JOURNAL OF BACTERIOLOGY, Sept. 1981, p. 944-948
0021-9193/81/090944-05$02.00/0

Vol. 147, No. 3

Characterization of 2-Aminoisobutyric Acid Transport in *Neurospora crassa*: a General Amino Acid Permease-Specific Substrate

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Received 27 January 1981/Accepted 29 May 1981

We report the characterization of an amino acid permease-specific substrate for *Neurospora crassa*. The neutral amino acid 2-aminoisobutyric acid was transported solely by the general amino acid permease and not by the neutral amino acid permease. Furthermore, this substrate was not metabolized after transport. The potential for a system-specific nonmetabolizable substrate as a tool in the analysis of amino acid transport and its regulation is discussed.

Several laboratories have been involved with the analysis of amino acid transport in the fungus *Neurospora crassa*. The early goals of this research were to determine the number and types of systems which were responsible for transporting amino acids. This approach defined the strategy for amino acid transport by *N. crassa* as involving a limited number of constitutive permeases which transport families of structurally related amino acids: a neutral (N) system which is specific for neutral aliphatic or aromatic amino acids, a basic (B) system which transports positively charged amino acids, and a general (G) system which handles all classes of amino acids (1, 2, 4–7, 9–11, 14).

Each family of amino acids is, therefore, transported both by the family-specific system and by the G system. Such overlap in substrate specificity between systems has made it difficult to ascribe particular properties to single systems. One approach to this problem involves the construction of mutant strains defective for the activity of one or more transport systems so that only the system of interest remains active. This approach has been very useful in defining the number of transport systems that exist and the physiological and kinetic properties of each. A second approach involves the identification and use of a system-specific amino acid substrate to monitor the activity of the permease of interest without eliminating the activities of the other permeases. This approach allows one to detect interactions among functional systems, an advantage not possible with the mutant approach. In addition, a permease-specific substrate is a useful tool, in combination with the mutant approach, for molecular studies of the components which comprise a particular transport system.

We describe here the transport of the amino acid 2-aminoisobutyric acid (AIB) and its properties as a general amino acid permease-specific substrate.

MATERIALS AND METHODS

Strains and growth of cultures. The wild-type strain 74a (FGSC 988) was obtained from the Fungal Genetics Stock Center, Humboldt State University, Arcata, Calif. The *pmn*, *pmb*, and *pmg* strains are defective for the neutral amino acid-specific permease, the basic amino acid-specific permease, and the general amino acid permease, respectively, and are isogenic with the wild type. Each of these strains is available from the authors. All strains were maintained in 1× Vogel medium N supplemented with 2% sucrose and solidified with 1.5% agar as previously described (2, 13).

Amino acid transport assays. For the conidial assays, cells were incubated at 25°C (0.1 mg of dry weight per ml of incubation medium, final concentration) in 1× Vogel medium N in which the NH_4NO_3 had been replaced with equimolar KNO_3. Radiolabeled substrate was present at a final concentration of 4×10^{-4} M (0.125 mCi/mmol) in the case of [^{14}C]AIB, 1×10^{-5} M (0.5 mCi/mmol) in the case of L-[^{14}C]-phenylalanine, and 5×10^{-5} M (1 mCi/mmol) in the case of L-[^{14}C]citrulline. Where utilized, the following metabolites were present in the incubation medium in these concentrations: 2,4-dinitrophenol (0.5 mM), sodium azide (0.5 mM), 3-*O*-methyl-D-glucose (4 mM), and uridine (4 mM). Assays were initiated by the addition of cells. Samples were withdrawn at 30-min intervals over a 2-h period, filtered onto glass fiber filters (Whatman GF/A), dried, and counted in a gas-flow proportional counter (Beckman).

The postconidial stage is an early developmental stage in which the activity of the amino acid transport systems is considerably amplified over that of the conidial stage (12). For the postconidial studies cells were incubated (0.1 mg of dry weight per ml of medium) at 25°C for 3 h in 1× KNO_3 Vogel salts containing 1% D-glucose. Assays were initiated by the addition

of radiolabeled substrate to the same final concentrations used in the conidial studies. Samples were withdrawn at 15-min intervals over a 1-h period, filtered, and counted as described above.

All nonradiolabeled chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). Uniformly labeled L-[^{14}C]phenylalanine and L-[^{14}C]citrulline were obtained from Schwarz/Mann (Spring Valley, N.Y.); 2-amino [3-^{14}C]isobutyric acid ([3-^{14}C]AIB) and [1-^{14}C]AIB were purchased from New England Nuclear Corp. (Boston, Mass.). The [3-^{14}C]AIB was used in all transport and chromatographic studies. The [1-^{14}C]-AIB was used only for the chromatographic studies.

