Identification and purification of the reconstitutively active glutamine carrier from rat kidney mitochondria

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The glutamine carrier from rat kidney mitochondria, solubilized in dodecyl octaoxyethylene ether ($C_{12}E_8$) and partly purified on hydroxyapatite, was identified and completely purified by Celite chromatography. On SDS/PAGE, the purified glutamine carrier consisted of a single protein band with an apparent molecular mass of 41.5 kDa. When reconstituted into liposomes, the glutamine carrier catalysed both the unidirectional flux of glutamine and the glutamine/glutamine countertransport, which were completely inhibitable by a mixture of pyridoxal 5′-phosphate and *N*-ethylmaleimide. The carrier protein was purified 474-fold with a recovery of 58 % and a protein yield of 0.12 % with respect to the mitochondrial extract. The glutamine carrier-mediated transport is quite specific for L-glutamine. L-Asparagine is the only other amino acid that is efficiently transported by the reconstituted carrier protein. D-Glutamine, L-glutamate and L-aspartate are very poor substrates. The transport activity was inhibited by several thiol-group and amino-group reagents.

INTRODUCTION

The inner membranes of mitochondria contain several transport proteins that catalyse the net flux or exchange of physiologically important metabolites, nucleotides and cofactors between the cytosol and the matrix of mitochondria [1–4]. Amino acid sequences have been published for the carriers of ADP/ATP [5], phosphate [6], 2-oxoglutarate [7], citrate [8], dicarboxylate [9], carnitine/acylcarnitine [10], ornithine [11] and fumarate/succinate [12] as well as for the uncoupling protein [13], which is an H^+ transporter. All these carriers have related primary structures and belong to the same carrier protein family, characterized by a tripartite structure, the presence of two hydrophobic stretches in each of the three domains and the 3-fold repetition of a distinct sequence motif [2–4].

So far, ten mitochondrial carrier proteins have been purified and functionally characterized after reconstitution into liposomes (reviewed in [3]). There are, however, many other transporters that have been identified and functionally differentiated in studies with intact mitochondria but not yet purified and sequenced (reviewed in [4]). An example is provided by the glutamine carrier, which catalyses the transport of glutamine into the mitochondrial matrix, where the enzymes responsible for the degradation of glutamine, glutaminase and glutamate dehydrogenase are located.

The transport of glutamine across the membrane of kidney, liver and brain mitochondria has been the subject of many investigations since 1970 [14–24]. This transport activity is inhibited by thiol reagents, and L-glutamine is transported much more efficiently than D-glutamine. There has been much controversy about the mode of transport of glutamine across the mitochondrial inner membrane. Although an electroneutral uniport of glutamine seems to be the most likely mechanism, other mechanisms such as energy-dependent uniport and glutamine/glutamate and glutamine/malate exchanges have been suggested. Several attempts have been made to measure the kinetics of glutamine transport in mitochondria from normal and acidotic rats. Goldstein and Boylan [18] and Kovacevic and Bajin [20] concluded that mitochondrial transport is not rate-limiting for glutamine hydrolysis. However, Lenzen et al. [25], in agreement with work reviewed in [26] and [27], suggested a regulatory role for transport in glutamine metabolism. In general, characterization of the glutamine carrier in intact mitochondria has encountered serious difficulties due to concurrent glutamine metabolism in the absence of an available specific inhibitor of glutaminase (see also [27,28]). To avoid complications due to glutamine metabolism, Sastrasinh and Sastrasinh [29,30] have studied glutamine transport in submitochondrial particles. They confirmed that glutamine is transported by a mersalyl-sensitive neutral uniport. Furthermore they showed that the rate of transport increased on reducing the pH from 7.5 to 6.5 and that this was an effect of pH itself: it was not due to any pH difference across the mitochondrial membrane.

The purification and reconstitution of the purified protein in artificial membranes is essential for the identification of a transport protein and for its detailed functional and structural characterization. Here we describe the purification and the identification of the glutamine carrier from rat kidney mitochondria by using functional reconstitution as a monitor of the carrier activity during the isolation procedure. Additional aspects of this carrier with respect to substrate specificity and inhibitor sensitivity are also reported.

