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ACIDIC AMINO ACID TRANSPORT IN *NEUROSPORA CRASSA* MYCELIA

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SUMMARY

1. L-Aspartate transport activity was optimally induced following 6 h growth on acetate minimal media.

2. The observed aspartate (and glutamate) transport activity was attributed to the general amino acid transport system since L-phenylalanine, but not L-cysteic acid, inhibited transport activity at all pH values tested.

3. Changes in K_m values for L-aspartate and L-glutamate transport following growth on acetate minimal media indicated that apparent "affinities" of the "acidic amino acid transport systems" dramatically increased during the stages of active growth.

INTRODUCTION

The transport of acidic amino acids into *Neurospora crassa* has been reported to occur *via* the following three systems: a system specific for acidic amino acid[1], a neutral[2], and a general amino acid transport system[2]. The latter two systems have been reported to transport the zwitterion species of L-aspartate and L-glutamate[3] (low pH) as opposed to the anionically charged species (high pH) which the system described by Pall[1] is presumed to transport.

The studies described above were undertaken in either conidia or starved mycelia with the result that little is known of acidic amino acid transport in actively growing mycelia. Knowledge of transport activity in this stage of the cell's cycle is particularly important since the acidic amino acid transport system described by Pall in starved mycelia is presumed to be absent in conidia and one (or both) of the systems described by Wolfinbarger *et al.*[2], in conidia may be absent in starved mycelia. Thus, it becomes apparent that a transition in systems available for acidic amino acid transport must occur somewhere between the conidial and starved (stationary) mycelial stages. Further, Wolfinbarger and Kay[4] have recently described a dicarboxylic acid transport system in actively growing mycelia of *Neurospora* which reportedly recognizes L-aspartate as a substrate molecule.

This study was undertaken in an attempt to resolve the available acidic amino acid transport activity in actively growing mycelia of *Neurospora*.

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METHODS AND MATERIALS

Strains employed

The wild type (SY $4f_8a$) and mutant strains used in this study have been previously described[2,3,5]. To facilitate manipulation by the Stock Collection centers the designations of the mutants *Pm⁻N*[22], *Pm⁻B*[37] and *Pm⁻NB* have been changed to *pm−n*, *pm−b* and *pm−nb* (*pm−n*; *pm−b*), respectively. Briefly, the *pm−n* and *pm−nb*, but not the *pm−b*, mutants are reduced in conidial transport activity for neutral amino acids at pH 5.8 and for L-aspartate and L-glutamate at pH values between 2.4 and 5.8.

Maintenance of stocks has been previously described[2].

Preparation of cells for transport studies

The techniques for growth and storage of conidia used in these studies have been described elsewhere[2]. Briefly, conidia were obtained from aerial hyphae following growth for 7 days, in the light at room temperature, on Vogel's minimal media N (ref. 6) supplemented with 2% sucrose and 2% agar.

For uptake studies the conidia were germinated for varying lengths of time, at 30 °C, in Vogel's minimal media N supplemented with either 20 mM potassium acetate or 2% sucrose. The original conidial inoculum was sufficient to give 0.1 mg dry wt conidia per ml of media. The resultant cell suspensions were harvested and stored as described[4].

Subsequent transport sudies were also as previously described[4]. Under all conditions of pH, substrate concentration, *etc.*, uptake remained linear for at least 8 min.

Chemicals

The non-labelled amino acids were obtained from either Sigma or Aldrich. L-[U-^{14}C]Aspartate and L-[U-^{14}C]glutamate were purchased from Amersham/Searle.

