Glutamine transport by vesicles isolated from tumour-cell mitochondrial inner membrane

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Mitochondrial-inner-membrane vesicles, isolated from Ehrlich ascites carcinoma cells by titration with detergents, accumulated L-glutamine by a very efficient transport system. The vesicles lack any phosphate-activated glutaminase activity, allowing measurement of transport rates without interference by L-glutamine metabolism. The time course of the transport was linear for the first 60 s, reaching a steady state after 120 min. L-Glutamine transport showed co-operativity, with a Hill coefficient of 2.2; the kinetic parameters $S_{0.5}$ and V_{max} had values of 5 mM and 26 nmol/30 s per mg of protein respectively. The pH-dependence curve showed a bell shape, with a pH optimum about 8.0. The uptake of L-glutamine was not affected by the presence of a 50-fold molar excess of D-glutamine, L-cysteine, L-histidine,

L-alanine, L-serine and L-leucine, whereas L-glutamate behaved as a poor inhibitor. The structural analogue L-glutamate γ -hydroxamate (5 mM) inhibited the net uptake by 68%; interestingly, other analogues (6-diazo-5-oxo-L-norleucine, acivicin and L-glutamate γ -hydrazide) were ineffective. The impermeant thiol reagent *p*-chloromercuriphenylsulphonic acid (0.5 mM) completely abolished the mitochondrial L-glutamine uptake; in contrast, other thiol reagents (mersalyl and *N*-ethylmaleimide) did not significantly affect the transport. These data confirm the existence of a specific transport system with high capacity for L-glutamine in the mitochondrial inner membrane, a step preceding the highly operative glutaminolysis in tumour cells.

INTRODUCTION

Tumour cells have been described as avidly consuming L-glutamine, even exceeding their own growth needs (for reviews see [1–3]). A century ago, Müller [4] reported a negative nitrogen balance in tumour-bearing patients. A nitrogen interchange between host tissues and tumour cells takes place, L-glutamine being the main non-toxic vehicle of nitrogen [2,5,6].

The major degradative pathway for L-glutamine is carried out in mitochondria and is initiated by the enzyme phosphate-activated glutaminase (PAG; EC 3.5.1.2) [7,8]. Before that, glutamine has to be transported through both plasma and mitochondrial inner membranes. L-Glutamine transport through the plasma membrane of Ehrlich ascites carcinoma cells has been studied by using native vesicles [9]; in these cells, there are two Na^+ -dependent transport systems implicated in glutamine transport, A and ASC. The derepressed system A seems to be the main pathway of glutamine uptake by the plasma membrane of tumour cells (for reviews see [10,11]).

With regard to the mitochondrial transport, Kovacevic et al. [12] firstly postulated the existence of a neutral uniport mechanism for L-glutamine in liver mitochondria; the uptake was inhibited by mersalyl [13]. Since the mitochondrial transport of metabolites is usually 1–2 orders of magnitude faster than the plasma-membrane-related transport [14], special problems arise in mitochondrial transport studies. So several experimental approaches have been attempted to study transport in intact mitochondria, including the inhibitor-stop technique and rapid centrifugation and filtration procedures [15,16]. Using the latter technique, Goldstein and Boylan [17] were able to obtain initial-velocity data in the 1 s range for glutamine transport in rat kidney mitochondria. However, a major drawback in that study was the strong interference by the very active mitochondrial glutamine metabolism. To avoid this problem, Kovacevic and Bajin [18] loaded rat liver mitochondria with [^{14}C]glutamine and

the efflux rates were determined at 0 °C in a P_i -free medium. Although the existence of a mitochondria glutamine carrier seems to be widely postulated, information about the structure and function of the transport system is almost completely lacking, as stated in a review on mitochondrial carriers [19].

The purpose of the present paper is to characterize the mitochondrial L-glutamine transport system of tumour cells. The use of native vesicles isolated from mitochondrial inner membranes was optimized to allow transport measurements without any interference by the mitochondrial glutamine metabolism. A very specific transport system with a high capacity, showing co-operativity and strongly inhibited by the thiol reagent *p*-chloromercuriphenylsulphonic acid (PCMPS) and the glutamine analogue L-glutamate γ -hydroxamate, is described.