EXPERIMENTAL

Materials

Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad, Celite 535 from Roth, Amberlite XAD-2 from Fluka, Sephadex G-50 and G-75 from Pharmacia, L-[3,4-^{3}H(N)]-glutamine, L-[1-^{14}C]ornithine and L-[1-^{14}C]ketoglutaric acid from NEN, L-[1,4(2,3)^{14}C]malic acid, [^{32}P]P_i, [1,5-^{14}C]citric acid, L-[U-^{14}C]-glutamic acid and L-[U-^{14}C]aspartic acid from Amersham International, egg yolk phospholipids (L-α-phosphatidylcholine) from fresh turkey egg yolk, Hepes, Pipes, Triton X-114, octylglucoside, Lubrol WX and tetraethylene glycol mono-octyl ether (C_8E_4) from Sigma, octaethylene glycol monododecyl ether

Abbreviations used: C_xE_y, alkyl(*x*)-poly(*y*)oxyethylene ether; LDAO, lauryl(dimethyl)amine *N*-oxide.

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($C_{12}E_8$), lauryl(dimethyl)amine *N*-oxide (LDAO) and Triton X-100 from Fluka. All other reagents were of analytical grade.

Purification of the glutamine carrier

Mitochondria from different tissues (12–14 mg of protein), prepared with standard procedures, were solubilized in 2% (w/v) $C_{12}E_8$/10 mM Hepes/10 mM Pipes (pH 6.5) and centrifuged at 100000 ***g*** for 15 min at 4 °C in a final volume of 1 ml. The glutamine carrier was purified from rat kidney mitochondria by hydroxyapatite and Celite chromatographies as follows: 500 μl of ultracentrifuge supernatant (extract) supplemented with 25 μl of 4 M sodium acetate, pH 6.5, was applied to a hydroxyapatite column (0.5 cm diameter containing 0.2 g of dry material) and eluted with the solubilization buffer. The first 0.5 ml, eluted at a rate of 2 ml/min, was discarded. The second fraction of 0.5 ml, eluted at 50 μl/min, was collected and applied to a second column of hydroxyapatite (0.5 cm diameter containing 0.35 g of dry material) that was eluted with the solubilization buffer. The first 0.5 ml, eluted at 2 ml/min, was again discarded. The second fraction of 0.5 ml, eluted at 50 μl/min, was collected and applied to a Celite column (0.5 cm diameter containing 0.2 g of dry material) that was eluted with the solubilization buffer. The first 0.5 ml was collected (Celite eluate). All the operations were performed at 4 °C.

Reconstitution of the glutamine carrier in liposomes

Protein eluates were reconstituted into liposomes by cyclic removal of the detergent with a hydrophobic column [31]. In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through the same Amberlite XAD-2 column. The composition of the initial mixture used for reconstitution was: 300 μl of purified protein (Celite eluate, approx. 2 μg of protein) (or 35 μl of mitochondrial extract or 150 μl of hydroxyapatite eluates), 10% (w/v) $C_{12}E_8$ up to a final concentration of 11.5 mg/ml, 100 μl of 10% (w/v) egg yolk phospholipids in the form of sonicated liposomes prepared as described previously [32], 30 mM L-glutamine (unless otherwise indicated) and 10 mM Hepes/10 mM Pipes, pH 6.5, in a final volume of 680 μl. After vortex-mixing, this mixture was passed 15 times through the same Amberlite column (0.5 cm × 3.5 cm), pre-equilibrated with the same buffer and with the same substrate present in the initial mixture. All the operations were performed at 4 °C, except the passages through Amberlite, which were performed at room temperature.

Transport measurements

The external substrate was removed by passing 550 μl of proteoliposomes through a Sephadex G-75 column (0.7 cm × 15 cm) pre-equilibrated with 50 mM NaCl/5 mM Hepes/5 mM Pipes (pH 6.5). The first 600 μl of the turbid eluate from the Sephadex column was collected, transferred to reaction vessels (each of 100 μl) and used for transport measurements by the inhibitor stop method [31]. Transport was initiated by adding 10 μl of 1.1 mM labelled substrate and stopped, after the desired interval, by the addition of 5 μl of 220 mM pyridoxal 5′-phosphate/5 μl 44 mM *N*-ethylmaleimide. In control sample, the inhibitors were added together with the labelled substrate at zero time. Each sample was run in duplicate; the assay temperature was 25 °C. The external radioactivity was removed by passing the samples (100 μl) through a Sephadex G-50 column (0.6 cm × 8 cm). The liposomes were eluted with 1.2 ml of 20 mM NaCl and were collected in 4 ml of scintillation mixture, vortex-mixed and counted for radioactivity. The experimental values were corrected by subtracting the respective control value.

