Transport of L-Glutamine by *Neurospora crassa*

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The transport of the neutral amino acid L-glutamine by *Neurospora crassa* occurred by means of two permeases: the neutral-specific permease and the general permease. For both transport systems, accumulation of L-glutamine was a saturable process that occurred against a concentration gradient and was dependent upon metabolic energy, suggesting that accumulation is a carrier-mediated active transport process. The transported glutamine was incorporated into cellular protein. The kinetic values $K_m$ and $V_{max}$ were determined for glutamine transport by both systems. The glutamine analogues methionine sulfoximine and $\gamma$-glutamyl hydroxamate appear to be transported by the same permeases that transport glutamine.

In addition to determining the physiological properties of glutamine transport, we examined whether the pmn;pmb;pmg;nit-2 strain could transport this amino acid. This strain is defective for constitutive amino acid transport and for the ability to utilize amino acids as sole nitrogen sources, with the exception of glutamine. No glutamine transport was detected, suggesting that glutamine utilization by this strain is not due to its ability to transport this amino acid.

INTRODUCTION

Considerable attention has been focused on the phenomenon of nitrogen metabolism and its regulation in micro-organisms (Magasanik, 1982; Marzluf, 1981). Several processes have been identified in the fungi as being under nitrogen metabolite repression and, in many cases, the amino acid glutamine has been implicated as the effector molecule (Arst & Cove, 1973; Chang & Sorger, 1976; Dantzig et al., 1978; Dubois et al., 1977; Dunn-Coleman et al., 1979; Dunn-Coleman & Garrett, 1980; Espin et al., 1979; Facklam & Marzluf, 1978; Hynes, 1974; Pateman et al., 1973; Pateman & Kinghorn, 1976; Premakumar et al., 1979; Reinert & Marzluf, 1975; Vichido et al., 1978). Each of these studies has in common the assumption that before exogenously supplied glutamine can exert its regulatory effects, the repressor must first gain entry into cells. The basic parameters for the transport of glutamine and its analogues are of interest to investigators using these molecules as repressors of nitrogen-regulated activities.

The route of glutamine entry is of added interest because a strain of the fungus *Neurospora crassa* that is defective in both amino acid transport and nitrogen regulation retains the ability to utilize glutamine as a sole nitrogen source. We previously determined that the pmn;pmb;pmg strain, which is defective for the neutral-aromatic, the basic and the general amino acid transport systems, utilized amino acids as its sole nitrogen source through the induction of an extracellular deaminase that degrades the amino acid to its ammonium ion and keto acid components (DeBusk & Ogilvie, 1982, 1984a). Induction of this enzyme requires the product of the nit-2 locus, which is believed to be the major nitrogen regulatory locus for *N. crassa* (Reinert & Marzluf, 1975). The pmn;pmb;pmg;nit-2 strain cannot induce the deaminase and cannot utilize amino acids as nitrogen sources (DeBusk & Ogilvie, 1984b). Glutamine, however, can be

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utilized as a nitrogen source by this strain, thereby suggesting that this amino acid or its metabolites may enter cells by a mechanism other than the deaminase mechanism described and, presumably, by a mechanism other than by transport via the three known permeases. In order to determine whether another distinct catabolic mechanism is operating in the case of glutamine utilization, it is first important to establish that glutamine behaves as a typical neutral amino acid and is not entering cells via a glutamine-specific system. We therefore investigated the ability of glutamine and its analogues to be transported by the neutral-specific and the general amino acid permeases of N. crassa and explored the basic physiological and kinetic parameters of glutamine transport by each permease. Additionally, we examined the ability of the pmn;pmb;pmg;nit-2 strain to transport intact glutamine under conditions where glutamine was capable of serving as a sole nitrogen source.

