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Peptide Utilization by Amino Acid Auxotrophs of *Neurospora crassa*

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The ability of auxotrophs of *Neurospora crassa* to grow on certain tripeptides, despite the presence of excess competing amino acids, suggests it has an oligopeptide transport system. In general, dipeptides did not support growth except in those instances where extracellular hydrolysis occurred, or where the dipeptide appeared to be accumulated by an uptake system which is sensitive to inhibition by free amino acids. Considerable intracellular peptidase activity toward a large number of peptides was demonstrated, including a number of peptides which could not be utilized for growth. The intracellular peptidase activity was shown to be selective for amino acid composition and sequence (N-terminal or C-terminal) within the peptide; glycine-containing peptides were particularly poor substrates for peptidase activity. Only a small amount of extracellular peptidase activity could be detected.

Neurospora crassa is known to utilize exogenous proteins as a sole carbon, nitrogen, or sulfur source for growth, by producing extracellular proteases (7, 8). The question therefore arises as to whether growth proceeds on the resultant peptides or on the individual amino acid molecules. Before growth on any of these compounds can occur, they must first be transported into the cells. A number of studies (5, 15, 19, 21, 22, 25) have revealed in *Neurospora* the presence of at least four distinct amino acid transport systems specific for (i) L-neutral amino acids, (ii) L-basic amino acids, (iii) acidic amino acids, and (iv) a "general" system for both D- and L-neutral and basic amino acids. A system apparently specific for L-methionine has also been reported (16). Provided the extracellular proteases were to cleave the proteins down to the individual amino acids, they could be readily transported by the various uptake systems and thus support growth. However, if the cleavage products of the proteases are primarily peptides, as suggested by the findings of Coffey and deDuve (4) for liver lysosomal enzymes, the question of the presence of transport systems for these peptides arises.

Peptide transport systems have been well characterized in bacteria and the reader is referred to the excellent review on this subject by Payne and Gilvarg (18). The study of peptide transport systems in eukaryotes is only just beginning (1, 4, 9, 20). Although the evidence is indirect, this study has suggested the presence of peptide transport systems in *Neurospora* and indicates that specificity for transport is exhibited regarding the composition and number of amino acid residues in the peptide. Furthermore, the transport of certain dipeptides appears to occur via a system subject to inhibition by either L-phenylalanine or L-arginine. The present work has also demonstrated that *Neurospora* possesses considerably intracellular peptidase activity toward a wide spectrum of di- and tripeptides.

MATERIALS AND METHODS

Strains and growth conditions. The strains employed in this study are listed in Table 1. The leucine auxotrophs, *leu-4*, *leu-2*, and *leu-1*, and the lysine auxotroph *lys-1* were obtained from the Fungal Genetics Stock Center (Arcata, Calif.). The remaining strains are from stocks of a collection maintained at Ohio State University, Columbus, Ohio, but are also available, with the exception of the derived mutants, from the Fungal Stock Center.

The growth media employed in maintenance and growth assays was Vogel minimal medium N (23) with 2% sucrose and supplemented with the required amino acid or peptide, usually at 0.33 mM.

Growth assays. To determine which peptides the amino acid auxotrophs would respond to, growth tests were run with 125-ml Erlenmeyer flasks containing 31 ml of liquid minimal medium, supplemented with various free amino acids or peptides. The flasks were inoculated with 1 to 2 drops of a nonturbid (approximately 2,000 to 3,000 conidia/ml) conidial suspension

TABLE 1. *Stocks of N. crassa used*

Designation	Allele	FGSC no.[a]
Wild type[b]	$SY4f_8a$	
leu-1	33757	28
leu-2	37501	1070
leu-3	R156	1124
leu-4	R108	175
me-1	38706	559
me-2	H98	283
me-3	36104	112
lys-1	33933	74
his-6	Y152M105	457
cys-3	P22	1089
his-6;hgu-4		
his-6;hgu-6		

[a] FGSC, Fungal Genetics Stock Center, Arcata, Calif.