Other methods

Polyacrylamide slab-gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS by the method of Laemmli [33]. A minigel system was used; gel sizes were 8 cm × 10 cm × 0.75 mm (thickness). The stacking gel contained 5% (w/v) polyacrylamide and the separation gel contained 17.5% (w/v) polyacrylamide, with an acrylamide-to-bisacrylamide ratio of 38:1. Staining was performed by the silver nitrate method [34]. Protein was determined by the Lowry method, modified for the presence of non-ionic detergents [35]. All the samples used for protein determination were subjected to precipitation with acetone and redissolved in 1% (w/v) SDS. The activity of other transport systems was assayed by the inhibitor-stop method with the following inhibitors: *N*-ethylmaleimide (phosphate, carnitine and glutamate carriers), butylmalonate (dicarboxylate carrier), phthalonate (2-oxoglutarate carrier), benzene-1,2,3-tricarboxylate (tricarboxylate carrier), carboxyatractyloside (ADP/ATP carrier), pyridoxal 5′-phosphate (aspartate/glutamate and ornithine carriers) and α-cyanocinnamate (pyruvate carrier).

RESULTS

Solubilization and purification of the glutamine carrier

The conditions for solubilization of the glutamine carrier in a functionally active state were optimized by measuring the reconstituted ^{3}H-glutamine/glutamine exchange activity in mitochondrial extracts of rat kidney, liver, brain and heart. As shown in Table 1, 2% $C_{12}E_8$ was the most effective detergent in solubilizing the active glutamine carrier. The non-ionic detergents Triton X-100, Lubrol WX and n-octyl polydisperse oligo-oxyethylene also solubilized the glutamine carrier in an active form, but they were considerably less effective. In the mitochondrial extracts obtained by using octyl glucoside, C_8E_4 and LDAO there was virtually no glutamine exchange activity. Table 1 further shows that kidney mitochondria possess the highest reconstituted activity of glutamine transport of all the tissues investigated. A lower activity was found in the extracts obtained from liver and brain mitochondria, whereas the activity was

Table 1 Effect of various detergents on the solubilization of the active glutamine carrier protein

Mitochondria were solubilized by various detergents at the indicated concentrations. A 35 μl sample of the extract was reconstituted into liposomes. The activity was measured as uptake of 0.1 mM [^{3}H]glutamine into proteoliposomes containing 30 mM glutamine. Abbreviations: RKM, rat kidney mitochondria; RLM, rat liver mitochondria; RBM, rat brain mitochondria; RHM, rat heart mitochondria, octyl-Poe, n-octyl polydisperse oligo-oxyethylene, n.d., not detected.

Detergent	Concentration (%, w/v)	Extracted protein (mg/ml)	Glutamine transport (μmol/10 min per g of protein)			
			RKM	RLM	RBM	RHM
Triton X-100	2	6.2	1.5	0.8	1.1	0.0
Triton X-100	3	8.5	0.2	0.0	0.0	n.d.
Triton X-114	2	6.8	0.2	0.0	0.1	0.0
Octyl glucoside	2	6.1	0.0	0.0	0.0	n.d.
Lubrol WX	2	5.9	0.5	n.d.	n.d.	n.d.
$C_{12}E_8$	2	7.0	5.8	1.2	4.3	0.1
$C_{12}E_8$	3	7.5	2.1	0.4	1.6	0.0
C_8E_4	2	2.8	0.0	n.d.	n.d.	n.d.
LDAO	2	5.3	0.0	0.0	n.d.	n.d.
Octyl-Poe	2	6.4	0.5	0.0	0.0	n.d.

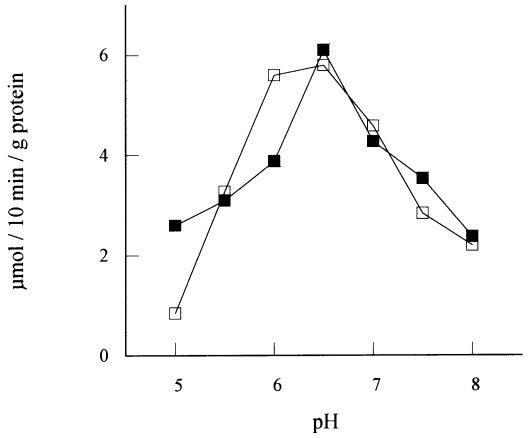

Figure 1 Effect of the pH of the solubilization medium and of the reconstitution mixture on the reconstituted glutamine carrier activity

The activity was measured in proteoliposomes reconstituted with 35 μl of the extract. The proteoliposomes contained 30 mM glutamine and the transport was started by adding 0.1 mM [^{3}H]glutamine. Symbols: □, solubilization was performed at the indicated pH values and reconstitution was conducted at pH 6.5; ■, solubilization was performed at pH 6.5 and reconstitution was conducted at the indicated pH values. The pH of the solubilization medium and of the reconstitution mixture was changed by using a mixture of 10 mM Pipes and 10 mM Hepes.

