Basic and Neutral Amino Acid Transport in *Aspergillus nidulans*

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SUMMARY

Arginine and methionine transport by *Aspergillus nidulans* mycelium was investigated.

A single uptake system is responsible for the transport of arginine, lysine and ornithine. Transport is energy-dependent and specific for these basic amino acids. The K_m value for arginine is 1×10^{-6} M, and V_{max} is 2.8 nmol/mg dry wt/min; K_m for lysine is 8×10^{-6} M; K_i for lysine as inhibitor of arginine uptake is 12 μ M, and K_i for ornithine is 3 mM.

On minimal medium, methionine is transported with a K_m of 0.1 mM and V_{max} about 1 nmol/mg dry wt/min; transport is inhibited by azide. Neutral amino acids such as serine, phenylalanine and leucine are probably transported by the same system, as indicated by their inhibition of methionine uptake and the existence of a mutant specifically impaired in their transport.

The recessive mutant *nap3*, unable to transport neutral amino acids, was isolated as resistant to selenomethionine and *p*-fluorophenylalanine. This mutant has unchanged transport of methionine by general and specific sulphur-regulated permeases.

INTRODUCTION

Several amino acid permeases have been described and characterized in the filamentous fungi *Neurospora crassa* and *Penicillium chrysogenum*. Results concerning amino acid uptake in *Aspergillus nidulans* are less complete. Robinson, Anthony & Drabble (1973*a*) and Pateman, Kinghorn & Dunn (1974) characterized an acidic amino acid permease. Sinha (1969) described a transport system for phenylalanine. Cybis & Weglenski (1969) demonstrated that lysine and arginine compete for entry into the *A. nidulans* mycelium, and suggested a single permease for transport of these two amino acids. Hackette *et al.* (1970) and Benko, Wood & Segel (1967) showed the development of a methionine transporting system during sulphur starvation and the derepression of uptake systems for amino acids resulting from nitrogen and carbon deficiency.

Contrary to the situation in *Neurospora* where mutants have been isolated for most of the transporting systems, no permease mutation is known for *Penicillium* and few for *Aspergillus*. In *A. nidulans*, Sinha (1969) described a dominant mutation which was impaired in the transport of phenylalanine and to some degree in the transport of most of the amino acids. Kinghorn & Pateman (1975) isolated recessive mutants blocked in the transport of acidic amino acids and dominant and recessive mutants impaired simultaneously in acidic and neutral amino acid uptake.

This paper characterizes the transport of arginine, lysine and ornithine by the basic amino acid permease of *A. nidulans*.

Data concerning the transport of methionine under different growth conditions are presented and discussed, and methionine transport by a neutral amino acid permease distinct from the sulphur- and nitrogen-regulated permeases is suggested. A recessive mutation specifically impaired in this system is described.

METHODS

Strains. All experiments were carried out with the same strain of *A. nidulans* *pabaAr, biAr* (auxotrophic for vitamins *p*-aminobenzoic acid and biotin). The same strain was used for mutagenesis.

Genetic methods and media were standard ones used for *A. nidulans* (Pontecorvo *et al.* 1953; Cove, 1966). Liquid media containing no nitrogen source (–N medium), no carbon source (–G medium) and no sulphur (–S medium) were also used.

Transport assay. In a typical experiment the standard minimal medium (MM) was inoculated with a heavy conidial suspension of about 10^7 conidia/ml and grown in an orbital shaker at 32 °C. Young mycelium (14 to 16 h old) was used, usually at about 1 mg dry wt/ml. Without changing the medium, 25 ml portions of cultures were transferred by pipette to an Erlenmeyer flask in a water bath; the flask already contained the radioactive substrate (0.2 mM, 0.1 to 0.2 mCi/assay). Samples (3 to 5 ml) were withdrawn with a pipette every 30 s during a 2 min period from shaken cultures, filtered under suction through pre-weighed Whatman GF 85 filters and washed immediately with 20 ml of ice-cold water. Filters with mycelium were dried for 24 h at 90 °C, weighed to calculate the dry weight of each mycelial sample, placed in vials with 5 ml of Packard Permablend III scintillation mixture, and the radioactivity was measured in a Beckman LS 150 scintillation counter. The quenching of the radioactivity was calculated for the conditions used and found to be 50 %. All results were corrected to absolute d.p.m. values.

Transport rates are expressed as nmol substrate/mg dry wt/min.

Protein incorporation. Two duplicate samples were taken every 30 s during a 3 min period, as for the transport assay. Samples were extracted immediately with 5 % (w/v) cold trichloroacetic acid (TCA), filtered, and radioactivity was measured in both filtrate and precipitate. Parallel untreated samples were counted for the total radioactivity accumulated.

