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The mitochondrial aspartate/glutamate and ADP/ATP carrier switch from obligate counterexchange to unidirectional transport after modification by SH-reagents

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The influence of various SH-reagents on the aspartate/glutamate carrier was investigated in the reconstituted system. When liposomes carrying partially purified carrier protein were treated with 5,5′-dithiobis(2-nitrobenzoic acid) or *N*-ethylmaleimide, antiport activity was strongly reduced. Several mercury compounds exerted a dual effect. They completely blocked the antiport and, in addition, induced an efflux pathway for internal aspartate. The maximum rate of this unidirectional flux was comparable to the original antiport activity. Induction of efflux always was coupled to inhibition of antiport. Efflux was neither due to unspecific leakage of proteoliposomes nor to a possible contamination by porin, but depended on active carrier protein, as elucidated by the sensitivity to proteinases and protein-modifying reagents. Besides efflux of aspartate, $HgCl_2$ and mersalyl also induced a slow efflux of ATP from liposomes carrying coreconstituted aspartate/glutamate and ADP/ATP carrier. The two efflux activities could be discriminated taking advantage of the differential effectiveness of several inhibitors and proteinases. Although basic carrier properties were changed by the applied mercurials (Dierks, T., Salentin, A. and Krämer, R. (1990) Biochim. Biophys. Acta 1028, 281), aspartate and ATP efflux could clearly be correlated with the aspartate/glutamate and the ADP/ATP carrier, respectively. When purifying these two translocators the respective efflux activity copurified with the antiporter, thus elucidating that the two different transport functions are mediated by the same protein. These results argue for a participation of the aspartate/glutamate and the ADP/ATP carrier in the generally observed increase of mitochondrial permeability after treatment with SH-reagents.

Introduction

Mitochondria possess a variety of specific carrier systems that mediate the exchange of metabolites across the inner membrane (for review see Refs. 1–3). Out of these, the Asp/Glu carrier plays an important role in energy metabolism as a central component of the malate/aspartate shuttle, which transfers reducing equivalents from the cytosol to the mitochondrial respiratory chain. Asp/Glu antiport is electrogenic due to a proton cotransported with glutamate but not with aspartate [4]. Thus, by means of this transport system both components of the protonmotive force, i.e., $\Delta\Psi$ and ΔpH, influence the redox state of nicotinamide adenine dinucleotides in the two compartments.

Investigation of transport kinetics of the Asp/Glu carrier in intact mitochondria [5–8] proved to be rather complex and led to contradictory results in several aspects of the overall transport mechanism [7–10]. We investigated Asp/Glu antiport in the reconstituted system using partially purified carrier protein [11–13]. The applied proteoliposomes contained functionally active carrier molecules of defined protein orientation [13]. The kinetic mechanism of antiport was found to be of the sequential type involving one substrate binding site on each membrane side [14]. These two sites have to be occupied by substrate molecules in order to form a ternary complex with the carrier protein. Upon a conformational change of this catalytic complex the con-

Abbreviations: Asp/Glu carrier, aspartate/glutamate carrier; BKA, bongkrekate; $C_{12}E_8$, dodecyl octaoxyethylene ether; CAT, carboxyatractyloside; DTE, dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; mersalyl, mersalyl acid; NEM, *N*-ethylmaleimide; PCMB, *p*-(chloromercuri)benzoate; PCMS, *p*-(chloromercuri)benzenesulfonate.

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certed transport of both substrates across the membrane occurs.

In order to gain further insight into the mechanism of carrier-mediated antiport, it is of basic interest to examine structural aspects of this process. The reconstituted system offers the advantage to selectively modify specific amino acids of the carrier protein under study and to correlate these residues with effects on transport function. By using various SH-reagents, cysteines were proven to be of functional importance in many carrier proteins. In the case of mitochondria, the phosphate carrier [15–19], the oxoglutarate carrier [20,21], the pyruvate carrier [22–24] and the carnitine carrier [25] are inhibited both by mercurials like mersalyl and by alkylating maleimides like *N*-ethylmaleimide (NEM) or, in the case of the oxoglutarate carrier, eosin maleimide [26]. The tricarboxylate [27] and dicarboxylate carrier [17,28–31] as well as the uncoupling protein [32] are sensitive to mercurials, the ADP/ATP carrier [33–35] and the glutamate carrier to NEM [36,37]. The activity of the Asp/Glu carrier is strongly reduced after treatment with DTNB [13] and NEM, and, as shown here, can be blocked completely by mercurials.

For more than 20 years a large number of publications have described efflux of different solutes from mitochondria after various treatments. Among others, particularly reagents affecting cysteines directly [38–47] or indirectly via glutathione [43,48,49] induced efflux. Thus, the redox state of SH-groups has been invoked to explain the general increase in the permeability of mitochondrial membranes. However, particular proteins containing the sulfhydryl groups relevant for this effect were not identified. Therefore, one aim of the present study was to clarify the specific involvement of the Asp/Glu carrier in efflux promotion by SH-reagents. This issue could only be addressed in the reconstituted system. Indeed, a reversible change of the Asp/Glu carrier and, in addition, of the ADP/ATP carrier from antiport function to unidirectional efflux activity was observed after modification of the carrier proteins by mercurials. Preliminary results have been described previously [50].

Material and Methods

Materials and their sources

Labelled compounds (Amersham Buchler) and $C_{12}E_8$(Kouyoh Trading Company, Tokyo). Turkey egg yolk phospholipid, cardiolipin, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dithioerythritol (DTE), mersalyl, *p*-(chloromercuri)benzoate (PCMB), *p*-(chloromercuri)benzenesulfonate (PCMS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), diethyl pyrocarbonate, carboxyatractyloside (CAT), Cibacron Blue 3GA, agaric acid, palmitoyl-CoA and 3,3′-diaminobenzidine tetrahydrochloride (Sigma). NEM, pyridoxal phosphate (Merck); Amberlite XAD2, Dowex 1-X8 (Fluka); hydroxyapatite (Bio-Gel HTP) (Bio-Rad); Sephadex, Phast Gel 10-15, Phast SDS buffer strips (Pharmacia); valinomycin, trypsin, pronase (Boehringer); subtilisin, Celite 545 (Serva); liquid gelatin (Norland, New Brunswick); peroxidase conjugated affinity pure goat anti-rabbit IgG (Fc) (Dianova). The antibodies against porin were a gift of Prof. D. Brdiczka (Constance) and those against the ADP/ATP carrier were provided by Dr. B. Seitner (Munich). The polyanionic inhibitor of porin was synthesized by Prof. T. König (Budapest) and put to our disposal by Prof. R. Benz (Würzburg). BKA was isolated by Prof. Berends (Delft). All other chemicals were of analytical grade.