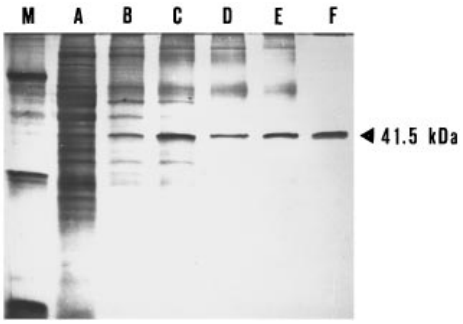

Figure 2 Purification of the glutamine carrier from rat kidney mitochondria

SDS/PAGE of fractions obtained by hydroxyapatite and Celite chromatography. Lane M, marker proteins (BSA, carbonic anhydrase and cytochrome *c*); lane A, mitochondrial extract; lanes B and C, first and second fractions respectively from the first hydroxyapatite column (100 μl); lanes D and E, first and second fractions respectively from the second hydroxyapatite column (200 μl); lane F Celite eluate (450 μl). Other conditions were as described in the Experimental section.

negligible in the extracts of heart mitochondria. In Figure 1 the effect of the pH of the solubilization medium and of the reconstitution mixture on the reconstituted glutamine/glutamine exchange activity was investigated. Optimal transport activity was obtained by solubilizing the mitochondria at pH 6.5 and by performing the reconstitution at the same pH (Figure 1). In other experiments we tested the influence of ionic strength on the solubilization of the active glutamine carrier from rat kidney, brain and liver mitochondria. For example, the reconstituted glutamine transport activity of kidney mitochondria, extracted with 2% $C_{12}E_8$ in the presence of 10 mM Hepes/10 mM Pipes, pH 6.5, was decreased from 5.6 μmol/10 min per g of protein to 4.1, 4.0 and 2.7 μmol/10 min per g of protein by the addition of 20 mM Na_2SO_4, 20 mM NaCl and 50 mM NaCl respectively. Because cardiolipin has been used successfully to improve the purification of several transport proteins [32,36–43], the effect of this lipid during solubilization and reconstitution was also analysed. It was found that, in proteoliposomes reconstituted with the extract obtained from rat kidney mitochondria, the activity of glutamine transport was about the same in the presence of cardiolipin during solubilization or during reconstitution as in its absence. As a result of the above-mentioned experiments, to obtain optimal solubilization of functionally active glutamine carrier, we decided to use rat kidney mitochondria as the starting material and to extract them with a solubilization buffer consisting of 2% (w/v) $C_{12}E_8$, 10 mM Hepes and 10 mM Pipes, pH 6.5, without further addition of salts.

