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CHARACTERISTICS OF A TRANSPORT-DEFICIENT MUTANT (*nap*) OF *NEUROSPORA CRASSA*

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SUMMARY

nap, previously characterized by Jacobson and Metzenberg as a neutral and acidic amino acid transport mutant, was found in this study to be reduced in transport activity for a variety of metabolites. Analysis of glycoprotein molecules involved in amino acid binding indicates that the deficiency is not at this level. The deficiency appears to be in some common component of all active transport systems.

INTRODUCTION

Several systems have been reported to be operative for the active transport of various metabolites and their analogues in bacteria and fungi. *Neurospora crassa* and *Saccharomyces cerevisiae* (yeast) exhibit several identical features in the transport of these metabolites and the former has been extensively studied in this laboratory.

Transport of sugars was reported to take place by a process of diffusion in both yeast and *N. crassa*. However, Scarborough[1] has described a high affinity active transport system operative for the transport of glucose in *N. crassa*.

DeBusk and DeBusk[2], Lester[3] and Stadler[4] have described an amino acid transport system (PmN) specific for neutral and aromatic amino acids in *N. crassa*. Baurele and Garner[5], and Roess and DeBusk[6] have observed a basic amino acid transport system (PmB) operating in *N. crassa*. Wolfinbarger and DeBusk[7], and Pall[8] have observed a general system (PmG) that is less specific and transports both neutral and basic amino acids. Pall[9] has described a specific permease system operative for acidic amino acids. Aspartic acid was reported by Wolfinbarger *et al.*[10], to be transported by the neutral amino acid system at low pH values.

Genetic control of these amino acid transport systems was evidenced by the isolation of mutants lacking particular transport activities. The neutral amino acid transport mutants include pm^{-N} (Wolfinbarger and DeBusk[11]) *mtr*-mutant (Lester[3]) and *un-t*-mutant (Kappy and Metzenberg[19]). pm^{-B} (Wolfinbarger and DeBusk[11]), *bat* (Thwailes and Pendyala[13]) and *Cr-10* (Roess and DeBusk[6]) were reported to be deficient in the basic amino acid transport activity.

Stuart and DeBusk[14] carried out an *in vitro* analysis of certain of these mutants employing the technique of an affinity chromatography. They have observed mutants

lacking the corresponding amino acid binding molecules which are glycoproteins.

Jacobson and Metzenberg[15] have isolated a mutant resistant to the amino acid analogues, 4-methyl-tryptophan and ethionine. It was found to be deficient in the transport activity of neutral and acidic amino acids and was called *nap* (neutral acidic permeability). The observation that *nap* was genetically unlike the other known neutral amino acid transport locus (pm^{-N}, *mtr* on L. G. IV), coupled with the observation that the latter is also responsible for most of aspartic acid transport in conidia, suggested the need for further studies of this mutant. We have observed *nap* to be partially but not completely deficient in the transport of various metabolites including glucose, uridine as well as amino acids.

METHODS

All strains employed were obtained from the Fungal Genetics Stock Center, Arcata, Calif. Tatum^{A} (SY7^{A}) was chosen as wild type standard in all these studies. *nap* (FGSC No. 1604^{a}) was isolated by Jacobson and Metzenberg[15]. All strains were maintained on silica gels (Brockman, H. E. and deSerres, J. F., (1962) *Neurospora Newsletter*). Cultures were grown on Vogel's minimal medium (N) supplemented with 2% sucrose as a carbon source and 2% agar at °C (Vogel, 1956).

All radioactive chemicals were obtained from Schwartz, New York or International Chemical and Nuclear Corporation, Calif. All amino acids were used in their L-form.

Transport studies were performed as described by Wolfinbarger and DeBusk[11]. The radioactive compound was added to give a final concentration of 0.01 μCi/0.1 μmole per ml. All transport experiments were done at 25 ± 1 °C. Incorporation of the labeled compound was determined by counting in a Beckman Low Beta counter.

For the study of "post conidial" transport activity[12], the cells were incubated in a reciprocal shaker bath, in Vogel's medium with 1% glucose as a carbon source for 180 min before the addition of the labeled compound.

