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Phenylalanine transport in *Aspergillus nidulans*: Demonstration of role of phenylalanine binding proteins

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Crude shock proteins extracted by two stage osmotic shock were further purified by affinity chromatography to obtain ligand (phenylalanine) specific binding protein (phebip) a component of phenylalanine (phe) transport system from wild type and a phe transport mutant *fpaD11* of *Aspergillus nidulans*. A new eluent 0.1 *M* Tris-HCl containing1.5 *N* NaCl and 0.5 *N* Na_2CO_3, *p*H 8 was used during the investigation. The elution profile of mutant phebip exhibited one simple and two compound peaks instead of three simple ones as exhibited by the wild type phebip. SDS-PAGE profile of mutant phebip showed faster electrophoretic mobility than that of wild type one. It is therefore evident that the mutant phebip has reduced molecular mass (M_r) due to deletion of a segment that somehow has bearing on the binding capacity of the active site of phebip. The resultant erosion in the binding capacity of the mutant phebip is in turn responsible for its incapability to stimulate transport of ligand across the plasma membrane.

Transport of metabolites across the membrane has long been recognised as of vital importance[1-3]. Investigations carried out during last few years led to the identification of more than one types of transport mechanisms, viz. active, passive, facilita-tive (active or passive), secondary (symport or anteport) and so on involved in the transport of metabolites[2].

Amino acids have however been found to be transported across the membrane by facilitative transport systems in prokaryotes[4-6] as well as in eukaryotes[7-9]. These transport systems have been increasingly identified as multimeric ones[10-12]. One of the essential components of these transport systems, traditionally named as binding proteins (bips) has been found to be located in periplasm and exhibited very high affinity with one or a group of amino acids both in prokaryotes[4-6,11-14] as well as in lower eukaryotes[7-9,15]. Contrasting reports of these bips to be capable of transporting amino acids under *in vitro* condition as monomeric systems both in prokaryote[4] and lower eukaryote[9] are also not very rare. These bips studied in bacteria have been found to be of low M_r[13-14] while in fungi they have been reported to be of low[8] or high[15] M_r. In either case however they do not show any enzymatic activity[14].

Selection of a mutant resistant to toxic amino acid analog has been widely used to isolate transport mutants[16-18]. Purification and biochemical characterisation of amino acid bips from wild type and transport mutant have helped greatly in the elucidation of the role of bips in the transport of amino acids[6-8,11,19]. *Aspergillus nidulans* is an ascomycetous fungus, whose importance as test system has been increasingly recognised since the discovery of parasexual cycle in this fungus[20].*p*-Fluorophenylalanine (FPA), analog of an aromatic amino acid, phe is toxic to this fungus. An FPA-resistant mutant *fpaD* isolated in this fungus has been found to have reduced ability to transport phe and a number of other amino acids[7]. The present paper deals with partial purification and characterisation of phebip from wild type and a transport mutant of *A. nidulans* to examine the role of phebip in the transport of phe in this fungus.

Materials and Methods

Strains and growth conditions—The wild type *riboAl, biAl* and the mutant *riboAl, biAl, fpaD11* strains were from our departmental culture collection. Minimal medium (MM) was a Czapek-Dox medium containing 1% glucose (w/v) as carbon source (*p*H 6.5). Liquid medium was MM minus agar agar (1.5% w/v). The MM was supplemented with the specific requirements of the strain. Liquid cultures were raised by inoculating approximately 1×10^8 conidia of the desired strain in liquid medium on a gyratory shaker (150 rpm) at 37°C. Mycelia were harvested on cheese cloth by filteration. Before weighing, mycelia were thoroughly rolled over filter paper till nearly constant weight was obtained.

Chemicals—Sepharose 4B (Phermacia), *N*-ethyl-N (3-dimethyl amino propyl) carbodiimide (EDC), Caproic acid and Cynogen bromide (all from sigma) were purchased from respective companies through CSIR, Centre for Biochemicals, New Delhi. All other chemicals were of

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analytical grade and obtained from different commercial sources.

