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Pore-like and carrier-like properties of the mitochondrial aspartate/glutamate carrier after modification by SH-reagents: evidence for a preformed channel as a structural requirement of carrier-mediated transport

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Upon modification of the reconstituted aspartate/glutamate carrier by mercuryreagents the antiporter was converted into a unidirectional efflux carrier (Dierks, T., Salentin, A., Heberger, C. and Krämer, R. (1990) Biochim. Biophys. Acta 1028, 268). In addition to this basic change in the mechanism, the mercurials, reacting with exofacial cysteines, also affected the internal binding site of the carrier leading to an unmeasurable high K_m and to a drastically reduced substrate specificity. The spectrum of efflux substrates comprised small anions from chloride to glutamate, but not cationic amino acids and ATP, hence resembling pore-like properties. However, in the efflux state important carrier properties were also observed. The activation energy (86 kJ/mol) was as high as for the antiport. Furthermore, efflux was inhibited by the presence of external substrate. This *trans*-inhibition strongly suggests that the external binding site of the carrier, prerequisite in the antiport mechanism, also is involved in conformational transitions during efflux function. However, antiport no longer is catalyzed after switching to the efflux state. Reversion of the induced efflux carrier to the antiport state was achieved using dithioerythritol, thereby further restoring substrate specificity and saturation kinetics. A model for antiport-efflux interconversion is presented suggesting that two reactive cysteines have to be modified in order to uncouple the inward and outward directed component of antiport. The pore-type characteristics of efflux are taken as evidence that a channel-like structure determines the selectivity of unidirectional transport. This intrinsic channel of the protein then is required for substrate translocation also during antiport function.

Introduction

Aspartate/glutamate antiport in mitochondria is catalyzed by an electrogenic carrier system mediating a $\Delta\Psi$- and ΔpH-modulated exchange, in which the negative charge of glutamate, but not of aspartate, is compensated by a cotransported proton [1]. A closer insight into the kinetics of this transport process was possible after the isolation of the carrier protein [2], which could be inserted with a defined transmembrane orientation into liposomal membranes [3,4]. Using this experimental system we characterized the kinetic mechanism of antiport to involve simultaneously two binding sites, i.e., one on each membrane side. Both binding sites have to be occupied by a substrate molecule in order to form a transport-competent complex [5]. Furthermore, evidence was obtained for a distinct proton binding site, protonation thereof leading to an increased affinity of the carrier for glutamate [5,6].

In the accompanying paper [7] an intriguing finding was described indicating that the transport mechanism of the Asp/Glu carrier is profoundly changed when cysteines of the carrier protein are modified by certain mercury-reagents. $HgCl_2$, mersalyl and *p*-(chloromercuri)benzenesulfonic acid (PCMS) in micromolar concentrations not only blocked Asp/Glu antiport completely, but also induced a unidirectional transport activity that could be measured as efflux of aspartate

Abbreviations: Asp/Glu carrier, aspartate/glutamate carrier; DTE, dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); mersalyl, mersalyl acid; PCMB, *p*-(chloromercuri)benzoate; PCMS, *p*-(chloromercuri)benzenesulfonate.

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from proteoliposomes in the absence of external substrate. The basic properties of this efflux phenomenon as described in the accompanying paper [7] revealed that the Asp/Glu antiporter was converted into an efflux-carrier system.

Most interestingly, mersalyl and $HgCl_2$ also induced efflux of ATP. This net-flux, however, was not due to the Asp/Glu carrier, but could be attributed to the ADP/ATP carrier, that also was present in the enriched Asp/Glu carrier preparation used for reconstitution [7]. When separating these two carrier proteins on hydroxyapatite, aspartate and ATP transport, efflux as well as antiport, copurified with the Asp/Glu and the ADP/ATP carrier, respectively.

The antiport and efflux activities of the respective translocator share several properties, such as the sensitivity to inhibitors or proteinases [7]. However, there exist profound differences between the two modes of transport. As elucidated in the present paper, pore-type characteristics were observed in the efflux state, although also fundamental carrier-type characteristics were retained after SH-modification. From this comparison of functional properties, it was possible to evaluate basic aspects of antiport coupling and also of substrate channeling through the carrier protein. A working model for antiport-efflux interconversion is presented in order to understand the efflux process on the basis of the established antiport mechanism.