Isolation of glycoprotein molecules involved in amino acid binding, employed the technique developed by Stuart and DeBusk[14]. Seven-day-old conidia were extracted with 4.98 M KCl and the resultant material was applied to a cyanogen bromide L-arginine affinity column prepared as described by Stuart and DeBusk[14]. The column was eluted first with distilled water and later with 0.01 M Na_2CO_3 and fractions were collected as 5-ml aliquots. They were read for absorbance at 260 nm wavelength in a Beckman DU spectophotometer.

RESULTS

Earlier studies with *nap* by Jacobson and Metzenberg[15] revealed that *nap* was resistant to the amino acid analogues, ethionine and 4-methyltryptophan. It was also found to be deficient in the transport activity of both neutral and acidic amino acids. Basic amino acid transport was reported to be normal. The former characteristics are confirmed by these studies but a further deficiency in basic amino acid transport was revealed in conidia.

Three-day-growth studies on medium supplemented with analogues of both

basic and neutral amino acids is presented in Table I. Tatum^A (wild type) was completely inhibited by *p*-fluorophenylalanine. Weak growth was observed on medium supplemented with thiosine (*S*-aminoethyl-L-cystine, a basic (lysine) amino acid analogue). *nap* exhibited a similar growth response to various amino acid analogues as Tatum^A except for its resistance to ethionine. A strain of *nap* was made partially isogenic with Tatum^A (a single spore colony was isolated from a cross between Tatum^A and *nap*. The isolate referred hereafter as Tatum/*nap*.) was found to be highly resistant to all the analogues tested (Table I). It exhibits all transport characteristics of *nap*. Further genetic analysis also indicates that *nap* is a single locus and characteristics of *nap* are controlled by a single gene product.

Fig. 1 illustrates conidial transport characteristics of *nap* and Tatum^A for phenylalanine and arginine. Compared to Tatum^A, *nap* exhibits 55% transport activity for either amino acid during the first 60 min of incubation. The observation that both neutral (phenylalanine) and basic (arginine) transport activity was deficient in *nap* suggested that an element common to both amino acids was altered in the mutant. The PmG (general) transport system which transport all amino acids was examined since it represents the most obvious common element. This can be done by determining the degree of competition between basic and neutral amino acids. Such studies indicate a similar degree of competition for the residual transport activity found in both *nap* and Tatum^A. This supports the conclusion that general system (PmG) of amino acid transport in *nap* is similar in properties but less active than wild type.

Fig. 2 represents transport studies with two other amino acids, methionine and aspartic acid. Both show similar reduction in transport as compared with wild type. Aspartic acid transport is carried out at pH 4, where transport of this amino acid is optimal[10]. Since there is rapid metabolic conversion of methionine, short term (initial rate) transport is examined.

Other metabolites were examined to determine if the reduced transport in

TABLE I

ANALOGUE RESISTANCE EXHIBITED BY TATUM^A, *nap* AND TATUM/*nap*

Analogue resistance exhibited by wild type (Tatum^A), *nap* and Tatum/*nap*. Growth tests were done on Vogel's minimal liquid medium supplemented with 2% sucrose as carbon source. Amino acid analogues were added at specified concentrations. Resistance to analogues were measured as the dry weight in milligrams of mycelial pad after 3 days of growth at 25 °C.

	Analogues	*Tatum^A*	*Strains*	
			nap	*Tatum/nap*
I)	Control (Vogel's minimal medium)	115	133	110
II)	*p*-fluorophenylalanine (2 μmoles/25ml)	0	0	35
III)	Thiosine (0.5 μmoles/25 ml)	20	16	68 (1 μmole/25ml)
IV)	Ethionine (0.2 μmoles/25 ml)	0	22	44

nap was restricted to amino acids or was due to an element common to several transport systems. This appeared to be the case since both glucose and uridine transport were equally reduced to about 45% of that observed in wild type conidia (Fig. 3).

It has been previously shown[12] that amplification (4–10-fold) of amino acid transport, occurs as conidia undergo developmental changes (post conidial), but prior to actual germination. Mutants have been previously isolated which fail to develop activities beyond the transport rate characteristic of conidia. The mutant *nap* appears to be reduced in post conidial activity for both neutral and basic amino acids (Fig. 4). However, transport is amplified beyond that of conidia and to similar extent in both *nap* and wild type.

