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Alternate Mechanism for Amino Acid Entry into *Neurospora crassa*: Extracellular Deamination and Subsequent Keto Acid Transport

RUTH MASON DEBUSK,* DAVID T. BROWN, A. GIB DEBUSK, AND ROBERT D. PENDERGHAST

Department of Biological Science, Florida State University, Tallahassee, Florida 32306

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The growth of the *pm nbg* mutant strain of *Neurospora crassa* was inhibited by the amino acid analog *para*-fluorophenylalanine despite the fact that none of the three constitutive amino acid permeases is functional in this strain. This observation led to the detection of both a deaminase which was released into the growth medium in response to *para*-fluorophenylalanine and a keto acid transport system which allowed entry of the resulting keto acid into the cell. The transported keto acid was recovered in cellular protein, suggesting its regeneration as the amino acid. The cooperative activity of these two systems represents an additional mechanism for the intracellular accumulation of amino acids, which is distinct from the known amino acid permeases.

Amino acid transport in the ascomycete *Neurospora crassa* is accomplished by means of the following three constitutive amino acid permeases: (i) the neutral permease system, which transports the class of amino acids with neutral aliphatic or neutral aromatic side chains; (ii) the basic permease system, which is specific for the class of amino acids whose side chains give net positive charges to the molecules; and (iii) a general permease system, which has broad specificity and can transport both the neutral and basic classes of amino acids (6, 7, 23). Each of these systems is under genetic control, and mutants have been isolated by selecting for strains which could grow in the presence of a particular amino acid analog as a result of a lesion in one of the transport systems (17, 22, 23, 26, 30). The triple permease-negative mutant *pm nbg* has been shown to be essentially devoid of amino acid transport activity during the early stages of development (22). Curiously, however, this strain is not completely resistant to the growth-inhibiting effect of *para*-fluorophenylalanine (FPA), an analog of the naturally occurring amino acid phenylalanine. This residual susceptibility to growth inhibition prompted us to investigate the possibility that one or more additional systems were operating for permeation of amino acids and their analogs. We describe here the cooperative efforts of an extracellular deaminase and a keto acid transport system which result in the entry of the keto analog into cells, where presumably the analog is restored to the amino acid form, which possesses growth-inhibiting properties. This report of a keto acid-specific transport system represents the first such observation in *N. crassa*.

MATERIALS AND METHODS

Strains. The wild-type strain 74a (FGSC 988) was obtained from the Fungal Genetic Stock Center, Humboldt State University, Arcata, Calif. The *pm n*, *pm ng*, and *pm nbg* strains are isogenic with the wild type. The original *pm nbg* mutant was constructed in this laboratory, and we deposited it with the Fungal Genetic Stock Center (FGSC 2606).

Growth of cultures. Cultures were grown on Vogel medium N (H. J. Vogel, Microb. Genet. Bull. 13:42-43, 1956) containing 2% sucrose and solidified with 1.5% agar as previously described (7).

Liquid growth assays. Growth assays were performed in 125-ml Erlenmeyer flasks containing 25 ml of Vogel medium N plus 2% sucrose and supplements as indicated below. In those experiments where we examined the effect of removing ammonium ions on a physiological process, the NH_4NO_3 of Vogel medium N was replaced with equimolar KNO_3.

Each growth flask was inoculated with 2 drops of a very dilute conidial suspension and incubated without shaking at 35°C under constant illumination. Mycelial pads were harvested with a glass rod and dried to constant weight. If the growth media were to be analyzed for deaminase activity, they were filtered through glass fiber filters (Whatman GF/A) after removal of the mycelial pads, and the filtrates were dialyzed extensively against distilled water.