Materials and Methods

Materials and their sources

Labelled compounds (Amersham Buchler); dodecyl octaoxyethylene ether (Kouyoh Trading Company, Tokyo); turkey egg yolk phospholipid, Triton X-114, dithioerythritol (DTE), mersalyl, diethyl pyrocarbonate (Sigma); pyridoxal phosphate (Merck); valinomycin (Boehringer); Amberlite XAD2, Dowex 1-X8 (Fluka); Dowex 50W-X8 (Bio-Rad); Sephadex (Pharmacia). All other chemicals were of analytical grade.

Preparation of Asp / Glu carrier

The preparation of the Asp/Glu carrier from bovine heart mitochondria was as described previously [2]. The protein fraction obtained after hydroxyapatite centrifugation chromatography and desalting on Sephadex G-25 was used for reconstitution (G-25 protein). This fraction contained both Asp/Glu and ADP/ATP carrier protein.

Protein incorporation into liposomes

Incorporation of protein into liposomes was carried out by hydrophobic chromatography of mixed micelles on Amberlite beads in a recycling procedure [3]. The exact reconstitution conditions were described by Dierks and Krämer [4].

Determination of transport activities

Reconstituted transport activities were determined by measuring export of ^{14}C-labelled substrate from proteoliposomes, as is described in the accompanying paper for the Asp/Glu carrier [7]. The prelabelling of the internal substrate pool (16 mM aspartate) usually was accomplished by means of the antiport activity (forward exchange), which equilibrates added isotope (0.5 μM [^{14}C]aspartate) between the two compartments without changing substrate concentrations. However, in those experiments where the specificity of the efflux carrier was analyzed, labelled substrate (0.3 μCi/ml) had to be entrapped in the liposomes during reconstitution. Export of internal label was initiated either by adding unlabelled substrate (backward exchange) or, for measuring net-flux activity, by addition of a mercury reagent as indicated. Transport was stopped by pyridoxal phosphate and, in case of efflux determinations, by DTE [7]. For removing exported radioactivity from the liposomes, generally anion exchange material (Dowex 1-X8, acetate form) was applied [7]. However, when cationic efflux substrates were tested, cation exchanger (Dowex 50W-X8, lithium form) was used instead; in the case of glucose, samples (50 μl) were passed through small columns (7 × 65 mm) of Sephadex G-50.

Antiport and efflux activities were evaluated using a computer fitting program, as is explained in the accompanying paper [7]. Apparent first-order rate constants (k) were calculated, which within a single experiment can be compared directly between liposomes containing identical substrate pools [4]. However, if the internal substrate concentrations had to be varied (Fig. 1), relative velocities (v') were calculated according to Dierks and Krämer [4] by multiplying the rate constant k with the corresponding internal substrate concentration ($v' = k \cdot S_{in}$).

The forward exchange technique, which was applied for determinations of external K_m values (Table I), measures import of labelled substrate. The performance of this method also is described in the accompanying paper [7].

Inhibition of oxoglutarate efflux by diethyl pyrocarbonate

The inhibition of oxoglutarate efflux by diethyl pyrocarbonate was measured using liposomes which carried also the oxoglutarate carrier besides the Asp/Glu and the ADP/ATP carrier. The oxoglutarate carrier was prepared according to Indiveri et al. [8]. As compared to the other experiments, the reconstitution conditions were changed only with respect to the detergent applying 0.05 mg Triton X-114 plus 1.7 mg dodecyl octaoxyethylene ether per mg phospholipid. The concentration of Triton X-114 in the oxoglutarate carrier preparation was decreased to 0.4% by passing the protein six times through Amberlite XAD-2 (approx. 20 mg/mg Triton X-114). The oxoglutarate carrier facilitated loading of

proteoliposomes (internal 20 mM oxoglutarate and 16 mM aspartate) with labelled oxoglutarate, which resulted in more reliable inhibition kinetics as compared to experiments where the label was entrapped during reconstitution (see above). After addition of 6 μM $HgCl_2$ or 100 μM mersalyl, efflux of oxoglutarate was observed in those liposomes carrying coreconstituted Asp/Glu carrier. This efflux was inhibited if proteoliposomes were preincubated (15 min) with 80 μM diethyl pyrocarbonate. Almost no efflux was measured in liposomes prepared under identical conditions carrying, however, only the oxoglutarate carrier.

