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Transport of L-glutamic acid in the fission yeast *Schizosaccharomyces pombe*

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Transport of L-glutamic acid into the fission yeast *Schizosaccharomyces pombe* grown to the early stationary phase and preincubated for 60 min with 1% D-glucose is practically unidirectional and is mediated by a single uphill transport system with a K_T of 170 μM and J_{max} of 4.8 nmol min^{-1} (mg dry wt.)$^{-1}$. The system proved to be rather non-specific since all the amino acids transported into the cells acted as potent competitive inhibitors. It has a pH optimum at 3.0–4.0, the accumulation ratio of L-glutamic acid is highest at a suspension density of 0.6–1.0 mg dry wt. per ml and decreases with increasing L-glutamic acid concentrations in the external medium. The system present in the cells after preincubation with D-glucose is unstable and its activity decays after washing the cells with water or after stopping the cytosolic proteinsynthesis with cycloheximide, with a half-time of 24 min in a reaction significantly retarded by phenylmethylsulfonyl fluoride, a serine proteinase inhibitor. The synthesis of the transport protein appears to be repressible by ammonium ions.

Introduction

Transport of amino acids in lower eukaryotes including yeasts is an active and practically unidirectional process. Probably all these organisms possess two types of transport system for amino acids: those that are specific for one or a few structurally related amino acids and a general system which is shared by most amino acids (see Ref. 1 for review).

Compared to *Saccharomyces cerevisiae* where amino acid transport is effected by approximately sixteen systems with different specificities, our knowledge of analogous systems of *Schizosaccharomyces pombe* lags behind. Only limited data are available, suggesting the existence of perhaps four different amino acid transport systems [2]. (i) A high-affinity system specific for basic amino acids, viz. L-arginine, L-histidine, D- and L-lysine, L-ornithine and L-canavanine, operating in the wild-type strain with either ammonium ions or L-glutamic acid as the nitrogen source during growth [3–5], Its activity is much reduced in the mutant can1, isolated as a resistant to L-canavanine, an L-arginine analogue [3]. (ii) The L-arginine-specific system which is absent in cells grown on ammonium ions as the sole nitrogen source. (iii) A system specific for most of the neutral but not the basic and/or acidic amino acids [6]. (iv) The general amino acid transport system assumed to exist on the basis of competitive inhibition of L-tyrosine transport by phenylalanine, histidine and L-glutamic acid. Its activity is apparently completely lost in the *pho1* mutant with highly reduced acid phosphatase activity [4].

We report here on the kinetic characterization of L-glutamic acid transport in the wild-type strain of *Schizosaccharomyces pombe* 972h$^-$.

Materials and Methods

Schizosaccharomyces pombe 972h$^-$ was grown in a YPD medium containing 1% yeast extract, 1% bactopeptone and 1% D-glucose at pH 4.5. The culture (50 ml) was placed in a reciprocal shaker at 29°C. Cells from the early stationary growth phase (26–28 h) were harvested by centrifugation, washed twice with distilled water and suspended in distilled water to a density of 5–8 mg dry wt./ml.

Preincubation of the cells with energy donors and measurement of the amino acid transport activity were carried out as described previously [5] using membrane filtration and liquid scintillation counting.

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All transport experiments were done at least in triplicate. The initial rates of uptake were estimated from the linear parts of the curves (generally up to 2 min). The K_T and J_{max} values were estimated from reciprocal plots and the mean values and deviations from 16 separate experiments were calculated. Inhibitor constants were calculated from the formula

$$K_i = i/(K_a/K_T - 1)$$

where i is the inhibitor concentration and K_a and K_i are the 'half-saturation' constants in the presence and absence, respectively, of the inhibitor.

Cycloheximide was from Fluka, Switzerland; diethylstilbestrol, phenylmethylsulfonyl fluoride, 2,4-dinitrophenol, *N,N'*-dicyclohexylcarbodiimide and carbonyl cyanide *m*-chlorophenylhydrazone were purchased from Sigma, FRG. Yeast extract and bactopeptone were from Difco, USA. All other chemicals were from local commertial sources and were of the highest purity. L-[U-^{14}C]Glutamate (4 MBq ml^{-1}, 8800 MBq mmol^{-1}) was obtained from the Institute for Research, Production and Uses of Radioisotopes, CSFR.