METHODS

Strains. The wild-type Neurospora crassa strain 74a (FGSC 988) was obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, Calif., USA. The pmn, pmg and pmn;pmg strains are defective for the neutral amino acid transport system, the general amino acid transport system, and both the neutral and general systems, respectively. The pmn;pmb strain is deficient in the neutral amino acid and the basic amino acid transport systems. The pmn;pmb;pmg;nit-2 strain is defective for each of these three constitutive amino acid permeases and for the major nitrogen regulatory locus of N. crassa (DeBusk & Ogilvie, 1984b). The mutant strains are isogenic with the wild-type and may be obtained from the authors.

Growth of cultures. Vegetative cultures were grown in 125 ml Erlenmeyer flasks containing minimal medium (1 × Vogel medium N; Vogel, 1964) plus 2% (w/v) sucrose solidified with 1-5% (w/v) agar as described by DeBusk & DeBusk (1980).

Cultures used for transport assays were harvested aseptically into cold sterile glass distilled water, filtered, and stored at 4 °C in an ice bath as previously described (DeBusk & DeBusk, 1980).

Glutamine accumulation assays. Assays were done at 25 °C in sterile 125 ml Erlenmeyer flasks containing minimal medium with KNO₃ as the nitrogen source rather than the standard NH₄NO₃. l-[U-¹⁴C]Glutamine was used at 0-02 μCi (0-74 kBq; 0-035 μmol) ml⁻¹ and l-[U-¹⁴C]lysine at 0-005 μCi (0-18 kBq; 0-01 μmol) ml⁻¹. Where the effect of glutamine analogues on glutamine transport was examined, the analogues were included in the incubation medium at a concentration of 350 μM or 3500 μM which is 10-fold or 100-fold greater, respectively, than the concentration of glutamine. Assays were initiated by the addition of conidia (vegetative cells) to a final concentration of 0-1 mg dry wt per ml of incubation medium. The flasks were shaken gently in a reciprocal shake-water bath. Portions (5 ml) were removed with a repeating syringe at 30 min intervals over a 2 h period, filtered onto glass fibre filters, rinsed three times with ice water, dried, and counted as previously described (DeBusk & DeBusk, 1980).

The extent of incorporation of glutamine into protein at any given time was determined by depositing a duplicate 5 ml sample into 10 ml 10% (w/v) TCA. After a period of at least 20 min, the TCA samples were filtered, washed, dried and counted as usual. The extent of 'free' label within cells was determined as the difference between the total label accumulated, as represented by the untreated sample, and the amount of label incorporated into protein (TCA-insoluble).

To determine whether glutamine transport was an active transport process, the effect of the metabolic inhibitors 2,4-dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone on glutamine transport by the wild-type strain was examined. Inhibitor was present at the initiation of the transport assay or added 60 min after the assay was initiated. The final concentration of inhibitor was 0-5 mM.

Determination of concentration gradient. Conidia were incubated under standard transport assay conditions for 8 min at which time duplicate samples were either filtered immediately or delivered into 10 ml 10% TCA and subsequently filtered after 20 min incubation. The external concentration of glutamine was known, and the internal concentration was calculated using the radioactivity present as free glutamine (as described above), a known specific activity, and a known packed cell volume. The procedure is described in detail by DeBusk & DeBusk (1980).

Kinetic analysis. The standard transport assay conditions were used with the exception that a range of glutamine concentrations was used, and the assays were done at 35 °C to maximize the amount of radioactivity transported. Samples were removed at 2 min intervals over an 8 min incubation period. The transport process was first-order under these conditions, and the velocity of transport was calculated from the initial rates determined at each glutamine concentration. The kinetic constants K_m and V_max were derived by computer analysis using the Hofstee transformation of the Michaelis–Menten equation as suggested by Dowd & Riggs (1965). Further details of the assays and the methods for data analysis are given in DeBusk & DeBusk (1980).
For inhibition kinetic analysis, L-[U-14C]phenylalanine or L-[U-14C]lysine was present at either 0.02 μCi (0.01 μmol) ml⁻¹ or 0.02 μCi (0.001 μmol) ml⁻¹. Nonradio-labelled L-glutamine was present at various concentrations in the range 0–300 μM. The velocity of transport was calculated from initial rate determinations as described above, the data were plotted as suggested by Dixon (1953), and the kinetic constant, Ki, was determined by computer analysis of these data. Details of the data analysis are presented in DeBusk & DeBusk (1980) and in Ogilvie-Villa et al. (1981).