Mitochondrial carrier proteins have been purified by using hydroxyapatite chromatography as the first and major step of purification (reviewed in [3]). In general, mitochondrial carrier proteins are not bound to hydroxyapatite in media of low ionic strength, in contrast with most other mitochondrial proteins. On this basis we first adopted the general procedure used for the purification of many mitochondrial transporters [31], i.e. we applied the mitochondrial kidney extract (0.5 ml, 6–8 mg of protein) to hydroxyapatite columns containing 0.6 g of dry hydroxyapatite and used the solubilization buffer for elution. Two fractions of the hydroxyapatite eluate (each of 0.5 ml) were collected and tested for reconstitutable activity of the glutamine carrier. Surprisingly, when reconstituted into liposomes, both fractions exhibited no glutamine/glutamine exchange activity, suggesting that the glutamine carrier had been bound by the resin. To check the possibility that the glutamine carrier was bound to hydroxyapatite under the conditions used above, we decreased the amount of hydroxyapatite used and added sodium acetate to the mitochondrial extract immediately before chromatography. In experiments investigating these conditions, we found that the elution of glutamine carrier activity applied to 0.2 g of hydroxyapatite (keeping the volume and the amount of protein applied to the column constant) increased with increasing the concentration of sodium acetate up to 200 mM. When using the 0.2 g hydroxyapatite column and the mitochondrial extract supplemented with 200 mM sodium acetate, the first two fractions of the hydroxyapatite eluate exhibited a significant reconstituted glutamine carrier activity (the total activities of the first and second fractions were 13.0 and 6.5 nmol/10 min per ml respectively). The specific glutamine transport activities of both fractions, however, were not substantially different from that of the mitochondrial extract (12.7 and 11.3 μmol/10 min per g of protein in the first and second fractions of the hydroxyapatite eluate respectively). These findings can be explained by the relatively large amount of protein eluted in the first and second fractions (1.0 and 0.58 mg/ml respectively). In comparison with the results obtained with other carriers, the purification of the glutamine carrier achieved by this procedure was very unsatisfactory. This was probably the consequence of the presence of the high salt concentration and of the detergent $C_{12}E_8$ (instead of Triton) during the hydroxyapatite chromatography. However, we then observed that the glutamine carrier activity present in the first fractions of the hydroxyapatite eluate varied inversely with the elution rate of the column. By changing this critical parameter in combination with the others mentioned above, we obtained a first fraction, eluted at a high rate (2 ml/min), containing several protein bands (Figure 2, lane B) and showing a low specific activity of glutamine transport (3.8 μmol/min per g of protein). The second fraction too, which was eluted at a low rate (50 μl/min), contained several protein bands (Figure 2, lane C), although fewer than those in the first fraction, but the specific

Table 2 Purification of the glutamine carrier

The proteoliposomes were loaded with 30 mM glutamine and the exchange reaction was started by adding 0.1 mM [^{3}H]glutamine. The incubation time was 10 min. HTP 1 and HTP 2 indicate the second fractions from the first and the second hydroxyapatite columns (see the Experimental section). The results are from a representative experiment; similar results were obtained in three independent experiments.

Stage	Protein (mg/ml)	Specific activity (μmol/10 min per g)	Total activity (nmol/10 min per ml)	Purification (fold)
Extract	7.4	5.6	41.7	–
HTP 1	0.517	59.2	30.5	10
HTP 2	0.101	358	36.1	64
Celite	0.009	2671	24.3	474

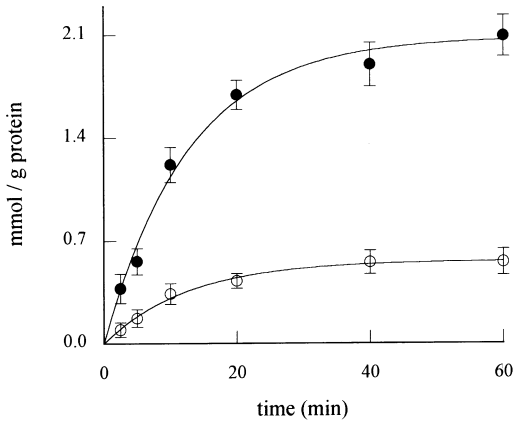

Figure 3 Time course of [^{3}H]glutamine uptake in reconstituted liposomes

[^{3}H]Glutamine (0.1 mM) was added at zero time to reconstituted liposomes with 30 mM glutamine as internal substrate (●), or to reconstituted liposomes without internal substrate (○). Transport was stopped at the indicated times and the intraliposomal radioactivity was measured. Results are means ± S.E.M. for four experiments.

activity of glutamine transport was 10-fold that of the mitochondrial extract (Table 2). For further purification of the glutamine carrier, the second fraction of the hydroxyapatite eluate was applied to a second hydroxyapatite column containing 0.35 g of dry hydroxyapatite, i.e. 1.75-fold more resin than in the first hydroxyapatite chromatography. This column was also eluted at two different rates, the first 0.5 ml at 2 ml/min and the second 0.5 ml at 50 μl/min. Both fractions contained few protein bands (Figure 2, lanes D and E). However, the glutamine carrier was present in a smaller amount in the first fraction (with a specific activity of 66.9 μmol/10 min per g of protein), whereas it was enriched in the second fraction (with a specific activity of 358 μmol/10 min per g of protein).

Final purification of the glutamine carrier protein was achieved by chromatography on Celite. By this purification step, all the contaminating proteins remained bound to the resin, whereas the glutamine carrier passed through. Thus the Celite eluate exhibited a glutamine transport activity of 2671 μmol/10 min per g of protein (Table 2), a value comparable to that of other purified and reconstituted mitochondrial transporters [38,40–43]. On SDS/PAGE, the Celite eluate showed a single protein band with an apparent molecular mass of 41.5 kDa (Figure 2, lane F). The entire procedure increased the specific activity 474-fold with respect to that of the mitochondrial extract (Table 2). Approx. 58 % of the total transport activity was recovered, with a protein yield of 0.12 %.

