Journal of Bioenergetics and Biomembranes, Vol. 23, No. 4, 1991

L-Glutamine Transport in Native Vesicles Isolated from Ehrlich Ascites Tumor Cell Membranes

Miguel A. Medina,[1] Ana R. Quesada,[1,2] and Ignacio Núñez de Castro[1]

Received October 25, 1990; revised January 2, 1991

Abstract

Native vesicles isolated from Ehrlich ascites tumor cells accumulate glutamine by means of Na^+-dependent transport systems; thiocyanate seems to be the more effective anion. The apparent affinity constant for the process was 0.38 mM. The Arrhenius plot gave an apparent activation energy of 12.3 kJ/mol. The structural analogs of glutamine, acivicin (2.5 mM) and azaserine (2.5 mM), inhibited the net uptake by 67 and 70%, respectively. The sulfhydryl reagents mersalyl, PCMBS, NEM, and DTNB also inhibited net uptake, suggesting that sulfhydryl groups may be involved in the activity of the carrier protein. A strong inhibition was detected when the vesicles were incubated in the presence of alanine, cysteine, or serine; in addition, histidine, but not glutamate or leucine, had a negative effect on glutamine transport.

Key Words: L-Glutamine; amino acid transport; plasma membrane vesicle; Ehrlich cells.

Introduction

Glutamine is described as a major substrate avidly consumed by both proliferating cells and tumors (Ardawi and Newsholme, 1985; Brand, 1985; Sauer and Dauchy, 1983). The role of glutamine as a vector of nitrogen between host and tumor, as well as its interactions with other energy substrates, has been extensively studied in Ehrlich ascites tumor cells (Carrascosa *et al.*, 1984; Medina *et al.*, 1988a, b; Medina and Núñez de Castro, 1990).

The first event in the utilization of glutamine by the cell is its uptake by plasma membrane transport systems. Kinetic experiments with intact cells

[1] Laboratorio de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Málaga, E-29071 Málaga, Spain.
[2] Antibióticos Farma, Antonio López 109, Madrid, Spain.
[3] Abbreviations used: AIB, α-aminoisobutyric acid; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; PCMBS, *p*-chloromercuribenzenesulfonic acid.

0145-479X/91/0800-0689$06.50/0

led to the conclusion that Ehrlich cells can transport glutamine very efficiently through two transport systems (Carrascosa *et al.*, 1984). This previous approach using intact cells must now be continued by studies using native and reconstituted vesicles. Although a number of papers deal with amino acid transport in both native (Colombini and Johnstone, 1974a, b; Im and Spector, 1980) and reconstituted vesicles (Bardin and Johnstone, 1978; McCormick *et al.*, 1984, 1985; McCormick and Johnstone, 1988a) obtained from Ehrlich cell membranes, the above-mentioned studies were carried out by using the nonmetabolizable amino acid AIB, or the amino acids alanine, serine, and/or leucine. At present, there is no complete study of glutamine transport system in tumor cells. The present paper reports the characterization of Na^+-dependent glutamine transport in native vesicles obtained from tumor cell membranes.

Materials and Methods

Chemicals

L-(G-^{3}H)-Glutamine (specific activity 2 TBq/mmol) was supplied by Amersham International, Amersham, England. Aquasol-2 scintillation mixture was purchased from NEN Chemicals, Dreieich, Germany. All other reagents were analytical grade bought from Merck, Darmstad, Germany, or from Sigma Chemical Co., St. Louis, Missouri.

Isolation of Plasma Membrane Vesicles

Ehrlich ascites tumor cells were maintained by intraperitoneal injection of albino Swiss mice OF1 (SPF Ico) and harvested between 9 and 12 days after the tumor transfer. All procedures were performed at 4°C, unless specified otherwise in the text. Tumor cells free of red blood cells were washed in 0.9% saline by centrifuging at 900 *g* for 30 s. The cells were washed twice more in the hypotonic homogenization buffer (buffer H), consisting of 15 mM Tris, pH 7.5, 15 mM NaCl, and 1 mM $MgCl_2$; they were then centrifuged at 2000 *g* for 3 min. Afterwards, cells were resuspended in buffer H and kept on ice for a minimum of 30 min. The swollen cells were homogenized in a glass–Teflon Potter. The homogenate was centrifuged at 650 *g* for 10 min. The supernatant was withdrawn and the pellet was resuspended in buffer H and centrifuged twice at 500 *g* for 5 min. The three resultant supernatants were centrifuged at 7500 *g* for 10 min. The pellet was resuspended in 15 mM Tris pH 75, 0.25 M sucrose, and 0.2 mM $CaCl_2$ (buffer S) and centrifuged at 1500 *g* for 10 min to discard the mitochondrial fraction. Both supernatants were centrifuged at 100,000 *g* for 20 min. The resulting membrane pellet was fractionated by using a Percoll gradient. The pellet was resuspended in a small volume of buffer S and diluted with 1.5 volumes of