MATERIALS AND METHODS

Materials

L-[3,4- $^3H(n)$]Glutamine (sp. radioactivity ~ 50 Ci/mmol) and [U- ^{14}C]sucrose (sp. radioactivity 667 mCi/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.). The ^3H_2O (sp. radioactivity 1.0 mCi/g) and the scintillation cocktail employed, Aquasol-2, were obtained from NEN-Dupont (U.S.A.). All other chemicals were from Sigma Química (Madrid, Spain). The filtration device Model 1225-multiple collector as well as the filters PHWP (0.30 μm pore) were from Millipore Corp. (U.S.A.).

Isolation of mitochondrial-inner-membrane vesicles

A hyperdiploid Lettré strain of Ehrlich ascites carcinoma cells was maintained as previously reported [20]. Cells were harvested on day 10–11 after tumour transplantation, in the stationary phase of growth. The mitochondria were isolated as described

Abbreviations used: DON, 6-diazo-5-oxo-L-norleucine; NEM, *N*-ethylmaleimide; PAG, phosphate-activated glutaminase; PCMPS, *p*-chloromercuriphenylsulphonic acid; POEE, polyoxyethylene ether W-1.

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elsewhere [21]; afterwards, mitochondria (30 mg of protein/ml) were suspended in 20 mM Tris/HCl/70 mM sucrose/210 mM mannitol/1 mM EGTA, pH 8.0 (buffer A), and incubated for 5 min in 0.075 % (w/v) digitonin and 0.25 mg/ml BSA. The reaction was stopped by adding 3 vol. of buffer A and centrifuging at 10000 *g* for 10 min. The pellet (crude mitoplasts) was washed with the same buffer and centrifuged again. The pellet (mitoplasts) was resuspended in buffer A at 30 mg/ml and incubated for 10 min with the Lubrol analogue polyoxyethylene ether W-1 (POEE; 0.16 mg/mg of protein). The detergent action was stopped by addition of 3 vol. of buffer A, followed by centrifuging at 144000 *g* for 50 min. The pellet fraction, mitochondrial-inner-membrane vesicles, was washed with buffer A, centrifuged again, suspended in a small volume of buffer, and frozen at -20 °C until use.

Enzyme assays

The following methods were used to determine mitochondrial marker enzyme activities: adenylate kinase [22], citrate synthase [23] and cytochrome *c* oxidase [24]. In order to evaluate the orientation of the inner-membrane vesicles, the F_0F_1 -ATPase was assayed by the method described by Pullman et al. [25]. The ratio of ATP hydrolysis in the presence to that in the absence of carboxyatractylsides, an inhibitor of the ADP/ATP translocase, indicates the percentage of sealed vesicles oriented inside-out. According to the original protocol [26], we assume that there are no leaky vesicles. Plasma-membrane contamination was evaluated by measuring the ouabain-sensitive Na^+, K^+ -ATPase activity [27].

Transport experiments were carried out at room temperature by mixing 40 μ l of vesicle suspension with 360 μ l of buffer A containing L-[3,4- ^3H]glutamine and unlabelled glutamine at the required concentrations. The final protein concentration was 0.87 mg/ml and the specific activity 44.4 d.p.m./pmol. Uptakes were terminated by rapid filtration of 360 μ l of vesicle suspension through 0.30 μ m-pore filters, by using a multiple collector connected to a vacuum line. The filters were immediately washed with 10 ml of ice-cold buffer A, dried at room temperature, and then placed into scintillation vials containing 5 ml of Aquasol-2. Radioactivity was determined with a Rack-Beta liquid scintillation counter from Pharmacia-LKB. Non-specific binding of L-glutamine to mitochondrial inner membrane and to the filter was determined by duplicate experiments at 4 °C; these values, considered as blanks, were always subtracted from the values of respective experiments. Similar values to those obtained for the blanks were found in experiments carried out in the presence of 1.0% Triton X-100. This detergent disrupts the structure of the vesicles, and consequently avoids the accumulation of radiolabelled L-glutamine into the vesicles. The results confirm that, under the experimental conditions described above, a real L-glutamine transport through the mitochondrial inner membrane is being evaluated. For the transport experiments in which the dependence on pH of the medium was studied, the vesicles were washed twice and resuspended in a buffer consisting of 20 mM Tris/Hepes, 70 mM sucrose, 210 nM mannitol and 1 mM EGTA and adjusted to the desired pH. Moreover, the vesicles were preincubated for 10 min with 2 μ M carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler of oxidative phosphorylation, in order to avoid any pH gradient during the transport study.

