JOURNAL OF BACTERIOLOGY, Mar. 1973, p. 1320-1325
Vol. 113, No. 3

Histidine Uptake in Mutant Strains of *Neurospora crassa* Via the General Transport System for Amino Acids

C. W. MAGILL, S. O. NELSON, S. M. D'AMBROSIO, AND G. I. GLOVER

Department of Plant Sciences, Genetics Section, and Department of Chemistry, Texas A&M University, College Station, Texas 77843

Received for publication 6 October 1972

A transport double mutant of *Neurospora crassa* has been isolated that has only one of the three transport systems capable of L-histidine uptake. The substrate specificity of the remaining transport system, termed the general transport system, has been fully characterized with regard to the contributions to binding of the side chain, the α-amino group, and the carboxylate group. The positively charged α-amino group is necessary for binding; the negatively charged carboxylate group is of less importance, since its replacement by a neutral carbonyl functional group does not completely abolish binding. The greatest structural latitude for binding was found in the side chain; affinity for α-amino acids was uniformly high except for L-aspartic and L-glutamic acids, L-asparagine, and L-proline. Thus, this transport system is "general" with these restrictions.

Amino acid uptake in *Neurospora crassa* has been shown to be a result of active transport by five systems (6, 10-14, 17). Previous investigators (5, 8, 9, 22) have shown that L-histidine uptake in *N. crassa* is mediated by three transport systems: the neutral, which transports neutral α-amino acids such as L-tryptophan and L-leucine; the basic, which transports L-lysine and L-arginine; and the general, which transports α-amino acids with a variety of side chains. Significant histidine uptake by the basic and neutral systems occurs only at millimolar concentrations of histidine, whereas the general permease operates at micromolar concentrations of histidine (8). Histidine, which has a pK_a of 6 for protonation of the imidazole ring, is uniquely suited for study of each of the transport systems, with the exception of the acidic (12) and the methionine (13) systems, which do not transport histidine.

The amino acid transport systems of *Neurospora* have been studied by kinetic analyses and by isolation of mutants with one of the transport systems altered or missing. Mutants lacking the neutral transport system have been described by Stadler (17), Wolfinbarger and DeBusk (21), and Magill et al. (8); Roess and DeBusk (14), Thwaites and Pendyala (18), Wolfinbarger and DeBusk (21), and Magill et al. (8) have characterized mutants lacking the basic transport system. Wiley and Matchett (20) studied the uptake of L-tryptophan by the neutral system showing inhibition of uptake only by those amino acids having neutral side chains. Pall (10) has defined the general permease by its ability to transport a wide range of natural amino acids with neutral, positively charged, and negatively charged side chains. Because of the complexity of studying transport in organisms possessing multiple transport systems for a given compound (even in the mutants described above), mutants were sought that lacked all but one of the transport systems for L-histidine. This paper describes the isolation of a mutant possessing only the general transport system for histidine and its substrate specificity. The mutant strain was compared to the strain *his-3*, which has neutral, basic, and general transport systems capable of transporting L-histidine.

MATERIALS AND METHODS

Chemicals. L-Amino acids were purchased from Nutritional Biochemicals Corp. β-2-Thienylalanine, L-1-methylhistidine, imidazole, 4-hydroxymethylimidazole, thiazolyl-DL-alanine, D-histidine, L-histidine hydroxamate, L-histidylglycine, histamine, histidinol, dihydrourocanic acid, glycyl-L-histidine,

urocanic acid, and β-alanine were the products of Sigma Chemical Co. The *N*-chloroacetyl amino acids, from Mann BioResearch Inc., were prepared free from the contaminating amino acid by passing aqueous solutions of the derivatives through a column of SE-Sephadex A-50, acid form, and lyophilizing the eluent. *N-acetyl*-L-histidine (1), L-histidine amide (7), L-histidine methyl ester (4), β-2-thienylalanine methyl ester (4), L-leucine chloromethyl ketone (2), and L-lysine chloromethyl ketone (15) were prepared as described in the references indicated.