75% (v/v) Percoll in buffer S. Once resuspended, it was added at the bottom of a centrifuge tube previously layered with 30% (v/v) Percoll, 10% (v/v) Percoll, and buffer S. The gradient was centrifuged at 20,000 *g* for 2 min. Two membrane rings were formed. The upper one was the plasma membrane-enriched fraction. This was collected with care by using a syringe with 40/8 needle. The collected membranes were diluted with buffer S and centrifuged at 100,000 *g* for 20 min. The final pellet was suspended again in buffer S, divided in 200–300 μl aliquots in Eppendorf tubes, frozen in liquid nitrogen, and stored at − 80°C until use. Vesicles were essentially free of mitochondrial contamination, as determined by the absence of the characteristic absorption peak for the cytochrome oxidase system in their difference spectra.

Protein was measured by the method of Bradford (1976).

The linear relationship observed between the amount of vesicle protein and transported glutamine into the plasma membrane vesicles from Ehrlich ascites tumor cells confirms the integrity and functioning of the vesicles (results not shown).

Transport Assay

Membranes were quickly thawed at 37°C. Unless otherwise stated, transport experiments were performed at room temperature (25°C). Native membrane vesicles (80 to 150 μg membrane protein) were mixed with an equal volume of 0.2 M NaSCN or KSCN in buffer S, plus 0.2 mM L-(G-^{3}H)-glutamine (500 dpm/pmol). The transport reaction was stopped by the addition of 1 ml ice-cold stop buffer (100 mM NaCl in buffer S). The diluted membranes were immediately filtered through a Millipore filter (HAWP, pore size 0.45 μm). After washing with 3 × 1 ml of stop buffer, the filters were air dried and radiactivity was measured in an LKB Rack-Beta scintillation counter. The apparent uptake of amino acids extrapolated to zero time was assumed to represent the unspecific glutamine binding and was subtracted from the total uptake to calculate the time course transport.

The membrane suspension was not preincubated with acivicin or azaserine before the uptake study. On the other hand, the membrane suspension was preincubated for 5 min with the sulfhydryl group inhibitors.

All incubations were carried out in duplicate or triplicate. All the experiments were repeated several times, and those carried out with different membrane vesicle preparations showed good reproducibility.

Results and Discussion

The time course of the glutamine uptake by Ehrlich cell plasma membrane vesicles in the presence of NaSCN or KSCN is depicted in Fig. 1. The

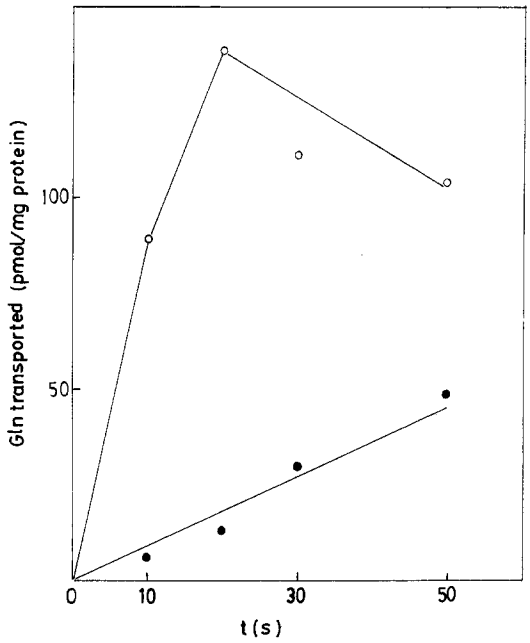

Fig. 1. Time course of L-glutamine uptake by native plasma-membrane vesicles from Ehrlich ascites tumor cells. Transport was measured in the presence of NaSCN (○) or KSCN (●) as described in Material and Methods. Apparent nonspecific binding was subtracted.

apparent uptake extrapolated to zero time was independent of Na^+ gradient and was taken to represent the unspecified binding of glutamine. This binding accounted for 40% of total maximum uptake. In the presence of NaSCN, the glutamine uptake was observed to reach a maximum after approximately 20 s, and then to decay. This overshooting is characteristic of active transport systems driven by a sodium electrochemical gradient and it is presumably a consequence of the dissipation of the gradient. In the presence of NaCl, the overshooting occurred after more than 30 s (results not shown). All the experiments reported in this paper, with the exception of that indicated in Table I, were carried out in the presence and absence of NaSCN for 20 s; a linear uptake for this period of time was assumed.