Synthesis and chromatographic analysis of [^{14}C]FPP. The keto acid analog *para*-fluoro[^{14}C]phenylpyruvate ([^{14}C]FPP) was synthesized from DL-[^{14}C]FPA by a modification of the Meister procedure (19). A 10-ml reaction mixture containing 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.5), 50 μCi of [^{14}C]FPA

(Schwarz/Mann, Orangeburg, N.Y.), 2 mg of *Crotalus adamanteus* L-amino acid oxidase (3 U/mg; Worthington Diagnostics, Freehold, N.J.), and 0.03 mg of catalase (3,000 U/mg; Mann Research Laboratory, New York, N.Y.) was incubated at 37°C under constant oxygenation for 6 h. Portions (approximately 50 μl) of octyl alcohol were added periodically throughout the incubation to prevent foaming. Protein was removed by passing the incubation mixture through an Amicon UM-2 ultrafiltration membrane (Amicon Corp., Lexington, Mass.). The mixture was then passed over an AG 50W-X2 cation exchange column (sodium form; Bio-Rad Laboratories, Richmond, Calif.). Fractions were eluted with water. A single peak of radioactivity was obtained, and peak fractions were pooled. FPP was identified by ascending thin-layer chromatography on precoated cellulose sheets (E. Merck AG, Darmstadt, Germany), using a 1-butanol–acetic acid–water (80:20:20) solvent. Phenylalanine, phenylpyruvate, and FPA served as standards since FPP was not available commercially. Amino acids were visualized with ninhydrin; keto acids were visualized with 1% $FeCl_3$. The putative FPP reaction product had an R_f value nearly identical to the R_f value of the keto acid phenylpyruvate, reacted strongly with $FeCl_3$ (as is characteristic of keto acids), and did not react with ninhydrin, which is specific for amino acids. The product was identified further as a keto acid by its quantitative reaction with phenylhydrazine. Preparation of FPP by this method yielded less than 1% contamination with the amino acid form.

Transport assays. The methods used for the conidial transport assays have been described previously by DeBusk and DeBusk (7). Conidial assays were performed at 25 or 35°C, as indicated in the figure legends. The amount of radiolabeled substrate incorporated into macromolecules after any given incubation time was determined by placing 5-ml samples into 10 ml of 10% (wt/vol) trichloroacetic acid, filtering after 20 min onto nitrocellulose filters, drying, and counting as usual.

For developmental studies, conidia were incubated for the desired length of time in Vogel medium N containing 1% D-glucose and either NH_4NO_3 or KNO_3. Cultures were shaken gently at 35°C in a constant-temperature reciprocal shaking water bath. At the end of this incubation, the cells were filtered onto nitrocellulose filters (type AA; 0.8 μm; diameter, 2.5 cm; Millipore Corp., Bedford, Mass.) and suspended to a concentration of 0.1 mg (dry weight) per ml in Vogel medium N containing radiolabeled substrate. Portions were sampled as previously described (7). Non-radiolabeled amino acids and keto acids were present at final concentrations of 2 mM where indicated. All radiolabeled amino acids were of the L stereoisomer, with the exception of DL-FPA. All of these amino acids were labeled uniformly and were obtained from New England Nuclear Corp., Boston, Mass., or Schwarz/Mann.

Deaminase assay. The assay used to detect deaminase activity was a modified version of the assay described by Woodward and Cirillo (33); 1 μl of 0.5 M HEPES buffer (pH 7.5), 5 μl of L-[^{14}C]phenylalanine (460 μCi/μmol; 10 μCi/ml), and 5 μl of test solution (which had been dialyzed three times against distilled water) were placed in a disposable microtiter plate and incubated at 35°C for 2 to 4 h as indicated in the figure legends. The incubation was terminated by pipetting the reaction mixture into a test tube containing 1 ml of distilled water and 2 drops of concentrated hydrochloric acid. Ethyl acetate (2 ml) was added to the mixture, which was then blended with a Vortex mixer for 30 s. The mixture formed an organic phase and an aqueous phase, and 0.5 ml of each phase was removed and counted by liquid scintillation.

