Functional reconstitution of the partially purified aspartate-glutamate carrier from mitochondria

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The aspartate/glutamate carrier from beef heart mitochondria has been solubilized with detergent. The transport protein was partially purified by chromatography on hydroxyapatite in the presence of dodecyl octaoxyethylene ether and high concentrations of ammonium acetate. During purification, the aspartate/glutamate carrier was identified by functional reconstitution into egg yolk phospholipid liposomes. After hydroxyapatite chromatography the protein is 30 fold enriched in aspartate/glutamate transport activity but still contains ADP/ATP-carrier and phosphate carrier. The reconstituted activity is specific for exchange of L-aspartate and L-glutamate and is similar to intact mitochondria with respect to substrate affinity and inhibitor sensitivity.

Aspartate/glutamate carrier *Beef heart mitochondria* *Liposome* *Reconstitution* *Detergent* *Kinetics*

1. INTRODUCTION

Among the variety of carrier proteins in the inner mitochondrial membrane the aspartate/glutamate carrier is one of the most interesting systems, not only due to its importance in the malate/aspartate cycle, in gluconeogenesis and in urea synthesis, but also because it is the only mitochondrial transport system to be regulated both by membrane potential and pH gradient. Although the properties of aspartate/glutamate exchange in mitochondria have been extensively studied (reviewed in [1]), there is still disagreement about the kinetic properties [2,3] and about the existence of microcompartmentation of substrates [4]. Furthermore, basic properties such as the mechanism of proton cotransport and functional asymmetry in the deenergized state are still not understood. And, what is most important, the carrier protein itself has not yet been identified.

Abbreviations: asp, aspartate; C_xE_y, alkyl(x)-polyoxyethylene(y)-ether; BKA, bongkrekate; CAT, carboxyatractylate; glu, glutamate; PalP, pyridoxalphosphate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)

To provide a basis for isolation and purification of this carrier, functional reconstitution has been used here. Several mitochondrial transport proteins have been previously reconstituted: the ADP/ATP carrier [4] from a pure, and the phosphate carrier [5,6] from a nearly-pure protein preparation; the citrate carrier [7] from an enriched fraction; the dicarboxylate [8] and the ornithine [9] carrier from crude solubilisates. This paper describes a 30-fold enrichment of the asp/glu carrier and may be considered as a step towards purification of this transport protein.

2. EXPERIMENTAL

Hydroxyapatite was purchased from BioRad, Dowex 1 × 8 and Amberlite XAD2 from Serva, all labelled compounds from Amersham. $C_{12}E_8$ was from the Kouyoh Trading Company (Tokyo) and $C_{13}E_{10}$ from Sigma. All other chemicals were of analytical grade. Beef heart mitochondria and egg yolk phospholipids were prepared as in [10].

Mitochondria (10 mg/ml) were preextracted with 2% $C_{13}E_{10}$, 20 mM Pipes (pH 7.0) for 10 min

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at 0°C. The pellet, after 20 min centrifugation at 175 000 × g, was solubilized with $C_{12}E_8$ at a protein/detergent ratio of 0.5 (w/w) in the presence of 2 M ammonium acetate (pH 7.0) for 10 min at 0°C. After ultracentrifugation (20 min at 175 000 × g) the supernatant was desalted on Sephadex G-25 in 0.1 M ammonium acetate. The eluate was applied to a hydroxyapatite column, equilibrated in 10 mM Pipes, pH 7.0 (5 mg protein/ml bed volume). After washing with 0.1 M ammonium acetate, elution was performed with 0.8 M ammonium acetate, 20 mM Pipes (pH 7.0), 0.6% $C_{12}E_8$, 0.1% egg yolk phospholipids. The eluted material was again desalted on Sephadex G-25. To the protein fractions used for reconstitution, egg yolk phospholipids (liposomes with 40 mM aspartate, 20 mM Pipes, pH 7.0) were added with a lipid/protein ratio of not lower than 75 (w/w). The ratio of detergent/lipid should not exceed a value of 0.03 (w/w). The freeze-thaw-sonication procedure and the transport assay were carried out as for the ADP/ATP carrier [10]. The sonication time was 20 s with pulses of 0.2 s; i.e., total sonication time 4 s. The stop inhibitor was PalP (15 mM). Instead of Dowex-Cl [11], Dowex-acetate columns were used to obtain complete retention of labelled aspartate.