[b] American Type Culture Collection accession no. ATCC 26193.

and incubated stationary and in the light at 30 C for 3 days. After this growth period, each mycelial mass was harvested, pressed dry of excess water, and dried overnight in a drying oven at 60 C prior to weighing. The data were recorded as milligrams (dry weight) per mycelial pad.

Cell-free extracts. Conidia were obtained from aerial hyphae, grown 7 to 10 days at 25 C, harvesting with a sterile wire loop into sterile distilled water (0 to 4 C) and stored on crushed ice until used (usually no longer than 4 days). Conidia were inoculated into Vogel minimal medium N (500 ml in a 1-liter Ehrlenmeyer flask) with 2% sucrose (plus required amino acid where necessary) to a concentration of 0.1 to 0.2 mg (dry weight)/ml and shaken overnight (16 to 18 h) on a reciprocal shaker at room temperature. The mycelial mass was cooled in ice water prior to filtering and washing with 1 volume of 0.1 M tris(hydroxymethyl-aminomethane (Tris)hydrochloride buffer (pH 7.0). The mycelial mass, usually 2 to 3 g (wet weight), was then resuspended in 10 ml of 0.1 M Tris-hydrochloride buffer (pH 7.0) with chloramphenicol (0.5 mg/ml) and homogenized at 0 to 4 C with an Omnimixer (Ivan Sorvall Co.) at top speed for 3 to 4 min. The cell homogenate was centrifuged for 10 min at 5,000 rpm and 4 C. The centrifugation was repeated three times with the pellet removed each time. The resulting supernatant was dialyzed overnight at 0 C against Tris-hydrochloride buffer. To determine extracellular peptidase activities, the growth medium was collected, concentrated 10-fold (at 4 C) using an Amicon Ultrafiltration cell, and dialyzed overnight against Tris-hydrochloride buffer. Protein concentrations of these extracts were determined by the method of Lowry et al. (10).

Peptidase assays. The protein concentration of the various cell extracts was adjusted with buffer to 0.8 to 0.9 mg of protein per ml prior to assay and chloramphenicol added to a final concentration of 0.5 mg/ml. Portions (0.1 ml) of the cell extract (or concentrated media) were incubated at 30 C with equal volumes of peptide solutions (10 mM). At various time intervals portions were removed and the reaction was terminated by freezing. The samples were stored frozen, until analyzed by spotting 5-μliter samples on thin-layer plates (Eastman chromatogram sheet, cellulose with fluorescent indicator) which had been hand scored to 19 individual channels, 1 cm wide by 20 cm long. The samples were then chromatographed with ethanol:acetic acid:water (65:1:34) solvent for 160 to 180 min. The sheet was then removed and air-dried, and the peptides and free amino acids were visualized with ninhydrin spray.

Mutant isolation. The *hgu* mutants to be described were isolated in a *his-6* background as nonutilizers of L-histidyl-glycine, to satisfy the requirement of histidine for growth.

Conidia of *his-6* were mutagenized with ultraviolet light and the mutants were selectively enriched by incubation in minimal medium plus 0.33 mM L-histidyl-glycine with frequent filtration (see reference 2 for complete technique). The "enriched mutants" were plated on sorbose minimal medium plates supplemented with L-histidine, and the resultant colonies were picked into minimal medium containing either no supplement, L-histidine, or L-histidyl-glycine. Mutants which are incapable of utilizing histidyl-glycine were obtained in this manner. Both the *his-6; hgu-4* and *his-6; hgu-6* strains were outcrossed to wild type. The cross involving *his-6; hgu-4* yielded the original *his-6* phenotype in about 10% of the total progeny suggesting that the *hgu-4* locus was linked, approximately 20 map units from the *his-6* locus on linkage group V. The *his-6; hgu-6* strain yielded equal numbers of all possible phenotypes (parentals and segregants) suggesting a random segregation of *hgu-6* from the *his-6* locus. Further mapping of the *hgu-6* locus has not yet been undertaken.

Transport assays. Conidia and germinated conidia for the transport assays were obtained as previously described. The transport assays for conidial and mycelial uptake were as previously described (26, 27).