Isolation of the deaminase. Media were collected from stationary-phase cultures of strain *pm nbg* grown in the presence of NH_4NO_3 and 0.2 mM FPA. The procedure used to isolate the deaminase from the culture media was a modification of the method of Aurich et al. (1). The mycelia were removed by filtration through Whatman no. 1 filter paper, and the filtrate was concentrated with an Amicon ultrafiltration unit fitted with a PM-30 filter. The retained proteins were taken to 80% saturation with $(NH_4)_2SO_4$ (25°C) and centrifuged at 10,000 × *g* for 20 min. The pellet was discarded, and the supernatant was taken to 100% saturation (25°C) and centrifuged as described above. The pellet was suspended in phosphate-buffered saline (0.01 M Na_3PO_4–0.15 M NaCl, pH 7.6, containing 10% glycerol) and then dialyzed extensively against this buffer; 2 ml of the 80 to 100% $(NH_4)_2SO_4$ fraction was placed on a Sephacryl S-200 column (2 by 130 cm) and eluted with phosphate-buffered saline. The flow rate was 10 ml/h.

Gel electrophoresis was performed by the method of Laemmli (15), using a 7% polyacrylamide non-dissociating discontinuous tube gel electrophoresis system in the absence of mercaptoethanol. The proteins were visualized by using Coomassie blue or a modification of the Hayes-Wellner specific activity stain (13), which contained 0.1 M HEPES buffer (pH 7.5), 0.35 mg of phenazine methosulfate per ml, 0.35 mg of 2,3,5-triphenyltetrazolium chloride per ml, and 0.001 M L-leucine or L-phenylalanine. This latter stain is specific for L-amino acid oxidases. The gel was incubated in the dark for 1 to 2 h, after which the protein bands appeared bright green against a yellow background.

RESULTS

Growth inhibition of strain *pm nbg* by FPA. FPA is a neutral amino acid analog, which exerts its effect by being inserted into proteins in place of the natural amino acid phenylalanine (20). We examined the growth of strain *pm nbg* in the presence of FPA at 25 and 35°C under conditions of nitrogen sufficiency (NH_4NO_3) and nitrogen insufficiency (KNO_3). Since this strain is defective for all three of the constitutive amino acid permeases, no significant growth inhibition was expected. Figure 1 shows that, although strain *pm nbg* was more resistant to growth inhibition by FPA than the wild-type strain, the presence of this analog was clearly deleterious. The FPA effect was further enhanced by the removal of ammonium ions from the growth medium. The results shown in Fig. 1 were ob-

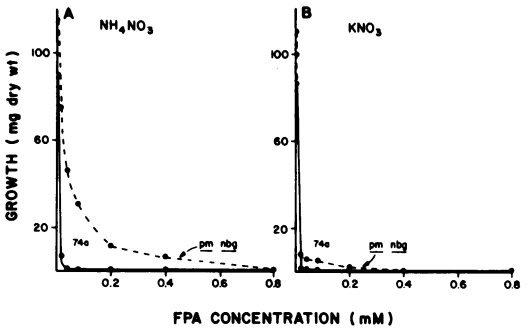

FIG. 1. *Growth of N. crassa wild-type strain 74a and mutant strain pm nbg in the presence of the amino acid analog FPA. The nitrogen source was NH_4NO_3 (A) or KNO_3 (B). The temperature was 35°C, and the duration of the growth assay was 72 h.*

tained at 35°C, but the same phenomena were observed at 25°C.

Transport of FPA by the *pm nbg* mutant. Since FPA exerts its effect at the protein synthesis level, this analog must enter cells in order to inhibit growth. As a neutral amino acid, FPA would be expected to enter via the two neutral amino acid permeases (neutral and general permeases). We confirmed this expectation by monitoring FPA transport in the wild-type strain, the *pm n* strain (neutral permease defective), and the *pm ng* strain, which lacks both the neutral permease and the general permease (Fig. 2).

Since FPA was inhibitory to the *pm nbg* mutant, we considered the unlikely possibility that this strain was capable of transporting FPA. We examined the ability of strain *pm nbg* to transport phenylalanine and the phenylalanine analog FPA during the first 24 h of development, a period in which cells are very active physiologically. Cells were grown in Vogel medium N containing KNO_3 and 1% D-glucose in the presence or absence of 0.4 mM FPA (or L-phenylalanine), washed, and exposed to radiolabeled FPA or L-phenylalanine for 60 min. The ammonium-free KNO_3-containing medium was chosen to enhance any residual general permease system activity which might have been present. No transport of either the natural amino acid or its analog was observed under any conditions in cultures sampled at 0, 6, 12, 18, and 24 h (data not shown; representative data for the zero-time sample are shown in Fig. 3). Thus, it appears that FPA was not transported by strain *pm nbg*.

