BBA 75718

ACTIVE TRANSPORT OF L-ASPARTIC ACID IN *NEUROSPORA CRASSA*

L. WOLFINBARGER, JR., H. H. JERVIS AND A. GIB DEBUSK

Genetics Group, Department of Biological Sciences, Florida State University, Tallahassee, Fla. 32306 (U.S.A.)

(Received May 3rd, 1971)

SUMMARY

Transport of L-aspartic acid in *Neurospora crassa* conidia is shown to be mediated by neutral and general amino acid transport systems. The transport activity is dependent on pH and results in accumulation of L-aspartic acid against a gradient. Mutants deficient in transport of L-aspartic acid are described.

INTRODUCTION

The presence of three genetically distinct amino acid transport systems in *Neurospora crassa* conidia has been previously described[1]. The neutral amino acid transport system transports neutral amino acids. The basic amino acid transport system transports basic amino acids. The general amino acid transport system is less specific, transporting both neutral and basic amino acids. These earlier studies were undertaken at pH 5.8 and failed to demonstrate appreciable transport activity for L-aspartic acid or competition by L-aspartic acid for L-arginine or L-phenylalanine transport. Since the existence of an aspartic acid transport system has been postulated[2] and mutants (*nap*[3] and *un-t*[4]) have been isolated and reported to be deficient in transport of neutral and acidic amino acids, further characterization of this apparent lack of L-aspartic acid transport was undertaken.

METHODS AND MATERIALS

Strains utilized

The amino acid transport characteristics (at pH 5.8) of the strains employed in this study have been previously reported[1]. At pH 5.8 the *Pm*$^{-}$*N*^{22} and *Pm*$^{-}$*NB* mutants are reduced, as compared to the Tatum a wild type, approximately 75 % in transport of the neutral amino acids, *i.e.* L-phenylalanine, L-leucine, *etc.* Transport of L-arginine by *Pm*$^{-}$*N*^{22} is normal. The *Pm*$^{-}$*B*^{37} and *Pm*$^{-}$*NB* mutants are reduced, again compared to the wild type, approximately 50 % in transport of the basic amino acids, *i.e.* L-arginine or L-lysine. Transport of L-phenylalanine by *Pm*$^{-}$*B*^{37} is normal. The *Pm*$^{-}$*NB* strain is a double transport deficient mutant obtained by crossing *Pm*$^{-}$*N*^{22} and *Pm*$^{-}$*B*^{37} phenotypes.

Preparation of cells for transport studies

The preparation of cells utilized in the transport studies has been described[1].

Measurement of amino acid transport

The basic incubation mixture contained the following components per 50 ml: 5 ml 10× VOGEL's[5] salts (adjusted to desired pH), radioactive amino acid (0.01 μC/0.1 μmole per ml, final concentration), conidia (0.1 mg dry wt per ml) and water to volume. The uptake was initiated by addition of an appropriate volume of conidia to the reaction mixture shaking at 25°.

5-ml samples were removed at intervals with a Cornwall syringe, immediately filtered on membrane filters (Millipore, Type AA, 0.8 μm, 25 mm diameter), washed with 3 vol. cold water, and glued to planchets for counting on a low beta thin-window gas flow proportional counter (Beckman).

The various pH values utilized in this study were obtained by substituting citric acid for sodium citrate or K_2HPO_4 for KH_2PO_4 in the basic VOGEL's salts buffer and adjusting to the desired pH with HCl or KOH.

The L-aspartic acid transport velocities in Figs. 1 and 3 were calculated from short-term (< 16 min) uptakes. The uptake of L-aspartic acid remains linear for up to 40 min with points taken after zero, 4, 8, 16, 20 and 40 min of transport (L. (WOLFINBARGER, unpublished data).

RESULTS

Titration studies with aspartic acid reveal that it is predominately a neutral molecule from pH 1.9 to 3.7 and an anionic molecule from pH 3.7 to 9.6. Conidial transport of L-aspartic acid as a function of pH is represented in Fig. 1. Employing a wild type standard (Tatum a, $SY4f_8a$) and the three previously characterized transport deficient mutants (*Pm⁻N*[22], *Pm⁻B*[37], and *Pm⁻NB*), the following results