Chemicals. The nonradioactive di-, tri-, and tetrapeptides were obtained from Sigma Chemical Co., Fox Chemical Co., or Bachem Inc. (Calif.). Purity of these peptides was confirmed by thin-layer chromatography.

Radioactive L-histidyl-glycine and L-phenylalanine were obtained from Nuclear Dynamics Corp., El Monte, Calif.

RESULTS

The experimental results basically consist of two types of studies: (i) the observed growth response of auxotrophs to various peptides, and (ii) assays for peptidase activity toward these same peptides. The concentrations of peptide supplements employed in the growth assays were 10 μmol of peptide per 31 ml of media. This concentration was chosen because a similar concentration of most free amino acids, e.g., L-leucine for the leucine auxotrophs, provided

for growth falling about midpoint of the linear portion of a plot of growth versus substrate (amino acid) concentration. Thus, if a peptide such as L-leucyl-L-leucine were to be cleaved externally prior to entry into the cell, the actual concentration of L-leucine would provide for approximately twice as much growth.

Leucine auxotrophs. Four different leucine auxotrophs were used in assays of growth on leucine-containing peptides. With the exception that *leu-1* and *leu-2* displayed growth on the dipeptide L-leucyl-L-leucine, the leucine auxotrophs were unable to utilize (or utilized poorly) various dipeptides containing leucine to fulfill their amino acid requirement. These same dipeptides do not inhibit growth of the wild type (Table 2).

The tripeptides (containing L-leucine) tested, except L-leucyl-glycyl-glycine, supported growth of all the auxotrophs to a level comparable to free leucine (Table 2). That growth on the tripeptides is not due to their extracellular breakdown to free leucine, with its subsequent utilization, is shown by the inability of excess (3.3 mM) L-phenylalanine to block growth of *leu-1*, *leu-2*, and *leu-3* on the leucine-containing tripeptides. Excess L-phenylalanine completely eliminates growth of these auxotrophs on free L-leucine (Table 3). This inhibition is presumably due to competition by phenylalanine for leucine transport by the neutral and general amino acid transport system (26).

TABLE 2. *Growth of leucine auxotrophs and wild type on peptides containing leucine*

Supplement[a]	Growth[b]				
	Wild type[c]	*leu-1*	*leu-2*	*leu-3*	*leu-4*
None	82.8	0	0	0	0
L-Leu	74.0	42.0	56.7	28.0	37.0
L-Leu-L-Leu	83.0	43.7	14.3	2.0	2.0
L-Leu-L-Leu-L-Leu		73.0	68.0	20.4	55.0
L-Leu-Gly-Gly	70.0	0.0	0.0	0.0	0.0
L-Leu-Gly	68.0	0.0	0.0	0.0	0.0
Gly-L-Leu	75.0	0.0	0.0	0.0	0.0
Gly-L-Leu-L-Tyr		33.5	52.0	19.8	26.0
L-Leu-L-Phe	68	2.0	0.0	0.0	0.0
L-Phe-L-Leu	56	6.5	3.5	2.0	3.0
L-His-L-Leu	72	0.0	0.0	0.0	0.0
L-Leu-β-Ala	78	0.0	0.0	0.0	0.0
Gly-D-Leu	84			0.0	
Gly-L-Ala-L-Leu		31.0	49.0	14.0	23.0
L-Met-L-Leu	74	0.0	0.0	0.0	2.0
L-Lys-L-Leu		0.0	0.0	0.0	0.0

[a] Supplements at 0.33 mM.
[b] Growth as milligrams (dry weight) after 3 days of growth at 30 C. Values represent the averages of from 3 to 10 separate determinations.
[c] Strain.