Results

As shown in the accompanying paper [7], modification of the Asp/Glu and the ADP/ATP antiporter by mercury reagents induced a unidirectional net-flux activity. This was measurable as efflux of aspartate or ATP, respectively, from proteoliposomes in the absence of external countersubstrate (cf. Fig. 2). The two efflux carriers were purified and identified. However, the pure protein fractions were unsuitable for a more detailed kinetic analysis due to methodical reasons explained in the first paper [7]. Therefore, in order to characterize the efflux-mediating carriers with respect to their mechanism of transport, experiments had to be carried out using an enriched protein preparation, which contained both the Asp/Glu and the ADP/ATP carrier (see [7]).

Substrate affinity and specificity

The results mentioned above are in agreement with two efflux-carrier systems transporting aspartate and glutamate on the one hand and ATP and ADP on the other, hence corresponding to the specificity of the respective antiporter. However, further analysis of functional properties revealed several important differences between efflux and antiport with respect to the internal substrate binding site. First, the aspartate efflux carrier could not be saturated with internal aspartate in the concentration range tested (1–50 mM), as can be visualized directly from the slope of the Eadie-Hofstee plots in Fig. 1. For comparison the original transport affinity of the antiporter is shown ($K_m = 2.8$ mM). Similar results were obtained for the ATP efflux carrier (not shown). Second, the substrate specificity, after efflux induction by mercurials, was also drastically reduced. In addition to efflux of aspartate and glutamate, efflux of oxoglutarate (Fig. 2), malate, malonate and even glucose could also be induced by mersalyl showing similar rates in all cases. Even faster rates were measured for sulfate (Fig. 2), sodium and in particular chloride. On the contrary, efflux of the cationic amino acids lysine (Fig. 2) and ornithine was only marginally enhanced as compared to the basic efflux of aspartate (Fig. 2), i.e.,

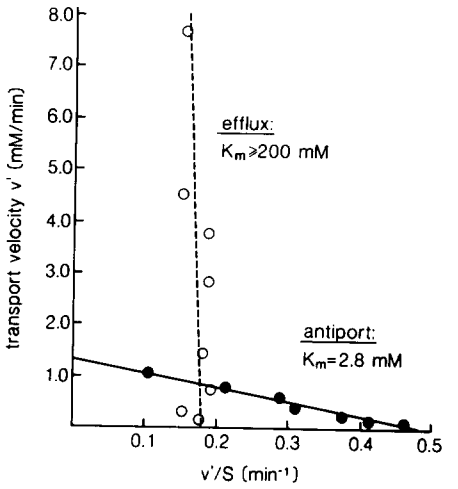

Fig. 1. Transport affinities of the Asp/Glu antiporter and the aspartate efflux carrier. Different preparations of proteoliposomes containing different internal aspartate concentrations (●, 0.25–10 mM; ○, 1–50 mM) were prelabelled using the antiport activity of the Asp/Glu carrier, which leads to an equilibration of added [^{14}C]aspartate between the internal and external compartment without changing substrate concentrations (see Materials and Methods). After removal of external substrate by size exclusion chromatography of liposomes, backward exchange was started by adding 8 mM external aspartate to proteoliposomes (●), whereas efflux was induced applying 100 μM mersalyl (○). Relative transport velocities (v') were calculated (see Materials and Methods), which are presented in an Eadie-Hofstee plot. The K_m values derived from the slope of the straight lines are 2.8 mM (backward exchange) and $\geq$ 200 mM (efflux).

the very low net-flux activity which always was measured without applying mercurials (cf. Ref. 7).

The observed efflux of all these ions was not due to an increased permeability of the membrane, as was tested with liposomes without protein. Diethyl pyrocarbonate, an effective inhibitor of the Asp/Glu carrier, clearly reduced efflux of oxoglutarate (67 ± 17% inhibition, data not shown); the inhibition of aspartate efflux under these conditions (see Materials and Methods) amounted to 76 ± 12%. Thus, the unspecific efflux at least of oxoglutarate is mediated by the Asp/Glu carrier and not by the coreconstituted ADP/ATP carrier which is insensitive to diethyl pyrocarbonate [7]. Fig. 2 shows the much slower efflux of ATP; this large substrate clearly is not accepted by the aspartate efflux carrier [7]. Furthermore, the efflux of UDP-glucose was tested, which proceeded another 5–10-times slower than the ATP efflux (not shown).