Determination of the intravesicular water space

The water space of the inner-membrane vesicles was calculated by the method described by Nicholls [28], modified as follows: a

solution of buffer A was prepared containing 0.5 μ Ci of [^{14}C]sucrose and 2.5 μ Ci of ^3H_2O . The inner-membrane vesicles were added and left 5 min at room temperature for equilibration. Samples (360 μ l) were then filtered and processed as described above. Blank samples were analysed in parallel, to account for non-specific binding to the filters. Total water space and the volume occupied by sucrose were obtained from the equation:

$$\text{space} = V / [(N/n) - 1] \mu\text{l}$$

where *V* is the volume of filtrate, *N* the total counts of the radioisotope in the sample before filtration and *n* the counts of the radioisotope retained on the membrane filter. The vesicle water space was calculated by subtraction of volume occupied by sucrose from the water-permeable space.

Calculation and expression of results

The uptake of glutamine was expressed as pmol/mg of protein. Protein was determined by a modified Lowry procedure with BSA as standard [29]. All values are expressed as means $\pm$ S.E.M. from at least three independent observations, with individual experiments performed in triplicate.

RESULTS AND DISCUSSION

Characterization of inner-membrane vesicles

The inner membrane of the mitochondria was selectively isolated by using detergents which allow a good subfractionation of mitochondria [26], except that POEE was substituted for Lubrol WX. This Lubrol analogue has also been routinely used to obtain enriched inner-membrane fractions [30]. Due to the high cholesterol content of the Ehrlich cell mitochondrial lipids [31], the digitonin concentration used was lower than that described in the original procedure for rat liver mitochondria [26]. After careful titration of detergent concentrations and incubation conditions, an optimal procedure was achieved which permits the isolation of submitochondrial fractions, with the degree of enrichment shown in Table 1. The inner membrane remained essentially intact during the preparation of mitoplasts (inner membrane + matrix), as revealed by the low amounts of the marker enzymes citrate synthase and cytochrome *c* oxidase detected in the fraction corresponding to the outer membrane + intermembrane space. The mitoplasts were then fractionated

Table 1 Subfractionation of Ehrlich mitochondria by the digitonin/POEE method

The marker enzymes used were: adenylate kinase (outer membrane + intermembrane space); citrate synthase (matrix); cytochrome (Cyt.) *c* oxidase (inner membrane). The recovery of the marker activities was greater than 75%. N.D., enzyme activity not detected in submitochondrial fraction.

Fraction	Marker enzyme (% of total activity)			
	Adenylate kinase	Citrate synthase	Cyt <i>c</i> oxidase	PAG*
Outer membrane + intermembrane space	96	7.5 $\pm$ 0.7	3.3 $\pm$ 0.4	4.5 $\pm$ 0.8
Matrix	4	85.1 $\pm$ 2.8	N.D.	6.5 $\pm$ 2.7
Inner membrane	N.D.	7.4 $\pm$ 1.6	96.7 $\pm$ 0.4	0.4 $\pm$ 0.3

* Most of the glutaminase activity (88.6%) was labile under the conditions used for the subfractionation.

Figure 1 Time course of L-glutamine uptake by mitochondrial-inner-membrane vesicles

The concentration of L-glutamine was 0.3 mM (sp. radioactivity 29.6 d.p.m./pmol). The final protein concentration in the assay was 0.5 mg/ml. Each point represents the mean $\pm$ S.E.M. from at least three different experiments performed in triplicate. Inset shows L-glutamine uptake during the initial 60 s.

with POEE, yielding an essentially pure inner-membrane fraction, accounting for 96.7% of the total cytochrome *c* oxidase activity. The vesicles were basically devoid of plasma-membrane contamination, as assessed by the marker enzyme Na^+, K^+ -ATPase (results not shown).