TABLE 3. *Growth of leucine auxotrophs on leucine or leucine-containing peptides in the presence and absence of competing amino acids*

Source of leucine[a]	Growth[b]			
	None[c]	L-*phe*	L-*arg*	L-*arg* plus L-*phe*
leu-3				
L-Leu	28	0	26	0
L-Leu-L-Leu	3	2	3	3
L-Leu-L-Leu-L-Leu	21	18	26	18
Gly-L-Leu-L-Tyr	20	20	30	19
L-Phe-L-Leu	2	3	2	2
leu-1				
L-Leu	46	0	46	0
L-Leu-L-Leu	51	3	62	6
L-Leu-L-Leu-L-Leu	45	30	49	26
Gly-L-Leu-L-Tyr	35	28	32	27
leu-2				
L-Leu	60	0	47	0
L-Leu-L-Leu	26	<1	18	<1
L-Leu-L-Leu-L-Leu	59	49	52	40
Gly-L-Leu-L-Tyr	57	41	53	34

[a] Sources of leucine at concentrations of 0.33 mM.
[b] Growth as milligrams (dry weight) of mycelial pad after 3 days of growth at 30 C.
[c] Inhibiting amino acid at concentrations of 3.3 mM.

Thus, it appears that the leucine-containing tripeptides can be utilized without involvement of the amino acid transport systems, which immediately suggests the presence of a separate uptake system capable of transporting tripeptides.

Growth of *leu-1* and *leu-2* on L-leucyl-L-leucine is almost completely inhibited by excess L-phenylalanine, but not by excess L-arginine (Table 3). This finding suggests that the utilization of the dipeptide L-leucyl-L-leucine by *leu-1* and *leu-2* involves its extracellular hydrolysis prior to transport. These two auxotrophs may possess a greater capacity for external hydrolysis of di-L-leucine than do *leu-3* or *leu-4*. Assays for peptidase activity with cell-free extracts of the wild-type strains and the *leu-3* mutant demonstrated that these strains contained sufficient intracellular peptidase activity to cleave all leucine containing peptides tested (listed in Table 2) except for L-lysyl-L-leucine and L-leucyl-L-phenylalanine. Thus, limitation of growth of leucine auxotrophs on the leucine-containing dipeptides may be due to an inability to transport them to a level required for growth.

Methionine auxotrophs. In contrast to the observations with the leucine auxotrophs, the methionine auxotrophs, *me-1*, *me-2*, and *me-3*, were able to use di-, tri-, and tetrapeptides as sources of L-methionine (Table 4). It was, however, more difficult to exclude the possibility that growth on these peptides was due to their extracellular cleavage to yield methionine, with its subsequent transport by one or more of the three known methionine transport systems (16, 26), than with the leucine-containing peptides. Table 5 reveals that excess L-phenylalanine, L-arginine, or both, do not completely inhibit growth of *me-1* on free L-methionine or any methionine-containing peptide. The failure to completely inhibit growth on methionine is presumably due to the presence of the specific transport system for methionine (16) which is not subject to inhibition by L-phenylalanine or L-arginine. Because L-phenylalanine partially inhibited growth of *me-1* on L-methionine (50% inhibition), L-methionyl-L-methionine (75% inhibition), and L-methionyl-L-alanyl-L-serine (30% inhibition), as well as on glycyl-L-methionine and L-methionyl-glycine, it is suggested that these peptides are at least partially hydrolyzed by extracellular peptidases prior to transport and utilization. To eliminate problems encountered because of the methionine-specific permease, we utilized the *cys-3* mutant, which lacks this particular transport system (16). *Cys-3* grows with methionine as its sole source of sulfur, but its growth with methionine can be completely eliminated by a 10-fold higher concentration of L-phenylalanine (Table 5). It is noteworthy that the *cys-3* mutant can also grow on several methionine-containing peptides. However, phenylalanine cannot completely eliminate their use as a sole source of sulfur. Although the inhibition of peptide usage by phenylalanine still suggests that external hydrolysis to free methionine occurs, it also suggests that *Neurospora* is capable of using either the intact tri- and tetrapeptides (or some smaller hydrolytic product, e.g., L-methionyl-L-alanine) as a source of sulfur and that these peptides are accumulated by an uptake system independent of amino acids. Cell extracts from the wild type were capable of hydrolyzing all of the methionine-containing peptides listed in Table 4.