Preparation of Asp / Glu carrier

The preparation of the Asp/Glu carrier from bovine heart mitochondria was performed as described previously [11]. In most experiments, the protein fraction obtained after hydroxyapatite centrifugation chromatography and desalting on Sephadex G-25 was used for reconstitution (G-25 protein). Further purification of the Asp/Glu carrier and the ADP/ATP carrier, both present in this preparation, was achieved by HPLC on a hydroxyapatite column (Pentax PEC 102, 7.5 × 100 mm) using a gradient HPLC system (ERC, Knauer). In order to purify the Asp/Glu carrier, a complex sequence of HPLC buffers was necessary, which was similar to that described by Krämer et al. [11]. The differences were as follows: Flow rate 1.0 ml/min; preequilibration of the column with 0.4% $C_{12}E_8$/300 mM ammonium acetate; injection of 2.5 mg G-25 protein (0.64% $C_{12}E_8$/600 mM ammonium acetate); linear phosphate gradient (10.5 ml, with a plateau of 3.5 ml) from 10–130 mM sodium phosphate keeping ammonium acetate at 50 mM; continuous reduction of phosphate to 10 mM and concomitant increase of cardiolipin from 0.05 to 0.1%; linear ammonium acetate gradient from 50–1200 mM (20 min) lowering the flow rate from 1.0 to 0.8 ml/min at the end of the gradient. The fractions collected at 650–850 mM ammonium acetate contained almost pure Asp/Glu carrier protein (cf. Fig. 6A).

Purification of ADP / ATP carrier

For purifying the ADP/ATP carrier, 1.7 mg G-25 protein (0.64% $C_{12}E_8$/300 mM ammonium acetate) were applied on the same HPLC column as described above. The pass-through fraction eluting with 0.4% $C_{12}E_8$/300 mM ammonium acetate/10 mM sodium phosphate was collected (cf. Fig. 6A). The protein preparation shown in Fig. 6B was obtained when collecting the pass-through of 2.5 mg applied G-25 protein that elutes with 0.4% $C_{12}E_8$/750 mM ammonium acetate/10 mM sodium phosphate.

Purification of porin

Porin was prepared from solubilized beef heart mitochondria by chromatography on hydroxyapatite/Celite columns according to De Pinto et al. [51].

Preparation of proteoliposomes

Incorporation of different protein preparations into liposomes was carried out by hydrophobic chromatography of mixed micelles on Amberlite beads in a recycling procedure [12]. The exact reconstitution conditions described by Dierks and Krämer [13], were also adapted for the reconstitution of porin using 1.7 mg Triton X-100/mg phospholipid instead of 1.75 mg $C_{12}E_8$/mg phospholipid. In the porin coreconstitution experiments (Fig. 4) 8 mg/ml phospholipid were used instead of 12 mg/ml and 1.6 mg Triton X-100 plus 0.1 mg $C_{12}E_8$ per mg phospholipid. The ratio of protein/phospholipid in the case of G-25 protein was about 0.04 mg/mg, but was much lower in the case of HPLC fractions (0.002–0.004 mg/mg) and in the case of porin (0.00065–0.0026 mg/mg).

The protein fractions analyzed in Fig. 6B were repeatedly incorporated into liposomal membranes in order to increase the amount of protein per liposome. For this purpose, proteoliposomes were sedimented at 140 000 × *g* for 40 min (4°C), solubilized again in mixed micelles of detergent and protein, and reconstituted a second and third time on Amberlite columns under adjustment of all substrate and buffer concentrations.

Determination of transport activities

Reconstituted transport activities were determined by measuring the flux of ^{14}C-labelled substrate. For monitoring the antiport activity of the Asp/Glu carrier two different techniques were applied. In forward exchange experiments (uptake of label) the assay was started by the addition of labelled aspartate to proteoliposomes containing unlabelled countersubstrate inside (16 mM aspartate). In backward exchange experiments (export of label) unlabelled substrate was added to proteoliposomes containing prelabelled internal substrate pools. In both modes of antiport measurement the reaction was stopped using pyridoxal phosphate (25–75 mM, depending on the external substrate concentration).

A similar inhibitor-stop technique was developed in order to measure unidirectional transport (efflux) activity with sufficient kinetic resolution. After removal of external substrate, the assay was started by adding SH-reagents to prelabelled proteoliposomes, as is indicated in the context of each experiment; a stopping mixture was applied containing 5 mM DTE and 40 mM pyridoxal phosphate, two inhibitors of aspartate efflux (see Results). For prelabelling the internal substrate pool (usually 16 mM aspartate) in efflux or backward exchange experiments, proteoliposomes were loaded with labelled substrate by antiport activity in the forward exchange mode (5–8 min) adding 0.5 μM aspartate of high specific radioactivity. However, in the experiments where we tested efflux from liposomes carrying no protein, inactive protein or porin, labelled substrate (0.3 μCi/ml) was entrapped in the liposomes during reconstitution.

Essentially the same experimental procedures were applied for determining reconstituted ATP transport activities. ATP/ATP antiport was measured in the backward exchange mode monitoring export of [^{14}C]ATP from prelabelled proteoliposomes (10 mM ATP) after addition of unlabelled ATP to the incubation medium. In the case of ATP efflux experiments, exit of labelled ATP (10 mM, unless otherwise indicated) was initiated by addition of 5 μM $HgCl_2$ or 100 μM mersalyl in the absence of external substrate. Antiport was stopped using a mixture of 33 μM CAT and 5 μM BKA, while 5 mM DTE/40 mM pyridoxal phosphate were applied in order to stop efflux of ATP.

At certain steps during the different experimental procedures, substrate (and isotope) gradients had to be generated. This was done by size exclusion chromatography of liposomes on Sephadex G-75 prior to the start of the assay and additionally, in the case of efflux and backward exchange determinations, prior to the prelabelling step. Finally, after treatment with the stop inhibitor each sample was passed through an anion exchange column (Dowex 1-X8, acetate form) in order to remove external radioactivity. For further experimental details see Ref. 13. All transport determinations were carried out at pH 6.5, except those involving proteinase treatment, where pH 7 was chosen throughout the whole experiment, i.e., first, incubation of prelabelled proteoliposomes for 1 h with subtilisin, trypsin or pronase (1 mg/ml, 25°C), second, size-exclusion chromatography for removal of proteinases and, third, efflux or backward-exchange measurements.

In both antiport and efflux assays, the time course of the flux of isotope was fitted by a computer program (ENZFITTER, Biosoft) according to a first order process (cf. Eqs. in Ref. 13). In the case of forward-exchange determinations initial velocities can be calculated directly, which are expressed in μmol/min per liter liposomes. For evaluating backward-exchange or efflux activities, apparent first-order rate constants were calculated (1/min) by the computer fitting program, which within a single experiment can be compared directly between liposomes containing identical substrate pools [13]. In Table I the back-exchange velocity (v) was calculated by multiplying the rate constant k with the corresponding internal substrate pool ($v = k \cdot S_{in}V_{in}$), which depends on the internal volume (V_{in}) of the active proteoliposomes [13]. When evaluating rate constants of backward-exchange or efflux kinetics measured after incubation with inhibitors or proteinases,

TABLE I

Inhibition of aspartate antiport and induction of aspartate efflux by various SH-reagents

In forward exchange experiments (see Materials and Methods) the SH-reagents were added to the proteoliposomes at indicated concentrations, 30 s before the addition of the external substrate (100 μM labelled aspartate). The capability of these reagents to induce efflux was tested in the absence of external substrate, starting the measurement by addition of the reagent to proteoliposomes that were previously loaded with labelled substrate. In the case of efflux, apparent first-order rate constants k were evaluated (see Materials and Methods). For comparison of efflux and antiport velocities, the rate constant of backward exchange was measured adding 100 μM aspartate instead of the SH-reagent. All data were determined using proteoliposomes of identical composition (16 mM internal aspartate). Prelabelling of the internal substrate pool as in Fig. 1.