RESULTS

Induction of L-*aspartate transport*

Previous studies of L-aspartate transport activity in *Neurospora* have been performed in either conidia[2,3], 3-h germinated conidia[3] or carbon/nitrogen-starved mycelia[1]. It was therefore decided to examine L-aspartate transport during conidial germination and outgrowth. Fig. 1 shows that L-aspartate transport activity in wild type cells (at pH 3.8) reached an optimum at approximately 6 h and declined thereafter (up to 10 h). Growth of wild type cells with acetate or sucrose as the sole carbon source resulted in similar patterns of transport activity. However, the transport rate of cells grown on acetate minimal media were higher than when cells were grown on sucrose minimal media. The reported induction of succinate transport in acetate grown cells occurs only after 9 h of growth[4]. Transport assays for growth times longer than 10 h were not performed because of the difficulty in obtaining homogeneous cell samples. Also, growth of cells on acetate minimal media was chosen for future assays because of cleaner mycelial samples, greater transport rates, and the possibility of comparing amino acid transport to C_4-dicarboxylic acid transport.

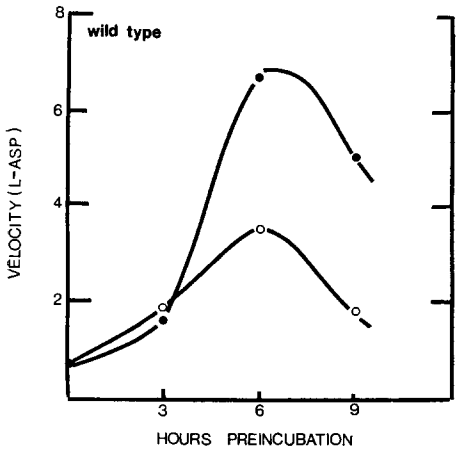

Fig. 1. Velocity (nmoles/min per mg dry wt cells) of L-aspartate (0.1 mM) transport by wild type as a function of time of preincubation. Cells grown in acetate minimal media (●—●), cells grown in sucrose minimal media (○—○). The L-aspartate transport was assayed at pH 3.8.