Chromatography of extracts. After 2 to 3 min incubation with radioactive substrates (0.2 mM, 0.2 mCi) the mycelium was harvested, washed with ice-cold water and extracted immediately with 5 % TCA. After centrifugation, the radioactivities of a portion of the supernatant and of the pellet were assayed. The supernatant was applied to a Dowex 50 H⁺ column and elution was carried out with 2 M-NH₄OH. The amino acid fraction eluted was evaporated to dryness and resuspended in 1/10 vol. of distilled water. All radioactivity applied was recovered from the columns in this procedure. Samples (5 ml) were then submitted to thin-layer chromatography on plates of MN 300 cellulose and developed with propanol–85 % formic acid–water (40:2:1, by vol.). Chromatograms were stained with ninhydrin and the positions of amino acids were identified using external and internal standards. Chromatograms were then scanned for radioactivity with a Berthold automatic thin-layer scanner.

Chemicals. L-[U-¹⁴C]arginine–HCl, specific activity 336 mCi/mmol, and L-methyl-[¹⁴C]-methionine, 60 mCi/mmol, were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. All other L-[U-¹⁴C]amino acids were from UVVVR, Czechoslovakia. The remaining chemicals were of commercial grade.

Fig. 1

Fig. 2

Fig. 1. Time-dependence of [¹⁴C]arginine and [¹⁴C]methionine transport by mycelia of the wild type and of mutant *aga65*, which lacks arginase. ○, [¹⁴C]arginine, wild type; □, [¹⁴C]arginine, mutant *aga65*; ●, [¹⁴C]methionine, wild type.

Fig. 2. Kinetics of [¹⁴C]methionine transport by mycelium. Direct plot of the initial velocity of transport as the function of varying concentrations of amino acid.

RESULTS

Transport of basic amino acids

Linearity of the assay and pH optimum. Figure 1 shows the linear time-dependence of [¹⁴C]arginine transport under standard conditions during the 2 min assay period. Transport of the amino acid was inhibited more than 90 % in the presence of 1 mM-sodium azide added 2 min before the substrate, indicating that transport is energy-dependent.

The optimal pH was about 5.0 to 5.5 (Fig. 3); the pH of the medium in standard experiments is about 6.5 to 7.0.

Metabolism of arginine during the transport assay period and incorporation into protein. About 95 % of the total transported radioactivity was found to be extractable into cold TCA, while about 5 % was found in the TCA precipitates during the 3 min following the addition of radioactive substrate.

Cycloheximide (10 μg/ml) inhibits protein synthesis in *A. nidulans* (Robinson, Anthony & Drabble, 1973*b*). No difference in velocity of transport was found in the presence and absence of the antibiotic (10 μg/ml) during the first 3 min following addition of [¹⁴C]-arginine. TCA extracts were prepared after 3 min incubation of mycelium with [¹⁴C]-arginine and desalted on a Dowex 50 H⁺ column. All radioactivity (within a 5 % range of error) was recovered from the column into the eluate. Only one peak of radioactivity was found on the chromatogram, identified as arginine. Thus, more than 90 % of the radioactivity in the extractable pool was still in the form of free, unmetabolized arginine.

The rate of arginine transport was unchanged (Fig. 1) in mutant *aga65*, which lacks arginase, the first enzyme of arginine catabolism (unpublished).

These results indicate that the initial velocity of transport measured is not dependent on arginine being catabolized and/or incorporated into protein.

Kinetics of [¹⁴C]arginine transport. The dependence of initial velocity of transport on

Fig. 3

Fig. 4

Fig. 3. [¹⁴C]arginine and [¹⁴C]methionine transport as a function of pH value. Citrate-phosphate, potassium phosphate and tris-HCl buffers were added to the medium at a final concentration of 0.1 M, to obtain the indicated pH. O, Initial rate of [¹⁴C]arginine transport; ●, initial rate of [¹⁴C]methionine transport.

Fig. 4. Kinetics of (O) [¹⁴C]arginine and (●) [¹⁴C]lysine transport by mycelium. Direct plot of the initial velocity of transport as the function of varying amino acid concentrations.

Table 1. *Effect of unlabelled amino acids on [¹⁴C]arginine and [¹⁴C]methionine uptake*

The [¹⁴C]arginine concentration was 25 μM, and the [¹⁴C]methionine concentration was 25 or 200 μM. Unlabelled amino acids, together with either arginine or methionine, were added at the same time as the labelled substrate.