As a further consequence of this unspecificity of the efflux carrier, all attempts to modulate unidirectional transport by energization of the membrane either with $\Delta\Psi$ (potassium diffusion potential) or ΔpH failed, due to the instability of the K^+-, Na^+- and H^+- gradients applied in these experiments. Hence, no criteria concerning the electrogenicity of aspartate, glutamate, ATP or ADP efflux could be obtained.

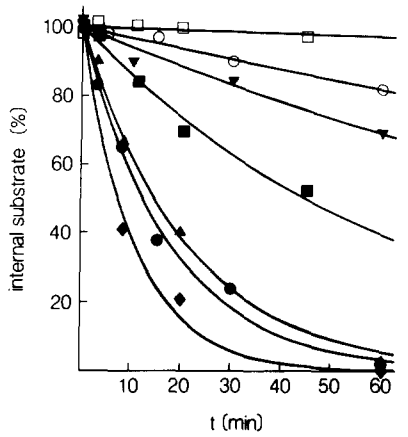

Fig. 2. Efflux of different solutes from proteoliposomes carrying Asp/Glu and ADP/ATP carrier. During reconstitution labelled substrate (○, ●, 16 mM [^{14}C]aspartate; ▲, 16 mM [^{14}C]oxoglutarate; ◆, 16 mM sodium [^{35}S]sulfate; ▼ 16 mM [^{14}C]lysine; and □, ■, 10 mM [^{14}C]ATP) was entrapped into liposomes (see Materials and Methods). After size-exclusion chromatography efflux was induced by 100 μM mersalyl. For comparison, the uninduced efflux of aspartate (○) and ATP (□) is shown, as observed without addition of mersalyl. Since the determinations were carried out in four different experiments, the internal substrate is expressed as percentage of the initial internal radioactivity that was calculated by fitting the data according to a first-order process (see Materials and Methods). The resulting values were normalized on the basis of the aspartate efflux control (●), which was measured in each experiment, by setting the value of internal aspartate extrapolated for infinite time to 0%. Thereby the measured efflux rate constants became directly comparable and could be calculated as follows: < 0.001 (□), 0.003 (○), 0.006 (▼), 0.015 (■), 0.048 (▲), 0.056 (●), 0.094 min^{-1} (◆).

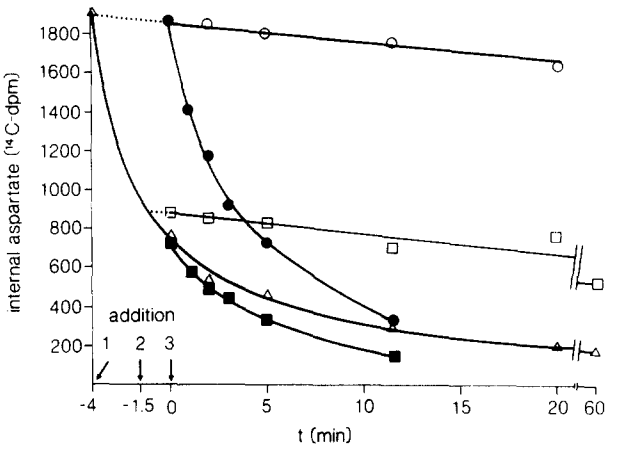

Fig. 3. Reversible antiport-efflux conversion. Efflux (open symbols) and antiport (closed symbols) of labelled aspartate from proteoliposomes was measured following three consecutive additions at indicated points in time. ○, basic efflux (addition of buffer); Δ, efflux induced by 5 μM $HgCl_2$ (first addition); □, stop of efflux by removing bound mercury with 4 mM DTE (second addition); ■, reactivation of antiport (backward exchange) by 2.5 mM aspartate (third addition); ●, backward exchange control (2.5 mM aspartate added after two additions of buffer). The following rate constants were calculated: 0.007 (○), 0.265 (Δ), 0.011 (□), 0.307 (■) and 0.284 min^{-1} (●). Prelabelling of the internal substrate pool as described *in* Fig. 1.

