SHORT COMMUNICATION

A Mutant of *Aspergillus nidulans* with a Reduced Level of Phenylalanine-binding Protein

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Partial purification of the phenylalanine-binding protein from *Aspergillus nidulans* mycelia, using Triton X-100 extraction and affinity chromatography on L-phenylalanine-CH-Sepharose, indicated that the *p*-fluorophenylalanine-resistant mutant, *fpaD11*, has a significantly reduced level of phenylalanine-binding protein compared with the wild type. This seems to be the cause of the reduced uptake of phenylalanine and consequent *p*-fluorophenylalanine-resistance of this mutant.

INTRODUCTION

Active transport of amino acids in fungi has been the subject of intensive investigations (Hunter & Segal, 1971; Wolfinbarger & Kay, 1973; Robinson *et al.*, 1973; Kinghorn & Pateman, 1975). Selection of mutants resistant to analogues of amino acids has been widely used in the study of active transport of amino acids (Grenson, 1972; Sinha, 1969; Kinghorn & Pateman, 1975). In *Aspergillus nidulans*, 15 loci controlling resistance to the phenylalanine analogue, *p*-fluorophenylalanine, have been identified (Singh & Sinha, 1979). Mutants at the *fpaD* locus have a reduced ability to take up phenylalanine and a number of other amino acids (Sinha, 1969), lose their resistance to *p*-fluorophenylalanine on poor sources of carbon and nitrogen (Singh *et al.*, 1977) and are unable to utilize efficiently amino acids as sole sources of carbon (Singh & Sinha, 1979).

Isolation and purification of an extrinsic protein showing affinity for a given metabolite offers a suitable system for the study of transport mechanisms. Recently, a few such proteins have been isolated and characterized in bacteria and fungi (Wiley, 1970; Stuart & DeBusk, 1971, 1973; Desai & Modi, 1975; Stepien, 1976; Schellenberg & Furlong, 1977).

This paper describes the partial purification of the phenylalanine-binding protein from wild-type mycelium of *A. nidulans*. A significantly reduced level of phenylalanine-binding protein in partially purified extracts from the *fpaD11* mutant seems to confirm the role of this protein in the active transport of phenylalanine into the cell.

METHODS

Strains and growth media. Strains (*riboA1*, *biA1*, and *riboA1*, *biA1*; *fpaD11*) were originally from the Glasgow stock. Media and growth conditions were as described previously (Sinha, 1969). After 10 h growth in minimal medium (MM) supplemented with riboflavin and biotin, mycelium was transferred to fresh MM containing 100 μ M-glycine and [3 H]glycine (1 mCi, 37 MBq) and incubated for another 8 h.

Preparation of mycelial extracts. Mycelium was collected on a cheese cloth and washed thoroughly with distilled water. The mycelial paste (10 g) was stirred for 15 min in 100 ml of extraction buffer (1 % Triton X-100, 10 mM-phenylmethylsulphonyl chloride and 1 mM-dithiothreitol in 0.025 M-citrate/phosphate buffer).

After extraction, the mycelium was filtered under vacuum on Whatman GF/A filters and the filtrate was used for affinity chromatography.

Affinity chromatography. Both CH-Sepharose and AH-Sepharose (Pharmacia) were used to prepare L-phenylalanine-Sepharose matrix. Coupling was performed according to Stepien (1976) with some modifications. Wet washed gel (from 2 g dry wt) was mixed with 6 ml 0.45 M-L-phenylalanine (Merck) and the pH was adjusted to 4.5. Then 2 ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (150 mg ml^{-1}) pH 4.5 was added drop by drop. The mixture was shaken gently on a gyrotory shaker; the pH was measured at 30 min intervals over a period of 8 h and adjusted to 4.5 with dilute HCl. Finally, the mixture was left in the shaker overnight. Coupling of phenylalanine to the gel was checked by estimating spectroscopically the amount of uncoupled phenylalanine; about 10 % of the added phenylalanine was bound to the gel. The gel was packed in a 2.0×1.0 cm column and washed thoroughly with extraction buffer until no phenylalanine could be detected in the eluate by the ninhydrin test. Extract of known radioactivity was loaded on to the gel which was equilibrated with extraction buffer and washed with the same buffer until the radioactivity in the wash was as low as the background radioactivity. The coupled protein was eluted from the gel by adding 6 M-guanidine hydrochloride, pH 6.0, in bovine serum albumin solution ($200 \mu\text{g ml}^{-1}$). All steps were carried out at 4°C . Fractions (0.5 ml) were collected and 0.1 ml of each was assayed for radioactivity in Bray's scintillation mixture (Bray, 1960).