Evidence for keto acid transport in the *pm nbg* mutant. Thus, it appeared that FPA must enter cells by some route other than an amino acid transport system. Previous assays by Brockman et al. (3) suggested the existence of a keto acid transport system. We investigated the possibility that the amino acid analog FPA was converted to a keto acid and transported into cells in the keto form. The radiolabeled keto acid FPP was synthesized from FPA. We as-

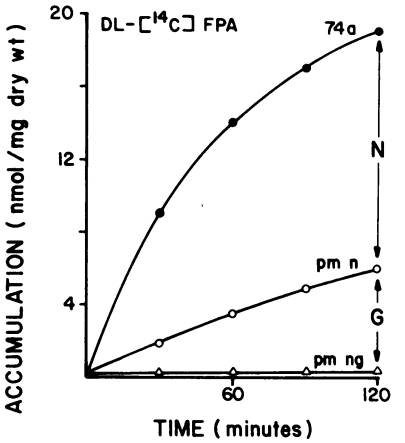

FIG. 2. *Transport of FPA by the wild-type, pm n, and pm ng strains of N. crassa. The pm n strain is defective for the neutral (N) permease, and the pm ng strain is defective for the neutral and general (G) permeases. The nitrogen source was KNO_3, the FPA concentration was 100 μM, and the temperature was 25°C.*

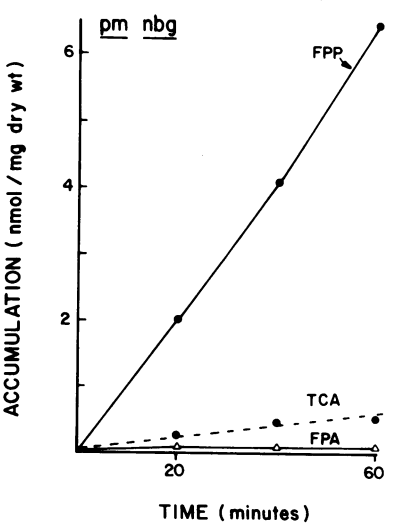

FIG. 3. *Conidial transport of the keto acid [^{14}C]FPP and the amino acid [^{14}C]FPA by the pm nbg strain defective for the neutral, basic, and general permeases. The transported [^{14}C]FPP recovered in macromolecules was determined as trichloroacetic acid-insoluble material (TCA). The nitrogen source was NH_4NO_3, the temperature was 35°C, the FPP concentration was 50 μM, and the FPA concentration was 100 μM.*

sayed the ability of strain *pm nbg* to transport these two compounds and found that FPP was transported and FPA was not (Fig. 3). Radiolabel from the transported FPP was detected in intracellular macromolecules, presumably protein, suggesting that the keto acid was restored to the amino acid form upon entering cells. A chromatographic analysis of the free radiolabel (trichloroacetic acid soluble) confirmed that the FPP given to cells could be recovered internally as FPA (data not shown).

FPP transport activity was reduced greatly in the presence of either the metabolic inhibitor sodium azide or an excess of another keto acid, such as phenylpyruvate or alpha-ketoisocaproate (Fig. 4). The amino acids phenylalanine and leucine failed to inhibit FPP transport, suggesting that FPP may be transported by an active transport system specific for keto acids.

Detection of an extracellular deaminase activity. In order for FPP uptake to be physiologically significant in explaining the growth inhibition of strain *pm nbg* by FPA, a mechanism must exist for the conversion of FPA to FPP. A chromatographic examination of the growth medium of strain *pm nbg* grown with FPA revealed the presence of the keto acid FPP. The medium was then examined for the presence of deaminase activity. An extracellular deaminase was released into the growth medium in response to different concentrations of FPA (Fig. 5). Although deaminase activity reached the same maximal level irrespective of the nitrogen source, the maximum was attained at an earlier time in the absence of ammonium ions (Fig. 6). The earlier release of deaminase activity in medium containing KNO_3 correlated with the increased growth inhibition observed with this medium (Fig. 1).