Reversibility of efflux induction

In view of these apparent pore-like characteristics, the possibility had to be taken into consideration that the applied mercury reagents exerted such drastic effects on the protein structure leading to partial denaturation, thereby allowing a transmembrane pathway to be opened for various solutes. Therefore we tested whether the efflux carrier, after induction with mercurials, could be reverted to the antiport state. For this purpose, the dithiol compound DTE was used, which showed no inhibitory or efflux-inducing effect (see accompanying paper, Ref. 7). DTE is known to remove bound SH-reagent from proteins, in this case by converting cysteinyl-mercaptides into cyclic dimercaptides of DTE (cf. Ref. 9). In Fig. 3 the efflux of aspartate after addition of 5 μM $HgCl_2$ is shown (first addition, open triangles). In parallel, 2.5 min after induction of efflux, DTE was added (second addition) which obviously removed bound mercury. As a result, the efflux of internal aspartate was reduced to a very low activity (open squares) showing rate constants almost identical to the basic, i.e., uninduced efflux control (open circles). Moreover, if external substrate was added to these liposomes (third addition), antiport was reactivated, as became obvious from the observed export of label from the interior of the proteoliposomes (closed squares) proceeding with nearly the same rate constant as the untreated backward exchange control (closed circles). Thus, Fig. 3 demonstrates that the modified cysteines, which are involved in the induction of net-flux activity and in the inhibition of antiport, could be reverted to the initial state by using dithiols like DTE.

The reverted Asp/Glu antiporter, i.e., after treatment first with mercurials (1 min) and second with DTE, no longer accepted oxoglutarate as a substrate. Moreover, it could again be characterized by transport

TABLE I

Transport affinities of the reconstituted Asp/Glu carrier after reversion from the efflux to the antiport state

Transport affinity constants (K_m values) were derived from Lineweaver-Burk plots that were obtained from forward- (external K_m values) or backward-exchange measurements (internal K_m values). The substrate concentrations were varied as follows: external K_m determinations, 20–140 μM aspartate (16 mM internal aspartate); and internal K_m determinations, 1–15 mM aspartate (1 mM external aspartate). No significant difference was observed between K_m values determined with proteoliposomes carrying untreated protein or protein that was converted into the efflux state by $HgCl_2$ (first addition) and, after 1 min, reverted by DTE (second addition).

First addition	Second addition	K_m (mM) for	
		external aspartate	internal aspartate
Buffer	5 mM DTE	0.045	2.7
10 μM $HgCl_2$	5 mM DTE	0.042	2.4

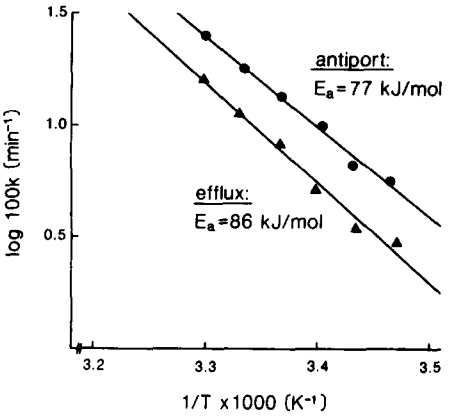

Fig. 4. Temperature dependence of efflux and antiport of aspartate. Efflux (▲) and antiport activity (●) were measured at various temperatures (15–30 °C) following addition of 200 μM mersalyl or 200 μM aspartate, respectively, to proteoliposomes preloaded with labelled aspartate. Apparent rate constants were evaluated (see Materials and Methods) and are given in an Arrhenius plot. From the slope of the obtained lines, activation energies of 86 and 77 kJ/mol were calculated for efflux and backward exchange, respectively. Prelabelling of the internal substrate pool as described in Fig. 1.

affinity constants. As shown in Table I, the K_m values determined were almost identical to those of the untreated carrier with respect to both the inner and the outer side of the membrane. Consequently, a completely reversible switching between antiport and efflux function was triggered by specific cysteine modification.

Activation energy of efflux

Efflux processes via transmembrane pores are characterized by low activation energies (16 kJ/mol for porin; Benz, R., personal communication). In order to test this criterion for the mersalyl-induced efflux carrier, the temperature dependence of aspartate efflux was investigated. From the Arrhenius plot shown in Fig. 4 the activation energy (E_a) was determined to be 86 kJ/mol (15–30 °C), which is in the range of values generally found for carrier-mediated processes. Using the same proteoliposomes but measuring aspartate/aspartate exchange, an activation energy of 77 kJ/mol (15–30 °C) was calculated (Fig. 4).

Influence of external substrate on efflux induction and efflux activity

We furthermore investigated whether the external binding site of the carrier, prerequisite for antiport function, also is involved in efflux function. Since in the efflux state the interaction of substrate with the internal binding site is unspecific, we had to test the possibility that the external binding site also had lost its specificity, which might allow 'unspecific antiport' to occur. This concept could explain efflux as exchange of internal aspartate for some not identified compounds from the incubation medium. Therefore we examined the influence of external substrate on the net-flux activity.