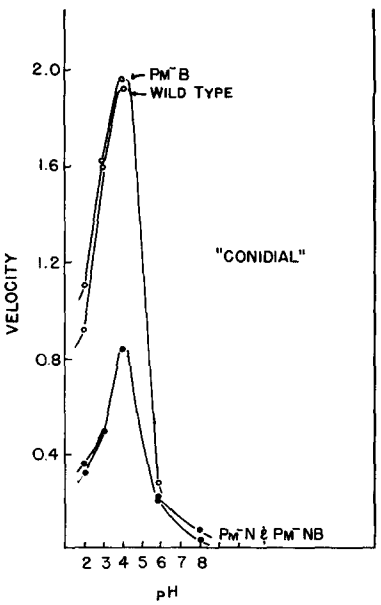

Fig. 1. Conidial L-aspartic acid transport as a function of pH. The transport velocity indicated is μmoles × 10^{-3}/mg dry wt. conidia per min. The external concentration of L-aspartic acid is 0.1 mM with a specific activity of 0.01 μC/0.1 μmole per ml.

were obtained: (1) L-aspartic acid (0.1 mM) transport is optimal at pH 3.7–4.0 in wild type and the mutants. (2) The total L-aspartic acid transport activity at pH 4.0, as compared to that at pH 5.8, increases over 6-fold in wild type and $Pm^{-}B^{37}$ and over 4-fold in $Pm^{-}N^{22}$ and $Pm^{-}NB$. (3) At all pH values tested, $Pm^{-}N^{22}$ and $Pm^{-}NB$, but not $Pm^{-}B^{37}$, are reduced (*i.e.* 60 % at pH 4.0) in L-aspartic acid transport. Since the *Pm N* locus has been implicated in transport of neutral aliphatic and aromatic, but not basic, amino acids[1], it must be assumed that L-aspartic acid is transported by the neutral transport system. This transport activity of the neutral system for an acidic amino acid is pH dependent and explains the low transport capacity for L-aspartic acid observed in the earlier studies performed at pH 5.8.

Since L-aspartic acid is a highly metabolizable molecule, it is not possible, *a priori*, to assume that this observed transport activity is "active". For example, rapid accumulation of label could occur through metabolic drag. Recovery of sufficient radioactivity as L-aspartic acid would indicate the ability of wild type conidia to concentrate this amino acid against a gradient.

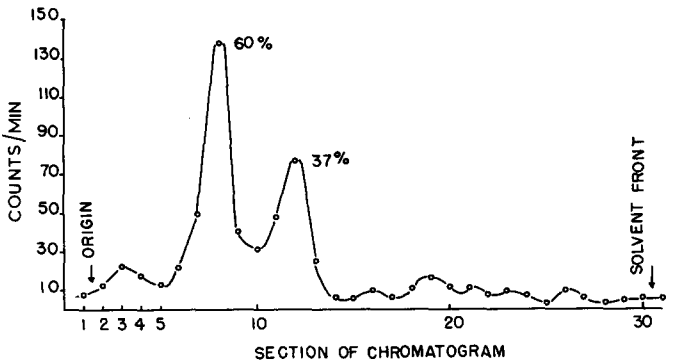

Fig. 2. Extraction and chromatography of accumulated L-aspartic acid. Following a 60-min incubation in 0.1 mM L-[^{14}C]aspartic acid at pH 4.0, the wild type conidia were filtered, washed and resuspended in boiling water for 30 min. The millipore filtered extract was spotted onto Whatman chromatographic paper and developed utilizing water saturated phenol. The developed chromatogram was cut into 0.5 cm × 1.0 cm sections, glued to planchets and counted in a Beckman gas-flow counter.

Extraction and chromatography of L-[^{14}C]aspartic acid accumulated by the wild type, as described in the legend to Fig. 2, revealed the presence of two peaks of radioactivity. Peak I, constituting approximately 60 % of the total label extracted, corresponded (chromatographically) to aspartic acid while Peak II, constituting approximately 37 % of the total label, was chromatographically similar to serine (Fig. 2). Calculation of the concentration of L-aspartic acid inside the cell was based on a conidial wet cell volume of 5.3 μl per 10 mg dry wt. of cells[6]. Comparison of counts attributable to [^{14}C]aspartic acid on a volume to volume basis (inside to outside the cell) indicated a 180-fold concentration of "free" (hot water extractable) aspartic acid inside the conidia.

The demonstration of saturation kinetics for L-aspartic acid transport has been more difficult than for other amino acids. At pH 4.0 there exist two molecular species of aspartic acid, a neutral and anionic species. By shifting to either pH 2.8 or 5.0, *i.e.* where aspartic acid exists predominately as either a neutral or anionic species,

respectively, half-maximal transport velocities may be obtained with 10^{-3} M concentrations of aspartic acid. Although saturation of transport can be demonstrated, the kinetics of L-aspartic acid transport as a function of pH are by no means simple. Such kinetics will be the subject of a future and more extensive study.