Determination of concentration gradient. Cells were incubated in the presence of 400 μM [^{14}C]AIB under standard conidial transport conditions. After 20 min of incubation, duplicate samples were removed: one was filtered onto a nitrocellulose filter (Millipore Corp., type AA, 0.8 μm, 25-mm diameter), and the duplicate sample was placed into 10% (wt/vol) trichloroacetic acid for 30 min and then filtered to remove trichloroacetic acid-insoluble material. The radioactivity present in the trichloroacetic acid-soluble fraction (total radioactivity − radioactivity in trichloroacetic acid-insoluble fraction) was used to determine the internal AIB concentration as outlined by DeBusk and DeBusk (2).

Kinetic analysis of AIB transport. The kinetic analyses employed initial rate measurements of [^{14}C]AIB accumulation over a 20-min period at 35°C. Transport was linear for at least 20 min. A detailed description of the assays and the determination of the kinetic parameters K_m and V_{max} are given by DeBusk and DeBusk (2), with the exception that the nitrogen source in the present study was KNO_3, and the radioactivity was detected by liquid scintillation counting.

Fate of transported AIB. Incorporation of transported [^{14}C]AIB (or L-[^{14}C]phenylalanine) into cellular protein was determined by sampling the conidial or postconidial incubation media as usual except that a duplicate sample was withdrawn at each time interval and added to 10% (wt/vol) trichloroacetic acid for 30 min before filtering, drying, and counting. The amount of radiolabel present in trichloroacetic acid-insoluble material was taken as the measure of the incorporation of transported amino acid into protein.

To determine the extent of metabolic conversion of AIB after transport, cells were incubated in the presence of radiolabeled AIB under standard conidial transport conditions. At the end of 1 h cells were filtered, washed with three 5-ml volumes of distilled water, and extracted in boiling water for 30 min. The extract was centrifuged, concentrated by drying at 60°C, and resuspended in 100 μl of distilled water. The extract was then spotted onto precoated cellulose sheets (E. Merck AG, Darmstadt, Germany) in 10-, 20-, and 30-μl quantities and subjected to ascending thin-layer chromatography. Radiolabel was localized by placing a sheet (5 by 7 in.; ca. 12.7 by 17.8 cm) of No-Screen medical X-ray film NS-5T (Eastman Kodak Co., Rochester, N.Y.) against the chromatogram, which was then incubated at −40°C for 2 days and subsequently developed by standard techniques for X-ray film.

The potential metabolic conversion products of AIB resulting from loss of the amino, carboxyl, or methyl groups from the alpha-carbon were anticipated, and various analyses were designed to detect these products. Radiolabeled compounds containing amino groups were localized by spraying the chromatograms with a commercially prepared ninhydrin solution. Both [1-^{14}C]AIB and [3-^{14}C]AIB were tested to detect possible conversion of AIB to isopropylamine. Three different solvent systems were used to test for the presence of alanine: *n*-butanol–acetic acid–water (80:20:20), *n*-butanol–95% ethanol–water (80:20:20), and saturated phenol. Each system clearly resolved AIB from alanine.

In addition, cell extracts were analyzed using a Beckman model 121 amino acid analyzer. Cells were incubated at 25°C at the usual density for 12 h in the presence of 1% D-glucose, 1× Vogel salts containing KNO_3 as the nitrogen source, and 400 μM AIB. Samples containing 25 mg (dry weight) of cells were filtered, washed with three 5-ml volumes of distilled water, extracted in boiling water for 30 min, and centrifuged. The pellet was again extracted and centrifuged, and the combined supernatants were filtered through a nitrocellulose filter and taken to dryness at 60°C. Two milliliters of 4% sulfosalicylic acid was added to precipitate macromolecules, and the extract was filtered and again taken to dryness at 60°C. The extract was then analyzed using the standard procedures set forth by Beckman Instruments for amino acid analysis.

RESULTS

Transport of AIB by the general amino acid permease. Since the transport of AIB had not previously been described for *N. crassa*, we wished to determine which transport systems were responsible for AIB accumulation. Therefore, transport of radiolabeled AIB was examined in the conidial stage in the wild-type strain and in mutant strains which were defective for one of the three amino acid transport systems: the *pmn* strain (N system defective), the *pmb* strain (B system defective), and *pmg* strain (G system defective). No transport of AIB was observed in the *pmg* strain (Fig. 1). Transport of AIB by the *pmb* and *pmn* strains was identical to that of the wild type (data not shown). We then examined AIB transport in another stage of the life cycle in which transport is quite active, the postconidial stage, and likewise observed that AIB transport was eliminated in the presence of the *pmg* mutation (Fig. 1).