Results

Conditions of preincubation

During growth on YPD media no transport of basic and neutral amino acids is measurable in *S. pombe* regardless of the growth phase. Cells must be preincubated with an appropriate source of energy to be able to transport them [4–6]. The stimulatory effects of such preincubations on L-glutamic acid transport activity (Table I) closely resembled those found earlier for L-leucine and L-lysine uptake, both as to the energy sources stimulating the transport (except for D-maltose which was ineffective here) and as to its suppressibility by ammonium ions and by cycloheximide, but not by chloramphenicol. Taken together, these data are in accordance with the view that the stimulation of L-glutamic acid uptake by certain energy sources is associated with cytosolic, but not mitochondrial protein synthesis of the appropriate transport proteins. Since the rate of L-glutamic acid uptake by the glucose-pretreated cells depends on the growth phase at which the cells were harvested, peaking at 15 h of growth and attaining constant value as the stationary phase was reached [7], all the experiments were carried out with stationary cells after preincubation with an appropriate energy source.

Factors affecting transport protein synthesis

If cycloheximide was added to the cell suspension preincubated with 1% D-glucose for 60 min, L-glutamic acid transport activity decreased with a half-time of 20 min (Fig. 1A). The loss of activity was fully protected by phenylmethylsulfonyl fluoride (PMSF) during the

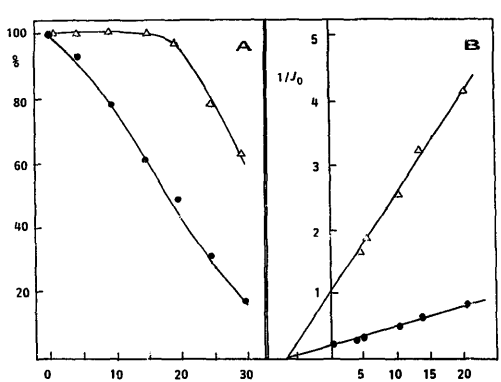

Fig. 1. L-Glutamic acid transport activity in cells preincubated for 60 min with 1% glucose and further incubated (starting at time zero) with 0.4 mM cycloheximide (○) or with cycloheximide plus 2 mM PMSF (△; A). Lineweaver-Burk plot of L-glutamic acid transport in cells preincubated with 1% glucose as in (A) and further incubated with cycloheximide for 1 min (●) or 30 min (▲; B). J_0, initial rate of transport in nmol min^{-1} (mg dry wt.)$^{-1}$; S_0, L-glutamic acid concentration in μmol l^{-1}.

TABLE I

Initial rate of 50 μM L-glutamic acid transport into cells preincubated for 60 min with different energy sources

The inhibitors and nitrogen sources were added to the suspension 30 min before labelled L-glutamic acid. The values shown are the means of five separate experiments.

Substrate	Concentration	Initial rate of transport (%)
Glucose	1%	100
Fructose	1%	98
Sucrose	1%	80
Ethanol, galactose, trehalose, maltose, α-methylglucoside, or none	1%	0
Glucose + cycloheximide	1% + 0.4 mM	63
Glucose + chloramphenicol	1% + 2.0 mM	103
Glucose + ammonium ions	1% + 4.0 mM	25
Glucose + cycloheximide + PMSF	1% + 0.4 mM + 2 mM	101
Glucose + ammonium ions + PMSF	1% + 4.0 mM + 2 mM	93
Glucose + PMSF	1% + 2.0 mM	98
Glucose + proline	1% + 1 mM	104
Glucose + allantoin	1% + 1 mM	90
Glucose + urea	1% + 1 mM	106

first 20 min of incubation and then a similar decline of activity was observed, probably due to fast hydrolysis of PMSF. Since in cells treated with cycloheximide decrease of the maximum rate of L-glutamic acid uptake J_{max} without K_T change may be observed (Fig. 1B), proteolysis of the corresponding transport protein appears to be the most probable explanation of such results.