Even though the biochemical and morphological characteristics of the inner-membrane vesicles prepared by the digitonin/Lubrol method are well known [26], it was very important to assess the sidedness of the preparation. By assaying the F_0F_1 -ATPase in the presence or absence of carboxyatractyloside, a grade of inversion ranging from 85 to 95% was determined. Therefore, the inner-membrane vesicles prepared by this technique were sealed and oriented mainly inside-out compared with mitochondria. This percentage of inverted vesicles was very similar to that obtained with submitochondrial vesicles prepared by sonication [32]; however, care must be taken to control the sonication conditions, because contamination by non-inverted material has been reported by several authors in vesicles prepared by sonication [33,34]. The fact that glutamine was accumulated in the mitochondrial vesicles, although they were sealed inside-out, indicates that mitochondrial glutamine transport is a fully reversible process, a common characteristic of a mediated transport system when it occurs via a simple protein carrier [35].

The use of sealed vesicles in transport studies has the advantage of avoiding further metabolism of the molecule transported; consequently, this method allows the isolation of the transport process, and the study of its kinetic characteristics. For the present work, the absence of any PAG activity, the first step of L-glutamine metabolism in the mitochondria, was critical, since this process interferes notably with glutamine transport studies [16]. It is noteworthy that, under the fractionation conditions used in this work, more than 88.6% of the PAG activity was labile (Table 1), and the inner-membrane fraction was always free of glutaminase (final contamination less than 0.5%). Moreover, the inactive PAG protein was detected with antibodies in the matrix fraction (M. Molina, J. A. Segura, J. C. Aledo, M. A. Medina, I. Núñez de Castro and J. Márquez, unpublished work). These results eliminate the possibility that the carrier and glutaminase are two different activities of the same protein. At

Figure 2 Kinetics of glutamine transport across the mitochondrial inner membrane

Transport of L-glutamine was measured over a 30 s period as described in the Materials and methods section. The protein concentration was 1 mg/ml. Values are means $\pm$ S.E.M. of triplicate determinations for five different experiments. The curve drawn through the experimental data represents the best fit to the Hill equation.

least in ascites carcinoma cells, they belong to different proteins; nevertheless, a non-covalent association between them cannot be ruled out in the whole native mitochondria [36].

Glutamine transport in mitochondrial-inner-membrane vesicles

Figure 1 shows the time course of uptake of 0.3 mM L-glutamine by inner-membrane vesicles. The transport was curvilinear, but the initial rate approximated to linearity for the first 60 s; similar results (not shown) were found in the presence of 0.1 mM glutamine. Therefore, a 30 s incubation period was selected for the following transport experiments.

The L-glutamine uptake reached a steady state within 120 min; at this time an accumulative transport value of 726.2 ± 68 pmol/mg was calculated. The transport capacity of tumour cells was one order of magnitude greater than the value reported for submitochondrial particles of rat kidney, where a plateau was reached at about 90 min [35]. On the other hand, the volume of the inner-membrane vesicles, calculated as the sucrose-impermeable water-permeable space, was 1.7 ± 0.3 μl /mg of protein. With this value and the data of Figure 1, the intravesicular glutamine concentration at equilibrium was calculated to be about 0.43 mM, similar to the glutamine concentration in the incubation medium (0.3 mM). So the data do not support concentrative transport under the experimental conditions.

The incubation medium in which transport experiments were carried out was Na^+ -free (buffer A); this suggests that transport of glutamine across the mitochondrial membrane does not seem to require Na^+ . The results are those expected from a physiological point of view, but contrast with the L-glutamine transport throughout plasma-membrane vesicles of Ehrlich cells, which is a truly Na^+ -dependent transport system [9].

