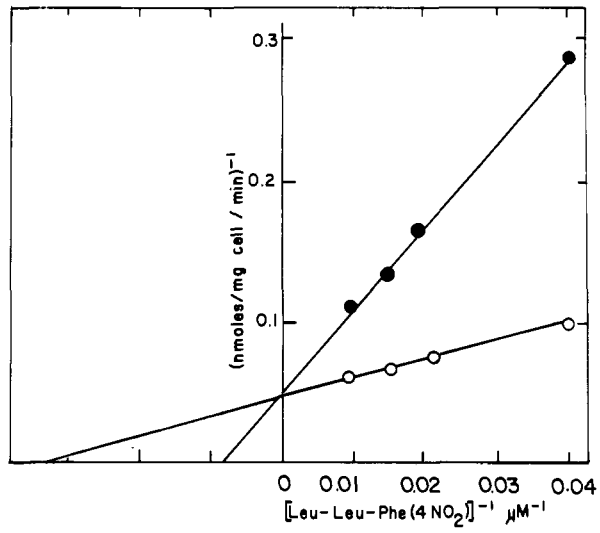

FIGURE 2: Kinetics of inhibition of trimethionine uptake by leucyl-leucyl-4-nitrophenylalanine. The initial rate of trimethionine uptake at various concentrations in the absence (○) and presence (●) of Leu-Leu-Phe($4NO_2$) was determined and plotted in a double-reciprocal graphical representation.

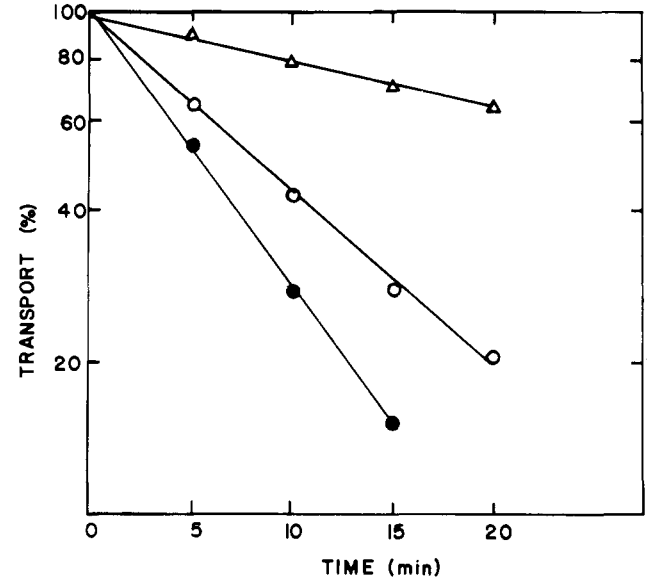

FIGURE 3: Time course of photoinactivation. Cells were photolyzed in buffer (△), Leu-*p*-NA at 2.4 μM (●), or Leu-Leu-Phe($4N_3$,$2NO_2$) at 2.4 μM (○) for various time periods as indicated. The initial rate of trimethionine transport after photolysis at the indicated times is represented.

extensively after photoinactivation did not recover the ability to transport peptide. A similar irreversible photoinactivation of peptide transport occurred upon photolysis of cells in the presence of Leu-Leu-Phe($4N_3$,$2NO_2$) (Figure 1). In the absence of light, repeated competition experiments with Leu-Leu-Phe($4N_3$,$2NO_2$) and trimethionine led to inconsistent results as to the type of competition. However, we did determine that a similar compound, Leu-Leu-Phe($4NO_2$), was a competitive inhibitor of peptide uptake (Figure 2). Approximately a 10-fold higher concentration of Leu-Leu-Phe($4N_3$,$2NO_2$) was required to give the same amount of photoinactivation as Leu-*p*-NA. A number of azido-containing compounds have been used for photoinactivation of transport systems (Henderson et al., 1979; Rudnick et al., 1975; Staros & Knowles, 1978). However, *p*-nitroanilides to our knowledge have not been used for photoaffinity labeling studies. Therefore, we carried out several experiments to determine whether side reactions or nonspecific interactions were leading to the inactivation of peptide transport.