In *S. pombe* preincubated with D-glucose ammonium ions decrease the initial rates of uptake of basic [5,8] and of 'hydrophobic' [6] amino acid transport mediated by at least two different systems to a considerable extent, this decrease being largely prevented by PMSF. Moreover, the ammonium ions increased the K_T value of L-lysine transport 4-fold, leaving the J_{max} of uptake practically unchanged while PMSF neutralized their influence [8].

When analogous experiments were carried out with L-glutamic acid transport, the following data were obtained. (i) If the ammonium ions and 1% D-glucose were added simultaneously at time zero of the preincubation period, no transport activity was measurable after 60 min. But when the ammonium ions were added after 'triggering' of the stimulation, the stimulation proceeded for a certain time, giving rise to higher

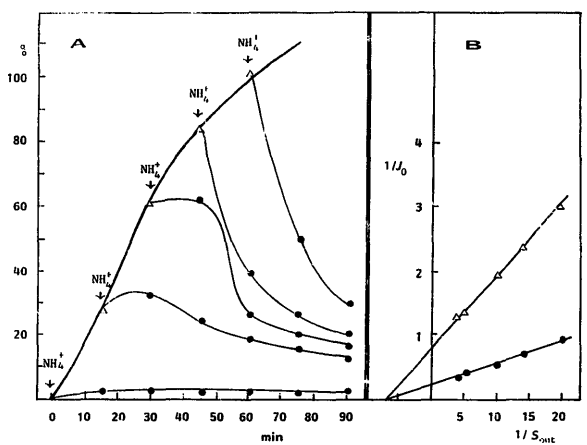

Fig. 2. Time course of L-glutamic acid (50 μmol l^{-1}) 'inhibition' by 4 mM ammonium ions added at different times of the preincubation period (A). The initial transport rate after 60 min of preincubation with 1% glucose is taken as 100%. Lineweaver-Burk plot of L-glutamic acid uptake in cells preincubated with glucose for 30 min and further with ammonium ions for 1 min (●) or 30 min (△). J_0 is expressed in nmol min^{-1} (mg dry wt.)$^{-1}$; L-glutamic acid concentration range from 50 to 250 μmol l^{-1}.

rate of uptake than at the time of inhibitor addition. Addition of ammonium ions at a later time stopped the stimulation more or less immediately and an exponential decrease in the initial rate of L-glutamic acid transport followed (Fig. 2A). (ii) The poor nitrogen sources, e.g., urea, allantoin, or L-proline, do not influence the stimulatory effects of D-glucose under analogous conditions. (iii) Ammonium ions decrease only the J_{max} of L-glutamic acid transport, leaving its K_T value unchanged (Fig. 2B). (iv) The decrease of L-glutamic acid transport by ammonium ions is prevented to a large extent by PMSF (Table I). Such results appear to be most plausibly explained by assuming that the ammonium ions act as a 'repressor' of L-glutamic acid transport protein synthesis and that the repression may be followed by its proteolytic degradation in a reaction prevented by PMSF, acting as a serine proteinase inhibitor.

Kinetic analysis

The transport of L-glutamic acid into glucose-pretreated *S. pombe* was generally linear with time for at least 2 min and after 10 to 12 min the net entry stopped at a constant value, higher at all L-glutamic acid concentrations used (0.02–2.5 mM) than the extracellular value (data not shown).

The efflux of L-glutamic acid (accumulated in 15 min from 50 μM) from cells was insignificant, regardless of whether the loaded cells were resuspended in water, uncouplers (2,4-dinitrophenol or carbonyl cyanide *m*-chlorophenylhydrazone), a transport inhibitor (uranyl nitrate) or nonlabelled L-glutamic acid at relatively high concentration (0.5 mM).

A reciprocal plot of L-glutamic acid transport was monophasic, composed of a single component with K_T of 170 ± 15 μM and maximum rate J_{max} of 4.8 ± 1.7 nmol min^{-1} (mg dry wt.)$^{-1}$. The properties of this transport system were now studied in more detail.