Strains. The strains of *N. crassa* used were either the histidine auxotroph *his-3* (K57) obtained from D. G. Catcheside with normal amino acid transport or the transport double mutant *his-3, neu*^{a}, *bas*^{a}. The latter strain was isolated from a cross of *his-3, bas*^{a} by *his-3, neu*^{a}, which lack the basic and neutral amino acid transport systems, respectively (8). The transport double mutant progeny were selected by their inability to grow on L-histidine-supplemented Vogel's (19) minimal medium (10 mg/100 ml) to which either L-arginine or L-methionine (50 mg/100 ml) was added. L-Arginine competitively inhibits L-histidine uptake by the general and basic permeases, and excess methionine prevents histidine uptake by the general and neutral amino acid transport systems.

Methods. Details concerning the media used and measurement of the accumulation of ^{14}C-L-histidine by germinated conidia have been previously described (8). Briefly, conidia were germinated for 5 h in the presence of histidine and were transferred to minimal medium for 2 h before kinetic assays were made. In all cases, the pH of the medium was 5.9. The amount of ^{14}C-L-histidine (4 μCi/μmol) accumulated in 3 min by 8 × 10^6 conidia in a 4-ml suspension was determined by collecting the conidia on a membrane filter, washing, drying with a heat lamp, and counting with a Beckman liquid scintillation counter with Omnifluor (New England Nuclear Corp.) as the scintillator.

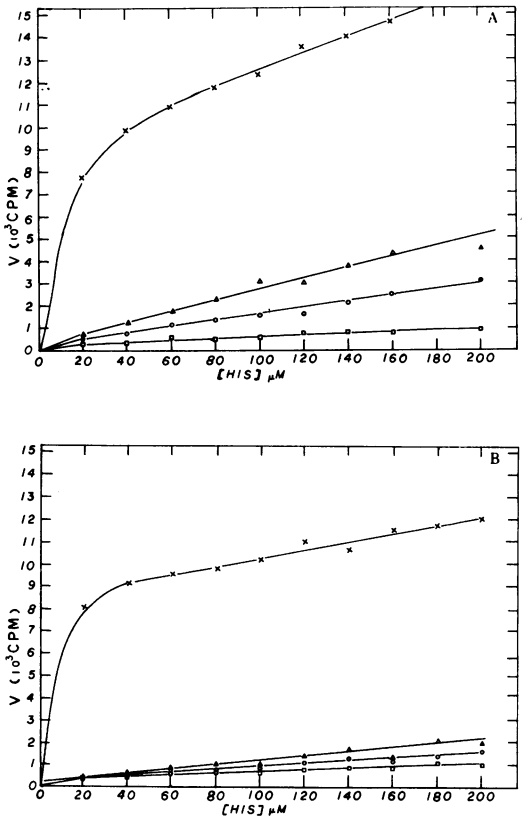

FIG. 1. *Initial velocity of histidine uptake by germinated conidia in the presence and absence of inhibitors. Velocity (V) is measured as counts/minute accumulated in 5 min by 8 × 10^6 conidia in a 4-ml suspension.* (×) *Histidine only (specific acitivity was 0.5 μCi/μmol);* (Δ) *histidine + 0.02 M arginine;* (○) *histidine + 0.02 M methionine;* (□) *histidine + 0.02 M arginine + 0.02 M methionine. A, his-3; B, his-3, neu*^{a}, *bas*^{a}.

RESULTS

The absence of the neutral and basic transport systems in the *his-3, neu*^{a}, *bas*^{a} double mutant strain was verified by measuring histidine accumulation in the presence and absence of other amino acids. Histidine uptake in *his-3, neu*^{a}, *bas*^{a} was inhibited to essentially the same extent by a basic (arginine) or a neutral (methionine) amino acid as by a combination of the two (Fig. 1B), whereas the *his-3* strain with normal transport alleles accumulated a significant concentration of histidine when only one of the two inhibitor amino acids was present (Fig. 1A).

The specificity of the general transport system was determined by inhibition of the uptake of 4 μM ^{14}C-L-histidine at several concentrations of selected L-α-amino acids, D-histidine, histidine analogues, β-alanine, carboxyl-modified amino acids. *N*-acyl amino acids, and simple derivatives of imidazole (Table 1). At this concentration, very little histidine uptake by the neutral or basic permeases was expected. This was confirmed by comparing uptake in the *his-3* strain to that in the *his-3, neu*^{a}, *bas*^{a} strain. No consistent differences were found for the two strains, indicating that only the general permease is being measured under these conditions in *his-3* as well as *his-3, bas*^{a}, *neu*^{a}. As the results for both strains were similar, they will be discussed together.