The deaminase was isolated from the FPA-containing growth media, partially purified, and subjected to polyacrylamide disk gel electrophoresis under non-dissociating conditions. Identification of the protein band which corresponded to the deaminase was achieved by using an activity stain specific for L-amino acid oxidases and Coomassie blue (Fig. 7).

Sparing of FPA inhibition by phenylpyruvate. If FPA entered strain *pm nbg* by means of deamination and subsequent keto acid transport, it follows that an excess of a keto acid in the growth medium should have prevented the inhibitory effects of FPA. Figure 8 shows the ability of the keto acid phenylpyruvate to prevent growth inhibition of strain *pm nbg* by FPA.

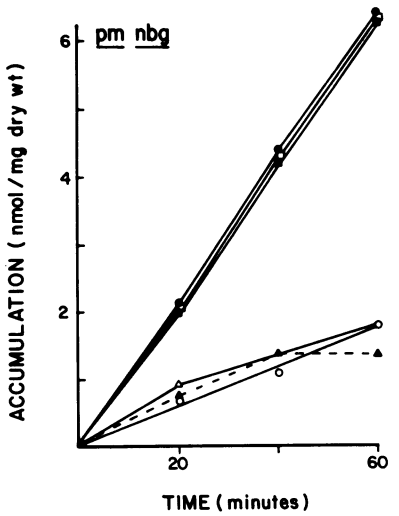

FIG. 4. *Conidial transport of the keto acid FPP in the presence of an amino acid (L-leucine or L-phenylalanine), a keto acid (alpha-ketoisocaproate or phenylpyruvate) or the metabolic inhibitor sodium azide. Symbols: ●, [^{14}C]FPP; □, [^{14}C]FPP plus L-leucine; ■, [^{14}C]FPP plus L-phenylalanine; △, [^{14}C]FPP plus sodium azide; ▲, [^{14}C]FPP plus alpha-ketoisocaproate; ○, [^{14}C]FPP plus phenylpyruvate. The nitrogen source was NH_4NO_3, and the temperature was 35°C. The [^{14}C]FPP concentration was 50 μM, the amino acid concentration was 2 mM, the keto acid concentration was 200 μM, and the sodium azide concentration was 0.5 mM.*

DISCUSSION

We propose the existence of a novel mechanism for the accumulation of intracellular amino acids in a strain of *N. crassa* that is devoid of the normal amino acid transport routes. We believe that amino acids are deaminated initally by an extracellular deaminase, transported into

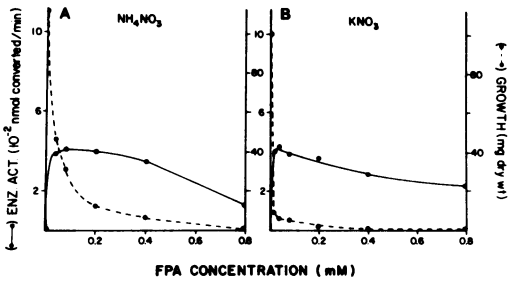

FIG. 5. *Deaminase activity detected in the medium of strain pm nbg grown in the presence of varying concentrations of FPA under nitrogen-sufficient (NH_4NO_3) conditions (A) and nitrogen-deficient (KNO_3) conditions (B). Enzyme activity (ENZ. ACT.) is expressed as nanomoles converted per minute per milliliter of culture medium. The temperature was 35°C, and the duration of the deaminase assay was 3 h.*

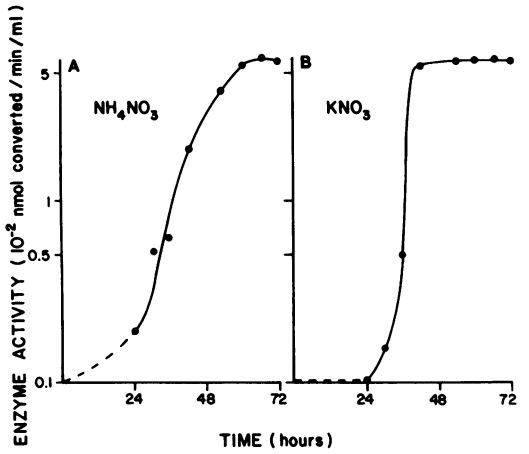

FIG. 6. *Time course of extracellular deaminase production by strain pm nbg grown in medium containing 0.2 mM FPA under nitrogen-sufficient (NH_4NO_3), conditions (A) and nitrogen-deficient (KNO_3) conditions (B). Enzyme activity is expressed as nanomoles converted per minute per milliliter of culture medium. The temperature was 35°C, and the duration of the deaminase assay was 4.5 h.*