3. RESULTS AND DISCUSSION

3.1. *Assay for asp/glu carrier by reconstitution*

Since no ligands of the asp/glu carrier are known which bind specifically and tightly enough to be used as a monitor during isolation, the method of choice is the functional reconstitution. To determine the reconstituted activity, a stop method for measuring the exchange kinetics had to be developed. This is, however, severely restricted by the lack of a specific inhibitor. Both filtration and ion exchange columns proved to be too slow for kinetic resolution. Mersalyl, which has been used in mitochondria for this purpose, as well as several other SH-reagents, are indeed able to block the asp/glu carrier activity. However, before stopping the transport reaction completely, they induce leakiness of the vesicles. On the other hand, pyridoxalphosphate was found to be an efficient stopping reagent when applied at 10–20 mM. It blocks the reaction in less than 5 s and has no harmful effect on liposome stability. With this technique, exchange kinetics with appropriate time resolution can be measured (cf. fig.1).

3.2. *Solubilisation and stability*

More than 30 different detergents have been

Table 1

Partial purification of the asp/glu carrier

Preparation	Protein (mg)	Reconstituted transport activity of		
		asp/glu carrier	ADP/ATP carrier	phosphate carrier
		(μmol·min^{-1}·g^{-1})		
Solubilized mitochondria[a]	258	6	186	n.d.
Second extraction[b]	178	7.5	158	8
Second supernatant	96	13	n.d.	n.d.
First G-25 column	72	16	145	7
Hydroxyapatite eluate	9	136	120	19
Second G-25 column	7	174	142	14
ADP/ATP carrier		10	2450	n.d.

The reconstituted nucleotide and phosphate exchanges were determined as in [4,6]. [a]To obtain an appropriate value for comparison of specific activities, mitochondria were solubilized with $C_{12}E_8$ as described for the second extraction. [b]All further preparations as described in section 2. [c]For comparison, the purified ADP/ATP carrier was reconstituted as in [12]

tested for solubilisation. Among these, sufficient solubilisation under non-denaturing conditions was found only with $C_{12}E_8$. To combine the stabilizing effect of this detergent with efficient solubilisation, it was necessary to add high salt concentrations. Of the many different salts tested, ammonium acetate proved to be the most effective and not harmful to the solubilized carrier protein. The presence of ammonium acetate in up to 2 M concentrations seemed to have a protective influence on the protein stability.

3.3. *Isolation and partial purification*

The enrichment of the asp/glu carrier as described in section 2 is documented in table 1. Preextraction with $C_{13}E_{10}$ followed by extraction with $C_{12}E_8$ and chromatography on hydroxyapatite lead to a substantial purification of this protein. However, the observed 30-fold increase in specific activity is not necessarily correlated with a 30-fold purification of the protein. Very likely the purification factor may be even higher due to partial inactivation of the transport function during the procedure. On

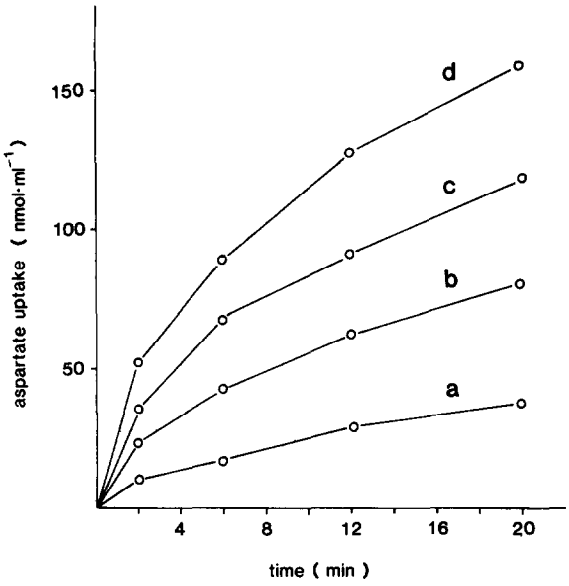

Fig.1. Kinetics of aspartate uptake with varying amounts of incorporated protein: (a) 0.06 mg/ml; (b) 0.12 mg/ml; (c) 0.18 mg/ml; (d) 0.31 mg/ml. External aspartate was 100 μM.