All α-amino acids with free amino and carboxyl groups were good inhibitors except L-aspartic acid, L-asparagine, and L-glutamic acid, which gave strong inhibition only at concentrations 100 to 1,000 times the concentration of ^{14}C-L-histidine. L-Proline gave essentially no inhibition at all concentrations stud-

TABLE 1. *Percent inhibition of the uptake of 4 μM L-histidine*

Inhibitor	*his-3*					*his-3, neu[a], bas**				
	2[a]	3	4	5	6	2	3	4	5	6
L-Lysine	—	99	92	53	15	—	100	93	60	26
L-Leucine	—	98	93	56	13	—	100	98	74	14
L-Valine	—	—	—	—	—	—	99	98	84	42
Glycine	—	—	—	—	—	—	97	79	29	7
L-Tyrosine	—	—	—	—	—	—	99	93	54	11
L-Serine	—	—	—	—	—	—	99	88	42	16
L-Cysteine	—	—	—	—	—	—	99	91	19	9
β-2-Thienylalanine	—	99	97	56	23	—	94	80	40	7
L-1-Methylhistidine	—	98	92	59	16	—	97	88	55	25
DL-Thiazolylalanine	—	96	85	36	12	—	92	91	63	46
L-Aspartic acid	98	51	12	4	0	—	72	51	—	—
L-Asparagine	—	93	67	21	4	—	—	—	—	—
L-Glutamic acid	—	71	24	8	8	—	—	—	—	—
D-Histidine	96	82	38	7	—	—	83	32	4	—
β-Alanine	—	88	59	16	4	—	—	—	—	—
L-Histidine amide	—	45	14	12	6	—	41	14	9	3
L-Histidine methyl ester	—	89	54	13	8	—	92	78	22	0
L-Histidine hydroxamate	81	22	4	0	—	94	51	11	11	—
L-Histidylglycine	84	46	—	—	—	87	53	32	22	—
Histamine	32	8	—	—	—	10	0	—	—	—
β-2-Thienylalanine methyl ester	—	52	10	18	1	—	68	19	5	3
L-Leucine chloromethyl ketone	—	93	57	21	12	—	88	48	29	25
L-Lysine chloromethyl ketone	—	—	—	—	—	—	21	7	5	0
N-acetyl histidine	16	0	0	0	—	8	0	2	1	—
N-chloroacetyl glycine	—	6	1	—	—	—	30	19	—	—
N-chloroacetyl L-leucine	—	11	9	—	—	—	28	28	—	—
N-chloroacetyl L-valine	—	14	9	—	—	—	26	31	—	—
N-chloroacetyl L-tyrosine	—	27	11	—	—	—	28	23	—	—
Urocanic acid	23	8	—	—	—	25	18	—	—	—
Dihydrourocanic acid	21	8	—	—	—	20	10	—	—	—
Glycyl-L-histidine	14	9	—	—	—	6	8	—	—	—
Histidinol	17	4	—	—	—	10	0	—	—	—
Imidazole	17	9	7	0	—	13	4	—	—	—
4-Hydroxymethylimidazole	—	—	—	—	—	7	2	—	—	—
L-Proline	—	—	—	—	—	—	3	5	2	—

* Molar concentration of inhibitor (−log).

ied. β-Alanine was about as effective as the acidic amino acids, which were moderate inhibitors. These results indicate a broad specificity for all side chains other than those of the acidic α-amino acids and their derivatives and the secondary α-amino acid, L-proline.

Results of modification of the amino or carboxyl groups were interesting. All *N*-acyl derivatives of α-amino acids were poor inhibitors, giving little inhibition even at the highest concentrations used. Complete removal of the amino group, as in the histidine analogues urocanic and dihydrourocanic acids, also resulted in poor inhibition.