Substrate specificity

The transport of 50 μM L-glutamic acid measured in the presence of other amino acids at 10-times higher concentrations (Table II) clearly shows that, except of L-proline and 2-aminoisobutyric acid, which are practically not transported by the wild-type strain of *S. pombe* below 1 mM [7], all the amino acids display a high degree of inhibition with respect to L-glutamic acid uptake. The inhibition by all the selected amino acids, e.g., by L-alanine, L-tyrosine, L-methionine, L-aspartic acid and L-arginine, was strictly competitive (Fig. 3) with $K_{i,Ala}$ of 400 ± 13, $K_{i,Tyr}$ of 273 ± 3.5, $K_{i,Met}$ of 132 ± 6.2, $K_{i,Asp}$ of 500 ± 3.8, and $K_{i,Arg}$ of 58 ± 2.9 μM, determined from three separate experiments.

TABLE II

Inhibition of the initial rate of entry of 50 μM L-glutamic acid by amino acids at 10-fold higher concentration added simultaneously

The values shown are the means of at least three separate experiments.

Amino acid	Inhibition (%)
Glycine	68
L-Alanine	62
L-Leucine	68
L-Isoleucine	61
L-Valine	64
L-Serine	68
L-Threonine	70
L-Asparagine	55
L-Glutamine	58
L-Proline	4
L-Arginine	84
L-Lysine	77
L-Histidine	82
L-Aspartic acid	54
2-Aminoisobutyric acid	7
L-Tyrosine	66
L-Tryptophan	69
L-Phenylalanine	71
L-Methionine	77
L-Cysteine	79

Effect of pH

The transport of L-glutamic acid showed a pH optimum at a pH 3.0–4.0 (Fig. 4), the value at which L-glutamic acid exists in an electroneutral, zwitterionic form.

Inhibition of L-glutamic acid uptake

Several selected inhibitors were active on the initial rate of L-glutamic acid transport (Table III). Sodium arsenate apparently decreased the intracellular ATP level and thus, among other things, diminished the efficiency of the plasma membrane H^+-ATPase. Carbonyl cyanide *m*-chlorophenylhydrazone and probably also 2,4-dinitrophenol and sodium azide, short-circuited the ΔpH across the plasma membrane as well as inner mitochondrial membrane and thus acted directly on the electrochemical potential difference at the plasma membrane and at the energy-converting inner mitochondrial membrane. The inhibition by diethylstilbestrol may be assumed to be an inhibition of plasma membrane H^+-ATPase. Also uranyl nitrate, a general inhibitor of amino acid and sugar transport in yeasts [9] predictably influenced L-glutamic acid uptake. All these data taken together are thus in agreement with a view that above all oxidation metabolism that provides energy for active L-glutamic acid transport.

Accumulation ratio of L-glutamic acid

Like with all actively transported solutes, the accumulation ratio of L-glutamic acid decreased with its

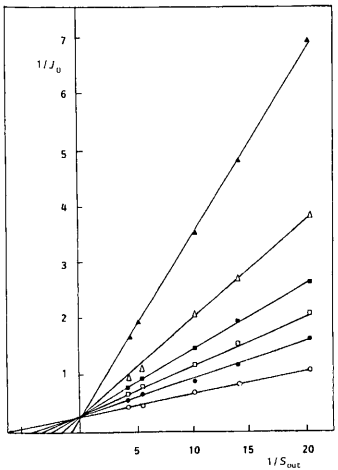

Fig. 3. Lineweaver-Burk plot of 50 μmol l^{-1} L-glutamic acid uptake in the presence of L-arginine (▲), L-methionine (△), L-tyrosine (■), L-alanine (□), and L-aspartic acid (●) at a concentration of 0.5 mmol l^{-1}, control (○).