The presence of external aspartate, on the one hand, could stimulate the hypothetical 'unspecific antiport', but, on the other hand, also could allow residual specific antiport activity (of unmodified carrier molecules) to take place simultaneously with net flux. In both cases a faster export of label from proteoliposomes would be expected. However, as shown in Fig. 5, addition of aspartate together with mersalyl did not lead to an enhanced export of labelled substrate as compared to the efflux and backward exchange controls; instead the transport activity was clearly reduced. Likewise, if external aspartate was added to the efflux carrier in the induced state, i.e., 1.5 min after addition of mersalyl, the efflux activity was reduced to relatively low rates (Fig. 5). These effects were specific for aspartate, as became evident from controls using oxoglutarate in place of aspartate (data not shown). The absence of an inward directed flux of substrate was substantiated, since no influx or 'influx exchange' of external [^{14}C]aspartate could be measured into liposomes containing no aspartate or [^{3}H]aspartate, respectively (not shown). These results, on the one hand, indicate that the applied mercury reagents induce unidirectional transport only in the absence of external substrate; otherwise the carrier becomes inhibited. On the other hand, external substrate does not reactivate residual antiport activity and, most important, reduces the efficiency of aspartate efflux mediated by the induced efflux-carrier. Consequently, the external binding site, although not directly participating in unidirectional transport, can still be detected, since its occupation by aspartate leads to a *trans*-inhibition of efflux function.

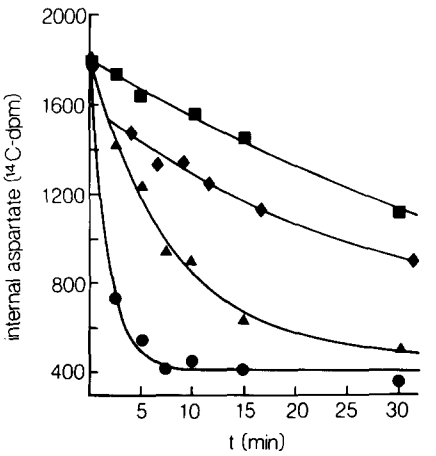

Fig. 5. Mutual interaction of aspartate and mersalyl with the Asp/Glu carrier. Efflux of labelled aspartate from proteoliposomes was induced by 100 μM mersalyl in the absence (▲) or presence of 5 mM aspartate, which was added simultaneously with mersalyl (■) or 1.5 min later (◆). The backward exchange control was measured adding 5 mM aspartate in the absence of mersalyl (●). The stop-inhibitor in all cases contained a mixture of 60 mM pyridoxal phosphate and 5 mM DTE. Prelabelling of internal substrate pool as described in Fig. 1.

Discussion

Both the reconstituted Asp/Glu and ADP/ATP antiporter could be converted into unidirectional efflux-carrier systems [7], hence suggesting that a common principle of mitochondrial carriers is affected by the applied mercury reagents. This conclusion is supported by other laboratories envisaging the existence of efflux carriers in intact mitochondria. In order to explain efflux of phosphate in the presence of mersalyl, a different conformational state of the mitochondrial phosphate carrier was postulated [10]. The *N*-ethylmaleimide-stimulated permeation of Cl^- and H^+ was attributed to the uncoupling protein [11,12]. In addition, the possibility was considered that the ADP/ATP carrier is involved in unidirectional efflux of adenine nucleotides induced by phosphate [13,14]. Also a mersalyl-induced release of ADP and ATP from mitochondria was reported [15]. From this list (see also Refs. 16–21) it may be concluded that different treatments lead to a change in the transport mechanism of different carriers.

The accompanying paper [7] elucidated an unequivocal correlation of aspartate efflux with the Asp/Glu carrier. In the present study we address questions concerning the transport mechanism of net flux (as compared to the antiport) and the antiport-efflux conversion. It should be pointed out that this profound change of functional properties was completely reversible (Fig. 3), thereby indicating that the induced efflux carrier is closely related to the original protein.