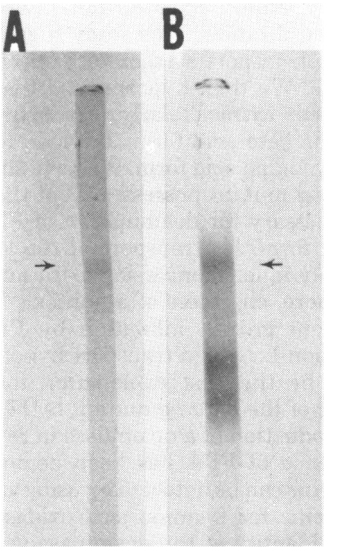

FIG. 7. *Detection of the FPA-induced deaminase with an activity stain specific for L-amino acid oxidase (A) and with Coomassie blue (B). The gel was first stained with the activity stain and subsequently stained with Coomassie blue. A 7% polyacrylamide non-dissociating discontinuous tube gel electrophoresis system was used. The arrows indicate the position of the deaminase on each gel.*

cells as keto acids, and restored to the amino acid forms internally. The investigation of this mechanism was prompted by the observation that the amino acid permease-defective strain *pm nbg* was subject to growth inhibition by the amino acid analog FPA, which it presumably could not transport. We examined several possibilities which could explain this growth inhibition. Since FPA exerts its effect through incorporation into protein in place of the natural amino acid phenylalanine, we assumed that FPA was able to enter strain *pm nbg*. Conceivably, this strain might retain residual activity for one of its permeases or might adapt to the presence of an amino acid or its analog over time and develop the ability to transport that molecule. Our evidence showed strain *pm nbg* was not capable of transporting FPA, suggesting that the activity of an amino acid permease (whether the residual activity of an existing permease or the activity of a newly assembled permease) was not the means of entry for this molecule.

Another explanation also involves adaptation in response to the FPA substrate, but in this instance it is proposed that FPA enters cells in the keto acid form. A previous report from this laboratory showed that growth of amino acid auxotrophs could be obtained by supplementing the growth medium with either the required amino acid or its keto acid precursor (3). Other keto acids interfere with the utilization of the required keto acid by the cells but do not interfere with utilization of the required amino acid;

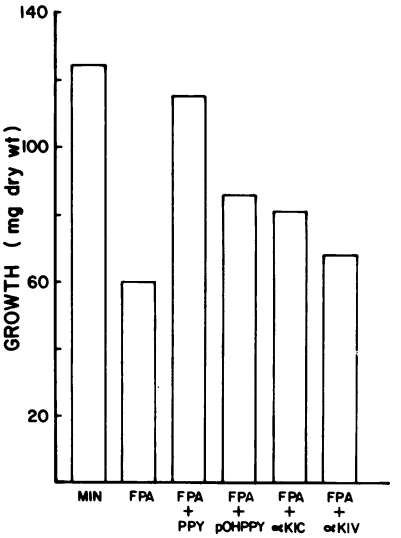

FIG. 8. *Effects of keto acids on the ability of FPA to inhibit growth of strain pm nbg. The nitrogen source was NH_4NO_3, the temperature was 35°C, and the FPA concentration was 0.04 mM. The following keto acids were present at final concentrations of 2.0 mM: phenylpyruvate (PPY), α-ketoisocaproate (αKIC), and α-ketoisovalerate (αKIV). The concentration of the keto acid para-hydroxyphenylpyruvate (pOHPPY) was 1.6 mM. The minimal medium (MIN) control contained no additives. The duration of the growth assay was 72 h.*