Metabolic fate of transported glutamine. Incorporation of transported glutamine into cellular protein was determined as described above for glutamine accumulation assays. The extent of metabolic conversion of transported glutamine during the initial rate period of the kinetic assays was determined by extraction of transported radio-labelled glutamine followed by TLC. Conidia were incubated in the presence of L-[14C]glutamine at a final concentration of 0.03 μCi (111 kBq; 0.035 μmol) ml⁻¹ under standard transport assay conditions. Samples were filtered onto nitrocellulose filters at intervals corresponding to those used for the initial rate determinations and washed with 3 vols distilled water. The glutamine was extracted in ethanol by the method of Espin & Mora (1978). The cell-free extracts were lyophilized and spotted onto pre-coated cellulose sheets (Merck) and subjected to ascending TLC with 1-butanol/acetic acid/water (4:1:1, by vol.) as the solvent. Radiolabel was localized by placing a sheet (about 12.7 × 17.8 cm) of No-Screen medical X-ray film NS-5T (Eastman Kodak) against the chromatogram, which was then kept at −70 °C for 7 d and subsequently developed by standard techniques for X-ray film.

Developmental studies. Conidia were incubated at 35 °C under standard growth assay conditions for 72 h in media containing NNV (no nitrogen Vogel's medium; Vogel, 1964) plus 15 mM-L-glutamine as the nitrogen source and 2% (w/v) sucrose as the carbon source. At 12 h intervals the cultures were filtered, rinsed three times with 1 × NNV, and resuspended in 25 ml 1 × NNV containing L-[14C]glutamine at 2 μCi (74 kBq; 5 μmol) ml⁻¹. Cultures were incubated at 35 °C with gentle shaking for 10 min, filtered, rinsed as above, placed in vials, and counted in a liquid scintillation counter. The total glutamine accumulated (nmol) was calculated by correcting for both nonspecific binding and quenching at each time period sampled. The extent of nonspecific binding was determined independently for both strains by incubating duplicate samples in incubation medium containing the metabolic inhibitor 2,4-dinitrophenol (0.5 mM). The extent of quenching was determined by counting a known amount of radiolabel in the presence and absence of culture at each time period for each strain.

Chemicals. All radio-labelled chemicals were purchased from Schwarz/Mann, Cambridge, Mass., USA. Nonradio-labelled chemicals, including the metabolic inhibitors and glutamine analogues, were obtained from Sigma and were of the highest purity available. All amino acids were L-stereoisomers.

RESULTS AND DISCUSSION

Physiological properties of L-glutamine transport

Transport of L-glutamine by N. crassa was examined in the wild-type strain and in strains defective for the transport of neutral amino acids. Both the neutral amino acid-specific (N) permease and the general amino acid (G) permease are capable of transporting neutral amino acids in this organism (DeBusk & DeBusk, 1980). The relative contribution of each permease to the total glutamine accumulation by the wild-type strain was investigated through the use of mutant strains defective for one or both of these permeases. The pmn strain lacks G system activity and was used to study glutamine transport by the N system. Similarly, the pnm strain lacks N system activity and was used to examine glutamine transport by the G system. Both the N and G systems were capable of transporting L-glutamine; no transport was observed in the pnm; pmn strain lacking both of these activities (Fig. 1).

Radiolabel from glutamine was recovered as both TCA-insoluble and TCA-soluble material (data not shown), the former representing incorporation into cellular protein.

