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Identification and purification of the aspartate/glutamate carrier from bovine heart mitochondria

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The aspartate/glutamate carrier from bovine heart mitochondria was solubilized with dodecyl-octaoxyethylene ether ($C_{12}E_8$) and purified by chromatography on hydroxyapatite and celite. On SDS gel electrophoresis, the purified aspartate/glutamate carrier consisted of a single protein band with an apparent M_r of 31 500. When reconstituted into liposomes the aspartate/glutamate carrier protein catalyzed an *N*-ethylmaleimide-sensitive aspartate/aspartate exchange. It was purified 620-fold with a recovery of 17.2% and a protein yield of 0.03% with respect to the mitochondrial extract. The properties of the reconstituted carrier, i.e. requirement for a counteranion, substrate specificity and inhibitor sensitivity, were similar to those of the aspartate/glutamate carrier as characterized in mitochondria.

Introduction

The aspartate/glutamate carrier from the inner mitochondrial membrane catalyzes a 1:1 exchange of aspartate for glutamate and plays an important role in several metabolic processes like urea synthesis, gluconeogenesis and the malate-aspartate shuttle [1,2]. Since aspartate is transported in the dissociated form and glutamate is co-transported with a proton, the aspartate/glutamate exchange is electrogenic and depends on both the membrane potential and the pH gradient [3]. Besides aspartate and glutamate, this carrier also accepts cysteinesulfinate, which is transported, like aspartate, as the anion [4]. It is inhibited by several SH reagents including NEM [1,5], by pyridoxal 5′-phosphate and by diethyl pyrocarbonate [6,7]. There is some disagreement about the kinetic mechanism in intact mitochondria [8,9]. However, studies in proteoliposomes provided convincing evidence that the reconstituted aspartate/glutamate carrier functions according to a sequential type of mechanism [10].

In 1986 the aspartate/glutamate carrier was substantially purified from bovine heart mitochondria by a relatively complicated procedure involving HPLC on hydroxyapatite and a series of elution buffers and gradiens [6]. In SDS-containing gel, the purified fraction consisted of a prominent band with a M_r of 68 000 and traces of contaminants mainly of M_r 31 000. The 68 kDa protein band was attributed to the aspartate/glutamate carrier and the 31 kDa band to the ADP/ATP carrier.

All mitochondrial carrier proteins so far purified have a similar M_r of 28 000–34 000 [11–22]. Furthermore, the four carriers the sequence of which is known, i.e. the ADP/ATP carrier, the uncoupling protein, the phosphate carrier and the oxoglutarate carrier, have a tripartite structure composed of related sequences of about 100 amino acids in length [23–26]. Since the aspartate/glutamate carrier activity has been attributed to a protein with a considerably higher M_r of 68 000, we reinvestigated the question of the identity of this important carrier protein.

In this paper we describe the identification and the purification of the aspartate/glutamate carrier from bovine heart mitochondria using functional reconstitution as a monitor of the carrier activity during the isolation procedure. Upon SDS gel electrophoresis the purified aspartate/glutamate carrier protein appears to be a single polypeptide with an apparent molecular mass of 31.5 kDa. When incorporated into liposomes it

Abbreviations: C_xE_y, alkyl(x)-poly(y)oxyethylene ether; EDTA, ethylenediaminetetraacetic acid; Pipes, 1,4-piperazinediethanesulphonic acid; SDS, sodium dodecylsulphate; NEM, *N*-ethylmaleimide.

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exhibits transport properties which closely resemble those described for the aspartate/glutamate carrier in intact mitochondria.

Materials and Methods

Materials. Hydroxyapatite (Bio-Gel HTP) and Dowex AG1-X8 were purchased from Bio-Rad; Celite 535 from Serva; Amberlite XAD-2, $C_{12}E_8$ and egg yolk phospholipids (phosphatidylcholine from eggs) from Fluka; Pipes, $C_{13}E_{10}$, L-glutamate and L-aspartate from Sigma; L-[U-^{14}C]glutamate and L-[U-^{14}C]aspartate from Amersham International (Amersham, UK); and Sephadex G-75 from Pharmacia. All other chemicals were of the highest purity commercially available.