Precipitation of extracts and the eluted fractions with trichloroacetic acid was carried out in order to determine the radioactivity specifically present in proteins; about 80 % of the total radioactivity was found to be present in proteins.

Uptake measurements. Portions (0.5 g) of 16 h-old mycelium were transferred to 250 ml flasks containing 25 ml minimal medium, 500 μM -L-phenylalanine and 0.2 μCi (7.4 kBq) L-[^3H]phenylalanine (2.9 Ci mmol^{-1} , $107 \text{ GBq mmol}^{-1}$). The mycelium was incubated at 37°C in a gyrotory shaker. Samples (5 ml) were taken at 1 min intervals and after rapid filtration under vacuum on pre-dried and weighed Whatman GF/A filter papers, the mycelium was washed with 25 ml cold water. The filters were dried overnight, weighed and assayed for radioactivity in 5 ml Bray's scintillation mixture in a Packard Tri-Carb scintillation counter.

RESULTS AND DISCUSSION

Triton X-100 present in the assay mixture did not affect the rate of uptake of L-phenylalanine by the wild-type mycelium. However, stirring the mycelium with Triton X-100 for 15 min prior to uptake measurements resulted in a complete loss of uptake ability (Fig. 1). Thus, the presence of the detergent has no effect on the function of phenylalanine permease *in vivo*, but stirring with Triton X-100 causes solubilization of the protein involved in the transport of phenylalanine, leading to loss of transport by the mycelium. Triton X-100 has been successfully used for the isolation of methionine-binding protein from *A. nidulans* mycelium (Stepien, 1976).

Affinity chromatography of the crude extract on the L-phenylalanine-CH-Sepharose column resulted in specific binding of the protein to the matrix. On the other hand, the L-phenylalanine-AH-Sepharose matrix did not show any difference between the loaded and the unloaded gel, and the non-specifically bound proteins could be eluted from both gels by washing them with the same volume of starting buffer. The empty gel exhibited non-specific binding equivalent to about 10 % of the specific binding on L-phenylalanine-CH-Sepharose.

The *fpaD11* mutant, which is resistant to *p*-fluorophenylalanine, ethionine and aminotyrosine (Sinha, 1969), was used for binding protein experiments. Specific adsorption to the gel matrix (% of the total counts loaded on the column) of proteins from the mutant and the wild-type were compared. The mutant extract showed only 3 % specific adsorption as against 12 % for the wild-type. Similarly, the elution pattern of proteins from the mutant adsorbed on the L-phenylalanine-CH-Sepharose column exhibited a much less pronounced peak compared with the pattern for the wild-type (Fig. 2). Both of these observations indicated that the *fpaD11* mutant has a significantly reduced level of phenylalanine-binding protein in its mycelium. Attempts to elute the binding protein with L-phenylalanine in order to obtain it in the native form were unsuccessful, probably because the protein is strongly

Fig. 1

Fig. 2

Fig. 1. Initial rate of phenylalanine transport in 16 h-old wild-type *A. nidulans* mycelium; ○, assayed in the absence of Triton X-100; Δ, assayed in the presence of Triton X-100; ●, mycelium treated with Triton X-100 for 15 min before assay.

Fig. 2. Elution pattern of proteins of *A. nidulans* bound to L-phenylalanine-CH-Sepharose gel, eluted with guanidine hydrochloride: ●, wild-type; ○, *fpaD11* mutant.

adsorbed to the matrix. Elution with 6 M-guanidine hydrochloride in albumin (0.2 mg ml⁻¹) as a carrier yielded the best results.

Earlier studies with the *fpaD11* mutant have indicated that it has a major defect in the phenylalanine-transport system because the recombinant *phenA2; fpaD11*, which lacks it, does not grow at all even on optimum phenylalanine, and the *fpaD11* mutant takes up practically no [³H]phenylalanine (Sinha, 1969). The *fpaD* locus may control the synthesis of phenylalanine-binding protein, a lack of which may result in the failure of phenylalanine transport in the mycelium. Comparable results have been obtained by Stepien (1976) with the *nap3* mutant of *A. nidulans* which is defective in the transport of methionine.

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