Purification of phebip—16 hr old mycelia raised in shake culture were harvested on cheese cloth and washed twice with cold distilled water and once with ice cold buffer 0.1 *N* Tris-HCl, *p*H 7.5 (washing buffer). The washed mycelia were subjected to osmotic shock as described by Wiley[15] with slight modification. One gm of washed mycelium was suspended in shock medium [SM]I containing 20% (w/v) sucrose and 0.01 *M* EDTA in washing buffer, *p*H 7.5 for 10 min. It was then filtered out and rapidly dispersed in the SM 11 which was washing buffer containing 1 m*M* dithiothreitol instead of water. $MgCl_2$ was added to the SM 11 within a minute to a final concentration of 1 m*M*. The SM 11 was centrifuged at 18,000 *g* for 30 min and supernatant containing shock proteins were further purified by affinity chromatography. In order to determine the maximum possible number of shock cycles (one cycle included suspending of mycelium in SM 1 plus resuspending in SM 11) to which the mycelium might be subjected to obtain the maximum amount of bip, the washed mycelium was passed through six shock cycles. L-phenylalanine-sepharose 4B matrix was prepared according to the manufacturer suggestion (Phermacia, Sweden). The coupled protein was eluted with the washing buffer containing 1.5 *N* NaCl and 0.5 *N* Na_2CO_3, *p*H 8. Fraction of 10 ml size was collected. Before collection, 1.0 ml acidified solution of $MgCl_2$ (5 m*M*) was poured in each collection tube. Fractions exhibiting high 260 nm absorbance were pooled and desalted on sephadex G-25 column (1.7×2.2 cm) previously incubated with washing buffer. Desalted fractions were further fractionated with finely ground ammonium sulphate powder to 60%, 80% and 100% saturations successively followed by centrifugation at 18,000 *g* for 30 min after first two saturations and at 97,000 *g* for 1 hr after last saturation. The pallet was dissolved in washing buffer containing 1 m*M* $MgCl_2$ and again desalted on sephadex G-25 column as above. All the operations were done at 4°C.

Marker enzymes—Isocitrate dehydrogenase and venadate sensitive ATPases were tested as marker enzymes in phebip solution at various levels of purification by the methods as described earlier[21].

UV spectra—The purified fractions of phebip were scanned for their absorbance in the wavelength range 900-190 nm with the help of UV-spectrophotometer (Hitachi model U3210) with inbuilt plotter.

SDS-PAGE—Discontinuous SDS-PAGE was carried out as described by Laemmli[22].

Estimation of Protein—Protein was estimated by the method of Lowry *et al.*[23] with BSA as standard.

Detection of carbohydrate moiety of bip—One ml of anthrone reagent [0.2% (w/v) anthrone dissolved in concentrated H_2SO_4] was poured in a test tube and 0.4 ml of sample was added slowly drop by drop. The reaction mixture was stirred with glass rod till uniform colour was developed (blue green). In case of non-glycoproteinous fraction (i.e. without carbohydrate) the reaction mixture turn into colourless or slightly yellowish and turbid solution. The colour was read at 620 nm against reagent blank to determine richness of bip in the fraction.

Results and Discussion

Shock proteins continued to be obtained through six shock cycles although the yield was lowered considerably after the third shock (not shown). The growth pattern of osmotically shocked and unshocked (control) mycelium is shown in Fig. 1A. The osmotic shock reduced subsequent