Since glutamine transport occurs by means of the N and G systems and transport by each of these systems is known to be an active process, we expected glutamine transport also to be dependent upon metabolic energy (DeBusk & DeBusk, 1965, 1980). This expectation was confirmed by examining glutamine transport by the wild-type strain in the presence of the metabolic inhibitor 2,4-dinitrophenol (Fig. 2). Similar findings were observed when glutamine transport by each system was examined using the appropriate pnm or pmn strain and when the inhibitor cyanide m-chlorophenylhydrazone was used (data not shown). In all cases both glutamine accumulation and retention were dependent upon metabolic energy (Fig. 2).

The N and G systems were then examined, using the appropriate mutant strains, for the ability to transport glutamine against a concentration gradient. The ratio of internal to external
Fig. 1. Accumulation of L-[14C]glutamine by wild-type *N. crassa* and by strains defective for the transport of neutral amino acids. The *pmn;pmg* strain is defective for both the neutral (N) and the general (G) permeases, the *pmg* strain is defective for G permease activity and retains N permease activity, and the *pmn* strain is defective for N permease activity and retains G permease activity.

Fig. 2. Accumulation of L-[14C]glutamine by wild-type *N. crassa* in the presence of the metabolic inhibitor 2,4-dinitrophenol (2,4-DNP; 0.5 mM). The inhibitor was added either at the time the transport assay was initiated or 60 min after initiation.

The concentration established after 8 min incubation in the presence of glutamine was $100:1$ for the N system and $10:1$ for the G system (data not shown).

The finding that glutamine is transported by one or more active transport systems is in keeping with the properties of glutamine transport described for both prokaryotes and mammalian systems (Benjamin *et al.*, 1980; Chapman & Meeks, 1983; Hunt & Hong, 1983; Kilberg *et al.*, 1980; Kovacevic *et al.*, 1979; Masters & Hong, 1981; Plate, 1979; Reynolds *et al.*, 1982; Simpson, 1980; Weiner & Heppel, 1971; Willis *et al.*, 1975). Unlike the situation in prokaryotes, however, there was no evidence for a glutamine-specific transport system.

**Transport of glutamine analogues**

Methionine sulfoximine and γ-glutamyl hydroxamate are commonly used as glutamine analogues in nitrogen regulatory studies. Methionine sulfoximine, in particular, has been useful for demonstrating the role of glutamine as the effector of nitrogen metabolite repression in *N. crassa* (Espin *et al.*, 1979; Premakumar *et al.*, 1980). Studies with *Escherichia coli* and *Salmonella typhimurium* have suggested that these compounds, in addition to their interference with metabolic reactions of glutamine, can also interfere with the entry of glutamine into cells by sharing one or more common transport systems (Betteridge & Ayling, 1975; Weiner & Heppel, 1971). We investigated whether in *N. crassa* these analogues were transported by the same permeases that handle glutamine by examining their ability to interfere with glutamine transport by either permease. Interference with glutamine transport by the N system was monitored in the *pmg* strain; the *pmn* strain was used to examine transport by the G system. A concentration of analogue 10-fold greater than that of glutamine interfered with glutamine
Neurospora crassa glutamine transport

Fig. 3. Ability of the glutamine analogues L-methionine sulphoximine (a, b, c) and γ-glutamyl hydroxamate (a', b', c') to interfere with the transport of L-[14C]glutamine by the neutral (N) and general (G) systems and of L-[14C]lysine by the basic (B) system. (a, a') Glutamine transport by the pmg strain which retains N permease activity for the transport of neutral amino acids; (b, b') glutamine transport by the pmn strain which retains G permease activity for the transport of neutral amino acids; (c, c') lysine transport by the pmn;pmg strain which retains B permease activity for the transport of basic amino acids: ○, control; □, 350 μM-analogue (10 x the concentration of glutamine); ■, 3500 μM-analogue.

transport by each permease and a 100-fold greater concentration interfered to an even greater extent (Fig. 3). At the lower concentration γ-glutamyl hydroxamate was a more effective inhibitor of both permeases than was methionine sulphoximine. The inability of these analogues to interfere significantly with the transport of lysine, a basic amino acid, suggests they have no nonspecific effects upon amino acid transport (Fig. 3).