TABLE 4. *Growth of methionine auxotrophs on methionine and peptides containing methionine*

Supplement[a]	Growth[b]		
	me-1[c]	me-2	me-3
None	0	0	0
L-Met	48	96	58
L-Met-L-Met	80	90	48
L-Met-L-Met-L-Met	38	51	38
L-Met-Gly-L-Met-L-Met	13	38	11
L-Met-L-Ala-L-Ser	34	68	51
Gly-DL-Met	8	8	40
L-Met-Gly	4	7	31
L-Met-L-His	2	4	2
L-His-L-Met	3	7	5
L-Met-L-Leu	35	52	33

[a] Supplements at concentrations of 0.33 mM.
[b] Growth data as milligrams (dry weight) of mycelial pad after 3 days of growth at 30 C. Values represent averages of 2 to 7 separate determinations.
[c] Strain.

TABLE 5. *Growth of me-1 and cys-3 on methionine or methionine-containing peptides in the presence or absence of competing amino acids*

Source of methionine[a]	Growth[b]			
	None[c]	L-*phe*	L-*arg*	L-*phe* plus L-*arg*
me-1				
L-Met	60	34	70	62
L-Met-L-Met	105	28	99	41
L-Met-L-Ala-L-Ser	36	28	42	33
Gly-DL-Met	11	2	9	2
L-Met-Gly	4	<1	12	4
cys-3				
None	0			
L-Met	69	<1	73	
L-Met-L-Met	66	4	85	
L-Met-L-His	0	0	0	
L-Met-Gly	60	0	<1	
L-Met-L-Met-L-Met	68	49	74	
L-Met-L-Ala-L-Ser	61	50	64	

[a] Source of methionine supplement at 0.33 mM.
[b] Growth as mg (dry weight) of mycelial pad following 3 days growth at 30 C. Values represent averages of 2 or more separate determinations.
[c] Inhibiting amino acid at concentration of 3.3 mM.

Lysine auxotrophs. A single lysine auxotroph, *lys-1*, was tested for growth on lysine-containing peptides. *Lys-1* grows quite well on the tripeptide L-lysyl-L-lysyl-lysine but poorly on the dipeptides L-lysyl-L-lysine, L-lysyl-glycine, glycyl-L-lysine, and L-lysyl-L-leucine (Table 6). The failure of *lys-1* to grow on these particular dipeptides may be related to the observed general inability (or reduced ability) of intracellular peptidases of *Neurospora* to cleave dipeptides containing glycine in either the amino or carboxyl position. Peptidase activity for the dipeptides L-lysyl-glycine and L-lysyl-leucine was not detectable whereas the tripep-

TABLE 6. *Growth of lys-1 on L-lysine and lysine-containing peptides in the presence or absence of arginine*

Source of lysine[a]	Growth[b]	
	Control	Plus L-*arg*[c]
L-Lys	31	5
L-Lys-Gly	1	0
Gly-L-Lys	4	0
L-Lys-L-Lys	1	0
L-Lys-L-Lys-L-Lys	52	40
L-Lys-L-Leu	2	1

[a] Source of lysine supplements at 0.33 mM.
[b] Growth as milligrams (dry weight) of mycelial pad after 3 days of growth at 30 C. Values represent averages of from 2 to 5 separate determinations.
[c] Arginine concentration at 3.3 mM.

tide L-lysyl-L-lysyl-L-lysine was hydrolyzed by both the cell extracts and concentrated growth media. Intracellular, but not extracellular, peptidase activity was shown for glycyl-L-lysine.

Inhibition of growth of lysine auxotrophs on lysine, by arginine, has been reported by researchers since the 1940s (6). This inhibition is primarily due to competition between the two amino acids for transport (19, 22). Referral to Table 6 reveals that 3.3 mM L-arginine reduced growth of *lys-1* on 0.33 mM L-lysine by approximately 85%. Higher concentrations of L-arginine completely inhibited growth on L-lysine. Growth on 0.33 mM tri-lysine was also inhibited by L-arginine, except that much higher concentrations were required, e.g., 3.3 mM L-arginine inhibited growth by only 25% while 16.5 mM L-arginine was required to inhibit growth approximately 80%. The requirement for a higher concentration of L-arginine to block growth on tri-lysine than on lysine may be explained if the tripeptide were largely hydrolyzed extracellularly prior to transport, since a threefold higher concentration of free lysine would be obtained upon hydrolysis of the tripeptide than was available when lysine itself was supplied (both lysine and tri-lysine were provided at the same concentration in the growth medium). Extracellular peptidase activity for tri-lysine was indeed found in the growth medium.