SH-reagent	Applied concn. (μM)	Forward exchange (μM/min)	Efflux rate constant k (1/min)
–	–	12.1	0.006
DTE	2000	11.0	0.008
NEM	5000	2.1	0.004
DTNB	200	3.0	< 0.003
PCMB	20	< 0.6	0.004
PCMS	20	< 0.4	0.018
$HgCl_2$	5	< 0.7	0.075
mersalyl	5	< 0.5	0.004
mersalyl	100	< 0.3	0.105
mersalyl/DTE [a]	100	11.9	0.007
mersalyl/PLP [b]	100	< 0.3	0.007
mersalyl/EDC [c]	100	< 0.2	0.012
mersalyl/DEPC [c]	100	< 0.2	0.010
backward exchange control		–	0.340 [d]

[a] 2 mM DTE was added to proteoliposomes preincubated with 100 μM mersalyl for 30 s.

[b] Mersalyl was added to proteoliposomes together with 20 mM pyridoxal phosphate (PLP).

[c] Proteoliposomes were pretreated for 20 min with 5 mM EDC or 100 μM diethyl pyrocarbonate (DEPC), respectively. Non-reacted reagent was removed during size exclusion chromatography of liposomes, which always preceded the addition of the SH-reagent (see Materials and Methods).

[d] Corresponding to an exchange activity of 11.8 μM/min (see Materials and Methods).

care must be taken that the computer extrapolates to the infinite value of the untreated control, which corresponds to the isotope equilibrium (back-exchange) or chemical equilibrium (efflux) of transported substrates. In order to define the initial time range corresponding to single exponential kinetics, also the graphical method described previously was applied (cf. Ref. 13 for further explanation).

Determination of protein concentration

The protein concentration was determined according to a modified Lowry method after precipitation by deoxycholate and trichloroacetic acid [52] because of the high ammonium acetate concentrations.

SDS-polyacrylamide gel electrophoresis

For SDS-polyacrylamide gel electrophoresis precipitated protein pellets (see above) were washed with 90% diethyl ether/100 mM Tris (pH 9) or, if extracted from proteoliposomes (Fig. 6B), with 90% acetone/100 mM Tris (pH 9) in order to remove coprecipitated detergent and phospholipid. Electrophoretic separation in the presence of SDS was carried out in 10–15% acrylamide gradient gels (Phast Gel) using an automated equipment (Phast System, Pharmacia). Gels were subjected to silver staining according to the protocol given by Olsson et al. [53], but using 0.25% silver nitrate for 13 min.

Western blot analysis

For Western blot analysis of the separated protein bands, the unstained gels were covered by a small sheet of nitrocellulose (Schleicher & Schüll BA 85) pre-wetted in water and heated to 70°C for 20 min on the separation bed of Phast System. The immunostaining of the nitrocellulose sheets was carried out after blocking (2 h) with 2% gelatin in phosphate buffered saline (pH 7.3) containing 0.05% Tween 20. Following 1 h of incubation, first with polyclonal antibodies directed against porin (1 : 300 in blocking buffer) or against the ADP/ATP carrier (1 : 1000), respectively, and second with peroxidase conjugated goat anti-rabbit IgG (Fc) (1 : 5000 in blocking buffer), the nitrocellulose sheet was developed by the peroxidase reaction using 3,3′-diaminobenzidine as a substrate in the presence of cobalt and nickel ions [54].

Results

Basic properties of efflux induction

Adding various cysteine-modifying reagents to proteoliposomes containing the Asp/Glu carrier led to two basically different observations. On the one hand, when measuring antiport activity by the forward-exchange method (uptake of labelled aspartate), more or less complete inhibition could be achieved by different classes of reagents (Table I). The corresponding IC_{50} values (inhibitor concentrations leading to half-maximum inhibition) were around 2 μM for $HgCl_2$, mersalyl, *p*-(chloromercuri)benzenesulfonate (PCMS) and *p*-(chloromercuri)benzoate (PCMB), but much higher for DTNB (about 125 μM) and NEM (about 1 mM). On the other hand, after preloading the proteoliposomes with labelled aspartate in order to measure antiport in terms of export of label (backward-exchange method), a drastic efflux of internal aspartate was detected upon addition of mercury compounds. However, this efflux of label could not be due to the antiport activity of the Asp/Glu carrier, since it occurred in the absence of external countersubstrate (Fig. 1), thus representing a unidirectional flow of aspartate.

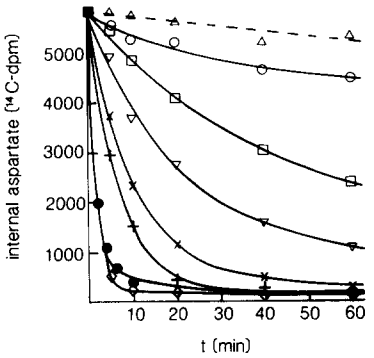

Fig. 1. Induction of aspartate efflux from proteoliposomes by mersalyl. The internal substrate pool of proteoliposomes (16 mM aspartate) was prelabelled by carrier-mediated equilibration with external [^{14}C]aspartate (see Materials and Methods). After removal of external substrate by size exclusion chromatography of liposomes, the decrease of internal label was monitored following different additions: buffer (basic efflux) (○), 200 μM DTNB (△), 10 μM (□), 26 μM (▽), 64 μM (X), 160 μM (+) and 400 μM (◇) mersalyl. For comparison also the antiport activity (backward exchange) was measured adding 3 mM aspartate without SH-reagent (●).

The concentration of reagents needed to promote this efflux at half maximal rate varied considerably between $HgCl_2$ (2 μM), mersalyl (100 μM), PCMS (about 40 μM) and PCMB (about 1 mM). Maximum induction by mersalyl, which was the most effective reagent (Table I), led to apparent efflux rates, that were in the range of the antiport activity (Fig. 1), i.e., 20–60-fold faster than the slow efflux always observed after removal of external substrate without addition of any reagent. DTNB inhibited this basic efflux component (Fig. 1), as was reported earlier [13]. Thus, completely different effects of different SH-reagents had to be discriminated, that all could be stopped by an excess of dithioerythritol (DTE), as is shown in Table I and, in more detail, in the accompanying paper [55]. Consequently, for the kinetic analysis of mercury-induced efflux (see Materials and Methods) DTE was used as stopping reagent in combination with pyridoxal phosphate, an unspecific inhibitor of the Asp/Glu carrier (Table I, Fig. 5).

Although the mercury-induced efflux of aspartate was always coupled to inhibition of aspartate/aspartate antiport this does not necessarily mean that efflux, like antiport, must be attributed to the reconstituted carrier protein. Therefore we had to prove that the efflux pathway is not due to unspecific leakage of substrate but in fact is a carrier-mediated process.