Conversion of the carboxylate group of histidine and other amino acids to an uncharged amide or ester resulted in moderate inhibition that was characteristic of aspartic and glutamic acids, asparagine, and β-alanine. Inhibition by these compounds is markedly better than the poor inhibition shown by the amino-modified derivatives discussed above. The absence of a carbonyl-containing group in histidine analogues such as histidinol and histamine resulted in weak inhibition comparable to that shown by the amino-modified derivatives. A substantial modification of the carboxyl group of histidine, found in L-histidyl-glycine,

gave an inhibitor comparable to L-histidine amide, whereas L-histidine hydroxamate, which is relatively small in size and is negatively charged like the carboxylate group of histidine, was no more effective as an inhibitor. Simple imidazole derivatives lacking both a carboxyl and an amino group were poor inhibitors.

The apparent affinity constants (K_i) for selected inhibitors were determined from Dixon-type plots (3) with the use of 4, 2.4, and 1.6 μM ^{14}C-L-histidine (Table 2). The constants were calculated by solving for intersection of lines fitted to the data by the weighted least squares method (16; Figure 2). The constants shown are similar for both strains and indicate that the general permease is intact in the transport double mutant *his-3,neu^a,bas^a*. The magnitude of the constants is in agreement with the inhibition data summarized in Table 1.

DISCUSSION

Isolation of a mutant lacking the two specific transport systems yielded a direct method of studying transport of L-histidine via the general system without possible interference due to the neutral and basic systems. Our results showed that the general systems in *his-3* and *his-3,neu^a,bas^a* behaved essentially the same with regard to inhibition by a wide variety of α-amino acids, α-amino acid derivatives, and α-amino acid analogues. The general transport system was retained intact by the transport double mutant, a result which indicates that other transport double mutants retaining either the basic or neutral transport systems will be useful in studying the specificity of these two systems.

All naturally occurring L-α-amino acids studied, with normal positively charged amino and negatively charged carboxylate groups, other than L-proline, L-aspartic and L-glutamic acids, and L-asparagine, are good inhibitors of histidine transport, in agreement with the observations of Pall (10). The affinity of β-2-thienylalanine, L-1-methylhistidine, and DL-thiazolylalanine is comparable to that of the natural amino acids, indicating a broad specificity for neutral side chains.

TABLE 2. *Apparent affinity constants (K_i) for the general transport system*

Inhibitor	K_i (μM)	
	his-3	*his-3,neu^a, bas^a*
L-Leucine	9	5
L-Lysine	8	6
β-2-Thienylalanine	3	4
L-1-Methylhistidine	7	6
Thiasolyl-DL-alanine	26	—
L-Histidine methyl ester	67	54
D-Histidine	200	—
L-Histidine amide	1,400	—

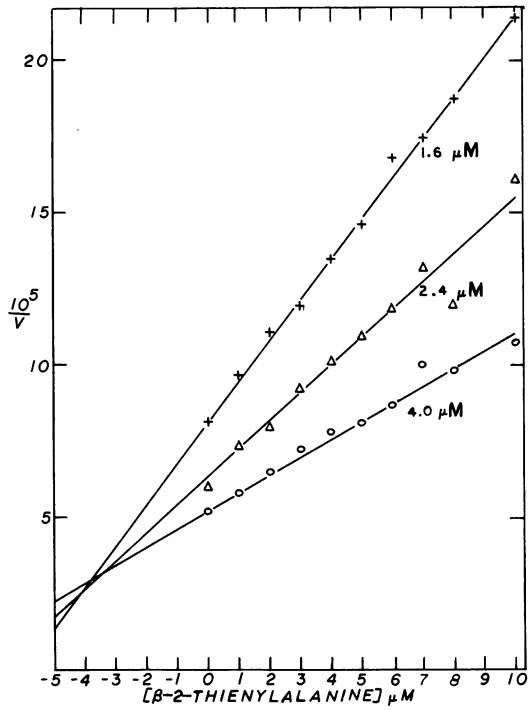

FIG. 2. *Inhibition of histidine uptake by β-2-thienylalanine at three concentrations of ^{14}C-L-histidine. Velocity (V) is measured as counts/minute accumulated in 3 min by 4 $\times$ 10^6 conidia in a 4-ml suspension. Symbols: +, 4.0 μM ^{14}C-L-histidine; Δ, 2.4 μM ^{14}C-L-histidine; O, 1.6 μM ^{14}C-L-histidine.*

The essentiality of the positively charged α-amino group in promoting strong binding was demonstrated by the fact that all *N*-acyl α-amino acids, including glycyl-L-histidine, were poor inhibitors of histidine uptake. The decreased binding is not due primarily to a steric effect (the *N*-acyl substituents are much bulkier than the free α-amino groups), since urocanic acid and dihydrourocanic acid, which are structural analogues of histidine lacking the α-amino group (they are less bulky than histidine at the α-position), are also poor inhibitors.