Hunter and Segal[21] have reported efflux of hydrophobic amino acids as keto acids in *Penicillium chrysogenum* through a carrier-mediated process sensitive to actidione. Preliminary studies indicate an efflux system to be operative in *N. crassa* conidia (Ferguson and Lindhiemer, personal communication). Neutral amino acids were reported to undergo efflux[9], but basic amino acids do not. Hence

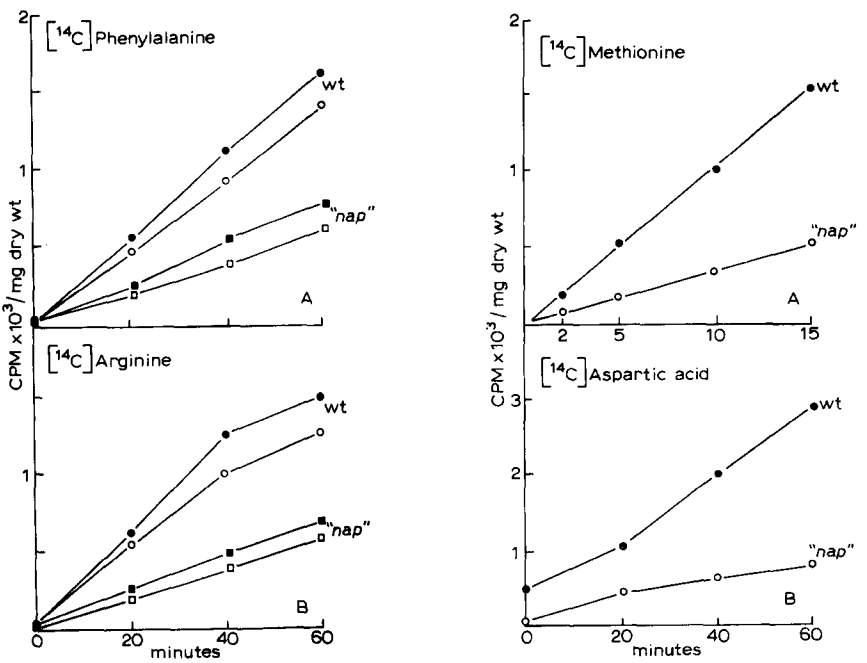

Fig. 1. (A) The transport of [^{14}C]L-phenylalanine (0.01 μCi/0.1 μmoles per ml) in wild type (Tatum^A) and *nap* (FGSC No. 1604). The uptake was initiated by adding conidia (0.1 mg/ml) to the uptake medium (pH 5.8) on a reciprocal shaker water-bath at 25 °C. (●, ■). Competition for PmG system was measured by addition of a 10-fold arginine to the incubation medium described above (○, □). (B) The transport of [^{14}C]arginine was measured as above (●, ■). The competition for PmG system was measured by the addition of a 10-fold concentration of cold phenylalanine to the incubation medium (○, □).

Fig. 2. (A) Transport of [^{14}C]methionine in wild type (Tatum^A) and *nap*. (B) The transport of [^{14}C]aspartic acid in wild type (Tatum^A) and *nap*. Cells were incubated in Vogel's medium of pH 4.

total accumulation of neutral amino acid is not a good measure of transport activity. Fig. 4 exhibits identical accumulation of phenylalanine by 60 min after 180 min of preincubation (post-conidial transport activity[12]). However rate of phenylalanine transport is low in the case of *nap* compared to Tatum^A (Fig. 4c).

To further study *nap* at permease level, amino acid binding molecules were isolated using the technique developed by Stuart and DeBusk[14]. A cyanogen bromide L-arginine affinity column was constructed and KCl extract of both Tatum^A (wild type) and *nap* were applied separately. The column was eluted first for three void volumes with glass-distilled water and later with 0.01 M Na_2CO_3. Three peaks