Table 3 Uptake of labelled substrates by reconstituted liposomes

The reconstitution mixture contained 30 mM L-glutamine (+glutamine$_{in}$) or no substrate (−glutamine$_{in}$). After the removal of external substrate by Sephadex G-75 columns, transport was started by the addition of the labelled substrates indicated, at 0.1 mM each. The incubation time was 10 min. The results are means ± S.E.M. for four experiments.

External substrate	Activity (μmol/10 min per g of protein)	
	+Glutamine$_{in}$	−Glutamine$_{in}$
[^{3}H]Glutamine	2432 ± 226	469 ± 44
[^{14}C]Glutamate	351 ± 23	97 ± 11
[^{14}C]Aspartate	304 ± 25	102 ± 9
[^{14}C]Malate	76 ± 11	73 ± 9
[^{14}C]Ornithine	73 ± 13	63 ± 13
[2-^{14}C]Oxoglutarate	65 ± 12	69 ± 12
[^{14}C]Citrate	72 ± 19	73 ± 13
[^{32}P]P_i	66 ± 18	71 ± 9

Functional properties of the reconstituted glutamine carrier

In the experiments described in this section, the purified glutamine carrier present in the Celite eluate shown in Figure 2 (lane F) was functionally characterized after incorporation into liposomes. Figure 3 shows the time course of uptake of 0.1 mM [^{3}H]-glutamine by proteoliposomes containing 30 mM glutamine or no substrate. The experimental data for the glutamine/glutamine exchange kinetics fitted a first-order equation with a rate constant, k, of 0.078 min^{-1}; the initial rate of the exchange, calculated as the product of k and the intraliposomal radioactivity at equilibrium, was 0.16 mmol/min per g of protein. The unidirectional transport of glutamine, i.e. the uptake of [^{3}H]glutamine by proteoliposomes without internal substrate, also followed first-order kinetics with a k of 0.089 min^{-1} and an initial rate of 0.045 mmol/min per g of protein. To confirm that glutamine uptake was carrier-mediated, controls were performed by using proteoliposomes that had been reconstituted with boiled protein. Under these conditions there was no uptake of [^{3}H]glutamine (results not shown).

The substrate specificity of the reconstituted glutamine carrier was first investigated by measuring the uptake of various labelled substrates by proteoliposomes in the presence and in the absence of internal glutamine. As shown in Table 3, [^{3}H]glutamine was efficiently taken up by proteoliposomes both in exchange for internal glutamine and in the absence of internal glutamine (see also Figure 3). To a much smaller extent than [^{3}H]glutamine, [^{14}C]glutamate and [^{14}C]aspartate also exchanged for internal glutamine. The still lower activity observed with different types of substrate, such as labelled malate, ornithine, 2-oxoglutarate, citrate and phosphate, represents the non-specific 'background', because the amount of radioactivity associated with proteoliposomes incubated with these substrates is approximately the same in the presence and in the absence of internal glutamine. Compared with this background there was also a very small, but significant, uptake of [^{14}C]glutamate and [^{14}C]aspartate by proteoliposomes in the absence of internal glutamine (Table 3).

The substrate specificity of the glutamine transporter was investigated further by measuring the uptake of [^{3}H]glutamine into proteoliposomes containing various substrates. The results in Table 4 show that the highest activity was observed with

Table 4 Dependence on internal substrate of glutamine transport in reconstituted liposomes

The reconstitution mixture contained each of the indicated substrates at 30 mM. After the removal of external substrate on Sephadex G-75 columns, transport was started by the addition of 0.1 mM [^{3}H]glutamine. The incubation time was 10 min. The results are means ± S.E.M. for four experiments.