Dependence on active protein. For measuring the stability of liposomes in the presence of the applied reagents, labelled aspartate was entrapped in the vesicles during reconstitution. Thus, different from the generally applied labelling by antiport activity (see Materials and Methods), all liposomes were loaded with labelled substrate including those without active carrier protein. After removing external aspartate by passage through Sephadex G-75, mersalyl was added in 1 mM concentration, i.e., at least 3-times higher than necessary for maximum induction of efflux. Fig. 2 shows that liposomes carrying no protein remained totally unaffected by this treatment for at least one hour. Likewise proteoliposomes retained internal label almost completely, if prepared from protein that was previously denatured with 4% SDS at high pH. Fig. 2 further demonstrates that the efflux activity of liposomes carrying intact Asp/Glu carrier protein could be inhibited, if preincubated with proteinases such as subtilisin. Correspondingly, aspartate/aspartate antiport was reduced to very low rates (see below). Similar inactivation of either transport activity was observed after pretreatment of proteoliposomes with the amino acid reactive reagents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and diethyl pyrocarbonate, which modify proteins at residues different from cysteines (Table I, Fig. 5). Thus, efflux of aspartate is essentially dependent on active protein.

Excluding the involvement of porin. The protein preparation used for reconstitution was enriched both in Asp/Glu and ADP/ATP carrier activity. These two proteins were attributed to the 68 kDa band and the double band at about 30 kDa, respectively [11], on a SDS-polyacrylamide gel (Fig. 3A, lane 1). The additional band at 44 kDa is not yet identified, but cannot be involved in promoting efflux, as became apparent when using purified proteins (see below). On the same gel (lane 2), purified porin, the outer membrane voltage-dependent anion channel (35 kDa), is compared with the Asp/Glu carrier preparation, thereby elucidating that the latter protein fraction comprised no 35 kDa porin. This could be confirmed by Western blot analysis

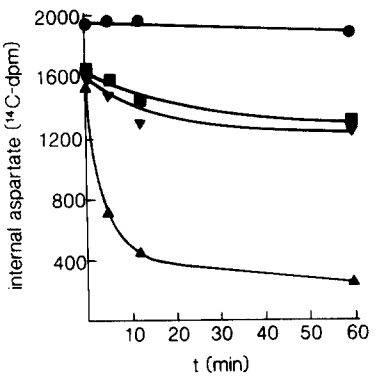

Fig. 2. Dependence of efflux on active carrier protein. During reconstitution labelled aspartate (16 mM) was entrapped into liposomes (see Materials and Methods), which then were subjected to size exclusion chromatography and treated with 1 mM mersalyl. Liposomes carrying no protein (●) or protein that was previously denatured by 4% SDS at pH 9 (■) showed no or only little efflux, respectively, as compared to proteoliposomes with intact protein (▲). Efflux activity could also be inhibited if proteoliposomes were preincubated with subtilisin (1 mg/ml) at 25°C for 60 min (▼).

using anti-porin antibodies (Fig. 3B). Applying this sensitive method no band was stained in the 35 kDa region. However, the antibodies also reacted with a faint band of about 66 kDa present in the porin control (lane 2). Likewise in lane 1 a band of this size was marginally stained, which on the SDS-gel (Fig. 3A) was impossible to discriminate from the Asp/Glu carrier showing the same mobility. Surprisingly, the applied antibodies detected the 66 kDa band also in preparations of the oxoglutarate and the ADP/ATP carrier purified according to Refs. 31 and 56, respectively (data not shown). Hence it appears that the immunostaining of the 66 kDa band is due to some unspecific reactivity of the antibodies and does not reveal the presence of porin dimers not dissociated by SDS.

A second line of evidence disproving the involvement of porin came from functional control experiments. In view of the high conductance of porin it might be put forward that a single molecule per liposome would lead to a loss of internal aspartate. However, in the proteoliposomes under study porin activity could not be detected, even though we looked for different aspects of porin function. (i) Porin switches to closed states if the membrane potential exceeds 10–30 mV [57,58]. On the contrary, no inhibition of induced or basic efflux could be found upon energization of the liposomal membrane by a K^+-diffusion potential of either polarity (data not shown). (ii) A polyanionic inhibitor of porin [59] did not inhibit the mercury-induced efflux. In control liposomes, carrying purified porin, the polyanion retarded

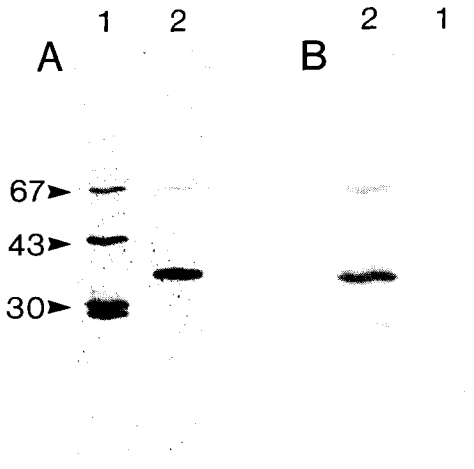

Fig. 3. Absence of porin from the Asp/Glu carrier preparation. The protein preparation used for efflux experiments (G-25 protein, lane 1) was compared with purified porin from bovine heart mitochondria (lane 2) using SDS-polyacrylamide electrophoresis (A) and Western blot analysis (B). Following electrophoretic separation of proteins and diffusion blotting to a nitrocellulose filter, protein bands were visualized by silver staining of the gel (A) and immunostaining of the filter using antibodies directed against porin from rat liver (B). For preparation of protein samples and for staining procedures see Materials and Methods. Arrows indicate the position of molecular mass standard proteins (in kDa).

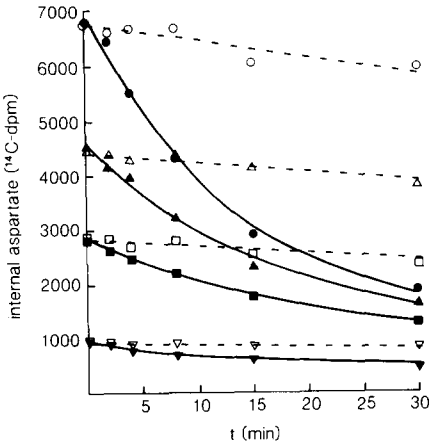

Fig. 4. Porin-mediated efflux is different from the mercury-induced efflux activity. A constant amount of Asp/Glu carrier (0.012 mg/mg phospholipid) and different amounts of purified porin (0–0.0026 mg/mg phospholipid) were coreconstituted (see Materials and Methods), the (monomeric) porin concentration in the reconstitution mixture corresponding to 0 (○, ●), 150 (△, ▲), 375 (□, ■) and 525 nM (▽, ▼). After prelabelling of the internal aspartate pool by Asp/Glu carrier activity (see Fig. 1), efflux was induced by 100 μM mersalyl (closed symbols). In parallel the basic, i.e., uninduced efflux was recorded (open symbols) following addition of buffer. The first-order rate constants of efflux (k) determined for the different preparations of proteoliposomes were very similar: 0.005 (○), 0.004 (△), 0.005 (□) and 0.004 min^{-1} (▽); 0.072 (●), 0.061 (▲), 0.057 (■) and 0.061 min^{-1} (▼).

the rapid efflux via porin (at least in the case of ATP, data not shown). (iii) These liposomes, in the absence of inhibitor, lost internal aspartate, ATP and sodium ions as soon as a transmembrane concentration gradient was established, which could not be resolved kinetically. Thus, the rapid efflux mediated by porin differed fundamentally from the much slower kinetics of the mercury-induced efflux (see also Fig. 4). (iv) Porin-mediated efflux was not influenced by mercurials (Fig. 4).