The kinetics of glutamine transport are shown in Figure 2. The data do not obey Michaelis-Menten kinetics, and the Lineweaver-Burk and Eadie-Hofstee plots were non-linear. However, the data were satisfactorily adjusted by a non-linear least-squares fit to the Hill equation, where a positive co-operativity was observed (Figure 2). The following kinetic parameters were obtained: Hill coefficient (*h*) 2.2 ± 0.3 ,

Figure 3 Effect of pH on L-glutamine transport into mitochondrial-inner-membrane vesicles

Transport was measured over a 30 s period as described in the Materials and methods section. FCCP ($2 \mu\text{M}$) was always included in the incubation medium. The time course of glutamine uptake was linear for 30 s at each pH studied. Values are means $\pm$ S.E.M. of four different experiments performed in triplicate.

$S_{0.5}$ 5.0 ± 0.3 mM, and V_{max} 26 ± 0.9 nmol/30 s per mg. Sigmoidal kinetics were also observed in rat kidney submitochondrial particles, with $S_{0.5}$ and V_{max} values of 19.5 mM and 3.76 nmol/15 s per mg respectively [35]. The comparison of the normalized tumour data with rat kidney data shows that the ratio $V_{\text{max}}/S_{0.5}$, a criterion of the transport efficiency, was about 16-fold greater in tumour mitochondria.

Compared with hyperbolic kinetics, a protein following sigmoidal kinetics would allow a more sensitive control of the glutamine transport rates. Indeed, the sigmoidal kinetics and the $S_{0.5}$ value inferred could be advantageous for the cellular glutamine metabolism, since mitochondrial glutamine uptake will be relatively slow until a concentration threshold is reached. This mechanism could avoid a rapid depletion of cytosolic glutamine essential for biosynthetic purposes.

Effect of pH

The effect of pH on mitochondrial glutamine transport is depicted in Figure 3. The uptake curve showed a bell shape, with maximum values at pH 8.0–8.5; alkalization or acidification from this point yielded a remarkable decrease in the uptake. This optimum pH of the glutamine carrier in tumour mitochondria is consistent with the value reported for glutaminase [8]. When the mitochondrial electron transport is activated, the matrix pH becomes slightly alkaline, and both the glutamine carrier and glutaminase can be fully operative. Actually, glutamine is a good respiratory substrate for tumour cells, even in the presence of glucose [37]. In contrast, a pH optimum of 6.5 has been reported in rat kidney submitochondrial particles [38]. In this system, the glutamine uptake increased more than 2-fold when the pH of the medium was decreased from 8.5 to 6.5, in accordance with the physiological role of renal glutaminolysis: the neutralization of H^+ excess.

Effect of amino acids and glutamine analogues

The specificity of the L-glutamine transport was confirmed by addition of an excess of different amino acids and glutamine analogues. The results are shown in Table 2; the transport of L-

Table 2 Effects of amino acids and glutamine analogues on glutamine transport into mitochondrial-inner-membrane vesicles

Uptake was measured over 30 s. The concentration of glutamine was 0.1 mM, and that of amino acids and analogues was 5 mM. The protein concentration was 1 mg/ml and the specific radioactivity 200 d.p.m./pmol. Results are means $\pm$ S.E.M. of triplicate determinations for at least three independent experiments.

Test substance	Transport (% of control)
None	100 $\pm$ 9
D-Glutamine	98 $\pm$ 3
L-Glutamine	66 $\pm$ 4
DON	84 $\pm$ 9
Acivicin	102 $\pm$ 8
L-Glutamate γ -methyl ester	93 $\pm$ 9
L-Glutamate γ -hydroxamate	32 $\pm$ 2
L-Glutamate γ -hydrazide	87 $\pm$ 7
L-Cysteine	103 $\pm$ 16
L-Histidine	93 $\pm$ 15
L-Alanine	103 $\pm$ 12
L-Leucine	106 $\pm$ 10
L-Serine	108 $\pm$ 18

glutamine was not affected by addition of a 50-fold molar excess of D-glutamine. This result indicates that the uptake was stereospecific, and confirms the protein nature of the carrier. Among the amino acids tested, only L-glutamate at 4 mM was weakly active (34%); L-cysteine, L-histidine, L-alanine, L-leucine and L-serine had no significant effect on the L-glutamine transport across the mitochondrial membrane. Interestingly, some of these amino acids (L-Ala, L-Ser, L-Cys and L-His) behave as inhibitors of L-glutamine transport through the plasma membrane of Ehrlich ascites cells [9] and rat liver cells (L-His) [39]. The result found for L-glutamate suggests that this amino acid might be transported, at least partially, by the same L-glutamine carrier. On the contrary, L-glutamate did not affect glutamine transport in rat kidney submitochondrial particles; however, a significant inhibition was unexpectedly found for L-alanine [35].