Isolation of the aspartate/glutamate carrier. Bovine heart mitochondria (20 mg protein), prepared as described in Ref. 27, were preextracted with 1% $C_{13}E_{10}$ (w/v), 1 mM EDTA and 100 mM NaH_2PO_4 (pH 6.5) for 10 min at 0°C in a final volume of 1 ml. After 20 min centrifugation at 140000 × g, the pellet was solubilized with 2.4% $C_{12}E_8$ (w/v), 1 mM EDTA and 100 mM NaH_2PO_4 (pH 6.5) in a final volume of 0.8 ml and at a concentration of about 16 mg protein/ml. After 10 min at 0°C the mixture (0.8 ml) was centrifuged at 100000 × g for 20 min to obtain a supernatant referred to as extract. 0.6 ml of the extract (8–9 mg protein) supplemented with 0.2 ml ammonium acetate (1 M final concentration) were applied to a 10 g dry hydroxyapatite column (2 cm diameter) and eluted with 1.2% $C_{12}E_8$, 0.5 mM EDTA, 1 M ammonium acetate and 50 mM NaH_2PO_4 (pH 6.5). The first 1.2 ml of the eluate from the hydroxyapatite column were collected, 0.8 ml of which were applied onto a dry celite column (pasteur pipette containing 0.5 g of dry material). Elution was performed with 0.3% $C_{12}E_8$, 0.125 mM EDTA, 0.5 M ammonium acetate and 12.5 mM NaH_2PO_4 (pH 6.5). Fractions of 0.6 ml were collected. Pure aspartate/glutamate carrier was present in fraction 3. All the operations were performed at 4°C, unless otherwise specified.

Reconstitution of the aspartate/glutamate carrier in liposomes. Liposomes were prepared as described previously [16] by sonication of 100 mg/ml egg yolk phospholipids in water for 60 min. Protein eluates were reconstituted by removing the detergent with a hydrophobic column [28]. In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through Amberlite XAD-2 columns. The composition of the initial mixture used for reconstitution was: 200 μl of the hydroxyapatite or celite eluate or 45 μl of the extract, 30 μl of 10% $C_{12}E_8$, 100 μl of 10% phospholipids in the form of sonicated liposomes, 14 μl of 1 M sodium L-aspartate or sodium L-glutamate, 50 μl ammonium acetate (to reach the final concentration of 1 M) in a final volume of 700 μl. After vortexing, this mixture was passed 15 times through the same Amberlite column (0.5 × 3.2 cm) preequilibrated with a buffer containing 10 mM NaH_2PO_4 (pH 7.0) and 20 mM of the substrate present in the starting mixture. All the operations were performed at 4°C, except for the passage through Amberlite, which was carried out at room temperature.

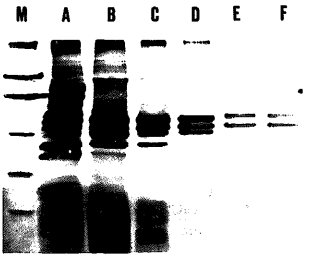
Transport measurements. In order to remove the external substrate, 650 μl of proteoliposomes were passed through a Sephadex G-75 column (0.7 × 15 cm) preequilibrated with 50 mM NaCl and 10 mM Pipes (pH 6.5). The first 600 μl of the turbid eluate from the Sephadex column were collected, transferred to reaction vessels (150 μl each), incubated at 25°C (for 4 min), and then used for transport measurements by the inhibitor stop method [29]. Transport was started by adding 0.1 mM L-[^{14}C]aspartate (10 μl, about 180000 cpm) or 0.1 mM L-[^{14}C]glutamate (10 μl, about 180000 cpm) and stopped after the desired time interval by addition of 0.5 mM NEM. In control samples, the inhibitor was added together with the labelled substrate. The assay temperature was 25°C. In order to remove the external radioactivity, 150 μl of each sample were passed through a Dowex AG1-X8 column, 50–100 mesh, acetate form (0.5 × 4 cm). The liposomes eluted with 1 ml of 50 mM sucrose were collected in 4 ml of scintillation mixture, vortexed and counted. The transport activity was calculated by subtracting the control from the experimental values.