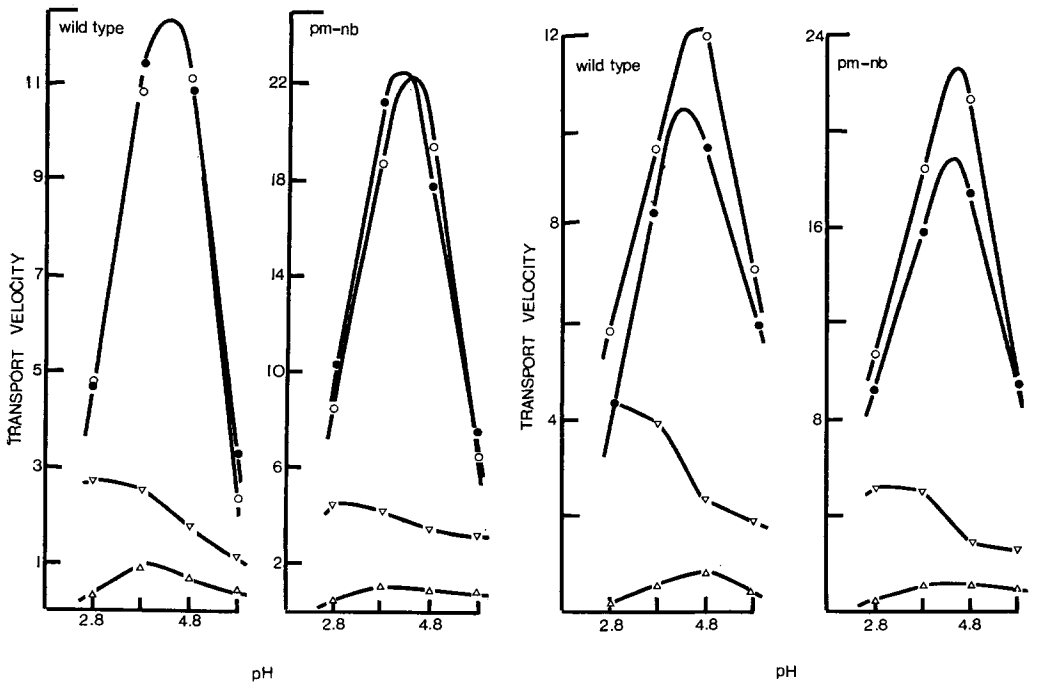

Fig. 2. Velocity (nmoles/min per 2 mg dry wt cells) of L-aspartate (0.1 mM) transport by wild type and *pm-nb* as a function of pH in the presence and absence of various inhibitors: control (●—●), with 1 mM L-cysteic acid (○—○), with 1 mM L-arginine (▽—▽), with 1 mM L-phenylalanine (△—△). Cells grown 9–10 h in acetate minimal media (see text) prior to transport assays.

Fig. 3. Velocity (nmoles/min per 2 mg dry wt cells) of L-glutamate (0.1 mM) transport by wild type and *pm-nb* as a function of pH in the presence and absence of various inhibitors: control (●—●), with 1 mM L-cysteic acid (○—○), with 1 mM L-arginine (▽—▽), with 1 mM L-phenylalanine (△—△). Cells grown 9–10 h in acetate minimal media (see text) prior to transport assays.

Transport as a function of pH

Previous studies[2,3] disclosed that optimal transport of aspartate and glutamate in *Neurospora* conidia occurred at low pH values. Figs 2 and 3 show that low pH values are also optimal for acidic amino acid transport in cells grown 9–10 h on acetate minimal media. As found with conidia[7], the optimal pH (pH 4.5) for glutamate transport in acetate grown cells was slightly higher than the optimal pH (pH 4.0) for aspartate transport (Figs 2 and 3).

Competitive inhibition of aspartate transport

The acidic amino acid transport system of *Neurospora* (described by Pall[1]) transported L-cysteic acid, L-glutamate, and L- and D-aspartate. If this system were responsible for the transport activity described here, L-cysteic acid (in 10-fold greater concentrations) should dramatically (greater than 90%) reduce transport of L-aspartate and L-glutamate. Figs 2 and 3 reveal that L-cysteic acid did not in fact significantly reduce the transport of L-aspartate or L-glutamate at any of the pH values tested. This absence of inhibition was particularly evident at pH 5.8 where transport of the negatively charged species of aspartate and glutamate would have predominated. In fact, L-cysteic slightly stimulated the rate of glutamate transport at most pH values tested. The reason for this stimulation is unknown as yet.

If the acidic amino acid transport activity being studied were due to, or had similar substrate specificities to, the systems previously described by Wolfinbarger *et al.*[2], L-phenylalanine should competitively inhibit aspartate and glutamate transport activity. A drastic reduction in acidic amino acid transport by a 10-fold greater concentration of L-phenylalanine was in fact demonstrated (Figs 2 and 3). At all pH values tested, L-phenylalanine reduced L-aspartate and/or L-glutamate transport by more than 90%.

Since previous studies have shown that arginine acts as a competitive inhibitor of the general amino acid transport system, the effect(s) of arginine on acidic amino acid transport was examined in the acetate grown cells. Referal again to Figs 2 and 3 shows that L-arginine also inhibits the uptake of both aspartate and glutamate, although not as effectively as does phenylalanine. Arginine appears to be a more effective inhibitor at the higher pH values.

Transport kinetics

The kinetics of acidic amino acid transport as a function of pH were examined to determine whether or not the zwitterion species, as opposed to the anionic species, of aspartate and glutamate are the substrates of the observed transport activity in acetate grown cells. The data presented in Table I reveal that the K_m values for wild type and the *pm−nb* double mutant are quite similar and increase with increasing pH, while the V values are consistently higher (about 2-fold) in the mutant compared with the wild type. (We have repeatedly observed that the *pm−nb* double mutant, but not the single mutants *pm−n* and *pm−b*, is higher in transport of most substrates, *e.g.* succinate, fumarate, malate, tested. The reason for this elevated transport activity is not clear, but may be due to the altered character of the "membrane" of this mutant as noted by Travis *et al.*[8]. They reported that the *pm−nb* double mutant was more easily disrupted by high concentrations of KCl than either the wild type or single transport mutants.)