Pore-like and carrier-like properties

Surprisingly, the applied mercurials not only changed the mechanism of transport, but besides exerted a *trans*-effect on the internal binding site of the Asp/Glu carrier upon reaction with exofacial sulfhydryl groups. This was reflected, first, by an unmeasurable low transport affinity (Fig. 1) and, second, by a drastically reduced substrate specificity (Fig. 2). Thus, in the efflux state the internal binding site cannot clearly be identified in terms of specificity and half-saturation constant, which is inconsistent with a classical carrier-mediated mechanism (uniport). The observation that ATP [7] and the cations lysine and ornithine are not, or very poor, efflux substrates, whereas much smaller ions like Cl^-, SO_4^{2-} or Na^+ are accepted, gives some indication that the appropriate substrates mainly are selected by size exclusion and electrostatic interaction, hence resembling more pore-like characteristics. However, as compared to normal pores or channels, the flux of substrate was vanishingly low and efflux rates only exceeded antiport rates at very high internal substrate concentrations (Fig. 1). Additionally, the activation energy for the unidirectional flow was as high as that of the counterexchange (Fig. 4), which clearly argues against a simple diffusion through aqueous pores formed by the carrier protein.

Net-flux of solutes could also be due to unspecific antiport, as had to be considered in view of the lacking specificity of the aspartate efflux carrier. As explained in the Results section, this concept would predict a stimulation of 'efflux-exchange' in the presence of external substrate; instead an inhibition was observed (Fig. 5). This *trans*-inhibition by external substrate indicates an indirect involvement of the (unoccupied) external binding site also in unidirectional transport. Further support comes from those protein-modifying reagents inhibiting antiport as well as efflux, namely diethyl pyrocarbonate, carbodiimidies and pyridoxal phosphate [7], which most probably react near the external binding site of the carrier, as evidenced by substrate protection studies (unpublished data). Consequently, the Asp/Glu carrier also in this respect retains important carrier-type characteristics after modification by mercurials.

Interestingly, a SH-dependent change in the transport affinity also was described for other transport systems (see Ref. 22 and references therein). Furthermore, studies of the net-flux activity of the erythrocyte anion exchanger, which occurs under physiological conditions, revealed that this activity, different from the counterexchange, could not be saturated with substrate and that also the anion selectivity was changed (see Ref. 23 for references).

Antiport-efflux interconversion

All mercurials capable of efflux induction obligatorily also inhibited Asp/Glu antiport. However, vice versa, some reagents (DTNB, *N*-ethylmaleimide) did not induce efflux, but exerted only the inhibitory effect [7]. Moreover, all mercurials blocked antiport already at 2 μM concentration but, with the exception of Hg^{2+}, induced efflux only when applied in a much higher concentration, as was observed particularly for mersalyl (100 μM) and PCMB (1 mM). Consequently, at least two different classes of reactive cysteines have to be modified in order to convert the antiporter into a unidirectional efflux carrier, as outlined in Fig. 6. If the reagent has access to only one class, inhibition of transport function results. Thus, an intermediate state between the antiport- and the efflux-carrier is identified which can be forced into the efflux state by $HgCl_2$, PCMS or 100 μM mersalyl (data not shown). Interestingly, a two-stage modification, also involving a non-transporting intermediate, was recently proposed for the maleimide-induced shift of the uncoupling protein into a high conductive state [12].

Removing bound reagents from the protein by DTE in all cases reverted the transport system to the original state, thereby restoring antiport properties (Fig. 3, Table I). Hence the reactive cysteines must be located in a hydrophilic environment at the external part of the carrier, which was reconstituted in an oriented manner [4]. In contrast to PCMS, PCMB promoted no efflux