Other methods. Polyacrylamide slab-gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli [30]. The stacking gel contained 5% acrylamide and the separation gel contained 17.5% acrylamide and an acrylamide/bisacrylamide ratio of 37.5. Staining was performed by the silver nitrate method [31]. The molecular weights were determined by comparison to standards obtained from Bio-Rad. Protein was determined by the method of Lowry et al. [32]. All the samples used for protein determination were subjected to acetone precipitation and redissolved in 1% SDS [22]. The activity of other transport systems was assayed by the inhibitor stop method as previously described: phosphate carrier [13], oxoglutarate carrier [16], dicarboxylate carrier [17], tricarboxylate carrier [18], pyruvate carrier [21], carnitine carrier [22] and ADP/ATP carrier [28].

Results and Discussion

Solubilization and purification of the aspartate/glutamate carrier

Bovine heart mitochondria were preextracted with $C_{13}E_{10}$ and then solubilized with $C_{12}E_8$, essentially as reported by Kramer et al. [6]. Inclusion of ammonium acetate in the solubilization buffer was found to be not



Total activity	0.099	0.097	0.094	0.089	0.077
Specific activity	274	350	668	1091	1020

Fig. 1. Purification of the aspartate/glutamate carrier in dependence of the amount of hydroxyapatite: SDS gel electrophoresis of the eluates (first 1.2 ml) obtained from columns containing 0.6 g (B), 2.5 g (C), 6 g (D), 10 g (E) or 12.5 g (F) hydroxyapatite. M, marker proteins (phosphorylase *b*, 97400; bovine serum albumin, 66200; ovalbumin, 45000; carbonic anhydrase, 31000; soybean trypsin inhibitor, 21500; and lysozyme, 14400); A, mitochondrial extract (5 μl); and B–F, hydroxyapatite eluates (50 μl). The numbers reported in the figure represent the specific activities in μmol/30 min per g protein and the total activities in μmol/30 min of the aspartate/aspartate exchange measured in each eluate as described in Materials and Methods.

necessary. However, when ammonium acetate was added to the mitochondrial extract before reconstitution the activity of the aspartate/aspartate exchange was increased 1.8-fold. Ammonium acetate was therefore always included in the reconstitution mixture at a concentration of 1 M.

In general, mitochondrial carrier proteins are not bound to hydroxyapatite (see Ref. 33 for a review). In the case of the aspartate/glutamate carrier, however, the transport protein is retained unless the ionic strength is increased [6]. We found that elution of the aspartate/glutamate carrier applied onto hydroxyapatite increased with increasing the concentration of ammonium acetate up to 1 M. The aspartate/glutamate carrier could also be eluted from hydroxyapatite by addition of other salts (NaCl, Na_2SO_4 and Na_2HPO_4), but in this case more contaminating proteins and less aspartate exchange activity was eluted.

When using the 0.6 g hydroxyapatite column, similar as described for the purification of several other carrier proteins [13,15,16,19–21], and the elution buffer including 1 M ammonium acetate (see Methods), the specific activity of the aspartate/glutamate carrier in the eluate was increased 15-fold as compared to that of the mitochondrial extract (Fig. 1, lane B). In comparison to the results obtained with other carriers, the purification of the aspartate/glutamate carrier achieved by this procedure was much less satisfactory. This was probably a consequence of the presence of the high salt concentration and of the detergent $C_{12}E_8$ (instead of Triton) during the hydroxyapatite chromatography.

For improving the purification procedure a main factor was found to be a significant increase in the amount of hydroxyapatite used. In experiments investigating these conditions, the amount of protein applied to the column was kept constant so that the protein/hydroxyapatite ratio decreased progressively. As shown in Fig. 1, lanes B–F, the number of polypeptides present in the hydroxyapatite eluate decreased on increasing the amount of hydroxyapatite. Using 10 g hydroxyapatite the SDS gel electrophoresis revealed the presence of only two protein bands (Fig. 1, lane E). Despite a substantial decrease in the protein content, the total activity of the aspartate/aspartate exchange remained nearly the same on increasing the amount of hydroxyapatite from 0.6 to 10 g (Fig. 1, lanes B–E). This resulted in a 4-fold increase of the specific activity of the aspartate/glutamate carrier with respect to that of the eluate obtained from chromatography on 0.6 g hydroxyapatite.