TABLE I

KINETIC CONSTANTS FOR ACIDIC AMINO ACID TRANSPORT BY WILD TYPE AND *pm-nb* AS A FUNCTION OF pH

The cells have been grown for 9–10 h on acetate minimal media as indicated in the text prior to transport analysis.

Amino acid	*K_m values (mM)*					*V values (nmoles/min per mg cells)*				
pH:	*2.8*	*3.8*	*4.8*	*5.8*	*6.8*	*2.8*	*3.8*	*4.8*	*5.8*	*6.8*
Wild type										
L-Glutamate	0.05	0.03	0.03	0.20	0.60	3.25	8.50	11.6	12.0	9.00
L-Aspartate	0.06	0.07	0.06	0.50	—	7.70	11.4	10.0	9.00	—
pm-nb										
L-Glutamate	0.05	0.04	0.05	0.10	0.40	7.00	15.0	19.0	15.0	11.0
L-Aspartate	0.04	0.01	0.02	0.50	—	8.80	25.0	17.0	17.0	—

The similarity of kinetic constants in the wild type and *pm−nb* double mutant would seem to suggest a single and common transport system for acidic amino acid transport in acetate grown cells. Since the *pm−nb* double mutant is deficient in the neutral amino acid transport system, it might be suggested that in acetate-grown cells the transport activity of the general amino acid transport system must develop to such an extent (in both the wild type and *pm−nb* double mutant) that the absence of the neutral amino acid transport system is negligible. If a single transport system is responsible for the uptake of the acidic amino acids in acetate-grown cells, one would predict that a Lineweaver–Burk plot of aspartate transport in wild type would be linear. Indeed, contrary to the situation found in conidia[3], one does obtain linear kinetics for L-aspartate transport by either wild type or *pm−nb* cells grown on acetate minimal media (Table I and Wolfinbarger, L., unpublished).

The increase in K_m values with increasing pH is more difficult to interpret. There is no correlation of K_m values with appearance or disappearance of the zwitterion or anionic species of acidic amino acid as was reported in *Neurospora* conidia[3]. Furthermore, the K_m values for L-aspartate transport (at pH 2.8 and 4.8, respectively) in conidia of the *pm−nb* double mutant were reported[3] to be 2.34 mM and 13.9 mM. In *pm−nb* cells grown on acetate minimal media for 10 h these respective K_m values have decreased to 0.04 and 0.02 mM, respectively (Table I). This decrease represents an apparent increase in "affinity"* of the residual transport activity in *pm−nb* of 50-fold at pH 2.8 and 700-fold at pH 4.8.

Stereospecificity of transport activity

In conidia the neutral and general amino acid transport systems have shown themselves to be consistently stereospecific for the L-forms of the amino acids[3,5,9]. However, in cells grown on acetate minimal media the transport activity seems to be less stereospecific as the D-forms of aspartate and glutamate reduced the transport

* For convenience in discussion the K_m constant is occasionally taken as a measure of the affinity (binding constant) of the transport system for substrate.

TABLE II

STEREOSPECIFICITY OF ACIDIC AMINO ACID TRANSPORT ACTIVITY IN WILD TYPE AND *pm-nb* AT pH 3.8

Values as nmoles/min per 2 mg dry wt cells. The molar concentrations of the labeled amino acids transported were 0.1 mM. The cells were grown 9–10 h in acetate minimal media (see text) prior to transport assays.