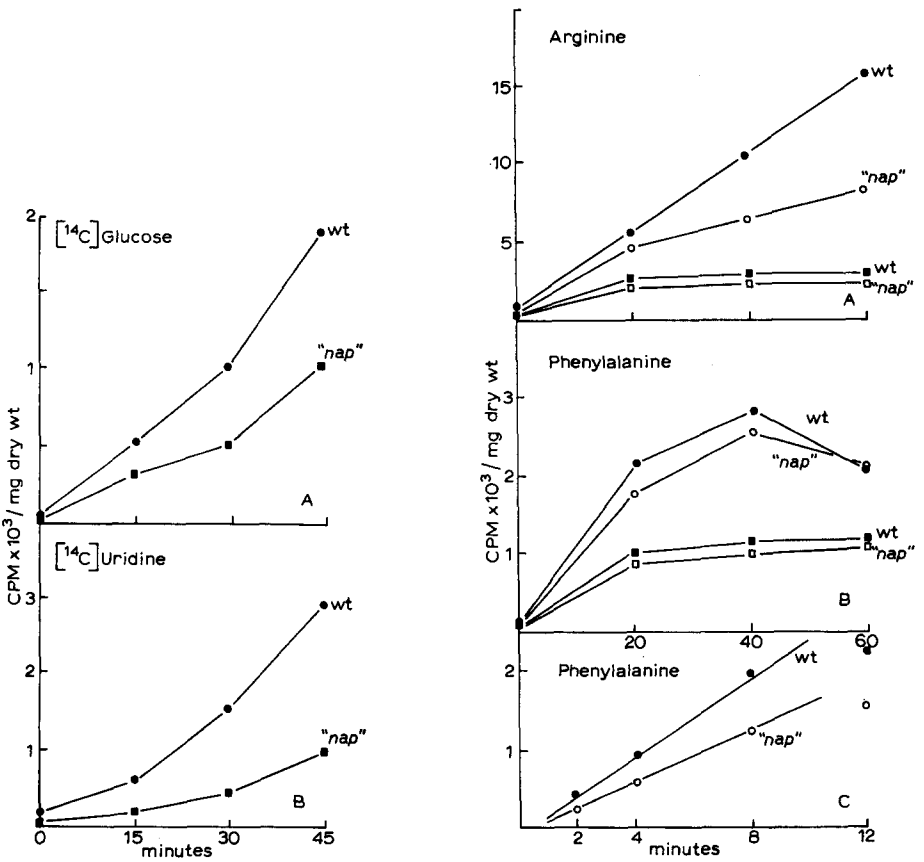

Fig. 3. (A) Transport of [^{14}C]glucose in wild type (Tatum^A) and *nap*. (B) Transport of [^{14}C]-uridine in wild type (Tatum^A) and *nap*.

Fig. 4. Post-conidial transport activity of phenylalanine and arginine in wild type (Tatum^A) and *nap*. Conidia were incubated in Vogel's medium with 1% glucose. Labeled amino acid was added to initiate transport after 180 min of incubation. (A) [^{14}C]arginine transport in wild type (Tatum^A) and *nap* (●,○). Competetion for PmG system was assayed by addition of 10-fold concentration of cold phenylalanine (■, □). (B) [^{14}C]phenylalanine transport in wild type (Tatum^A) and *nap* (●,○) PmG system competition was assayed by adding 10-fold concentration of cold arginine (■, □). (C) Initial rates of phenylalanine [^{14}C]transport in wild type (Tatum^A) and *nap*. Velocity of transport calculated from slope value of the curve.

of 260 nm absorbing material was found to be eluted from the column, both in the case of Tatum^{A} (wild type) and *nap*. This suggests that a major difference in the amino acid binding molecules does not exist between Tatum^{A} and *nap*.

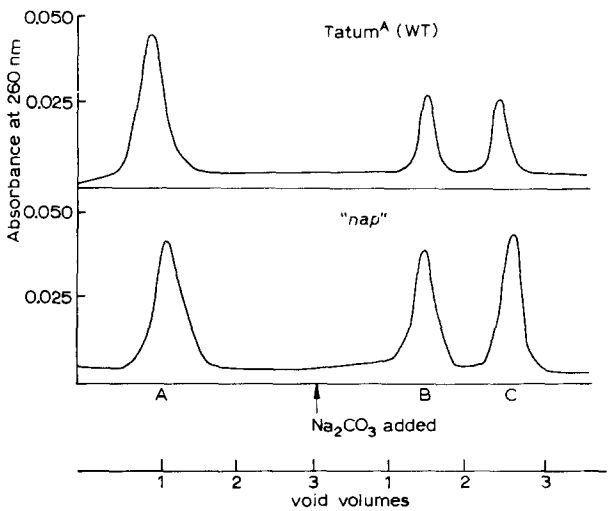

Fig. 5. Cyanogen bromide L-arginine affinity column chromatographic profiles of KCl crude extracts obtained from wild type (Tatum^{A}) and *nap*. (See Methods, Stuart and DeBusk[14]).