The extent of photoinactivation of peptide transport in the presence of either Leu-*p*-NA or Leu-Leu-Phe($4N_3$,$2NO_2$) was a function of photolysis time (Figure 3). In the absence of photolabel, significant inactivation of transport activity (30%) did not occur until 15–20 min of photolysis (Figure 3). Under identical conditions in the presence of photoaffinity label, much higher inactivation was achieved. The viability of the cells was not effected by irradiation under the conditions used for photoinactivation as measured by the ability of photolyzed cells to form colonies after plating on minimal or complex media. The count of viable cells was approximately 1×10^7 colony-forming units before and after 5 min of photolysis. *p*-Nitroaniline was a possible hydrolysis product of leucyl-*p*-nitroanilide. However, at 10^{-3} M, *p*-nitroaniline had no effect on peptide transport, and even photolysis of cells in the presence of high concentrations of *p*-nitroaniline (10^{-3} M) caused no effect on peptide uptake.

Cells were irradiated in the presence of Leu-*p*-NA at 2.4×10^{-5} M, and the activity of cell-bound aminopeptidase was determined as described previously (Parker et al., 1980). Photolysis was carried out at pH 5.5 under the conditions described. The aminopeptidase was assayed at pH 7.0, the optimum for aminopeptidase activity, since at pH 5.5 cell-bound aminopeptidase is not detectable. There was no effect of photolysis with Leu-*p*-NA on cell-bound aminopeptidase activity. Furthermore, photolysis of a soluble leucine aminopeptidase (Sigma Chemical Co., St. Louis, MO) in the presence of Leu-*p*-NA did not have any effect on the initial rate of hydrolysis of the substrate by the enzyme.

An experiment was carried out to test the possibility that a photoproduct of leucyl-*p*-nitroanilide might be toxic to the cell, thereby causing an apparent photoinactivation of the peptide transport system. A solution of leucyl-*p*-nitroanilide (2.4×10^{-5} M) was photolyzed and then added to cells simultaneously with radioactive tripeptide. No effect was detected on peptide transport.

The specificity of photoinactivation in the presence of Leu-*p*-NA and Leu-Leu-Phe($4N_3$,$2NO_2$) was determined by measuring amino acid and sugar transport after photolysis (Figure 4). The uptakes of the amino acid analogue aminoisobutyric acid (AIB), a nonmetabolized substrate in yeast (Kotyk & Rihova, 1972), and 2-deoxy-D-glucose (2-DOG), a nonfermentable glucose analogue (Seaston et al., 1973), were measured before and after photolysis of *S. cerevisiae* 139 with the photoaffinity labels. Neither high concentrations (10^{-4} M) of Leu-*p*-NA or Leu-Leu-Phe($4N_3$,$2NO_2$) nor photolysis for relatively long time periods (up to 10 min) raised the inhibition of AIB or 2-DOG transport above that seen in the absence of photolabel.

In the presence of trimethionine, the cells were protected from inactivation of Leu-*p*-NA and Leu-Leu-Phe($4N_3$,$2NO_2$) (Table I). The protection required a 500-fold molar excess of trimethionine to Leu-*p*-NA to reduce photoinactivation from 55% to 24%. Similar protection of Leu-Leu-Phe($4N_3$,$2NO_2$) photoinactivation required a 10-fold molar excess of trimethionine.

An exogenous energy source is not required for photoinactivation. Photolysis of cells in the presence of Leu-*p*-NA or Leu-Leu-Phe($4N_3$,$2NO_2$) in the presence or absence of glucose led to the same amount of photoinactivation. Cells held under partially anaerobic conditions (incubation of cells in deaerated medium by flushing with nitrogen, and then holding under nitrogen during photolysis) still were inactivated irreversibly by photolysis of Leu-*p*-NA. Sodium azide is a