Modification of the carboxylate group of histidine and other α-amino acids resulted in moderate inhibitors about as effective as the acidic amino acids, L-asparagine, D-histidine,

and β-alanine. The apparent affinity constants for D-histidine and L-histidine methyl ester, for example, were 200 and 67 μM, respectively, compared to 9 and 8 μM for L-leucine and L-lysine, respectively (Table 2). The carboxylate-modified inhibitors were significantly better inhibitors than the amino-modified inhibitors, indicating that binding of the carboxylate group is not completely dependent on the negative charge. Complete removal of the carbonyl group, as in histamine, resulted in poor inhibition; thus, the significant binding generally observed in this group of derivatives is probably due to the fact that the carbonyl group can still serve as the acceptor of a hydrogen bond with a hydrogen bonding donor in the carboxylate binding site. In support of this hypothesis is the fact that L-histidine hydroxamate, which is negatively charged and not appreciably larger than other carboxylate-modified inhibitors, is not a better inhibitor. L-Histidylglycine shows moderate affinity for the transport system, indicating a rather large binding site for the carboxylate function, so that steric interference with binding cannot be the principal reason for reduced binding in this group.

The moderate binding of D-histidine may be a reflection of the apparent greater contribution to binding by the positively charged α-amino group and the negatively charged carboxylate group relative to the side chain, since the carboxyl group and the amino group could occupy the proper binding sites, whereas the side chain would not be in the proper position for normal binding in this enantiomer.

Whereas L-leucine and L-lysine show similar behavior as inhibitors of L-histidine uptake, L-leucine chloromethyl ketone is a much better inhibitor than L-lysine chloromethyl ketone. This may be due to the fact that the lengthy side chain of L-lysine must fold back on itself for good binding, placing the positively charged α- and ε-amino groups in close proximity. In lysine, the positive charge on the ε-amino group can be at least partially neutralized by the carboxylate group, but folding of the side chain of the corresponding chloromethyl ketone places the positive charges of the two amino groups in close proximity with no significant stabilization by the neutral ketone carbonyl group.

Although the general transport system has a broad specificity for neutral, hydrophilic or hydrophobic, and for positively charged side chains, the acidic amino aicds, L-aspartic acid and L-glutamic acid, are only moderate inhibitors. Pall (10) has suggested that transport of these amino acids may be due to a high affinity for the small amount of undissociated acids at pH 5.9. However, L-asparagine is also a moderate inhibitor and has an uncharged form of the side chain carboxyl group. An explanation consistent with the observations is that the side chains of aspartic and glutamic acids are folded so that the carboxylate groups form hydrogen bonds with the α-amino group. This would greatly decrease the positive charge on the α-amino groups of these acidic amino acids, resulting in the decreased affinity observed, since it has already been determined that the positive charge is essential for binding. A second consideration is that the folded, hydrogen-bonded conformations of these amino acids resemble the structure of L-proline sufficiently that binding is also reduced. L-Proline showed essentially no inhibition at all concentrations studied, indicating that conformations of other amino acids similar in structure to this amino acid would show decreased binding. L-Asparagine can also fold and form a hydrogen bond with the α-amino group, reducing the positive charge and adopting an L-proline-like conformation to give the moderate binding observed.

As expected, in view of the above discussion, imidazole and its derivatives which lack both the α-amino and α-carboxylate groups are poor inhibitors (Table 1).

The general permease has high affinity for primary L-α-amino acids with neutral, hydrophobic or hydrophilic, and positively charged side chains but not for the acidic amino acids or their derivatives. This generalization also applies to analogues of natural L-α-amino acids such as thiazolyl-DL-alanine and β-2-thienylalanine. The positively charged α-amino group is essential for binding, whereas the carboxylate group can be modified to an uncharged amide, ester, or chloromethyl ketone with only moderate decreases in binding. Absence of the carbonyl group in any form reduces binding to that observed by loss of the α-amino group.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation grant GB-34245, The Research Corporation, and the R. A. Welch Foundation. One of us (S.O.N.) is an R. A. Welch Foundation Predoctoral Fellow.