Arginine				Methionine		
Inhibitor	Percentage inhibition*			Inhibitor	Percentage inhibition*	
	10	100	1000		10	100
Lysine	90			Leucine	40	60
Ornithine	0	10	65	Alanine	65	75
Histidine	0	13	20	Serine	62	85
Methionine	11	10		O-acetylserine	85	
Leucine	3	12		Phenylalanine	63	
Phenylalanine	15	25		Glutamine	60	
Alanine	0	0		Arginine	10	
Glutamate	0	0		Glutamate	0	
Proline	0	0		Proline	0	

* Percentage inhibition at inhibitor:substrate ratios of 10, 100 or 1000.

substrate concentration is shown in Fig. 4. The system was saturable and only one system seemed to be involved for the concentrations tested. The K_m , calculated from the reciprocal Lineweaver-Burke plot, was about 10 μM and V_{max} was about 2.8 nmol/mg dry wt/min.

Kinetics of [¹⁴C]lysine transport. Figure 4 shows the direct plot of velocity-concentration dependence for transport of [¹⁴C]lysine. The K_m calculated from this was about 8 μM, and V_{max} was about 2 nmol/mg dry wt/min.

Specificity of the arginine transport system. The effect of several unlabelled L-amino acids on the uptake of [¹⁴C]arginine is shown in Table 1. Only lysine was a good inhibitor. The

Fig. 5

Fig. 6

Fig. 5. Competitive inhibition of [^{14}C]arginine transport by unlabelled L-lysine and L-ornithine. $\circ$, No inhibitor; $\blacksquare$, 0.1 mM-lysine present; $\square$, 0.2 mM-lysine present; $\blacktriangle$, 4 mM L-ornithine present; $\triangle$, 12 mM L-ornithine present.

Fig. 6. Competitive inhibition of [^{14}C]methionine transport by unlabelled L-serine and O-acetyl-L-serine. $\circ$, No inhibitor; $\square$, 2 mM L-serine present; $\bullet$, 10 mM O-acetyl-L-serine present.

basic amino acids ornithine and histidine did not inhibit transport at 100-fold excess (i.e. at 100 times the substrate concentration), though inhibition by ornithine was observed at 1000-fold excess. The results of competition studies (Fig. 5) show that inhibition by the basic amino acids ornithine and lysine is of the competitive type. Dixon plots of inhibition data gave a K_i value for lysine as inhibitor of arginine uptake of 12 μM , and a K_i value for ornithine as an inhibitor of arginine transport of about 3 mM. The K_m values for arginine and lysine and the K_i of lysine as the inhibitor of arginine were roughly equal, which suggests competition for the same site of permease, which had equal affinity for these amino acids. Given the competitive nature of inhibition of arginine transport, it may be assumed that the same basic amino acid permease is responsible for transporting ornithine but probably with a distinctly lower activity.

Transport of methionine

Linearity of the assay and pH optimum. Figure 1 shows the linear time-dependence of [^{14}C]methionine transport. The transport rate was linear during the 2 min assay period. Sodium azide (1 mM) added 2 min before the labelled substrate inhibited transport more than 90 %, indicating that transport is energy-dependent.

The pH optimum was about 4.0 to 4.5 (Fig. 3).

Metabolism of methionine during the assay period. Incorporation of radioactivity into the protein was assayed as described in Methods. Within the first 3 min following the addition of [^{14}C]methionine the total amount of radioactivity in the TCA precipitates did not exceed 1 to 2 % of the total radioactivity accumulated in the mycelium. TCA extracts prepared after 3 min incubation of mycelium with [^{14}C]methionine were applied to thin-layer

chromatography plates. On the chromatogram about 60 % of the radioactivity applied was found in the peak corresponding to free methionine, and the rest was in other unidentified compounds. Thus, during the 2 min assay about 60 % or more of transported methionine still remained in the unmetabolized form.

Kinetics of methionine transport. Figure 2 shows the direct plot of velocity-concentration dependence of methionine transport. For the range of concentration used only one system seems to be involved. From Fig. 2, the K_m was calculated to be about 10 mM and V_{max} to be 0.8 to 1.0 nmol/mg dry wt/min.

Specificity of the transporting system. Table 1 shows the results of inhibition tests performed with several unlabelled L-amino acids added simultaneously with [14 C]methionine at 10 and 100 times the concentration of the substrate. There was no more than 10 % inhibition by basic and acidic amino acids, and by proline. Robinson *et al.* (1973*a*) demonstrated that there is a highly specific permease for acidic amino acids in *Aspergillus*. Proline does not inhibit neutral, basic or acidic amino acid transport and is probably transported by its own specific system (unpublished). The lack of inhibition by arginine is in agreement with results presented in this paper on transport of basic amino acids. Neutral amino acids tested inhibited methionine transport by at least 40 % at 10-fold excess, suggesting their transport by a common system. The character of inhibition of methionine transport was tested further for serine and O-acetylserine (Fig. 6), and was of the competitive type.