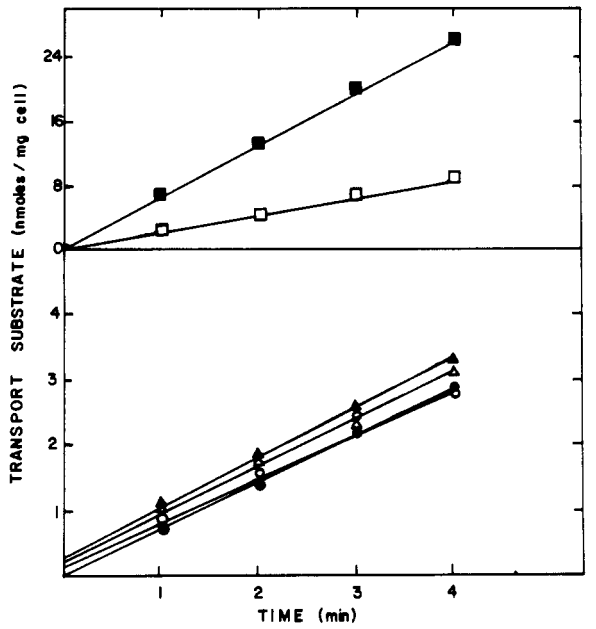

FIGURE 4: Effect of photolysis with Leu-*p*-NA on the transport of peptide, aminoisobutyric acid, and 2-deoxy-D-glucose. The upper panel represents trimethionine transport after a 5-min photolysis in the presence (□) of 2.4 μM Leu-*p*-NA. The lower panel is aminoisobutyric acid (○) and 2-deoxy-D-glucose (△) transport after a 5-min photolysis with Leu-*p*-NA at 2.4 μM. Closed symbols represent transport after photolysis in the absence of Leu-*p*-NA.

Table I: Protection by Substrate from Photoinactivation[a]

conditions	transport rate [nmol min^{-1} (mg of cell)$^{-1}$]	inhibition (%)
control	5.8	
Leu-*p*-NA	2.6	55
Leu-Leu-Phe($4N_3$,$2NO_2$)	2.4	58
Leu-*p*-NA + $(Met)_3$	4.5	24
Leu-Leu-Phe($4N_3$,$2NO_2$) + $(Met)_3$	4.7	19

[a] Cells were photolyzed for 5 min with 2.4 μM Leu-*p*-NA or 24 μM Leu-Leu-Phe($4N_3$,$2NO_2$), and the initial rate of trimethionine transport was determined. During photolysis with photolabel, trimethionine was present at 1.2 or 0.24 mM for Leu-*p*-NA or Leu-Leu-Phe($4N_3$,$2NO_2$), respectively.

reversible inhibitor of trimethionine transport in *S. cerevisiae* 139 (Becker & Naider, 1977). This compound partially protected *S. cerevisiae* 139 from inactivation by photolysis with Leu-*p*-NA (Table II).

Discussion

Photoaffinity reagents are useful for probing membrane carriers because of the high reactivity of the photochemically derived species. Investigators have used reagents containing photoactivatable functional groups such as azides (Chowdhry & Westheimer, 1979) and nitrophenyl ethers (Kaczorowski et al., 1980) to irreversibly inactivate various transport systems. Particularly relevant to the present study was the use of glycyl-4-azido-2-nitro-L-phenylalanine to inactivate the dipeptide transport system in *Escherichia coli* (Staros & Knowles, 1978). Our study utilizes a nitrophenylanilide to photoaffinity label the peptide transport system in *S. cerevisiae*. To our knowledge, this is the first use of such a compound as a photoactivatable reagent.

The peptide transport system in *S. cerevisiae* was irreversibly inactivated by two different reagents, leucyl-*p*-nitroanilide and

Table II: Effect of Sodium Azide on Photoinactivation[a]

conditions	transport rate [nmol min^{-1} (mg of cell)$^{-1}$]	inhibition (%)
control	6.0	
Leu-*p*-NA	2.7	55
sodium azide	5.4	10
Leu-*p*-NA + sodium azide	4.4	27

[a] Cells were photolyzed for 5 min with Leu-*p*-NA at 2.4 μM with or without sodium azide at 0.5 mM. The cells were washed to remove unreacted inhibitor, and trimethionine uptake was determined.

leucylleucyl-4-azido-2-nitrophenylalanine. We found that Leu-*p*-NA has a K_i equal to 2 μM for trimethionine transport whereas Leu-Leu-Phe($4NO_2$), a compound similar in structure to Leu-Leu-Phe($4N_3$,$2NO_2$), has a K_i equal to 15 μM. It is not surprising, therefore, that Leu-*p*-NA is a more effective photoinactivator than Leu-Leu-Phe($4N_3$,$2NO_2$). The relatively high concentration of substrate needed to protect $(Met)_3$ uptake from irreversible inactivation in the presence of Leu-*p*-NA (Table I) may be due to the hydrophobic nature of the Leu-*p*-NA which results in concentration of this compound in the membrane. Competition experiments in *S. cerevisiae* 139 (Parker et al., 1980) indicated that the affinity of Leu-*p*-NA for the peptide transport system is 10–25-fold higher than that of $(Met)_3$, and high concentrations of $(Met)_3$ are necessary to displace Leu-*p*-NA from the membrane.