Inhibiting amino acid (1 mM)	*Wild type*		*pm-nb*	
	L-*Glutamate*	L-*Aspartate*	L-*Glutamate*	L-*Aspartate*
None	17±1.0	11±0.7	32±1.9	24±1.4
L-Aspartate	3±0.2	2±0.1	—	—
L-Glutamate	2±0.1	2±0.1	—	—
D-Aspartate	12±0.7	11±0.7	21±1.3	13±0.8
D-Glutamate	8±0.6	8±0.5	17±1.0	12±0.7

velocities of the L-forms (Table II). For example, 1 mM D-glutamate reduced the transport velocity of 0.1 mM L-glutamate or 0.1 mM L-aspartate in either wild type or *pm−nb* by approximately 50% (at pH 3.8). With the exception that D-aspartate does not significantly inhibit L-aspartate transport by the wild type, D-aspartate inhibits transport of L-acidic amino acids to a degree comparable to D-glutamate. The reason(s) D-aspartate does not inhibit L-aspartate transport in the wild type are unknown at present.

The possibility that D-glutamate might be inhibiting transport of L-glutamate (or L-aspartate) by its known inhibition of the glutamate dehydrogenase enzyme of *Neurospora*[10] was explored by examining the kinetics of inhibition of transport. In all instances tested, inhibition of transport of the L-form of aspartate or glutamate by the L- or D-forms of these two amino acids was in fact strictly competitive in character. These observations are in agreement with those in conidial transport[2] and suggest that L-aspartate and L-glutamate share a common transport system(s) in acetate grown cells. That this transport activity may in fact be due to the appearance of entirely new systems for acidic amino acid transport, however, was not ignored.

DISCUSSION

The results of this study are difficult to reconcile with any of the previous studies of acidic amino acid transport in *Neurospora*. The similarity of pH optima, susceptibility to inhibition of L-aspartate and L-glutamate transport by L-phenylalanine and similarity of kinetic constants for acidic amino acid transport in the wild type and *pm−nb* double mutant suggests that the transport activity in acetate-grown cells might be attributed to the general amino acid transport system. However, in order to do this one must reconcile the enormous decrease in K_m values, independence of the K_m values from change in concentrations of charged *versus* zwitterion species of substrate amino acid with pH and alteration in stereospecificity such that the D-forms of amino acids are now recognized.

Alternatively, one might suggest that the observed transport activity could be

due to a completely new transport system(s) for acidic amino acids, inducible on acetate, with similar substrate specificities to those of the general (and/or neutral) amino acid transport system, but with vastly higher "affinities" for the respective substrate. We are hesitant to accept this second possibility as it would bring to 5 the number of transport systems reported to recognize L-aspartate as a substrate molecule. (See ref. 4 for the other four systems.)

As its name implies the general amino acid transport system is known to transport a wide variety of substrate molecules. Thus, it might not seem too unreasonable to suggest that following growth on acetate minimal media it may transport both the charged and zwitterion species of the acidic amino acids. This would explain the apparent independence of the K_m values from change in pH since substrate would no longer become limiting at a given pH. Also, the general system may be modified according to the dictates of the environment, principally with respect to an alteration in the apparent "affinity" for transport. Growth of cells on acetate as the sole carbon source could easily alter the chemical composition of the membrane with a resultant change in K_m values of transport systems. The mechanism whereby this change could occur is unknown, however the effects of nutritional or environmental changes on membrane composition —particularly the lipid composition— and hence membrane function in microorganisms is well documented[11–16]. Also of interest is the possibility that the properties of the general amino acid transport system could be altered during conidial outgrowth by the appearance of a specific protein (or glycoprotein) subunit or "K_m" factor.

It is of interest that the V values, as a function of pH, indicate a pK_H of the transport system(s) for L-glutamate around pH 4.8 and for L-aspartate around pH 3.8. These values are of course subject to confirmation pending elucidation of any contribution by titration of the substrate molecules.

In summary, it would appear that the acidic amino acids are transported primarily, if not exclusively, by the neutral and/or general amino acid transport systems in conidia and actively growing mycelia. We are still not certain when the acidic amino acid transport system described by Pall[1] becomes an effective transport system for these molecules. With the vast increase in the K_m values of the general amino acid transport system, one wonders whether the acidic amino acid transport system is real or simply a residual amount of the general system not completely inhibited by L-arginine.

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