Neutral amino acid transport by a mutant

Selection and characterization of the mutant. *Aspergillus nidulans* is highly resistant to most of the amino acid analogues used for the selection of permease mutations (e.g. canavanine or ethionine). We found that selenomethionine (S-Meth), a methionine analogue, slows the growth of *A. nidulans* at a concentration of 0.2 μ M and at 100 μ M stops the growth completely. The effect is specific, as indicated by reversion of toxicity by equimolar methionine. *p*-Fluorophenylalanine (PFA) is a toxic analogue of phenylalanine, widely used in studying the genetics of *A. nidulans*. Mutants resistant simultaneously to 0.1 mM-S-Meth and 1 mM-PFA were selected after u.v. mutagenesis and one of them (*nap3*) was studied further.

Mutant *nap3* was tested in heterokaryons and diploids against the wild type and was found to be totally recessive. The strain bearing the *nap3* mutation was crossed with the wild type and the mutation was found to be monogenic.

*Transport of amino acids by the *nap3* mutant.* Transport rates of various amino acids under different growth conditions were studied. [14 C]methionine, [14 C]phenylalanine and [14 C]leucine were not transported by mycelium of *nap3* growing on the standard minimal medium, whereas [14 C]arginine and [14 C]proline were.

Under nitrogen starvation, mutant *nap3* became capable of normal transport of [14 C]-methionine and [14 C]phenylalanine, and transport of these amino acids was inhibited 90 % by a 10-fold excess of unlabelled arginine. In the wild type and mutant *nap3* after 9 h of nitrogen starvation, the transport rate of arginine increased and it became sensitive to inhibition by methionine.

When the *nap3* strain was starved on -G medium it became capable of [14 C]methionine and [14 C]phenylalanine transport. Transport on both -N and -G media was inhibited by 1 mM-azide. The general, non-specific system(s) of amino acid transport remained unchanged in the mutant.

When *nap3* was subjected to sulphur starvation it developed the capability to transport methionine, as in the wild type, but not phenylalanine. This last result enabled us to distinguish between the specific permease regulated by sulphur level in the medium, and the

system of transport of neutral amino acids by which methionine, leucine and phenylalanine enter during growth on minimal medium. The results indicate that, on minimal medium, the sulphur-regulated permease is not present.

DISCUSSION

The results indicate that on minimal medium the same permease is responsible for the active transport of arginine, lysine and ornithine; arginine and lysine compete with equal affinity for the same site. Such a system seems to be similar to the basic amino acid permeases of other filamentous fungi such as *Penicillium* (Hunter & Segel, 1971) and *Neurospora* (Pall, 1970); K_m values found for arginine and lysine are of same order as those of *Penicillium*. This demonstration of a single permease for arginine, lysine and ornithine in *A. nidulans* is in agreement with the results of the growth studies of Cybis & Weglenski (1969).

At neutral pH and under the standard conditions of growth used in our experiments, histidine did not significantly inhibit uptake of arginine even at 1000-fold excess. Basic permeases of both *Penicillium* and *Neurospora* have a very low affinity for histidine and this amino acid is probably mainly transported by a general system (Pall, 1970; Hunter & Segel, 1971).

Derepression of methionine transport during sulphur, nitrogen or carbon starvation, and a highly specific methionine permease operating in sulphur-starved mycelium have been described for both *Penicillium* and *Aspergillus* by Benko *et al.* (1967). In *Neurospora*, on minimal medium, a single permease is mainly responsible for the transport of methionine and all neutral amino acids. In *Penicillium*, Benko *et al.* (1967) showed the lack of such a neutral amino acid permease and suggested that the highly specific methionine permease, derepressing upon sulphur starvation, is probably also responsible for methionine entry during the growth on minimal medium. However, Hunter & Segel (1973) suggested that, on minimal medium, neutral amino acid uptake may be due to free diffusion through the lipophilic membrane.

The results on the specificity of permeases developing in wild type and mutant *nap3* in different growth conditions suggest that methionine is transported by a neutral amino acid permease during growth in nutrient-sufficient medium. This permease is absent from mutant *nap3* and is different from the general permease derepressed during nitrogen or carbon starvation and also different from the system for methionine transport which is derepressed during sulphur starvation.

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