Histidine auxotroph. Growth responses of the histidine auxotroph, *his-6*, on peptides containing histidine did not permit generalizations regarding peptide use. *his-6* grew well on L-histidyl-L-leucine, moderately well on L-histidyl-glycine, L-histidyl-L-methionine, L-leucyl-L-histine, and L-methionyl-L-histidine, and poorly on glycyl-L-histidine, L-alanyl-L-histine, β-alanyl-L-histidine, and the tripeptide L-hystidyl-glycyl-glycine (Table 7). Wild type and *his-6* possess considerable intracellular peptidase activity for all of these peptides except L-histidyl-glycyl-glycine. The peptides L-hystidyl-glycine and glycyl-L-histidine were hydrolyzed at a much slower rate than the other peptides, and in some cases the hydrolysis products were not always discernible on the thin-layer plates. (The reader will note that peptidase activity in vivo must exist for L-histidyl-glycine because *his-6* will utilize this dipeptide as a source of histidine.)

It has been repeatedly shown that inhibition of growth of histidine auxotrophs on L-histidine requires the presence of both a neutral and a basic amino acid (references 3, 13, and Table 8). This requirement is due to the ability of L-histidine to be transported as both a neutral and a basic amino acid by the neutral, basic, and general amino acid transport systems (12, 26). However, growth on L-histidyl-glycine, as well as other histidine-containing dipeptides, is subject to inhibition by either L-phenylalanine or L-arginine. The ability of these two amino acids to individually inhibit growth on histidine-containing dipeptides suggests that these particular dipeptides may be transported by a system (or systems) subject to either inhibition or repression by L-phenylalanine and L-arginine (and perhaps other amino acids).

Peptide nonutilizing mutants. The enrichment technique of Catcheside (2) was used to isolate mutants (in a *his-6* background) which were unable to use L-histidyl-glycine. The mutants were separable into two groups based on

TABLE 7. *Growth of histidine auxotrophs on histidine and peptides containing histidine*

Supplement[a]	Growth[b]		
	his-6	*his-6 hgu-4*	*his-6 hgu-6*
None	0.0	0.0	0.0
L-His	84.0	47.5	64.6
L-His-Gly	29.3	1.8	2.7
Gly-L-His	1.6	0.0	2.0
L-His-L-Leu	60.6	29.8	44.0
β-Ala-L-His	3.0	0.0	2.5
L-His-L-Met	12.5	<1	47.3
L-Met-L-His	14.0	<1	5.3
L-Ala-L-His	5.5	0.0	9.0
L-His-Gly-Gly	3.3	<1	4.0

[a] Supplements at concentrations of 0.33 mM.
[b] Growth data as milligrams (dry weight) of mycelial pad after growth for 3 days at 30 C. Values represent averages of from 3 to 10 separate determinations.

TABLE 8. *Growth of his-6 on L-histidine or peptides containing histidine in the presence or absence of competing amino acids*

Source of histidine[a]	Growth[b]			
	None[c]	L-*phe*	L-*arg*	L-*arg* plus L-*phe*
L-His	81.5	86.3	98.0	0.0
L-His-Gly	39.7	1.0	1.3	0.0
Gly-L-His	1.5	0.0	0.0	0.0
L-His-L-Leu	64.7	3.3	34.7	5.0
L-His-L-Met	14	2	7	4
L-Met-L-His	26	1	2	1

[a] Sources of histidine at concentrations of 0.33 mM.

[b] Growth as milligrams (dry weight) of mycelial pad after 3 days of growth at 30 C. Values represent averages of from 3 to 5 separate determinations.

[c] Inhibiting amino acid at concentrations of 3.3 mM.

their growth response to other histidine-containing peptides. The two types of mutants, represented by *hgu-4* and *hgu-6*, also segregate differently from the *his-6* locus in a genetic cross (see Materials and Methods).