conversely, amino acids do not interfere with keto acid utilization. This study suggested that there is a transport system with specificity for keto acids. We then hypothesized that FPA is deaminated extracellularly, transported into cells in the keto acid form, and restored to the inhibitory amino acid form. We have shown that the *pm nbg* mutant possesses all of the components necessary for deamination of FPA to its keto acid form, for transport of the keto acid, and for subsequent conversion to the amino acid. Furthermore, an excess of a nontoxic keto acid can prevent growth inhibition by FPA. This deamination-keto acid transport hypothesis appears to be the best explanation for growth inhibition of the *pm nbg* mutant by FPA.

The production of a deaminase in response to the presence of FPA has been demonstrated. This enzyme can be detected by using an activity stain specific for L-amino acid oxidases. However, the identity of the deaminase with the L-amino acid oxidase known to be produced by *N. crassa* in response to biotin limitation, to the amino acid analog ethionine, or to nitrogen insufficiency (5, 14, 29) has not been established; this is presently under investigation.

The alpha-keto acid transport component of this deaminase-keto acid mechanism is reported here for the first time. Until now, the transport of α-keto acids has been investigated in only a few mammalian systems and in a single bacterium. Mechanisms appear to exist for the accumulation of keto acids by rat intestines (31), by rat brain mitochondria (10, 16), and by cultured lymphoblasts (28). In bacteria, a single system capable of transporting branched-chain α-keto acids has been reported for *Bacillus subtilis* (9). Currently, we are examining the properties of this transport system in *N. crassa*.

The physiological purpose of deaminating an amino acid analog, subsequently transporting the keto acid, and restoring it to the amino form is not clear. In the case of the amino acid analog canavanine, the presence of the analog in the growth medium triggers a detoxifying mechanism (18). We do not feel that the deaminase-keto acid mechanism represents a similar detoxification response since the production of the deaminase does not occur only in the presence of analogs. This enzyme is also produced in response to natural amino acids (Brown and Penderghast, unpublished data).

There is increasing evidence concerning the ability of *Neurospora* to activate different transport mechanisms in response to the extracellular environment, including methionine transport in response to sulfur starvation (21), glucose transport in response to carbon starvation (24, 25), and phosphate transport systems which operate coordinately to preserve a constancy in the phosphate accumulation rate over a wide range of phosphate concentrations (2, 4). All of these mechanisms function in response to environmental solute fluctuations and have the goal of maintaining an optimal intracellular metabolite supply.

The deaminase-keto acid mechanism most clearly parallels an additional mechanism which is triggered when cells are given protein as the sole carbon, nitrogen, or sulfur source. An extracellular protease is elaborated, which can hydrolyze the protein to a mixture of peptides (8, 11, 12). The peptides then may be degraded further and transported into the cells via the amino acid permeases, or they may be transported by a specific peptide transport system and subsequently degraded by intracellular peptidases (27, 32). The cooperative efforts of the protease, transport systems, and intracellular peptidase components result in a supply of intracellular amino acids, which thereby prevents starvation.

The deaminase-keto acid mechanism shares many similarities with the adaptive mechanisms described above. However, with many of these examples starvation for a required nutrient appears to promote scavenging for metabolites which prevents starvation. One might expect carbon starvation to trigger keto acid intake, and, in fact, we have observed marked stimulation of this transport activity under conditions of carbon starvation (Brown, unpublished data). However, starvation does not appear to be essential since the strains in this study were grown in carbon-sufficient medium for all growth assays. Similarly, nitrogen insufficiency might trigger deamination and subsequent ammonium ion transport, with the keto acid being a less important by-product. However, the FPA-induced deaminase is produced at high levels even in a nitrogen-sufficient medium.

Therefore, the physiological purpose of the deaminase-keto acid mechanism is not obvious. However, it is clear that cells are able to adapt to the absence of the normal amino acid permease routes by activating two systems which act in concert to provide the cells with carbon and nitrogen in the form of amino acids. Studies designed to characterize further the keto acid transport system and to compare the FPA-induced deaminase with the known L-amino acid oxidases of *N. crassa* are in progress.

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