Internal substrate	Glutamine transport (μmol/10 min per g of protein)
None (NaCl present)	505 ± 43
L-Glutamine	2403 ± 224
D-Glutamine	562 ± 53
L-Asparagine	1284 ± 136
L-Aspartate	591 ± 41
L-Glutamate	604 ± 52
L-Glutamate methyl ester	472 ± 46
L-Alanine	491 ± 18
L-Glycine	479 ± 25
L-Leucine	481 ± 39
L-Proline	498 ± 39
L-Serine	424 ± 27
L-Ornithine	485 ± 36
L-Malate	499 ± 63
2-Oxoglutarate	470 ± 37
Phosphate	455 ± 31
ADP	469 ± 56

internal L-glutamine. To a smaller extent, [^{3}H]glutamine was exchanged for internal L-asparagine. A low activity was found in the presence of internal D-glutamine, L-glutamate or L-aspartate. This activity was only slightly higher than the unidirectional glutamine transport activity (with internal NaCl), indicating that these amino acids are very poor substrates for the carrier. No exchange was observed with internal glutamate methyl ester, L-alanine, L-glycine, L-leucine, L-proline, L-serine, L-ornithine, L-malate, phosphate, 2-oxoglutarate or ADP. The residual activity in the presence of these substrates was virtually the same as the unidirectional glutamine transport activity (with internal NaCl).

To compare the inhibitor sensitivity of the isolated protein with that of the glutamine carrier in mitochondria and to characterize the purified protein further, we investigated the effect of known inhibitors of mitochondrial carriers [27] on the reconstituted glutamine/glutamine exchange activity (Table 5). At 0.1 mM external [^{3}H]glutamine, the inhibition by 0.5 mM mersalyl, 0.5 mM *p*-hydroxymercuriobenzoate and 2 mM 4,4′-di-isothiocyanate stilbene-2,2′-disulphonate was nearly complete. *N*-ethylmaleimide, eosin 5-maleimide, pyridoxal 5′-phosphate and *N*-ethyl-5-phenylisoxazolium 3′-sulphonate (Woodward's reagent K), at the concentrations indicated in Table 5, also strongly inhibited the carrier activity, although less efficiently than the mercurials and 4,4′-di-isothiocyanate stilbene-2,2′-disulphonate. In contrast, bathophenanthroline, phenylglyoxal and phenylsuccinate were poor inhibitors. Furthermore, benzene-l,2,3-tricarboxylate, carboxyatractyloside and α-cyanocinnamate, which specifically inhibit the citrate [44], ADP/ATP [45] and pyruvate [46] transporters respectively, did not inhibit the glutamine carrier at all. The best inhibition was obtained by a mixture of 2 mM *N*-ethylmaleimide and 10 mM pyridoxal 5′-phosphate, which was used as the stop inhibitor in all the experiments described in this paper. The same inhibition pattern was observed on measuring the unidirectional uptake of [^{3}H]-glutamine, instead of the glutamine/glutamine exchange.

In further experiments (results not shown), we found that the fraction shown in Figure 2 (lane F), consisting of a single protein band with an apparent molecular mass of 41.5 kDa, when reconstituted into liposomes did not catalyse the exchange reactions phosphate/phosphate (phosphate carrier), malate/phosphate (dicarboxylate carrier), citrate/citrate (tricarboxylate carrier), 2-oxoglutarate/2-oxoglutarate (oxoglutarate carrier), pyruvate/pyruvate (pyruvate carrier), carnitine/carnitine (carnitine carrier), ornithine/citrulline (ornithine carrier) and ADP/ADP (adenine nucleotide carrier). In contrast, very low pyridoxal 5′-phosphate/*N*-ethylmaleimide-sensitive aspartate/aspartate and glutamate/glutamate exchange activities, corresponding to 3–6 % of that of the glutamine/glutamine exchange, were also present. Therefore any contamination of our purified glutamine carrier preparation by the aspartate/glutamate carrier and/or by the glutamate/H^+ carrier would at most be very small. Most probably, the very low activities of aspartate and glutamate homo-exchanges observed in this paper are accounted for by the ability of the glutamine carrier to transport aspartate and glutamate at a very low efficiency. This interpretation is supported by the fact that the substrate specificity and the inhibitor sensitivity of our glutamine carrier preparation (Tables 3–5) are drastically different from those reported for the aspartate/glutamate carrier and the glutamate/H^+ carrier [47–49].

DISCUSSION

The present investigation represents, to our knowledge, the first report of a procedure that yields a highly purified preparation of functional glutamine carrier protein from mitochondria. This procedure resembles the purification scheme used in our laboratory for the isolation of other mitochondrial transporters involving solubilization with non-ionic detergents and chromatography on hydroxyapatite and Celite [31]. However, several important modifications had to be introduced for successful purification of the functionally active glutamine carrier. Among the several parameters varied during solubilization and purification, in particular the ratio of solubilized membranes to hydroxyapatite, the presence of 200 mM sodium acetate during chromatography on hydroxyapatite and the rate of elution of the hydroxyapatite column were found to be of major importance. Under appropriate conditions, the glutamine carrier, which (unlike most mitochondrial transporters) binds to hydroxyapatite, is eluted almost specifically, allowing a substantial purification of the glutamine carrier before passage over Celite. The highly purified nature of the final glutamine carrier fraction (i.e. the Celite pass-through) is demonstrated by the SDS/PAGE analysis, which indicates the presence of one main band with an apparent molecular mass of 41.5 kDa.