Finally, positive controls were also carried out. By coreconstituting Asp/Glu carrier (about 300 nM during reconstitution) and purified porin (0–600 nM) into the same liposomes we simulated a contamination by porin. As demonstrated in Fig. 4, the internal [^{14}C]aspartate measured at zero time decreased with increasing porin 'contamination'. Thus, the loading of the liposomes with labelled substrate became less efficient because porin, when present in the same liposome, led to an immediate loss of label imported by the Asp/Glu carrier. As a consequence, only those liposomes containing the carrier but no porin participated in unidirectional transport induced by mercurials. This can be recognized from Fig. 4 on the basis of two observations. First, the rate constants (not the rates!) of mersalyl-induced efflux determined with or without 'contaminating' porin were almost identical ($k = 0.061 \pm 0.006$ min^{-1}, $n = 5$). Second, the basic efflux rate constant measured in the

absence of mersalyl was not increased by the presence of porin ($k = 0.005$ min^{-1}). Furthermore, the data of Fig. 4 demonstrate that a considerable amount of added porin was necessary in order to reconstitute a significant portion of liposomes containing at least one single porin molecule, which thus led to a decrease of the zero-time value. Therefore, a minute contamination of the Asp/Glu carrier preparation by porin, that might have been overlooked in the Western blot of Fig. 3, can not explain the efflux of aspartate from 80–90% of all liposomes, which in general was observed after addition of mercurials (cf. Fig. 1). Even if present, traces of porin would not at all interfere with the kinetics of the mercury-induced efflux.

Identification of the efflux-mediating protein

On the basis of these results, we investigated whether the unidirectional efflux in fact was mediated by a carrier protein. In the protein preparation used for reconstitution (Fig. 3) the only carriers identified, the Asp/Glu and the ADP/ATP carrier, until now were classified as obligate antiport carriers. Interestingly, however, we found that mersalyl and $HgCl_2$ also induced efflux of ATP, albeit with much lower rates as compared to the efflux of aspartate (Table II). For an unequivocal correlation of efflux activity with the Asp/Glu or the ADP/ATP carrier, respectively, it would be appropriate to carry out experiments using the purified carrier proteins. Unfortunately, the pure fractions obtained after HPLC on hydroxyapatite (see below) were very diluted in protein concentration. As a consequence, the reconstituted transport activities, although sufficient to be accurately quantified (Table III), were too low for a kinetic analysis of transport inhibition, as performed below. Therefore, in order to characterize the efflux-mediating carriers comparative studies on efflux and antiport function had to be undertaken using the same protein preparation as above.

Competition studies. The substrate specificity of the aspartate and/or the ATP efflux carrier was severely reduced as compared to the corresponding antiport carriers, as will be described in the accompanying paper [55]. In order to discriminate, nevertheless, between efflux mediated by the Asp/Glu carrier and by the nucleotide carrier several kinetic approaches were carried out. It turned out that efflux of aspartate and glutamate showed a marked pH optimum in the range of 6.5–6.8, whereas efflux of ATP was stimulated below pH 6.0 (data not shown). This may reflect that different SH-groups have to be modified to induce efflux of aspartate or ATP, respectively. In order to provide optimum conditions for efflux of aspartate, the pH of 6.5 was not changed.

In competition studies, one specific substrate of either (antiport) carrier, aspartate and ATP, respectively, was present simultaneously inside the proteoliposomes at different concentration ratios. Using one substrate in labelled form, it was tested whether the second (unlabelled) substrate inhibits efflux of label, which would indicate a common efflux carrier for aspartate and ATP. A kinetic analysis of efflux inhibition investigating effects on K_m and V_{max} could not be carried out, because the efflux process did not show Michaelian properties (see accompanying paper, Ref. 55). Nevertheless, an inhibitory effect of aspartate on the rather slow efflux of ATP, and vice versa, would be expected if both substrates use the same transmembrane pathway competing at a common rate-limiting step. However, Table II clearly shows that neither efflux of aspartate nor of ATP was significantly reduced by the presence of the respective second substrate.

TABLE II

Mutual influence of internal ATP and aspartate on efflux of aspartate and ATP

Proteoliposomes were prepared containing various concentrations of aspartate and ATP, as indicated. After prelabelling the internal aspartate- or ATP-pool by the antiport activity of the coreconstituted Asp/Glu and ADP/ATP carrier, respectively (see Materials and Methods), efflux was initiated adding 100 μM mersalyl. Rate constants (k) are given for the efflux of the first (labelled) substrate determined in the presence or absence of the respective second (unlabelled) substrate.

First substrate	Second substrate	Efflux of first substrate k (1/min)
10 mM aspartate	–	0.153
	2.5 mM ATP	0.172
	5 mM ATP	0.156
	10 mM ATP	0.187
10 mM ATP	–	0.040
	4 mM aspartate	0.053
	8 mM aspartate	0.048
	16 mM aspartate	0.046

Inhibitor studies. Several Inhibitors of the ADP/ATP carrier like carboxyatractyloside (CAT), bongkrekate (BKA), palmitoyl-CoA, *N*-ethylmaleimide, agaric acid, Cibacron Blue 3GA and pyridoxal phosphate were applied to block efflux of ATP, but not efflux of aspartate. Out of these, the highly specific inhibitors CAT (50 μM) and BKA (17 μM) exerted a 50% inhibition on ATP efflux, whereas aspartate efflux was not inhibited (Fig. 5, data for BKA not shown). Most effective were Cibacron Blue 3GA, an inhibitor described to be specific for the nucleotide carrier [60], and pyridoxal phosphate, which inhibits both the ADP/ATP [61] and the Asp/Glu carrier [62]. Correspondingly, pyridoxal phosphate (20 mM) showed a similar effect on aspartate and ATP efflux (95% inhibition), whereas Cibacron Blue (0.1–0.5 mM) inhibited only efflux of ATP (75 ± 10%), but not of aspartate (Fig. 5). Surprisingly, Cibacron Blue inhibited also aspartate/aspartate antiport activity (83 ± 5%), although less pronounced than antiport of