The double mutant *his-6;hgu-4* will not grow on any dipeptide tested except L-histidyl-L-leucine, and growth on this dipeptide is reduced approximately 50% (Table 8). Growth of *his-6;hgu-4* on L-histidine is also reduced almost 50% compared to the *his-6* parent. By contrast, the double mutant *his-6;hgu-6* exhibits growth on histidine-containing dipeptides other than L-histidyl-glycine comparable to its *his-6* parent. Neither of the mutants *his-6;hgu-6* or *his-6;hgu-4* (or the recombinant *hgu-4* or segregant *hgu-6*) are sensitive to L-histidyl-glycine.

All of the *hgu* mutants are comparable to *his-6* and the wild type in intracellular peptidase activities. We have been unable to consistently detect peptidase activity for L-histidyl-glycine in an in vitro assay with wild type or any of the mutant strains, or to detect transport activity for L-[^{3}H]histidyl-glycine in conidia (26), acetate grown cells (27), or sucrose-grown (18 h) mycelia (27). It seems unlikely, however, that the *hgu-4* mutation affects a peptidase(s) since *his-6;hgu-4* does possess significant peptidase activity for the other dipeptides, e.g., L-alanyl-L-histidine, which it cannot use for growth. Furthermore, the *his-6;hgu-4* double mutant segregates prototrophic recombinants, presumably *hgu-4* (from a cross with wild type) that are resistant to the growth-inhibitory properties of D-glutamate. This amino acid has been reported (15, 28) to be recognized by the general amino acid transport system as a potential substrate for transport.

Peptidase assays. The peptidase assays utilized were qualitative in nature and were designed to determine whether or not a cell extract possessed peptidase activity for a given peptide. The results obviously cannot answer the interesting question concerning the number and specificities of different peptidases possessed by *Neurospora*, although current studies in our laboratory have this objective.

Intracellular peptidase activity was found for all peptides tested (see Tables 2, 4, 6, and 7) except glycyl-L-histidine, L-lysyl-glycine, L-lysyl-L-leucine, L-leucyl-L-phenylalanine, L-histidyl-glycine, and L-histidyl-glycyl-glycine. These exceptions do not necessarily mean that no cellular peptidase activity exists for these peptides (note that *his-6* grows quite well on L-histidyl-glycine), but simply that no activity was detected under the conditions employed. Hydrolysis of nearly all dipeptides containing glycine, e.g., L-methionyl-glycine, glycyl-L-lysine, etc., was markedly slower than other peptides not containing glycine.

To detect possible extracellular peptidase activity, we examined the growth medium from which the cells had been removed by filtration. Assays of the concentrated and dialyzed media in which cells had grown did indeed reveal extracellular peptidase activity for the peptides L-methionyl-L-methionine, L-methionyl-L-alanyl-L-serine, L-leucyl-L-leucyl-L-leucine, L-leucyl-L-leucine, L-leucyl-glycyl-glycine, L-methionyl-glycyl-L-methionyl-L-methionine, and L-lysyl-L-lysyl-L-lysine, but not for the remainder of the peptides tested. This peptidase activity is present only in limited quantities as indicated by the necessity to concentrate the media 10- to 20-fold as well as to allow the enzyme assays to proceed for 6 to 8 h at 30 C. We do not know, as yet, whether the presence of peptidase activity in the media is due to a specific secretion of enzyme or to loss through cell death and subsequent cell lysis. However, failure to obtain extracellular peptidase activity toward a number of peptides for which there was considerable intracellular activity suggests that either specific secretion of particular peptidases occurs or that the peptidase(s) may have altered activities or stabilities in the different environment.

DISCUSSION

The results presented here suggest the *N. crassa* possesses a peptide transport system capable of handling tripeptides. Growth on 0.33

mM concentrations of dipeptides, in general, did not occur except in those instances where extracellular hydrolysis to amino acids occurred, or where the dipeptide seemed to be transported by an uptake system which is subject to inhibition by amino acids.