We compared the transport of AIB in these two stages with that of the neutral amino acid L-citrulline, which had previously been proposed as a G system-specific substrate (11). Unlike AIB transport, citrulline transport was not eliminated by the *pmg* mutation and could only be eliminated by the addition of a second mutation, *pmn*. The *pmn pmg* strain, defective for both the general and the neutral permease activities,

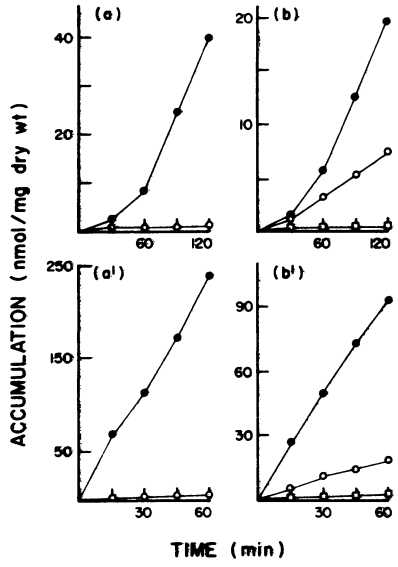

FIG. 1. *Amino acid permease specificity of AIB and L-citrulline transport by N. crassa. Accumulation of AIB by the wild type (●) and pmg (○) strains in the conidial (a) and postconidial (a') developmental stages. Accumulation of L-citrulline by the wild type (●), pmg (○), and pmn pmg (□) strains in the conidial (b) and postconidial (b') stages.*

was unable to transport L-citrulline in either the conidial or the postconidial stage (Fig. 1).

Properties of AIB transport. Several lines of evidence suggested AIB transport by wild-type *N. crassa* was an active transport process. Transport occurred against a concentration gradient, with the internal concentration/external concentration ratio being 23:1 after 20 min of incubation. Transport was dependent upon metabolic energy since no transport of AIB was observed in the presence of the metabolic inhibitors 2,4-dinitrophenol or sodium azide (data not shown).

As would be expected for any G system substrate, transport of AIB could be eliminated by the simultaneous presence in the incubation medium of an excess of either a neutral or a basic amino acid known to be transported by the G system, but was unaffected by the presence of the sugar 3-*O*-methyl-D-glucose or the pyrimidine uridine, which are not G system substrates (data not shown).

Although the data suggested the G system as the route of entry of AIB, these studies were performed at a single concentration of AIB which might be too low to allow us to detect the activity of a second system if such existed. Therefore, we examined the velocity of transport by the wild-type strain over a wide range of concentrations. Only a single activity was detected, and the kinetic parameters of that activity were generated by a Hofstee transformation of the data (Fig. 2). The apparent affinity (K_m) and the maximal velocity (V_{max}) were determined to be 98.7 μM and 0.534 nmol per min per mg of dry weight, respectively.

Since many substrates are known to inhibit G system activity at concentrations greater than 100 μM (2), the velocity of transport was plotted as a function of AIB concentration to determine whether substrate inhibition was a property of the G system with respect to AIB. No substrate inhibition was observed (Fig. 2).

Metabolic fate of transported AIB. Typically, transported amino acids are readily incorporated into trichloroacetic acid-insoluble material (1). Examination of the metabolic fate of a representative neutral radiolabeled amino acid, phenylalanine, revealed that 30% of the label had been incorporated into cellular protein after 120 min of incubation (Fig. 3). When the

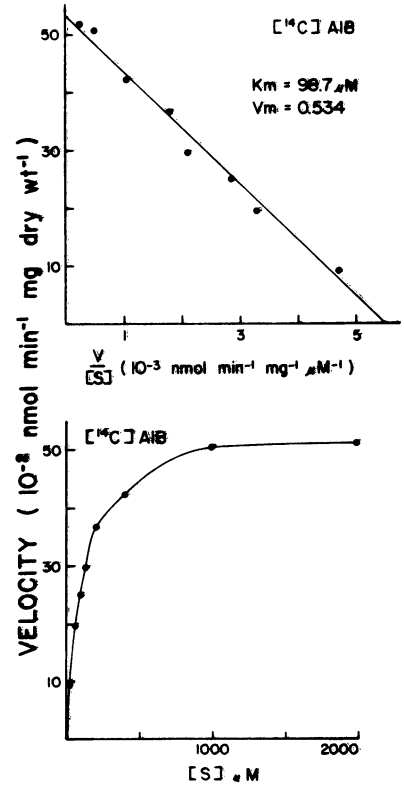

FIG. 2. *Kinetic analysis of AIB transport by wild-type conidia. A Hofstee transformation was used to generate the kinetic parameters K_m and V_{max}. V_{max} is expressed as nanomoles per minute per milligram of dry weight. The velocity of transport was plotted as a function of the AIB concentration to determine whether substrate inhibition was operative.*

extent of AIB incorporation was examined over this same time period, no significant incorporation occurred (Fig. 3). Less than 1% of the accumulated AIB was present in cellular protein. Similarly, no incorporation of AIB was observed in the postconidial stage (data not shown).