On incorporation into phospholipid vesicles, the purified glutamine carrier catalyses both the unidirectional transport of glutamine and the glutamine/glutamine exchange with similar first-order rate constants. The initial rate of the exchange is higher than that of the unidirectional transport, indicating trans-stimulation of the transport process. In this respect the glutamine carrier resembles the mitochondrial phosphate [50] and carnitine [51] transporters. Our results further indicate that the purified glutamine carrier displays a very narrow substrate specificity, as demonstrated by the finding that only L-asparagine can be used as countersubstrate and that D-glutamine, L-glutamate and L-aspartate are very poor substrates. The observation that alanine and other neutral amino acids such as serine, glycine, proline and leucine do not exchange for glutamine demonstrates that the glutamine carrier is not a broad-specificity neutral amino acid transporter, as suggested previously (see [28]). Clearly the L-configuration of the α-carbon and the amide group in the γ- or δ-position of the amino acid are essential requirements for the

Table 5 Effect of inhibitors on the reconstituted glutamine transport

Proteoliposomes were loaded with 30 mM L-glutamine; transport was started by adding 0.1 mM [^{3}H]glutamine. The incubation time was 10 min. The inhibitors were added 1 min before the labelled substrate at the concentrations indicated. The results are means ± S.E.M. for four experiments. The control of uninhibited glutamine/glutamine exchange was 2545 ± 159 μmol/10 min per g of protein.

Inhibitor	Concentration (mM)	Inhibition (%)
Mersalyl	0.5	90 ± 7.5
p-Hydroxymercuriobenzoate	0.5	86 ± 6.8
N-Ethylmaleimide	1	66 ± 3.7
Eosin 5-maleimide	0.5	69 ± 6.1
Pyridoxal 5′-phosphate	10	76 ± 4.5
4,4′-Di-isothiocyanate stilbene-2,2′-disulphonate	2	87 ± 7.1
Pyridoxal 5′-phosphate + *N*-ethylmaleimide	10 + 2	100
N-Ethyl-5-phenylisoxazolium 3′-sulphonate	2	58 ± 6.0
Bathophenanthroline disulphonate	10	23 ± 1.5
Phenylglyoxal	2	26 ± 2.8
Phenylsuccinate	10	20 ± 3.4
Benzene-1,2,3-tricarboxylate	10	1 ± 0.7
Carboxyatractyloside	0.02	6 ± 3.4
α-Cyanocinnamate	0.5	4 ± 2.7

specific interaction with the substrate-binding site of the glutamine carrier.

The amino acid modification studies reported here indicate that the purified and reconstituted glutamine carrier is substantially inhibited by organic mercurials, suggesting that one or more cysteine residues are essential for glutamine transport. This finding is in agreement with previous observations concerning the strong sensitivity of this carrier to mersalyl in intact mitochondria [20]. At variance with early studies we also observed an inhibition of glutamine transport by *N*-ethylmaleimide and eosin 5-maleimide. In addition we found that the purified glutamine carrier is highly sensitive to reagents that are relatively selective for lysyl (pyridoxal 5′-phosphate and 4,4′-di-isothiocyanate stilbene-2,2′-disulphonate) and carboxy (*N*-ethyl-5-phenylisoxazolium 3′-sulphonate) residues.

Despite the basic functional relationship of the glutamine carrier to the other mitochondrial transporters, this carrier differs from those members of the mitochondrial carrier family that have as yet been biochemically characterized in having a higher apparent molecular mass (41.5 kDa compared with 28–34 kDa; see [3] for refs.). There are, however, proteins (for example in the yeast genome [9]) of unknown function with a molecular mass of approx. 40 kDa that are considered members of the mitochondrial carrier family on the basis of their sequence features. Clearly, a knowledge of the primary structure of the glutamine carrier is needed to decide whether this metabolically important transporter belongs to the mitochondrial carrier family. It is hoped that the purification and reconstitution procedures described here will provide a useful basis for further characterization of the glutamine carrier at a molecular level.

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