For further purification, we have applied the hydroxyapatite eluate onto various resins which already have been successfully used for other mitochondrial carriers (see Ref. 33 for a review and Refs. 18–20). Only celite, a resin capable of binding proteins independently of the ionic strength, was effective for purification of the aspartate/glutamate carrier protein in functionally active form.

The final purification of the aspartate/glutamate carrier was achieved by elution with increasing concentrations of the detergent. Whereas in the presence of 0.1% $C_{12}E_8$ in the elution buffer all proteins remained bound to celite, we found that the aspartate/glutamate carrier could be specifically eluted from celite in the presence of 0.3% $C_{12}E_8$ in the elution buffer (Fig. 2). The first and the second fractions (lanes C and D) of the celite eluate were not active in reconstituted aspartate transport and did not contain protein. Fraction three (lane E) exhibited an aspartate/aspartate exchange activity of 11752 μmol/30 min per g protein (at 0.1 mM aspartate) and upon SDS gel electrophoresis showed a single protein band of 31.5 kDa. Fraction four (lane F) again was not active and did not contain protein. Lane G of Fig. 2 shows elution of the residual proteins from the celite column by applying 1% SDS. The total reconstituted aspartate/glutamate carrier activity present in the third fraction of the celite eluate represented 48% of that applied to celite (0.06 μmol/30 min per g protein).

The final purification factor (Table I) of the celite fraction containing the aspartate/glutamate carrier was 620 with respect to the mitochondrial extract. Approxi-

Fig. 2. Purification of the aspartate/glutamate carrier: SDS gel electrophoresis of fractions obtained by hydroxyapatite and celite chromatography. M, marker proteins (see Fig. 1); A, eluate from a column containing 0.6 g hydroxyapatite (50 μl); B, eluate from a column containing 10 g hydroxyapatite (50 μl); C–F, first four fractions of celite eluate (100 μl); and G, residual eluate of the celite column eluted with 1% SDS (100 μl).

mately 17.2% of the total transport activity was recovered with a protein yield of 0.03%.

Properties of the reconstituted aspartate / glutamate carrier

The experiments described in this section have been performed with the celite fraction (Fig. 2, lane E) containing the purified aspartate/glutamate carrier protein.

Fig. 3 illustrates the time course of the NEM-sensitive [^{14}C]aspartate uptake by proteoliposomes which were loaded with unlabelled aspartate. The uptake of aspartate increased linearly with time for about 5 min at a rate of 733 μmol/min per g protein at 25°C (at 0.1 mM aspartate). In the absence of internal substrate, aspartate uptake was negligible. There was virtually no activity without incorporation of the carrier protein into the liposomes (not shown). The time course of [^{14}C]aspartate/aspartate exchange as shown in Fig. 3 represents an exponential approach to isotopic equilibrium, which is demonstrated by the straight line obtained by plotting the natural logarithm of the fraction

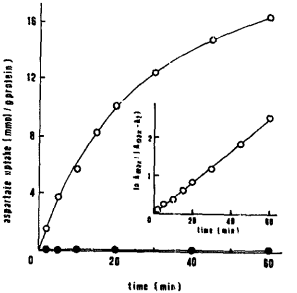


Fig. 3. Time course of aspartate uptake in reconstituted liposomes. 0.1 mM [^{14}C]aspartate was added at time zero to reconstituted liposomes loaded with 20 mM aspartate (○) or with 20 mM NaCl (●). The inset represents the logarithmic plot of $\ln A_{max}/(A_{max} - A_t)$ where A_{max} is maximum aspartate exchange per g protein and A_t is the aspartate exchange at time t, according to the relation $\ln A_{max}/(A_{max} - A_t) = kt$. The value of A_{max} (17.88 mmol/g protein) was extrapolated at infinite time by a computer non-linear regression analysis.

of equilibrium aspartate$_{max}$/(aspartate$_{max}$ − aspartate$_t$) against time [34]. This means that the exchange of aspartate in proteoliposomes follows first-order kinetics. The first-order rate constant, k, extrapolated from the slope of the logarithmic plot, was 0.041 min^{-1}. Similar results were obtained when the uptake of [^{14}C]glutamate was measured in proteoliposomes. The first-order constant for the glutamate/glutamate exchange was 0.034 min^{-1}. Furthermore, also in this case there was virtually no uptake in the absence of intraliposomal substrate. Both the absolute dependence on an appropriate counteranion and the inhibition by NEM proved that the purified aspartate/glutamate carrier has been reconstituted.