Intracellular peptidase activity shows specificity regarding the constituent amino acids and their position within the peptide. For example, wild-type cell extracts were quite active in hydrolyzing L-phenylalanyl-L-leucine, but were virtually inactive (under identical conditions) in hydrolyzing L-leucyl-L-phenylalanine. Similarly, wild-type cell extracts were able to hydrolyze glycyl-L-lysine, but not L-lysyl-glycine; L-leucyl-glycyl-glycine, but not L-histidyl-glycyl-glycine; and L-histidyl-L-leucine and L-phenylalanyl-L-leucine, but not L-lysyl-L-leucine. Similar specificities were reported for a proteolytic enzyme produced by the fungus *Endothia parasitica* in that it hydrolyzed L-leucyl-L-phenylalanine amide, but not L-phenylalanyl-L-leucine amide (24).

The general inability of cell extracts to hydrolyze (or hydrolyze poorly) peptides containing glycine, as opposed to peptides without glycine, further suggests that either separate peptidases exist for these two classes of peptides, or that a "single" peptidase occurs which exhibits a differential specificity. Patterson, Gatmaitan, and Hayman (17) reported that exceptionally lower rates of hydrolysis were observed with an ascites tumor dipeptidase toward N-terminal gly-dipeptides because of higher K_m values for these substrates.

The results obtained with *Neurospora* are similar to those obtained with yeast (1, 14) in that certain peptides could not be used for growth even though intracellular peptidase activity for these same peptides was present. However, *Neurospora* seems to release some peptidase activity into the medium, whereas yeast does not. The presence of extracellular peptidase activity in *Neurospora* for tri-L-lysine may be the reason for the differential growth response of the two microorganisms toward this peptide.

The inability of various auxotrophs to grow on certain peptides, while possessing intracellular peptidase activity capable of hydrolyzing those same peptides, tends to support the conclusion obtained in yeast (1) that, in general, peptidase activity is not extracellular nor located within the cell envelope (leading to peptide hydrolysis at the cell surface). Our results suggest that the ability to transport many of these peptides determines whether or not they can be utilized by *Neurospora*, since this organism does possess intracellular peptidase activity for nearly all of the peptides which were studied.

Perhaps one of the more interesting observations reported here is that the *his-6* auxotroph will grow on L-histidyl-L-leucine whereas the leucine mutants will not grow on this dipeptide. We think the explanation for this apparent contradiction, although complex, depends primarily upon the intracellular concentration of a particular amino acid which is required to support growth of an auxotroph. The *his-6* mutant grows maximally with an extracellular concentration of 0.33 mM L-histidine whereas *leu-3* requires a leucine concentration exceeding 1.5 mM to achieve maximal growth.

Thus, a small amount of uptake of L-histidyl-L-leucine, followed by its intracellular hydrolysis, may provide sufficient histidine for relatively good growth, and yet yield too little leucine to support growth of a strain requiring this amino acid. The leucine auxotrophs might be able to transport and metabolize leucine-containing dipeptides if they were provided at higher extracellular concentrations than employed in this study.

We initially suspected that the histidine-containing dipeptides were accumulated by the general amino acid transport system since growth of the *his-6* auxotroph on these dipeptides was inhibited by either phenylalanine or arginine. Magill et al. (11) had also earlier reported that both L-histidyl-glycine and glycyl-L-histidine inhibited histidine uptake by the general amino acid transport system. Since the *hgu-4* mutant prevented utilization of the histidine-containing dipeptides and also led to resistance to D-glutamate, an amino acid reported to be transported by the general system, it seemed possible that this mutant specifically affected the general amino acid transport system. However, we have been unable to attribute the almost 50% deficiency in transport of most amino acids by *hgu-4* to any one transport system (L. Wolfinbarger, unpublished data). Thus, we have concluded that the *hgu-4* mutant, in all probability, results in a general impairment in transport activity. Nevertheless, it still seems possible that the dipeptides may enter via the general amino acid transport system. One recent finding, however, which argues against this possibility is that the L-histidyl-glycine inhibition of L-phenylalanine transport via the general transport system (assayed in the doubly deficient transport mutant *pm-n;pm-b*) occurs in an "uncompetitive" rather than competitive manner.

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