Table I illustrates that the transient L-glutamine accumulation into the vesicles was an ion-specific process. For equal external cation concentrations, maximum glutamine transport was obtained with gradients of sodium ions. Ammonium ions, but not potassium or choline ions, can substitute for sodium ions; similar results have been found in our lab for the transport of L-serine (unpublished results). On the contrary, Sips *et al.* (1980) found in plasma membrane vesicles from rat liver that a gradient of ammonium ions

Table I. Effects of Ion Gradients on L-Glutamine Uptake in Ehrlich Ascites Tumor Cell Plasma Membrane Vesicles[a]

Addition	Percent of L-glutamine uptake
NaSCN	100
KSCN	14 ± 3
Choline chloride	26 ± 7
NH_4Cl	40 ± 7
NaCl	51 ± 4
NaH_2PO_4	16 ± 4
Sodium citrate	67 ± 2

[a]The uptake of 0.1 mM L-(G-^{3}H)-glutamine was determined in the presence of several salts as listed below. Transport was terminated after 20 s. The apparent nonspecific binding was subtracted. The results are given as percentages, taking uptake in the presence of NaSCN as 100%. Concentrations of cations were 100 mM in all the cases. Data are mean ± SEM of at least three different determinations.

was not effective in the stimulation of amino acid transport. In the same way, the anion accompanying Na^+ clearly influences the accumulation of L-glutamine. The maximum rate of glutamine transport was obtained in the presence of the anion thiocyanate, which easily moves through the membrane and rapidly establishes a membrane potential (Shirazi Beechey *et al.*, 1988). Chloride, phosphate, and citrate were much less effective. Similar results are reported for other plasma membranes (Sips *et al.*, 1980, 1982; Lynch and McGivan, 1987), although there was a smaller difference in the transport in the presence of thiocyanate in comparison with chloride ions.

The Na^+-dependent uptake of L-glutamine clearly shows a saturation kinetics, although it does not fit well to a michaelian hyperbole (Fig. 2). The value obtained for the apparent affinity constant in membrane vesicles was 0.38 mM, and this result is consistent with that obtained for intact Ehrlich cells (Carrascosa *et al.*, 1984).

Acivicin, an analog of glutamine, has been shown to be a reversible and competitive inhibitor of glutamine transport in rat kidney brush border membrane vesicles (Sastrasinh and Sastrasinh, 1986). The results of the present work in Ehrlich cell vesicles shown in Table II reveal a significant inhibition of glutamine transport by both glutamine analogs acivicin and azaserine. Recently, Huber *et al.* (1988) report inhibition of acivicin and azaserine transport in tumor cells by glutamine. Table II shows that mersalyl, DTNB, PCMBS, and NEM also inhibit glutamine transport in isolated vesicles. Because all these compounds are described as inhibitors acting on sulfhydryl groups, the results could suggest that one or more sulfhydryl groups of the carrier molecule might be fundamental to glutamine transport. Nevertheless, this inhibition could also be due to general membrane damage

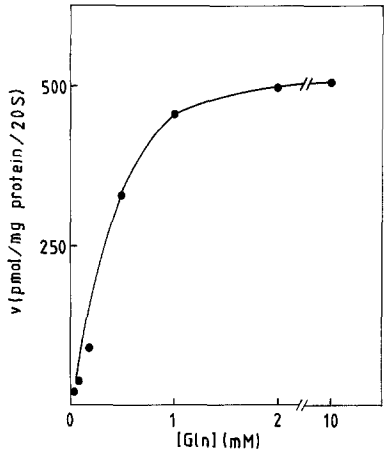

Fig. 2. Kinetics of Na^+-dependent L-glutamine uptake. Transport in the presence of different concentrations of L-glutamine was measured as described in Material and Methods. The uptakes in the presence of NaSCN were corrected for nonspecific transport and binding by subtraction of the corresponding values in the presence of KSCN.