The specificity of [^{14}C]aspartate and [^{14}C]glutamate exchange with respect to intraliposomal counteranions was further investigated in proteoliposomes loaded with

TABLE I

Purification of the aspartate / glutamate carrier

The proteoliposomes were loaded with 20 mM L-aspartate or 20 mM L-glutamate and the exchange was started by adding 0.1 mM external [^{14}C]aspartate (Asp/Asp exchange) or [^{14}C]glutamate (Glu/Glu exchange), respectively. Other conditions as described in Materials and Methods. Activity of the reconstituted exchange is expressed in μmol/30 min per g protein (specific activity) and μmol/30 min (total activity).

	Protein (μg)	Asp/Asp exchange		Glu/Glu exchange		Purification (fold)	
		specific activity	total activity	specific activity	total activity	Asp/Asp exchange	Glu/Glu exchange
Mitochondrial extract	8392	18.97	0.163	11.41	0.929	1	1
Hydroxyapatite eluate	79.8	1065	0.085	639	0.051	56	56
Celite eluate	2.4	11667	0.028	7083	0.017	615	620

a variety of substrates. The intraliposomal concentration of the anions used was 20 mM and the exchange time was 30 min. The data reported in Table II show that labelled aspartate and glutamate could be transported not only against L-aspartate and L-glutamate but also against internal L-cysteinesulfinate. Some activity was also observed with the D-stereoisomers of aspartate, glutamate and cysteinesulfinate. On the other hand, there was no significant exchange of aspartate and glutamate when substrates of other mitochondrial carriers (phosphate, malate, 2-oxoglutarate, citrate and ADP) were present inside the liposomes. This result is in agreement with the narrow specificity of the aspartate/glutamate carrier as characterized in mitochondria [1].

Table III, experiment 1 shows the effect of various inhibitors on the reconstituted aspartate/aspartate exchange. The reconstituted activity was nearly completely inhibited by the SH reagents NEM, mersalyl and *p*-hydroxymercuribenzoate at a concentration of 1 mM. Likewise, pyridoxal 5'-phosphate, phenylglyoxal and diethyl pyrocarbonate, which are known to be rather specific reagents for lysine, arginine and histidine, respectively, were found to decrease the reconstituted aspartate/aspartate exchange activity strongly. In contrast, carboxyatractyloside, 1,2,3-benzenetricarboxylate, phthalonate, butyl malonate and phenyl succinate, which inhibit other mitochondrial transport systems, were not effective. In Table III, experiment 2, the sensitivity of the aspartate/aspartate exchange to externally-added substrates was investigated. The aspartate exchange was strongly inhibited by L-aspartate,

TABLE II

Dependence of [^{14}C] aspartate and [^{14}C] glutamate transport in reconstituted liposomes on internal substrate

The proteoliposomes were loaded with 20 mM of the indicated substrate. Transport was initiated by the addition of 0.1 mM [^{14}C]aspartate or 0.1 mM [^{14}C]glutamate. The data are from a representative experiment. Similar results were obtained in four different experiments.

Internal substrate (20 mM)	Substrate uptake (μmol/30 min per g protein)	
	[^{14}C]-aspartate	[^{14}C]glutamate
None (Cl^- present)	290	320
L-Aspartate	10470	5850
L-Glutamate	6110	6280
L-Cysteinesulfinate	5900	5260
D-Aspartate	1290	990
D-Glutamate	820	1130
D-Cysteinesulfinate	970	1160
Phosphate	270	230
Malate	190	240
2-Oxoglutarate	270	310
Citrate	330	150
ADP	260	280

TABLE III

Effect of inhibitors and externally added substrates on the reconstituted aspartate/aspartate exchange