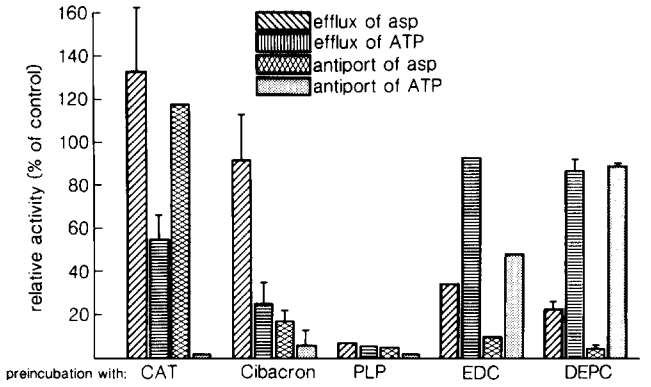

Fig. 5. Differential inhibition of transport mediated by the Asp/Glu carrier and by the ADP/ATP carrier using various inhibitors. Relative efflux and antiport (back-exchange) activities of the two core-constituted carriers are compared. They were determined after incubating proteoliposomes, preloaded with labelled substrate, for 10 min with 20 mM pyridoxal phosphate (PLP) or Cibacron Blue 3GA (0.1–0.5 mM), or for 15 min with 80 μM diethyl pyrocarbonate (DEPC) or 3 mM EDC (7.5 min incubation with EDC in case of antiport determinations). Non-reacted diethyl pyrocarbonate or EDC was removed by size exclusion chromatography of liposomes. In the case of carboxyatractyloside (CAT, 50 μM), the inhibitor was already added during reconstitution of the carrier proteins. Efflux and backward exchange were initiated by adding 100 μM mersalyl or 1 mM external substrate (aspartate or ATP), respectively, to inhibitor-treated proteoliposomes. Prelabelling of internal substrate pools (16 mM aspartate and 10 mM ATP) was carried out as described in Table II. Since all activities were determined in at least two independent experiments, they are given in percentage of the untreated control. Standard errors are indicated in the case of transport inhibition by CAT (efflux, four experiments), Cibacron Blue (efflux, eight experiments; antiport, five experiments) and diethyl pyrocarbonate (three experiments).

ATP (94 ± 7%). Nevertheless, inhibition by this anthraquinone dye made possible a clear differentiation with regard to the two efflux processes.

Furthermore, Fig. 5 demonstrates that EDC, a recently introduced inhibitor of the Asp/Glu carrier [63], and also diethyl pyrocarbonate, a histidine reagent, inactivated efflux of aspartate but not of ATP. Applying 80 μM diethyl pyrocarbonate prior to the addition of the mercurials led to a pronounced inhibition of both aspartate efflux (77 ± 4%) and aspartate antiport (96 ± 2%), whereas ATP efflux and antiport were nearly unaffected. Similar results were obtained when using EDC.

Sensitivity to proteinases. Further support for two different proteins being involved in efflux of aspartate and ATP, respectively, came from investigations on the differential inactivation of the two carriers by proteinases. Incubation of proteoliposomes with trypsin or pronase (see Materials and Methods), on the one hand, reduced aspartate/aspartate antiport activity by about 85%, whereas ATP/ATP antiport was affected not more than 25% (data not shown). On the other hand, also efflux of aspartate, especially when using pronase, was much more strongly inhibited (65 ± 10%) than the ATP efflux (about 15%). Subtilisin led to an even stronger inhibition of both efflux and antiport of aspartate (about 75%); however, the discrimination between the Asp/Glu and the ADP/ATP carrier was not improved.

Generally antiport was more severely inactivated than efflux, as was observed for the proteinases and also for the various inhibitors shown in Fig. 5. This finding is easily explained, since all the applied agents could not penetrate the liposomal membrane and hence only affected external parts of the carrier proteins. These domains obviously are essential for antiport but less important for efflux function. Consequently, the principal result of these studies is that the sensitivity of aspartate transport, antiport as well as efflux, to pronase, trypsin, subtilisin, EDC and diethyl pyrocarbonate in all cases was more pronounced than the inhibition of the corresponding ATP transport process.

Studies using purified carrier proteins. If antiport and efflux activity are intrinsic functions of the same carrier, both modes of transport should be closely correlated during purification of either of the two proteins, Asp/Glu or ADP/ATP carrier, respectively. In Table III specific first-order rate constants of efflux and backward-exchange activities of several protein fractions are compared. Using liposomes reconstituted with the enriched protein preparation (G-25 protein), ATP efflux in the two different determinations was 6.5- or 10.6-times slower than ATP/ATP exchange; on the contrary, the rate constants of aspartate efflux and exchange were not very different (Table III). After purification of the Asp/Glu carrier on a hydroxyapatite HPLC column it turned out that both transport activities (specific for aspartate) were copurified to the same extent, showing again similar rate constants for antiport and efflux. From the activity data a copurification factor (see Table III) of 0.82 was calculated, which is close to unity. Also, the ADP/ATP carrier was purified on the same HPLC column. In this case again a copurification of efflux and antiport specific for ATP was observed (copurification factor 1.11). The SDS-gels of the purified protein fractions are shown in Fig. 6A.

A direct test, whether efflux of aspartate can also be mediated by the ADP/ATP carrier or whether efflux of ATP also occurs via the Asp/Glu carrier, could not be carried out. For such experiments the labelled aspartate (or ATP) had to be entrapped in the liposomes during reconstitution, since a specific prelabelling by the activity of the corresponding Asp/Glu (ADP/ATP) carrier, which had been removed during purification, was no longer possible. The protein concentrations in the HPLC fractions, however, were extremely low. Thus, the total efflux that could be measured, corresponding to the very small portion of actively transporting proteoliposomes, was too low in these cases for evaluating significant efflux rates.

In a completely different approach we used HPLC protein from another fraction that contained both

TABLE III

Copurification of efflux and antiport activity of the Asp/Glu and the ADP/ATP carrier

Efflux and antiport activities were determined after purification of the Asp/Glu carrier and the ADP/ATP carrier from the protein preparation that was used in all other experiments (G-25 protein, cf. Fig. 3). The Asp/Glu carrier and the nucleotide carrier were separated on a hydroxyapatite HPLC column (see Fig. 6A), the ADP/ATP carrier eluting in the pass-through fraction (HPLC p.th.) and the Asp/Glu carrier eluting during a salt gradient at about 800 mM ammonium acetate (HPLC AA800). (A) Following reconstitution of the different fractions with 10 mM ATP and 16 mM aspartate as internal substrates, apparent rate constants of efflux and backward exchange were measured adding 100 μM mersalyl or 1 mM external substrate (aspartate or ATP), respectively. For comparison between different protein fractions, specific first-order rate constants of antiport and efflux were calculated. Two separate sets of data are presented. (B) Based on the values given in A, the purification factors of either antiport or efflux activity were calculated for the purified Asp/Glu and ADP/ATP carrier. Furthermore, a copurification factor of antiport and efflux activity was derived according to [antiport(HPLC)·efflux(G-25)]/[antiport(G-25)·efflux(HPLC)]. Provided that its value is close to unity, this factor indicates that antiport and efflux were copurified.