rather than specific inhibition of the transport protein itself, since it has been previously reported that mercurials can interact with one or several sulfhydryl groups located on the cytoplasmic side, or within the membrane. In this way, specific channels for sodium ions are generated through which the electrochemical sodium gradient that energizes sodium-dependent solute transport quickly dissipates (Will and Hopfer, 1979; Biber and Hauser, 1979). In reconstituted vesicles, the inhibition caused by blocking sulfhydryl

Table II. Effect of Inhibitors on L-Glutamine Transport in Plasma Membrane Vesicles[a]

Inhibitor	Percent of inhibition of Na^+-dependent transport of L-glutamine
Acivicin (2.5 mM)	67 ± 4
Azaserine (2.5 mM)	70 ± 20
Mersalyl (0.5 mM)	77 ± 5
NEM (2 mM)	29 ± 1
PCMBS (0.5 mM)	58 ± 4
DTNB (1 mM)	78 ± 4

[a]Plasma membrane vesicles (80–150 μg of protein) were preincubated for 5 min at room temperature with sulfhydryl group inhibitors in a total volume of 15 μl. Transport was initiated by adding an equal volume of transport medium as described in Materials and Methods. Transport was determined after 20 s. The uptakes in the presence of NaSCN were corrected for nonspecific transport and binding by subtraction of the corresponding values in the presence of KSCN. The results are given as percentages of inhibition of L-glutamine uptake in the presence of 0.1 M NaSCN. Data are mean ± SEM of at least three different determinations.

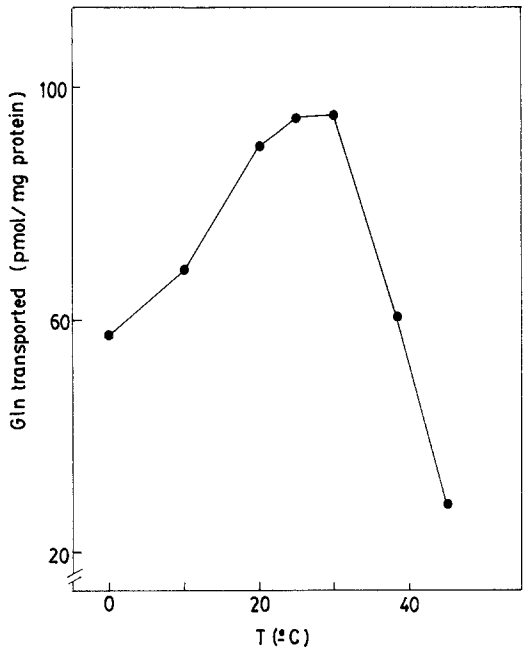

Fig. 3. Effect of temperature on L-glutamine uptake by plasma-membrane vesicles. Transport of 0.1 mM L-glutamine was measured at several temperatures for 20 s, as described in Material and Methods.

groups seems to be more specific. Recently, Quesada and McGivan (1988) report inhibition of system A amino acid transport in reconstituted vesicles from rat liver by NEM, but not by PCMBS. In reconstituted vesicles from Ehrlich cells, however, the inhibition is caused by PCMBS and not by NEM (McCormick and Johnstone, 1988b).

Figure 3 shows the effect of temperature on the uptake of L-glutamine in Ehrlich cell plasma membrane vesicles. Maximum uptake occurred at 30°C. The Arrhenius plot of the inclined part of the curve gave an apparent activation energy of 12.3 kJ/mol. This low activation energy value might be the cause of the high efficiency of the process of L-glutamine transport through the plasma membrane of Ehrlich cells. On the other hand, this is not the first low activation energy reported for a carrier-mediated transport; for instance, Herero *et al.* (1987) report that tryptophan transport by synaptosomal plasma membranes shows an activation energy of 13.6 kJ/mol above 23°C.

Table III reveals a strong inhibition of glutamine transport in the presence of alanine, serine, and cysteine. Although these amino acids are not exclusively transported by ASC, these data might be considered to be an

Table III. Inhibitory Effect of Other Amino Acids on L-Glutamine Transport[a]

Unlabelled amino acid	Na^+-dependent L-glutamine uptake (%)
No addition	100
L-Alanine	17 ± 10
L-Serine	20 ± 14
L-Cysteine	23 ± 2
L-Histidine	17 ± 9
L-Glutamate	86 ± 6
L-Leucine	93 ± 7

[a]Plasma membrane vesicles were incubated in the presence of 0.1 mM L-(G-^{3}H)-glutamate and 2.5 mM unlabelled amino acids as described in Material and Methods. Transport was terminated after 20 s. The uptakes in the presence of NaSCN were corrected for nonspecific transport and binding by subtraction of the corresponding values obtained in the presence of KSCN. The results are expressed as percentages of the uptake in the controls. Data are mean ± SEM of at least three different determinations.

additional argument in favor of the previous suggestion of two transport systems for glutamine in intact cells (Carrascosa *et al.*, 1984). Interestingly, glutamine transport is also inhibited by the basic amino acid histidine, but not by glutamic acid, or branched-chain amino acids such as leucine; nevertheless, it must be considered that under the experimental pH conditions, more than 80% of histidine is not protonized, and might be taken up by the transport systems for neutral amino acids.