The proteoliposomes were loaded with 20 mM aspartate and the exchange was started by adding 0.1 mM [^{14}C]aspartate. All the inhibitors (Experiment 1) were added 2 min before the labelled substrate at a concentration of 1 mM (SH reagents), 10 mM (pyridoxal 5'-phosphate, phenylglyoxal and diethyl pyrocarbonate), 2 mM (1,2,3-benzenetricarboxylate, phthalonate, butyl malonate and phenyl succinate) and 0.1 mM (carboxyatractyloside). The substrates (Experiment 2) were added together with [^{14}C]aspartate at a concentration of 1 mM. The control values of uninhibited aspartate exchange were 10470 and 9930 μmol/30 min per g protein in Expts. 1 and 2, respectively. The data are from representative experiments. Similar results were obtained in three different experiments.

Addition	% Inhibition
Experiment 1	
N-Ethylmaleimide	100
Mersalyl	93
p-Hydroxymercuribenzoate	96
Pyridoxal 5'-phosphate	95
Phenylglyoxal	91
Diethyl pyrocarbonate	96
Carboxyatractyloside	9
1,2,3-Benzenetricarboxylate	14
Phthalonate	8
Butyl malonate	13
Phenyl succinate	7
Experiment 2	
L-Aspartate	86
L-Glutamate	84
L-Cysteinesulfinate	77
D-Aspartate	25
D-Glutamate	28
D-Cysteinesulfinate	23
N-acetylaspartate	9
N-Acetylglutamate	4
L-Asparagine	13
L-Glutamine	10
Phosphate	8
Malate	6
2-Oxoglutarate	12
Citrate	10
ADP	5

L-glutamate and L-cysteinesulfinate, and, to a much lower extent, by the D-stereoisomers of the same amino acids. In contrast, the aspartate/aspartate exchange was not significantly affected by the substrate analogues *N*-acetylaspartate, *N*-acetylglutamate, asparagine and glutamine, and by substrates of other mitochondrial carriers like phosphate, 2-oxoglutarate, malate, citrate and ADP. The same inhibition pattern was observed in mitochondria or in liposomes reconstituted with partially purified aspartate/glutamate carrier [1,4–7].

In further experiments (not shown), we found that the fraction shown in Fig. 2, lane E, consisting of a single protein band with an apparent M_r of 31500, when reconstituted into liposomes did not catalyze the

exchange reactions ADP/ADP (adenine nucleotide carrier), phosphate/phosphate (phosphate carrier), malate/phosphate (dicarboxylate carrier), citrate/citrate (tricarboxylate carrier), 2-oxoglutarate/2-oxoglutarate (oxoglutarate carrier), pyruvate/pyruvate (pyruvate carrier) and carnitine/carnitine (carnitine carrier). Only occasionally, depending on the particular batch of hydroxyapatite used in the first step of purification, some ADP/ADP exchange activity, corresponding to 10–15% of that of the aspartate/aspartate exchange, was also present. Thus, the purified aspartate/glutamate carrier is not contaminated by other mitochondrial transport systems, including those which are normally not adsorbed by hydroxyapatite.

The data reported above demonstrate that the polypeptide of 31.5 kDa, which represents the single protein band present in the SDS gel electrophoresis of the preparation described in this paper, is the aspartate/glutamate carrier of the inner mitochondrial membrane. In a previous paper [6] the aspartate/glutamate carrier was identified as a 68 kDa polypeptide. It is possible that in that preparation the aspartate/glutamate carrier was present as a highly active contaminant. Another possibility is that the 68 kDa polypeptide was a dimer of the carrier protein formed during the purification procedure and/or during the gel electrophoresis. In this respect it should be noted that the mitochondrial carrier proteins have a tendency to dimerize when the gel electrophoresis samples contain a considerable amount of cardiolipin as in the case of Ref. 6. The M_r of 31 500 assigned to the aspartate/glutamate carrier in this report falls into the very narrow range of molecular masses shown by all mitochondrial metabolite carriers isolated so far [11–22] and suggests that the aspartate/glutamate carrier may also belong to the mitochondrial carrier protein family [24–26]. It is hoped that the simple and rapid purification procedure described here will provide a useful basis for further characterization of the aspartate/glutamate carrier at a molecular level.

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