Table 2

Specificity of the reconstituted asp/glu carrier

Experiment	Internal substrate	External		Substrate uptake (μmol·min^{-1}·g^{-1})
		labelled substrate	inhibitor	
1	L-asp	L-asp		122
2	L-glu	L-glu		69
3	L-asp	L-asp	+ PalP (15 mM)	<1
4	Na_2SO_4	L-asp		1.3
5	D-asp	L-asp		21
6	D-asp	L-asp	+ PalP (15 mM)	<1
7	ATP	L-asp		1.6
8	citrate	L-asp		2.3
9	phosphate	L-asp		2.1
10	malate	L-asp		1.8
11	glutamine	L-asp		1.3
12	L-asp	L-asp	+ CAT and BKA (0.1 μM each)	131
13	L-asp	L-asp	+ α-cyanocinnamate (1 mM)	108
14	L-asp	L-asp	+ butylmalonate (10 mM)	117

The transport rates of labelled external substrates (100 μM) were determined by the inhibitor stop method as described. In experiments 3, 6, 12, 13 and 14 the inhibitors were added before the substrate. Internal substrates were 30 mM

the other hand, a possible loss of inhibiting factors can also lead to an overestimation of the purification. Nevertheless, a protein fraction significantly enriched in functionally active asp/glu carrier is obtained. However, small amounts of activity of some of the transport systems from the inner mitochondrial membrane are still present (e.g., phosphate carrier and nucleotide carrier, cf. table 1). For comparison, the purified ADP/ATP carrier was reconstituted into the same liposomes (table 1). Electrophoresis on SDS-gels, as expected, shows several protein bands, the main components being in the region of 26–36 kDa (not shown). No correlation of the asp/glu carrier with one single band is possible at this stage of purification.

3.4. *Characterization of the reconstituted exchange activity*

The linear dependence of reconstituted activity on the amount of incorporated protein, a fundamental prerequisite, is shown in fig.1. Furthermore, this figure demonstrates the time course of exchange kinetics, which can be analyzed mathematically as described for the ADP/ATP carrier [11]. There are several lines of evidence which confirm that the observed transport rates are solely correlated to the reconstituted asp/glu carrier (table 2: (a) the exchange activity depends on the presence of internal substrate (experiments 1 and 4); (b) the exchange is more than 5-times faster with L-asp than with D-asp; the residual activity with D-asp is also blocked by PalP (experiments 1, 5 and 6); (c) uptake of external substrate is catalyzed only against internal asp or glu, not against internal ATP, phosphate, citrate, malate or glutamine (experiments 1, 2, 7–11); (d) the uptake of L-asp is not inhibited by CAT and BKA (inhibitors of ADP/ATP exchange), by α-cyanocinnamate (inhibitor of pyruvate transport), or by butylmalonate (inhibitor for dicarboxylate transport); (e) the reconstituted carrier is further characterized by a transport affinity constant of 80 μM and a pH optimum of around pH 7.0 (both for asp/asp exchange, not shown). These results correlate well with the properties of the carrier in intact mitochondria.

In conclusion, it has been shown that a protein fraction with at least a 30-fold enriched specific transport activity contains the functionally active asp/glu carrier. The reconstituted protein shows correct specificity, affinity and exchange kinetics. The specific activity of asp/asp exchange, obtained in the proteoliposomes, cannot be correlated quantitatively to the original mitochondrial activity, since the purity of the reconstitution protein fraction has not been determined so far. However, the V_{max} of the reconstituted asp/glu carrier is in the same order of magnitude when compared with the reconstituted citrate carrier [7] and definitely lower than the reconstituted adenine nucleotide carrier [12] or phosphate carrier [5,6], a relation which resembles the ratio of activities in mitochondria.

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