Because previous transport studies with the acidic amino acids have been carried out in the mycelial stage of fungal development, the pH optimum for post-conidial[7] L-aspartic acid transport has been determined. The post-conidial transport activity was measured following 3 h incubation at 25° in VOGEL's[5] salts (pH 5.8) *plus* 1 % glucose. Studies in the mycelial stage have been avoided because of the difficulty in obtaining representative mycelial samples.

Fig. 3 shows that in the post-conidial transport stage: (1) L-aspartic acid is optimally transported at pH 3.7–4.0. (2) Total transport activity increases over 4-fold in wild type and *Pm⁻B*[37] and over 6-fold in *Pm⁻N*[22] and *Pm⁻NB* at pH 4.0 as compared to transport in conidia at this pH. This increase in transport activity is consistent with a development of post-conidial transport activity[7]. (3) The mutants *Pm⁻N*[22] and *Pm⁻NB*, but not *Pm⁻B*[37], are reduced, as compared to the wild type, in L-aspartic acid transport.

In order to determine whether the residual L-aspartic acid transport remaining in the *Pm⁻N*[22] and *Pm⁻NB* mutants is due to the previously described[1] general transport system or a yet uncharacterized acidic amino acid transport system, competition experiments utilizing L-arginine or L-phenylalanine as competing amino acids were performed. Transport of L-phenylalanine and L-arginine in wild type and

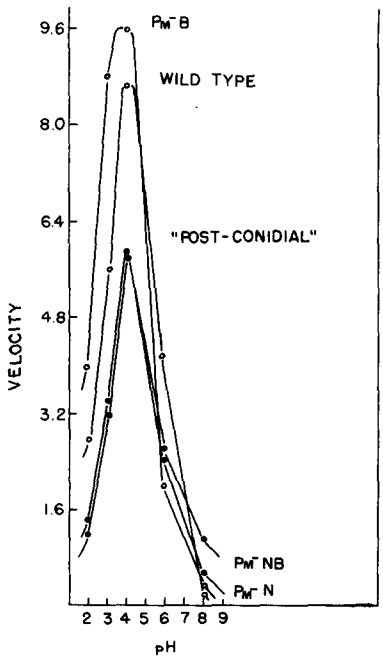

Fig. 3. Post-conidial L-aspartic acid transport as a function of pH. Conditions as described in the legend for Fig. 1 except for development of cells to a post-conidial transport activity as described in the text.

TABLE I

AMINO ACID COMPETITION FOR TRANSPORT AT pH 4.0 BY CONIDIAL AND POST-CONIDIAL TRANSPORT ACTIVITY

Values represent counts/min per mg dry wt. conidia after 40 min of [^{14}C]amino acid transport with or without unlabeled competing amino acid. The labeled amino acid is at an external concentration of 0.1 mM. Values are the mean of five experimental values.

Unlabeled amino acid added (1.0 mM)	*Conidial transport activity*					
	L-*Arg transport*		L-*Phe transport*		L-*Asp transport*	
	Wild type	*Pm⁻ NB*	*Wild type*	*Pm⁻ NB*	*Wild type*	*Pm⁻ NB*
None	1426 ± 26	710 ± 24	2680 ± 121	660 ± 14	1038 ± 89	402 ± 20
L-Asp	1178 ± 214	368 ± 14	2002 ± 76	490 ± 14	—	—
L-Arg	—	—	2586 ± 108	280 ± 13	472 ± 31	109 ± 7
L-Phe	844 ± 19	112 ± 25	—	—	139 ± 5	118 ± 7
	Post-conidial transport activity					
None	8997 ± 185	9179 ± 234	12 956 ± 1360	6043 ± 840	6932 ± 114	4616 ± 132
L-Asp	5801 ± 208	3563 ± 165	10 084 ± 606	3298 ± 39	—	—
L-Arg	—	—	11 249 ± 420	1797 ± 380	3472 ± 51	996 ± 26
L-Phe	4966 ± 178	523 ± 34	—	—	408 ± 24	610 ± 24

the three mutants remains relatively constant from pH values of 3.5 to 6.0 (WOLFINBARGER, unpublished data).