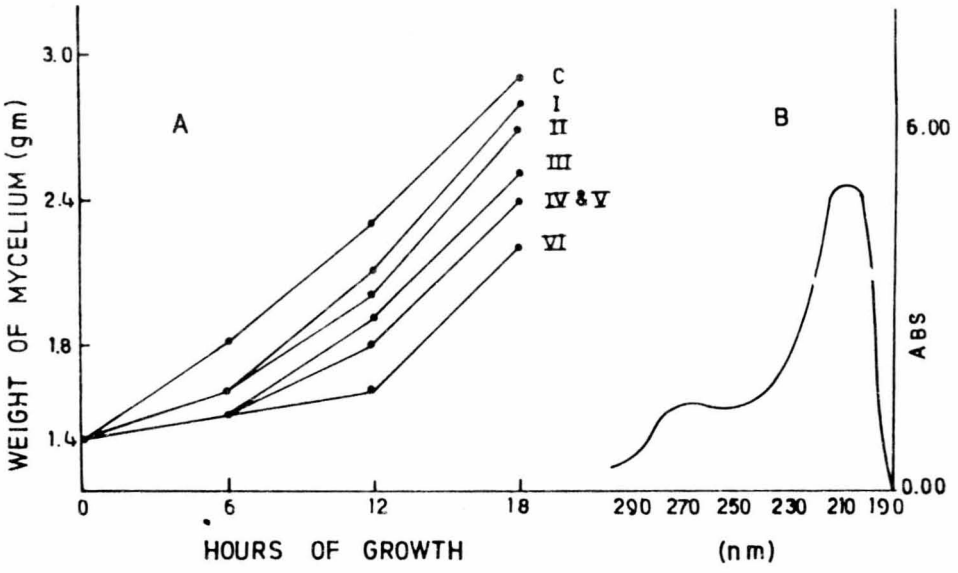

Fig. 1—(A) Growth pattern of shocked mycelium of *A. nidulans* subjected to different number of shock cycles [C,-Control (unshocked mycelium); I, II, III, IV, V & VI indicated one, two, three, four, five and six shock cycles respectively]. (B) UV-spectrum of phebip.

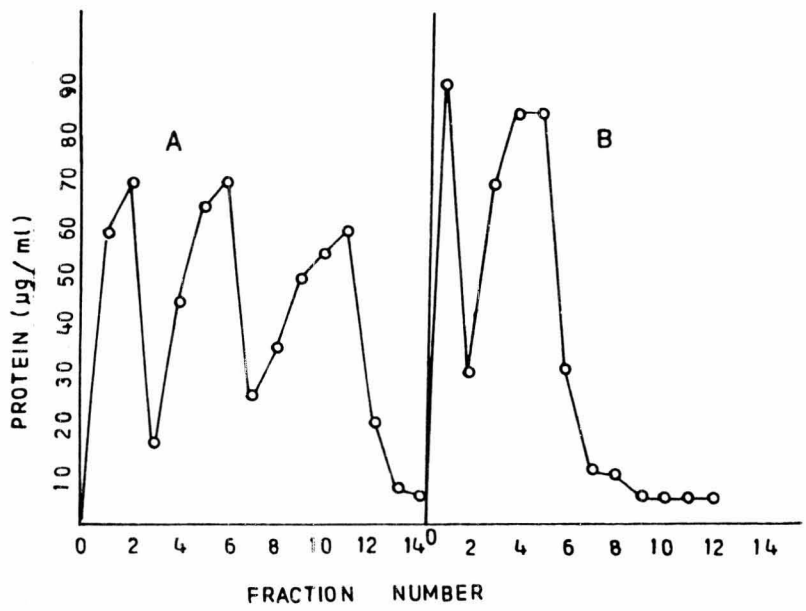

Fig. 2—Elution profile of wild type [A] and mutant [B] phebips from l-phenylalanine-sepharose 4B columns (column size 1.2 x 15 cm; equilibrated with 0.1 *M* Tris-HCl, *p*H 7.5; flow rate 15 ml hr^{-1}; column was eluted with 0.1 *M* Tris-HCl containing 1.5 *N* NaCl and 0.5 *N* Na_2CO_3, *p*H 8).

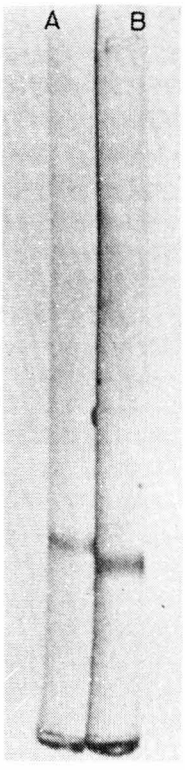

Fig. 3—SDS-PAGE profile of wild type [A] and mutant [B] phebips.

growth rate of the mycelium during first 6 hr of growth period suggesting a positive correlation between loss of periplasmic proteins including bips and reduction of growth which in turn seemed to be due to non-availibility of bips for binding to various metabolites including phe, whose transports are carrier mediated. A similar result was obtained earlier in case of tryptophan bip of *N. crassa*[15]. This is important to note that osmotic shock did not destroy the cellular integrity of the mycelium. The shocked cells remain biologically active and their genetic machineries competent enough to compensate the lost extrinsic proteins.

The elution profile of wild type and mutant phebips on l-phenylalanine-sepharose 4B are shown in Figs. 2A & B respectively. The phebip(s?) from wild type exhibited three simple peaks while corresponding second and third peaks of phebip(s?) from mutant tended to merge so that only two peaks were obtained in this case. The merger of the peaks occurred possibly due to reduction in the binding capacity of these peaks' materials and their consequent weak binding with the ligands in the column. When tested for their absorbance at 260 nm the second and the third peak materials in case of wild type and the second peak material in case of mutant phebip showed higher 260 nm activity.