**Kinetic properties of glutamine transport**

Both systems exhibited Michaelis-Menten saturation kinetics with respect to glutamine transport. Kinetic analysis yielded an apparent affinity constant (Kₘ) of 268.4 μM and a maximum velocity (Vₘₐₓ) of 2.39 nmol min⁻¹ (mg dry wt)⁻¹ for glutamine transport by the N system and a Kₘ of 13.0 μM and a Vₘₐₓ of 0.11 nmol min⁻¹ (mg dry wt)⁻¹ for the G system (Fig. 4).

**Substrate inhibition of the general permease by glutamine**

DeBusk & DeBusk (1980) reported that the activity of the G system decreases when substrate concentrations exceed approximately 100 μM, a phenomenon referred to as substrate inhibition. To determine whether either of the amino acid transport systems capable of
transporting glutamine was subject to substrate inhibition by this amino acid, we examined the velocity of transport for both systems as a function of the concentration of glutamine presented to cells. Only the G system exhibited substrate inhibition with respect to glutamine transport (data not shown). These data are consistent with the ability of a number of other amino acids to inhibit G system activity but not N system activity.

Inhibition kinetic analysis

Since glutamine is a potential effector for nitrogen metabolite repression and amino acid transport is subject to such control (Facklam & Marzluf, 1978; DeBusk & DeBusk, 1980), we were interested in determining whether glutamine could inhibit transport of amino acids by either of the systems for which it is a substrate. The pmg strain was used to examine the effect of glutamine on the ability of the N system to transport L-phenylalanine. To examine the effect of glutamine on transport by the G system, the pmn;pmb strain defective for lysine transport through the basic (B) system and of glutamine transport through the N system was used. The ability of glutamine to inhibit transport of these substrates competitively would suggest common or overlapping binding sites as a preparatory step in the transport of amino acids by either the N or G systems. Noncompetitive inhibition, however, would suggest that the inhibitor acted at a site distinct from the substrate binding site. By inhibition kinetic analysis it was determined that glutamine interacted competitively with both lysine and phenylalanine (Fig. 5). No evidence of a distinct site for glutamine inhibition was detected over the concentration range examined.

Transport of L-glutamine during early development

We previously observed that the pmn;pmb;pmg;nit-2 strain could utilize glutamine as a sole nitrogen source (DeBusk & Ogilvie, 1984). This strain is defective for constitutive amino acid
transport because of the presence of the permease mutations and, because of the nit-2 mutation, it is unable to produce the extracellular deaminase that removes the α-amino nitrogen from the amino acid. One possible explanation for the ability of this strain to utilize glutamine is the existence of a previously undetected transport system that is capable of transporting the intact molecule. Therefore, we examined glutamine transport by the pmn;pmb;pmg;nit-2 strain under standard 72 h growth conditions. Transport was detected in the nit-2 strain but not in the pmn;pmb;pmg;nit-2 strain (Fig. 6).

It would appear, therefore, that glutamine transport occurs by means of the N and G systems with no evidence for a glutamine-specific system and that the ability of the pmn;pmb;pmg;nit-2 strain to utilize glutamine as a nitrogen source is not due to its ability to transport the intact molecule. These findings lead us to consider mechanisms by which glutamine could be degraded externally before entry into cells. Two possible candidates are an external glutaminase that would convert glutamine to glutamic acid plus ammonium and a transaminase that converts glutamine to γ-ketoglutaramide followed by further conversion to ammonium plus α-ketoglutarate through the action of an ω-amidase. The degradation of glutamine via the ω-amidase pathway occurs in other systems (Cooper & Meister, 1977). The critical enzymes have been detected in N. crassa as well (Monder & Meister, 1958), and Espin et al. (1979) proposed a metabolic sequence whereby glutamine could be both degraded and regenerated through such a pathway. Either mechanism would supply the required ammonium, and we are presently testing for the production of the critical enzymes by the pmn;pmb;pmg;nit-2 strain.

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