Glutamine is efficiently taken up by Ehrlich cells. On the basis of the previous studies with whole cells, the kinetic behavior, the strong inhibition of transport by serine and cysteine, and by comparison with the transport of L-serine (unpublished results), it is suggested that at least two transport systems could be implicated in the uptake of L-glutamine, which is rapidly metabolized by tumor cells even in the presence of glucose (Medina *et al.*, 1988a). Further studies in reconstituted vesicles are needed to clearly discern the roles of the different glutamine carriers in Ehrlich cells.

Acknowledgments

This work was supported by grant PB/88/0445 from the DGICYT. Thanks are due to R. Cameselles for drawings.

References

Ardawi, M. S. M., and Newsholme, E. A. (1985). *Biochem. J.* **231**, 713–719.
Bardin, C., and Johnstone, R. M. (1978). *J. Biol. Chem.* **253**, 1725–1732.

Biber, J., and Hauser, H. (1979). *FEBS Lett.* **108**, 451–456.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Brand, K. (1985). *Biochem. J.* **228**, 353–361.
Carrascosa, J. M., Martínez, P., and Núñez de Castro, I. (1984). *Cancer Res.* **44**, 3831–3835.
Colombini, M., and Johnstone, R. M. (1974a). *J. Membr. Biol.* **15**, 261–276.
Colombini, M., and Johnstone, R. M. (1974b). *J. Membr. Biol.* **18**, 315–334.
Herrero, E., Giménez, C., and Aragón, M. C. (1987). *Life Sci.* **41** 643–650.
Huber, K. R., Rosenfeld, H., and Roberts, J. (1988). *Int. J. Cancer* **41**, 752–755.
Im, W. B., and Spector, A. A. (1980). *J. Biol. Chem.* **255**, 764–770.
Lynch, A. M., and McGivan, J. D. (1987). *Biochim. Biophys. Acta* **899**, 176–184.
McCormick, J., and Johnstone, R. M. (1988a). *J. Biol. Chem.* **263**, 811–819.
McCormick, J., and Johnstone, R. M. (1988b). *Proc. Natl. Acad. Sci. USA* **85**, 7877–7881.
McCormick, J., Tsang, D., and Johnstone, R. M. (1984). *Arch. Biochem. Biophys.* **231**, 355–365.
McCormick, J., Silvius, J. R., and Johnstone, R. M. (1985). *J. Biol. Chem.* **260**, 5706–5711.
Medina, M. A., and Núñez de Castro, I. (1990). *Int. J. Biochem.* **22**, 681–683.
Medina, M. A., Sánchez-Jiménez, F., Márquez, J., Pérez-Rodríguez, J., Quesada, A. R., and Núñez de Castro, I. (1988a). *Biochem. Int.* **16**, 339–347.
Medina, M. A., Sánchez-Jiménez, F., Quesada, A. R., Márquez, J., and Núñez de Castro I. (1988b). *Biochimie* **70**, 833–834.
Quesada, A. R., and McGivan, J. D. (1988). *Biochem. J.* **255**, 963–969.
Sastrasinh, S., and Sastrasinh, M. 1986). *J. Lab. Clin. Med.* **108**, 302–308.
Sauer, L. A., and Dauchy, R. T. (1983). *Cancer Res.* **43**, 3497–3503.
Shirazi-Beechey, S. P., Gorvel, J. P., and Beechey, R. B. (1988). *J. Bioenerg. Biomembr.* **20**, 273–287.
Sips, H. J., van Amelsvoort, M. M., and van Dam, K. (1980). *Eur. J. Biochem.* **105**, 217–224.
Sips, H. J., de Graaf, P. A., and van Dam, K. (1982). *Eur. J. Biochem.* **122**, 259–264.
Will, P. C., and Hopfer, U. (1979). *J. Biol. Chem.* **254**, 3806–3811.