DISCUSSION

The genetic control of amino acid transport has been clearly established in yeast and *N. crassa* by isolating mutants deficient in particular transport activities. The deficiency was further related to the absence of single gene product. A different class of mutants was reported by Surdin *et al.*[18] in yeast called *aap* which exhibits reduced transport activity for all amino acids. This indicates a common factor involved in the active transport of all amino acids.

Several amino acid transport mutants were obtained in *N.crassa* by their resistance to corresponding amino acid analogue. The resistance was attributed to non-transport of the analogue, which would otherwise be lethal to the organism. Two genetically distinct (unlinked) loci have been described controlling neutral (PmN) and basic (PmB) amino acid transport[7,11]. pm^{-N} (isolated by its resistance to *p*-fluorophenylalanine) shows 75% reduction in neutral amino acid transport, while pm^{-B} (isolated by its resistance to canavanine, analogue of arginine) shows 50% reduction in basic amino acid transport. As more than one system are operative in the transport of these metabolites, a residual transport activity is still observed[7,11].

nap which was isolated and characterized by Jacobson and Metzenberg[15] as a neutral acidic permease mutant, appears to be a mutant without specificity for any single system of active transport. A strain of *nap* made isogenic with Tatum^{A} (Tatum/*nap*) exhibits complete resistance to all the amino aicd analogues tested (Table I), indicating a general deficiency for the transport of all amino acids. It exhibits a general reduction of transport acitivity to about 50% for all metabolites tested. Competition studies between neutral and basic amino acids imply that

this deficiency is not limited to any particular system of amino acid transport, with PmN, PmB, and PmG all systems equally affected. Post-conidial transport activity and the rates of transport for phenylalanine and arginine over long incubation times indicate a slow accumulation process in the case of *nap*. However, the total accumulation of these amino acids appears similar both in Tatum^{A} and *nap* after sufficient incubation times. The reduction in transport activity of glucose and uridine, indicates a general deficiency in the transport capabilities of this mutant for a variety of metabolites.

Active membrane transport is characterized as a carrier-mediated process associated with energy coupling. Modification of any of these functions may results in transport-deficient mutants.

Examination of amino acid binding glycoproteins was made using Cyanogen bromide L-arginine affinity column chromatography. Earlier studies[14] with known permease mutants pm^{-N} and pm^{-B} (reduced in neutral and basic amino acid transport activities[7,11], respectively,) indicated loss of certain fractions of 260 nm-absorbing material that were observed in Tatum^{A} (wild type). Peak-B material was associated with basic amino acid transport activity, for its greater binding affinity with arginine than phenylalanine. Further Peak-B material was missing in the mutant pm^{-B} which lacks basic amino acid transport activity. However it was reported to be present in the mutant pm^{-N} which has normal basic amino acid transport activity. Peak A was thought to be associated with PmG system as the double mutant pm^{-NB} (deficient in both neutral and basic amino acid transport activity) is lacking both Peak-B and -C material but not Peak-A material. Thus the binding affinity of these complexes for amino acids was shown to be under genetic control of two unlinked genes.

Identical chromatographic profiles of KCl extracts from Tatum^{A} and *nap* indicate that the deficiency is not at the level of amino acid binding glycoprotein molecules. This evidence is further supported by identical thin layer chromatographic profiles of KCl extracts from Tatum^{A} and *nap* which are unlike other transport mutants tested (Stuart, unpublished). The transport deficiency in *nap* thus may be due to a structural difference in the cell wall and membrane components or due to an inefficient energy coupling mechanism.

Pall and Kelly[20] have described a process of transinhibition in *N. crassa* which was attributed to non recycling of carrier molecules. Even though direct experimental evidence is not provided, the reduced rates of transport observed in *nap* could be attributed to an inefficient energy coupling mechanism, resembling the process of transinhibition.

nap belongs to a special category of single gene mutants, in which the deficiency appears to be in a common component of all active transport systems tested. The common component can be a process like energy coupling.

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