Further purification was achieved by ammonium sulphate fractionation. The precipitates obtained by 60% and 80% saturations were very less in amount and exhibited very little 260 nm activity while those obtained by 100% saturation absorbed very strongly in 260 nm region. These higher 260 nm absorbing fractions were believed to be rich in bip[8].

The UV-spectrum of wild type phebip is shown in Fig. 1B. It exhibited two peaks, one in 200 nm and other in 260 nm regions. This characteristic UV-spectrum was also found earlier for tryptophan bip in case of *N. crassa*[9]. The mutant phebip yielded similar UV-spectrum (not shown). The purified phebip otherwise altered or inactivated

showed variously changed spectra (Sahay unpublished). This suggested that the mutant phebip underwent minor change in terms of its structure, not sufficient enough to be manifested in the spectrum.

A strong 260 nm activity was used as a characteristic feature to identify the phebip during the investigation. The positive anthrone test and the characteristic uv-spectrum[8] were taken as supplementary evidences for the same.

In order to check the level of contamination by cytosol proteins and membrane fraction, Isocitrate dehydrogenase and venadate sensitive ATPases activities were tested respectively as marker enzymes. No significant increase in the extinction at 340 nm (Isocitrate dehydrogenase) or 740 nm (ATPases) were observed which confirmed that the phebip thus purified was free from cytosol or membrane located proteins. The 60% and 80% ammonium sulphate precipitation were important steps that could exclude high M_r molecules including enzymes, if any leaving behind only low M_r proteins. Almost insignificant 260 nm activity in 60%- and 80%- precipitates further indicated that the osmotic shock method used during this investigation could not release any major intrinsic membrane located or cytosol proteins and was very selective in the extraction of low M_r periplasmic proteins.

The SDS-PAGE result of the purified phebip from wild type (A) and mutant (B) strains is shown in Fig. 3. The mutant phebip exhibited slightly higher mobility as compared to wild type one. This higher mobility might be due to deletion of some part of the protein. In another result the SDS-PAGE M_r of the purified phebip was found to be 17 kd[24]. This suggested that the phebip in this fungus was a low M_r protein similar to methionine bip[8] and different from tryptophan bip of *N. crassa*[15] which was found to be of high M_r. The reduced growth rate of shocked mycelium, the altered electrophoretic mobility and elution profile of phebip from transport mutant are strong evidences to conclude that phebip is essentially involved in the transport of phe in this fungus. Even in case of higher eukaryotes, although corresponding bips have not been purified and characterized, yet it has been reported by kinetic studies that amino acids are transported by facilitative transport mechanism[25,26].

In earlier study the phe transport system has been genetically characterised to be multimeric protein[10], the phebip seemed to be the part of this larger system responsible for binding of the ligand for its subsequent transport across the membrane and that the locus *fpaD* is a structural gene for phebip, a mutation (possibly a deletion one) at which has reduced the binding capacity of this protein, a reason for the uptake defect in this mutant. This weakening in binding capacity might also be responsible for the recovery of lesser amount of phebip in case of *fpaD* as eluted from phenylalanine-sepharose 4B column after washing it[7]. There seems to be two possible reasons for the erosion of binding capacity of the active site of bip due to the mutation namely, loss of a segment of bip constituting the binding site or deletion in some other part of bip that somehow has bearing on the binding capacity of the bip. Mutation leading to transport defects has been reported earlier both in prokaryotes[11,19,27] as well as in eukaryotes[10].

The *in vitro* studies have shown that amino acids are transported in higher eukaryotes by Na^+-dependent or Na^+-independent facilitative transport system while in case of bacteria by secondary transport system (H^+/alanine symport system)[4]. Similarly uptake of glycine by erythrocyte has been reported to be dependent on the presence of inwardly directed concentration gradients of both Na^+ and Cl^- (ref. 28). Earlier in *A. nidulans* ΔpH (pH gradient) has been found to be the driving force in the transport of acidic[29] as well as basic[30] amino acids. In light of these reports and present findings[19], efforts are underway to purify other components (intrinsic?), their assembly (by incorporating into liposome) and subsequent assay of the assembled transport system in order to unravel the exact mechanism of phe transport in this fungus.

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