Protein preparation	Transport of aspartate			Transport of ATP		
	antiport	efflux	antiport/efflux	antiport	efflux	antiport/efflux
A: Specific rate constants ($min^{-1} \cdot mg^{-1}$)						
G-25	12.76	10.13	1.26	25.16	3.87	6.50
HPLC AA800	115.1	111.6	1.03	4.72	n.d. [a]	–
G-25	12.53	8.40	1.49	14.09	1.33	10.59
HPLC p.th.	2.93	n.d. [a]	–	80.11	6.84	11.71
B: Purification factors (fold)						
HPLC AA800	9.02	11.02	0.82 [b]	0.19	–	–
HPLC p.th.	0.23	–	–	5.67	5.14	1.11 [b]

[a] n.d., cannot be determined.
[b] Copurification factor, i.e., purification factor (antiport)/purification factor (efflux).

ADP/ATP and Asp/Glu carrier, but no further contaminations (see Fig. 6B). In order to yield sufficient transport activity, this protein fraction was repeatedly inserted into the same liposomes (see Materials and Methods). Incubation of these liposomes with trypsin led to an almost complete inhibition of aspartate transport (antiport and efflux), whereas ATP transport was only affected by 42% (data not shown). After extraction

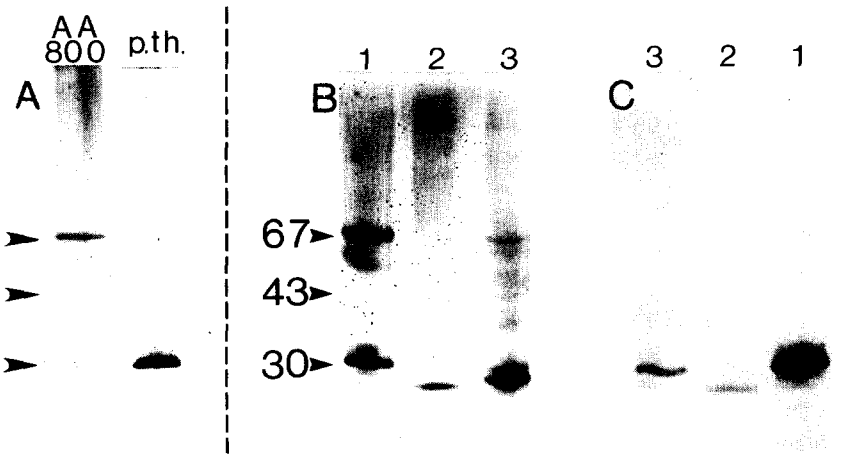

Fig. 6. Immunological identification of the proteinase-resistent ATP efflux carrier. The G-25 protein preparation (cf. Fig. 3) was subjected to further purification by HPLC on hydroxyapatite (see Materials and Methods). On the SDS-gel in panel A, fraction AA800 and the pass-through are shown containing purified Asp/Glu carrier (68 kDa) and purified ADP/ATP carrier (30 kDa), respectively. The protein fraction shown in panel B contained both Asp/Glu and ADP/ATP carrier (lane 1). After repeated reconstitution (see Materials and Methods) of this protein fraction and incubation of proteoliposomes with trypsin (1 mg/ml), which subsequently was removed by size exclusion chromatography, proteins were reextracted from the liposomes (see Materials and Methods) and separated electrophoretically (lane 2); the extracted but untreated control is shown in lane 3. Prior to silver staining of the gel (B), the proteins were transferred by diffusion blotting to a nitrocellulose filter and subjected to Western blot analysis (C). Immunostaining of the nitrocellulose filter was carried out using polyclonal antibodies directed against the ADP/ATP carrier from bovine heart mitochondria. For preparation of protein samples and for staining procedures see Materials and Methods. Arrows indicate the position of molecular weight standard proteins: bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa).

of the proteins from the proteoliposomes and SDS-gel electrophoresis it turned out that both the 68 kDa band (Asp/Glu carrier) and the 30 kDa band (ADP/ATP carrier) disappeared completely, and only a tryptic fragment of 27–28 kDa was detected (Fig. 6B). Upon Western blot analysis this tryptic fragment was still identified by specific anti-ADP/ATP carrier antibodies, albeit with reduced sensitivity (Fig. 6C). These results can be explained by assuming that the nucleotide carrier, after treatment with trypsin, was attacked to only some extent without being completely inactivated. On the basis of this correlation, the proteinase-sensitive efflux (and antiport) of aspartate has to be attributed to the Asp/Glu carrier, since only the 68 kDa band was completely degraded by trypsin.

Discussion

Robillard and Konings [64] presented a hypothesis for the importance of thiols in membrane proteins also comprising solute carriers. Although the main principle of this hypothesis, namely the regulation of substrate affinities via $\Delta\tilde{\mu}_{H^+}$-induced dithiol–disulfide interchanges, was rejected in subsequent studies [65–67], cysteines, as a matter of fact, are of almost ubiquitous importance in carrier proteins. Therefore, in order to identify essential amino acids we treated the mitochondrial Asp/Glu carrier especially by SH-modifying reagents. Using the reconstituted system offered the advantage to selectively modify the isolated protein reinserted into a lipid bilayer and to investigate effects on functional properties. In the present study we describe the sensitivity of Asp/Glu antiport to sulfhydryl-reagents, thereby adding a further transport system to the list of mitochondrial carriers inhibited by these compounds (see Introduction). Our main purpose, however, is to demonstrate a fundamental conversion of the carrier's transport function switching from counterexchange of substrates to unidirectional net-flux upon modification by certain mercurials.

Phenomenon of carrier-mediated efflux

Several arguments prove that the mercury-induced efflux phenomenon is not due to unspecific leakage of proteoliposomes. Efflux clearly depended on functionally active carrier protein (Fig. 2). Most convincingly, efflux induction was coupled to antiport inhibition and was nearly abolished after proteinase treatment of the intactly reconstituted carrier. Both transport activities were sensitive also to EDC, diethyl pyrocarbonate and pyridoxal phosphate, three reagents modifying proteins at residues different from cysteines (Table I). In the literature a model for the passive efflux from erythrocytes was described involving leakage through lipid–protein interfaces induced after crosslinking of sulfhydryls of the cytoskeleton and other integral proteins [68]. This possibility can be excluded for the reconstituted Asp/Glu carrier, since for explaining efflux inhibition one would have to assume that hypothetical leaks at the contact sites were specifically sealed by the applied proteinases or inhibitors. Likewise, the activation energy of the efflux process, as determined in the accompanying paper [55], argues against diffusion through any kind of aqueous pores.

A possible contamination of the protein preparation by porin, which might be put forward as an explanation of our results, was thoroughly examined and unambigiously rejected by both immunological and functional analysis (Figs. 3 and 4). A further candidate for possible artifacts could be the inner mitochondrial anion channel (IMAC) [69,70]. However, while efflux of aspartate is induced by mercurials showing a pH-optimum below pH 7, IMAC is activated at alkaline pH and is inhibited by mersalyl doses higher than 10 nmol/mg mitochondrial protein [71]. Furthermore, some inhibitors of IMAC, like palmitoyl-CoA [72] and agaric acid [73], showed no inhibitory influence on mercury-induced efflux.