It was previously shown that at pH 5.8 L-aspartic acid does not compete with L-arginine or L-phenylalanine for transport. However, at pH 4.0 L-phenylalanine reduces conidial L-aspartic acid transport in wild type and in *Pm⁻NB* by 87 % and 70 %, respectively (Table I). Analogous experiments utilizing 10 × L-arginine as the competing amino acid show a reduction in L-aspartic acid transport in wild type and in *Pm⁻NB* of 55 % and 73 %, respectively. Further, L-aspartic acid reduces L-arginine and L-phenylalanine transport in both the wild type and the *Pm⁻NB* mutant.

Examining the competition patterns of post-conidial transport activity (at pH 4.0) one finds competition for transport similar to that observed for conidial transport activity. L-Phenylalanine reduces post-conidial L-aspartic acid transport in wild type and in *Pm⁻NB* by 94 % and 87 %, respectively. Analogous experiments with 10× L-arginine as the competing amino acid show a reduction in L-aspartic acid transport in wild type and in *Pm⁻NB* of 50 % and 79 %, respectively (Table I). These competition patterns (and the L-aspartic acid transport studies described above) suggest that at pH 4.0 L-aspartic acid and L-phenylalanine, but not L-arginine, are transported by the neutral transport system, while L-aspartic acid, L-phenylalanine, and L-arginine are transported by the general transport system. The basic transport system does not transport L-aspartic acid at any pH value tested.

It should be noted that the *Pm⁻NB* mutant is not reduced (as compared to the wild type) in post-conidial transport of L-arginine. THWAITES AND PENDYALA[8] reported a similar masking of transport deficiency in their *bat* mutant. They proposed that development of a general transport system, in the absence of the *arg*-12^{s} locus, restored L-arginine transport capabilities of a mutant lacking a basic amino acid transport system. Our observations support their hypothesis. That the "restored"

transport activity is due to a general transport system is shown by the 94 % reduction of L-arginine transport in *Pm⁻NB* by a 10× concentration of L-phenylalanine. L-arginine transport in the wild type is reduced only 45 % by a 10× concentration of L-phenylalanine (Table I). This latter observation suggests some interrelationship(s) between the basic and general transport systems during development.

DISCUSSION

The acidic amino acid transport system reported in Neurospora mycelia by PALL[2] is not present in Neurospora conidia. It is proposed that in conidia (and non-germinated conidia), L-aspartic acid is transported by the neutral and general amino acid transport systems. Thus, any study of L-aspartic acid transport in Neurospora must account for possible transport of this amino acid by a minimum of two and a maximum of three active transport systems.

These findings raise the important question of the interrelationship(s) between the *nap*, the *un-t* and the *Pm⁻N* loci. These three loci have been mapped in different linkage groups (*nap* in V, *un-t* in I and *Pm N* in IV), yet they all appear to be involved in neutral and acidic amino acid transport. If, as suggested for the basic transport system[9], some component of the neutral system is a glycoprotein, these loci could represent structural or assembly, *i.e.* glycosylation, mutations. Or, an alternative hypothesis could be one of subunits assembled into a larger permease molecule.

The developmental relationships of the different transport systems or indeed even the transport systems themselves are far from being resolved. Neurospora does not grow exclusively at pH 5.8 (or even at 25°). Therefore, specificity ascribed to transport systems must remain flexible. A transport system present in conidia could well be modified by developmental changes such that it would then transport an entirely different group of amino acids or metabolites.

ACKNOWLEDGMENTS

This work was supported in part by a National Institutes of Health Public Health Service Traineeship in Genetics (2 To1 GM01316–06).

REFERENCES

1 L. WOLFINBARGER, JR. AND A. G. DEBUSK, *Arch. Biochem. Biophys.*, 144 (1971) 503.
2 M. L. PALL, *Biochim. Biophys. Acta*, 211 (1970) 513.
3 E. S. JACOBSON AND R. L. METZENBERG, *Biochim. Biophys. Acta*, 156 (1968) 140.
4 M. S. KAPPY AND R. L. METZENBERG, *J. Bacteriol.*, 94 (1967) 1629.
5 H. J. VOGEL, *Microbial Genet. Bull.*, 13 (1956) 42.
6 W. B. ROESS, Ph.D. Thesis, Florida State University, 1966.
7 J. H. TISDALE AND A. G. DEBUSK, *J. Bacteriol.*, 104 (1970) 689.
8 W. M. THWAITES AND L. PENDYALA, *Biochim. Biophys. Acta*, 192 (1969) 455.
9 W. D. STUART AND A. G. DEBUSK, *Arch. Biochem. Biophys.*, 144 (1971) 512.