Our results indicate that photoinactivation is specific for the peptide transport system since amino acid and sugar transport was not affected. Also, photoinactivation was not due to a general effect of photolysis on the cells. Irradiation in the absence of photoactivatable compound for up to 10 min had little effect on transport in *S. cerevisiae* 139. Previous studies have explored the effect of ultraviolet irradiation on *S. cerevisiae* transport (Doyle & Kubitschek, 1976). Near-ultraviolet light irradiation at 365–366 nm for 150 min led to 90% inactivation of the transport of sorbose in yeast. After 15 min of irradiation, only a 20% reduction in sorbose uptake was observed. Since greater than 80% inhibition of peptide transport was observed during a 10–15-min irradiation in the presence of low concentration of either Leu-*p*-NA or Leu-Leu-Phe($4N_3$,$2NO_2$), it is reasonable that we observed no loss of sugar or amino acid uptake.

It is pertinent to mention that we have examined peptides containing chemically reactive groups such as *p*-nitrophenyl esters, *N*-hydroxysuccinimide esters, or *N*-bromoacetamides as potential inactivators of $(Met)_3$ uptake in *S. cerevisiae* 139 (unpublished results). In contrast to the photoactivatable reagents discussed in this paper, none of these peptides resulted in the specific irreversible inactivation of peptide uptake, despite the fact that methionylmethionylmethionine *p*-nitrophenyl ester and methionylmethionine *p*-nitrophenyl ester were both competitive inhibitors of $(Met)_3$ uptake in *S. cerevisiae* 139. Apparently, the protein(s) recognizing $(Met)_3$ during its translocation into yeast contain(s) binding sites with side chains that either are unreactive toward active esters or bromoacetyl moieties or are in the incorrect geometry for a productive reaction to occur.

The irreversible photoinactivation of $(Met)_3$ uptake by Leu-*p*-NA was not a result of the hydrolysis of this amino acid derivative or the general toxicity of the compound or its hydrolysis products toward *S. cerevisiae*. Although previous studies have utilized nitrophenylazido groups, nitrophenyl

groups, and nitrophenyl ethers as photoactivatable reagents, there are no reports in the literature of photoinactivation by nitroanilides. Interestingly, the effect of Leu-*p*-NA on peptide uptake seems quite specific since photolysis in the presence of this substrate does not inactivate either commercial leucine aminopeptidase or the cell-bound aminopeptidase of *S. cerevisiae*. Thus, loss of peptide transport is independent of peptide hydrolysis and probably involves a component(s) of the transport system which either recognizes or translocates trimethionine, or provides energy for the uptake process.

At present, the mechanism leading to inactivation by photolysis with Leu-*p*-NA is unknown. Electron spin resonance studies do not detect free radicals after photolysis of Leu-*p*-NA at room temperature (unpublished results). We are attempting to conduct EPR studies in liquid helium in order to discern short-lived, highly reactive radicals. Whether photolysis generates a reactive species that leads to covalent bonding of Leu-*p*-NA to a cellular component or whether Leu-*p*-NA acts as a photosensitizer, leading to cross-linking or intramolecular reactions that result in inactivation, remains to be determined.

Investigations from other laboratories have shown that yeast cells are protected from ultraviolet-induced killing by azide (Ito, 1977). Azide anion is a known quencher of singlet oxygen and appears to protect cells and free enzyme (Makinen et al., 1982) from photoinactivation via a type II (singlet oxygen) mechanism. In the present study, low concentrations of azide anion (0.5 mM) partially protected the cells from photoinactivation of $(Met)_3$ uptake by Leu-*p*-NA (Table II). Higher azide concentrations could not be explored due to their direct influence on $(Met)_3$ uptake. The partial protection by azide suggests the possibility that singlet oxygen has a role in the photoinactivation of $(Met)_3$ uptake by Leu-*p*-NA. Clearly, additional studies are required to verify this hypothesis.

The findings reported herein provide the first example of the specific inactivation of a peptide transport system in a eukaryotic cell. We are currently preparing radiolabeled photoinactivators in order to tag the component which is inactivated and to determine its role in the transport of peptides by yeast.

Acknowledgments

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