As for the glutamine analogues, only L-glutamate γ -hydroxamate at 5 mM inhibited appreciably (68%); a similar result was found in the plasma membrane of rat liver cells [40]. The glutamine anti-metabolites 6-diazo-5-oxo-L-norleucine (DON) and acivicin were without effect; in contrast, acivicin was a potent transport inhibitor in plasma-membrane vesicles of Ehrlich cells [9] and renal tubules [41]. The results obtained with amino acids and structural analogues support the existence of a mitochondrial glutamine carrier in tumour cells, different from the two transport systems (A and ASC) implicated in the glutamine uptake across the plasma membrane [10,11].

Effect of thiol reagents

The inhibitory effect of the thiol reagents *N*-ethylmaleimide (NEM), mersalyl and PCMPS on glutamine transport was also evaluated. Before the transport assay, vesicles were incubated for 5 min at 25 °C with the indicated concentrations of thiol reagents, and the reaction was stopped with a 20-fold molar excess of 2-mercaptoethanol. Vesicles exposed to 1 mM NEM or 0.5 mM mersalyl were unaffected (values of 101 ± 8 and 100 ± 6 % of control respectively); however, incubation with 0.5 mM PCMPS yielded a full inhibition of the glutamine uptake. Taking into account the facts that the vesicles are inverted and PCMPS is a non-permeant thiol reagent, it could be suggested that essential

thiol group(s) of the carrier are located on the matrix side of the inner membrane.

On the other hand, the differential inhibition pattern obtained supports the notion of a specific effect on the carrier, arguing against a non-specific membrane perturbation. In previous studies, inhibition of the mitochondrial L-glutamine uptake was detected for rat liver [42] and kidney [43] in the presence of 20 nmol of mersalyl/mg of protein, a proportion much exceeded in our study; this may reflect a different susceptibility to the thiol reagents of the mitochondrial carrier in tumour cells. Furthermore, the inhibition pattern of the mitochondrial carrier was markedly different from that shown by glutaminase in mitochondria, where 1 mM NEM completely abolished the enzyme activity (M. Molina, J. A. Segura, J. C. Aledo, M. A. Medina, I. Núñez de Castro and J. Márquez, unpublished work).

An important result of the present work is the validation for transport experiments of the inner-membrane vesicles obtained by mild treatment with detergents, under less drastic and more controlled conditions than those employed to obtain particles by sonication. The technique allows us to obtain highly reproducible transport data in the absence of any interference by mitochondrial glutamine metabolism, the major drawback found in previous studies.

The intracellular glutamine concentration is undetectable during the exponential growth phase of Ehrlich tumour cells [5]. The glutaminolysis process seems to be regulated by the glutaminase expression, which also reaches a maximum during the exponential growth of the cells [44]. With the kinetic data obtained here for the carrier, it is tempting to speculate about one of the most conflicting points regarding the mitochondrial glutamine metabolism, i.e. which is the rate-limiting step: the carrier, the glutaminase or both? Comparison of the normalized values of $V_{\max}/S_{0.5}$ for the carrier and glutaminase [45] indicates that glutaminase has a 15-fold higher catalytic efficiency than the carrier. This may indicate a putative regulatory role for the carrier in the glutaminolytic process, characteristic of the neoplastic transformation. A similar result has been reported for rat kidney [35]; however, in rat liver cells, glutaminase was identified as the major control site of mitochondrial glutamine metabolism [40]. The present results confirm the existence of a very efficient stereospecific carrier for L-glutamine in the mitochondrial inner membrane of Ehrlich tumour cells, different from the carriers previously described in the plasma membrane.

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