TABLE III

Inhibition of the initial rate of 50 μM L-glutamic acid uptake by metabolic and transport inhibitors added to the yeast suspension 1 min before labelled L-glutamic acid

Inhibitor	Concn. (μM)	Inhibition (%)
2,4-Dinitrophenol	100	96
	250	97
	500	97
Sodium azide	10	1
	50	44
	100	71
	500	93
Carbonyl cyanide *m*-chlorophenylhydrazone	10	47
	20	45
Diethylstilbestrol	100	39
	200	68
N,N'-Dicyclohexylcarbodiimide	20	12
	50	11
	200	12
Sodium arsenate	2500	95
Uranyl nitrate	2500	89
	5000	93

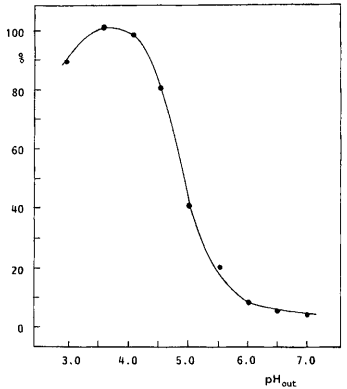

Fig. 4. Uptake of 50 μmol l^{-1} L-glutamic acid as a function of pH. Uptake was measured at various pH values in 0.1 M phthalic acid-triethanolamine buffer.

external concentration (Fig. 5), apparently due to local depletion of the energy source at the carrier [10].

Moreover, Kotyk [11] and Kotyk and Michaljaničová [12] described that suspension density plays a role in the capacity of various transport (as well as metabolic) events in yeasts. The L-glutamic acid transport system is no exception. The accumulation ratio of L-glutamic acid showed a clear maximum at a cell density of 0.6–1.4 mg dry wt. per ml (Fig. 6), distinctly lower than

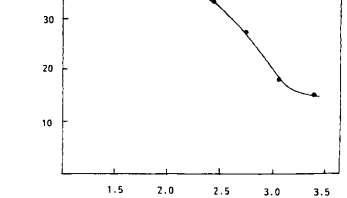

Fig. 5. Dependence of the accumulation ratio (S_i/S_o) of L-glutamic acid on its external concentration S_o in μmol l^{-1}.

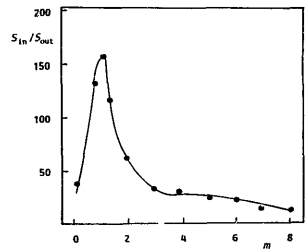

Fig. 6. Dependence of the accumulation ratio of L-glutamic acid at an initial concentration of 50 μmol l^{-1} on the density of yeast suspension m, in mg dry wt. per ml.

the 'optimum' density found for the accumulation of L-lysine [5] and L-leucine [6] in *S. pombe* and of L-proline in *S. cerevisiae* [11], respectively.

Discussion

The transport of L-glutamic acid in *S. pombe* resembles in many respects that of basic and neutral amino acids in the same yeast species, mediated by two specific systems [5,6] in being unidirectional, active (these features are also shared by most of the amino acid transport systems of *S. cerevisiae* and of eukaryotic microorganisms generally; see Ref. 1), nonconstitutive in the sense that its activity is measurable only in cells pretreated with an appropriate energy source regardless of the growth phase at which cells are harvested for amino acid transport assays and highly sensitive to proteolytic attack.

Our experiments disclosed at least two peculiar features of the L-glutamic acid transport system. The first is its nonspecificity and relatively low affinity for all substrates tested and the fact that it is composed of only one kinetic component (at least in the range of L-glutamic acid concentrations used, i.e. 0.02–2.5 mM). The second lies in its mechanism of regulation at the level of synthesis by ammonium ions both with and without phenylmethylsulfonyl fluoride, a compound known to play dual role, acting as a serine proteinase inhibitor or as a group-specific modifier of serine groups of proteins [13].

The finding of a single system for L-glutamic acid transport across the *S. pombe* plasma membrane was unexpected in the light of data on *S. cerevisiae* showing that this amino acid may be the substrate of up to five systems with different specificities and mechanisms of regulation [14–19]. Whether such differences are due to the real absence of analogous systems in *S. pombe* or whether they exist but are not detectable or functional under our experimental conditions, remains to be elucidated. The system characterized here appears to be similar to, if not identical with, the system for L-tyrosine transport in *S. pombe* competitively inhibitable by selected neutral, basic and acidic amino acids with comparable K_i values [4].