To determine whether AIB was metabolized after transport, wild-type *N. crassa* was incubated with radiolabeled AIB for 60 min, extracted, chromatographed, sprayed with ninhydrin, and subjected to autoradiography. Extracts of the wild type incubated for an extended period of time in AIB (12 h) were also analyzed with an amino acid analyzer. The analytical techniques chosen were designed to detect converison products resulting from the loss of the amino, carboxyl, or methyl groups from the AIB molecule. No significant metabolism of AIB was observed regardless of the length of incubation in AIB, the solvent system employed, or the position of the radiolabel in the molecule.

DISCUSSION

Transport of the neutral amino acid AIB by *N. crassa* occurs solely by the general amino acid permease (G system). Neutral amino acids typically enter *N. crassa* by means of the neutral amino acid-specific N system and the G system. With respect to AIB, however, only a single activity could be detected in the wild-type strain, and this activity was completely eliminated by the presence of the *pmg* mutation.

We compared the specificity of AIB transport with that of the neutral amino acid L-citrulline, which had previously been reported to be a general amino acid permease-specific substrate (11). In contrast to AIB, citrulline was transported by both the N and the G systems. The presence of the *pmg* mutation caused a reduction in citrulline transport, but the complete elimination of citrulline transport required the addition of a second mutation which affected N system activity. Our data displayed an increased G system activity in the postconidial stage as compared with N system activity. It may be that N system activity continues to decline so that by the late developmental stages examined by Thwaites and Pendyala (11) this system contributes negligibly to citrulline transport.

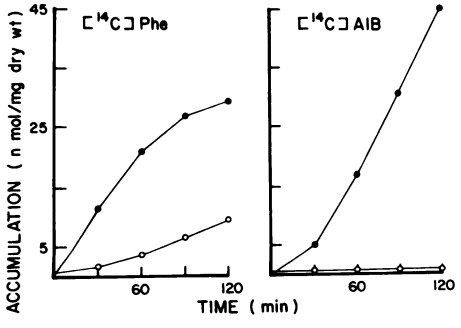

FIG. 3. *Ability of wild-type conidia to incorporate transported phenylalanine and AIB into macromolecules.* (●) *Accumulation of radiolabeled phenylalanine or AIB;* (○) *transported radiolabel recovered in cellular protein (trichloroacetic acid-insoluble material).*

Most of the properties described for AIB transport by the G system are typical of other amino acids transported by this system. Two properties, however, are distinct. AIB transport is not subject to substrate inhibition even at a concentration of 2,000 μM, in contrast to other substrates which inhibit G system activity at concentrations exceeding 100 μM (2). Second, whereas transported amino acids are usually rapidly incorporated into protein or subsequently metabolized, we detected neither metabolism nor incorporation in the case of AIB, suggesting that the ability to transport an amino acid is not dependent upon either of these activities. These features appear to be characteristic of AIB, since Ring and Heinz (8) have reported that no metabolism of transported AIB could be detected in *Streptomyces hydrogenans*, and Kotyk and Rihova (3) have reported similar findings for *Saccharomyces cerevisiae*.

The existence of a G system-specific amino acid is potentially a valuable tool for both regulatory and molecular studies of amino acid transport. Ultimately, we would like to understand the molecular architecture of each of the transport systems: the number and types of components which comprise each system, the extent to which the components are similar among the three systems, how the components of each permease interact to achieve a functional transport system, and which components are involved in the regulation of permease activity. The discovery of a system-specific substrate will allow us to begin to dissect the molecular composition of the general system by isolating the binding component responsible for the initial step in the transport of an amino acid by the G system.

Our earlier findings suggested that the three transport systems may interact at the functional level (2). To dissect complex interactions, it becomes essential to have all of the transport systems functional and to examine the effect on the activity of a particular system of transporting an amino acid through a second system. Previously, investigators were unable to monitor general permease activity without eliminating (by mutation) the activity of one or both of the other permeases. The consequences of this approach may be to eliminate possible coordinated inter-

actions among systems. With AIB it becomes possible to transport an amino acid through the G system and simultaneously to monitor the effects on the activities of the N and the B systems.

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