In summary, the arguments obtained are in line with the Asp/Glu carrier to be responsible for the mercury-inducible efflux of aspartate. Most important, induction of aspartate efflux always was coupled to inhibition of aspartate antiport, and, vice versa, antiport inhibition by proteinases or by inhibitors also led to an inhibition of efflux.

Two independent efflux pathways mediated by the Asp/Glu and ADP/ATP carrier

Since in addition to an efflux of aspartate (and glutamate) also a slow efflux of ATP could be induced by $HgCl_2$ or mersalyl, the possibility had to be tested that both the Asp/Glu and the ADP/ATP antiporter can be converted into a unidirectional carrier. However, a clear correlation of aspartate efflux and ATP efflux with the respective carrier was impeded by two findings. First, the mercurials also reduced the substrate affinity and specificity severely (see accompanying paper [55]). Thus, the aspartate efflux carrier could not be identified on the basis of its substrate spectrum or affinity constants. Second, an investigation of the electrogenicity (aspartate) or proton-compensated electroneutrality (glutamate) of efflux was impossible, since also a protein-dependent pathway for small ions was induced by the applied mercurials (see Ref. 55) leading to instable proton or potassium gradients in these experiments.

Nevertheless, the aspartate as well as the ATP efflux carrier could be identified taking advantage of the differential sensitivity of the Asp/Glu and the ADP/ATP carrier, respectively, to several inhibitors (Fig. 5) or proteinases. In these studies the two carriers were unambigiously addressed on the basis of their specific antiport activity. To summarize, each of the tested

inhibitors or proteinases, preferentially inhibiting efflux of aspartate (ATP), without exception also inhibited antiport of aspartate (ATP) stronger than antiport of ATP (aspartate). In particular diethyl pyrocarbonate and EDC nearly exclusively inhibited aspartate transport (efflux and antiport), which, on the contrary, was not affected by CAT, BKA or Cibacron Blue 3GA, three inhibitors of ATP transport. Thus, efflux of aspartate is solely mediated by the Asp/Glu carrier and there is no evidence for a participation of the nucleotide carrier. The corresponding data concerning the ATP efflux carrier are in agreement with this model. The experiments using Cibacron Blue indicate that at least 75% of the total flux of ATP from inside to outside is mediated by the ADP/ATP carrier.

In order to demonstrate that antiport and efflux function reside within the same protein, the essential experiment was to purify the two carriers. This was achieved by HPLC on hydroxyapatite, thereby reducing the contaminating activity of the respective second carrier to below 5% (Table III). Both aspartate efflux and ATP efflux activity copurified with the Asp/Glu and ADP/ATP carrier, respectively, hence providing clear evidence that either carrier can be converted from the antiport to the unidirectional efflux state.

HPLC protein furthermore was used to attribute aspartate or ATP transport to a distinct protein band on a SDS-gel (Fig. 6). The differential proteinase sensitivity of the two carriers, concerning antiport as well as efflux function, also became evident after electrophoresis of proteins extracted from liposomes. Mainly the Asp/Glu carrier was degraded by proteinase treatment, whereas the less sensitive ADP/ATP carrier obviously was cleaved only at one of its termini, as evidenced by immunoblot techniques. Hence a consistency of structural and functional data could be found. It should be mentioned that minor protein fragments may not have been recovered during extraction from proteoliposomes.

Thus, despite the restrictions concerning the possibility to test the specificity of either of the two efflux carriers (see Results section), conclusive data were compiled based on different experimental approaches that clearly are in favor, on the one hand, of an aspartate efflux carrier 'translocating' aspartate, glutamate and probably other metabolites of appropriate size (cf. accompanying paper, Ref. 55), and, on the other hand, of a second efflux carrier for ATP. These unidirectional transport activities are catalyzed by the Asp/Glu carrier and the ADP/ATP carrier, respectively, after modification by mercurials.

SH-induced efflux from mitochondria

Our results obtained in the reconstituted system appear to be relevant also to many observations made in intact mitochondria during the last 20–30 years. Many different treatments were described that led to efflux of a variety of solutes from the matrix space. Following application of different SH-reagents (mersalyl, Hg^{2+}, *p*-hydroxymercuribenzoate, PCMB, NEM, DTNB, diamide, hydroperoxides, arsenite or phenylarsine oxide) an increased permeability was observed for anions like Cl^- [39,40,42,74], phosphate [74,75] and sulfate [74], as well as for cations such as K^+ [38–40,42,45,46] and especially Ca^{2+} [43–47,76]. It may be pertinent in this connection that also a mersalyl-induced release of the much larger compounds ADP and ATP from mitochondria was reported previously [43]. However, a direct involvement of the ADP/ATP carrier was not considered, although BKA reduced the loss of adenine nucleotides. Instead, several distinct hypotheses have been put forward to explain these different efflux phenomena. For obvious reasons the redox state of SH-groups of the inner mitochondrial membrane was considered to be a central aspect in efflux induction [39–46]. A release of Ca^{2+} from mitochondria could also be evoked by providing conditions of increased oxidation of nicotinamide nucleotides, hence suggesting that SH-groups are oxidized due to a decline in reduced glutathione [43,48,49]. However, another group maintains that this efflux of Ca^{2+} is due to ADP-ribosylation of a specific membrane protein involving hydrolysis of NAD^+ [77,78]. A further hypothetical mechanism implies the action of free radicals [45], possibly oxygen radicals [79,80], which cannot be sufficiently removed after modification of reducing SH-groups [79] and thus lead to lipid peroxidation. A different explanation for the occurence of efflux infers an accumulation of lysophospholipids in the membrane, which is due to inhibition of enzymes needed for reacylation or complete deacylation of lysolipids [44,81]. It should be mentioned that these and other efflux phenomena described in the literature were observed especially in the presence of Ca^{2+} and/or phosphate.

Different from these divergent hypotheses, which mostly attribute efflux of solutes to a changed osmotic integrity of the mosaic bilayer, we considered the involvement of carrier proteins. In the present paper a carrier-mediated efflux of aspartate and ATP is demonstrated, that can be induced by treating the reconstituted Asp/Glu and ADP/ATP carrier with certain mercurials. Since these two carriers, when in the antiport state, specifically translocate aspartate, glutamate or ADP and ATP, respectively, it appears that the mercury-induced efflux activities can only explain the reported efflux of adenine nucleotides but not of K^+, Ca^{2+}, Cl^-, phosphate or sulfate. However, as will be shown in the accompanying paper [55] not only efflux of aspartate and glutamate, in addition to ATP, can be induced upon modification of the reconstituted carrier proteins, but also efflux of other solutes including small anions and cations. The direction of net flux measured in mitochondria and in our reconstituted system is

identical, since in the case of the Asp/Glu carrier the orientation in the liposomal membrane is right-side-out as compared to intact mitochondria (unpublished results) and not inside-out as suggested previously [13] on the basis of published data from mitochondria. Moreover, from studies on the phosphate carrier [75], the uncoupling protein [74,82], the ADP/ATP carrier [83–85], the carnitine carrier [86] and the Ca^{2+} uniporter [87,88] it must be concluded that also further mitochondrial transport proteins can be converted into efflux-carrier systems.

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