The present and earlier [5,6,8] studies on the influence of ammonium ions alone and in combination with PMSF on amino acid transport in two yeast species, *S. pombe* and *S. cerevisiae* led us to predict the existence of at least three kinetically distinct regulatory mechanisms they exert. According to the first mechanism, based on the ability of ammonium ions alone (but not in the presence of PMSF) to increase only the K_T value of L-lysine transport, ammonium ions inactivate the corresponding transport protein by changing to a less active form in a reaction, prevented by PMSF, acting presumably here as a group-specific modifier of its serine hydroxyl groups. In contrast, when analogous experiments were done with the basic amino acid transport in *S. cerevisiae*, ammonium ions decreased the J_{max} value, leaving the K_T values of L-lysine and L-arginine unchanged. Moreover, the decrease was not prevented by PMSF. The decrease of J_{max} without a change of K_T was also observed with L-glutamic acid transport in *S. pombe* but there the PMSF protected the uptake system to a large extent. It is suggested that the decrease of J_{max} of amino acid transport mediated by both systems may be due to a reduction of the effective concentrations of the corresponding plasma membrane transport proteins by repression of their syntheses followed in the case of L-glutamic acid transport by a proteolytic attack of serine proteinase, inhibited by PMSF. But since no proteinase has been isolated and characterized from *S. pombe*, the suggestion of proteolysis involvement is still speculative. Moreover, the results do not exclude unambiguously the possibility of ammonium ions acting as trans-inhibitors or as competitors with amino acids for a common source of energy for the active transport. The ability of PMSF to protect the uptake system against the negative influence of ammonium ions has also been observed with xanthine transport in *S. pombe* [20]. But here the data were interpreted in terms of a combination of inactivation (protected by PMSF) and of repression synthesis of the corresponding transport protein, being resistant to the protective role of PMSF.

References

1 Horák, J. (1986) Biochim. Biophys. Acta 864, 223–256.
2 Goffeau, A., Coddington, A. and Schlesser, A. (1989) in Molecular Biology of the Fission Yeast (Nasim, A., Young, P. and Johnson, B.P., eds.), pp. 397–429, Academic Press, New York.
3 Fantes, P.A. and Creanor, J. (1984) J. Gen. Microbiol. 130, 3265–3273.

4 Coddington, A. and Schweingruber, M.E. (1986) Curr. Genet. 11, 113–117.
5 Sychrová, H., Horák, J. and Kotyk, A. (1989) Biochim. Biophys. Acta 978, 203–208.
6 Sychrová, H., Horák, J. and Kotyk, A. (1989) Yeast 5, 199–207.
7 Sychrová, H., Horák, J. and Kotyk, A. (1989) Folia Microbiol. 34, 279–285.
8 Horák, J., Sychrová, H. and Kotyk, A. (1990) Biochim. Biophys. Acta 1023, 380–382.
9 Kotyk, A., Říhová, L. and Ponec, M. (1971) Folia Microbiol. 16, 445–450.
10 Kotyk, A. and Stružinský, R. (1977) Biochim. Biophys. Acta 470, 484–491.
11 Kotyk, A. (1987) Yeast 3, 263–270.
12 Kotyk, A. and Michaljaničová, D. (1987) Folia Microbiol. 32, 459–464.
13 Hayashi, R., Bai, Y. and Hata, T. (1975) J. Biochem. (Tokyo) 77, 1313–1318.
14 Gregory, M.E., Keenan, M.H.J. and Rose, A.H. (1982) J. Gen. Microbiol. 128, 2557–2562.
15 Grenson, M. and Dubois, E. (1982) Eur. J. Biochem. 121, 643–647.
16 Darte, C. and Grenson, M. (1973) Biochem. Biophys. Res. Commun. 67, 1028–1033.
17 Joiris, C.R. and Grenson, M. (1969) Arch. Int. Physiol. Biochim. 77, 154–156.
18 Grenson, M., Hou, C. and Crabeel, M. (1970) J. Bacteriol. 103, 770–777.
19 Rytka, J. (1975) J. Bacteriol. 121, 562–570.
20 Seipel, S. and Reichert, U. (1980) FEBS Lett. 115, 289–292.