LITERATURE CITED

1. Bergman, M., and L. Zervas. 1928. Uber katalische racemization von aminosauren und peptiden. Biochem. Z. **205**:280.
2. Birch, P. L., H. A. El-obeid, and M. Akhtar. 1972. The preparation of chloromethylketone analogues of amino acids: Inhibition of leucine aminopeptidase. Arch.

Biochem. Biophys. **148**:447-451.
3. Dixon, M., and E. C. Webb. 1964. Enzymes, 2nd ed. Academic Press Inc., New York.
4. Greenstein, J. P., and M. Winitz. 1961. Chemistry of the amino acids, vol. 2. John Wiley & Sons, Inc., New York.
5. Haas, F., M. B. Mitchell, B. N. Ames, and H. K. Mitchell. 1952. A series of histidineless mutants of *Neurospora crassa* whose growth is inhibited by a combination of neutral and basic amino acids. Genetics **37**:217-226.
6. Lester, B. 1966. Genetic control of amino acid permeability in *Neurospora crassa*. J. Bacteriol. **91**:677-684.
7. Levington, L., V. E. Price, and J. P. Greenstein. 1950. A resolution of histidine, cystine, and alanine by asymmetric enzymatic hydrolysis of the racemic amides. J. Biol. Chem. **184**:55.
8. Magill, C. W., H. Sweeney, and V. W. Woodward. 1972. Histidine uptake in strains of *Neurospora crassa* with normal and mutant transport systems. J. Bacteriol. **110**:313-320.
9. Mathieson, M. J., and D. G. Catcheside. 1955. Inhibition of histidine uptake in *Neurospora crassa*. J. Gen. Microbiol. **13**:72-83.
10. Pall, M. L. 1969. Amino acid transport in *Neurospora crassa*. I. Properties of two amino acid transport systems. Biochim. Biophys. Acta **173**:113-127.
11. Pall, M. L. 1970. Amino acid transport in *Neurospora crassa*. II. Properties of a basic amino acid transport system. Biochim. Biophys. Acta **203**:139-149.
12. Pall, M. L. 1970. Amino acid transport in *Neurospora crassa*. III. Acidic amino acid transport. Biochim. Biophys. Acta **211**:513-520.
13. Pall, M. L. 1970. Amino acid transport in *Neurospora crassa*. IV. Properties and regulation of a methionine transport system. Biochim. Biophys. Acta **233**:201-214.
14. Roess, W. B., and A. G. DeBusk. 1968. Properties of a basic amino acid permease in *Neurospora crassa*. J. Gen. Microbiol. **52**:421-432.
15. Shaw, E., and G. Glover. 1970. Further observations on substrate derived chloromethyl ketones that inactivate trypsin. Arch. Biochim. Biophys. **139**:298-305.
16. Smith, E. D., and D. M. Mathews. 1967. Least square regression lines: calculations assuming a constant per cent error. J. Chem. Educ. **44**:757.
17. Stadler, D. R. 1966. Genetic control of the uptake of amino acids in *Neurospora*. Genetics **54**:677-685.
18. Thwaites, W. M., and L. Pendyala. 1969. Regulation of amino acid assimilation in a strain of *Neurospora crassa* lacking basic amino acid transport. Biochim. Biophys. Acta **192**:455-461.
19. Vogel, H. J. 1964. Distribution of lysine among fungi: evolutionary implications. Amer. Natur. **98**:435-466.
20. Wiley, W. R., and W. H. Matchett. 1966. Tryptophan transport in *Neurospora crassa*. I. Specificity and kinetics. J. Bacteriol. **92**:1698-1705.
21. Wolfinbarger, L., Jr., and A. G. DeBusk. 1971. Molecular transport: in vivo studies of transport mutants of *Neurospora crassa* with altered amino acid competition patterns. Arch. Biochem. Biophys. **144**:503-511.
22. Woodward, C. K., C. P. Read, and V. W. Woodward. 1967. *Neurospora* mutants defective in their transport of amino acids. Genetics **56**:598.