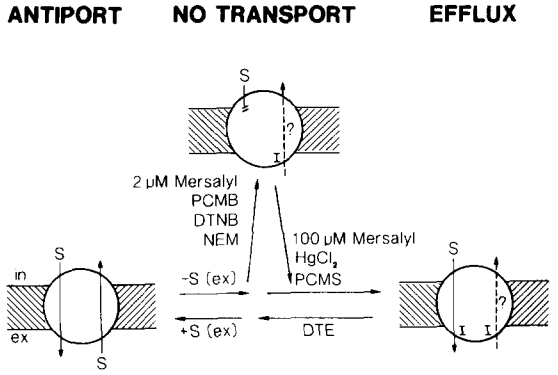

Fig. 6. Functional model of antiport-efflux interconversion of the Asp/Glu carrier. The antiporter is depicted as an integral unit in the membrane catalyzing a concerted countertransport of internal and external substrate (S) by a conformational change of the strictly coupled complex [S(in)-Carrier-S(ex)]. After removal of external substrate, addition of the indicated SH-reagents (I) leads to a weakened coupling of the inward- and outward-directed component of antiport. At least two sulfhydryl groups of the carrier protein have to be modified in order to achieve uncoupled transport activity (efflux). In the efflux state the carrier no longer showed antiport activity, if external substrate was present. However, without external substrate, i.e., during efflux, the unloaded external binding site might be involved in the conformational transitions during translocation ('slippage', dashed arrow). Some SH-reagents (DTNB, *N*-ethylmaleimide (NEM), PCMB and low concentrations of mersalyl) are not capable to induce efflux, but like the efflux-inducing mercurials inhibit antiport completely (no transport). This blocked carrier, just as the untreated antiporter, can also be converted into the efflux state by $HgCl_2$, PCMS and higher concentrations of mersalyl. The efflux carrier as well as the blocked intermediate are reverted to the initial antiport state, if DTE is added to remove bound reagent.

activity [7], possibly because the more hydrophobic PCMB reacts with additional, more deeply buried cysteines thereby hindering efflux induction. After pretreatment with PCMB, only Hg^{2+} stimulated efflux to its maximum extent (not shown).

The second SH-group, which is crucial for efflux induction, only is exposed in the absence of external substrate. On the contrary, the first SH-group was accessible for all tested reagents also in the presence of aspartate. If substrate was added after efflux induction, hence in principle allowing residual antiport activity to occur, the total flux of label from inside to outside was clearly reduced instead of being stimulated (Fig. 5). Consequently, antiport activity is no longer catalyzed once the carrier is in the efflux state. Antiport-efflux interconversion thus has to be rationalized as a complete functional switching between two exclusive modes of transport. The observation that external substrate even inhibits the induced efflux carrier suggests that also in the absence of substrate the outward facing binding site participates in the conformational transitions during the efflux process ('slippage'). This possibility is indicated in Fig. 6 by a dashed arrow. According to this model, external substrate may cause a *trans*-inhibition of the slippage step rendering the carrier efflux incompetent. Such an outline of the efflux carrier resembles the model for the Asp/Glu antiporter involving one binding side on each membrane side, as elucidated previously [6] (cf. Introduction). The main effect of the applied mercurials then would be to uncouple the inward and outward directed component of antiport. According to transition-state theory the tightness of coupling depends on the increase of binding energy in the transition intermediate during translocation [24]. Hence any disturbance of intrinsic binding could lead to uncoupled transport and possibly also to pore-like properties.

General consequences for carrier-mediated transport

The term 'slippage' as an explanation for uncoupled antiport involving conformational changes of, at least partially, unloaded transport proteins has been used since early investigations [25] on the erythrocyte anion exchanger, which shows net-flux activity in the physiological state. Thorough studies of this activity, however, led to different net-flux models named 'transit' [26], 'tunneling' [27] or 'diffusive flow' [28], which all included a more channel-like pathway. The main component of net flux, besides slippage, was described as transport without a conformational change ('tunneling'), thus representing a molecular slip in antiport coupling [23]. Since carrier-mediated transport needs a conformational transition, 'tunneling' may be regarded as a functional link between a carrier and a channel. Actually, the net flux of the anion exchanger cannot be compared in every respect to the artificially induced efflux activity of the Asp/Glu carrier, as is exemplified by a reduced (but still high!) activation energy (70 [27] instead of 96–136 kJ/mol measured for anion exchange [29]). In addition, *trans*-inhibition by external substrate was less complete [27], which, however, may be explained by the single-site model favored for anion exchange [30] in contrast to the two-site model of the Asp/Glu carrier [5].

On the basis of our data a mechanism of efflux has to be envisaged combining aspects of the uniport/'slippage' model on the one hand, and of the pore/'channel' model on the other. *trans*-Inhibition and high activation energy are good arguments in favor of 'slippage'. Alternatively, when following the channel hypothesis, external substrate may bring about a conformational change into a non-efflux-competent state. For explaining the high activation energy, additional transfer steps of the substrate, moving from the superficial binding site to inner-channel sites (cf. Ref. 31), may become rate-limiting after alteration of the transport mechanism. As a compromise, the Asp/Glu carrier is visualized assuming a narrow channel spanning part of the total transport pathway. This hybrid channel can only be penetrated by substrates after recognition at a specific binding site, which then triggers a carrier-like

gating mechanism. Such a model is similar to the gated pore model for the ADP/ATP carrier [32,33], although two binding sites would have to be assumed in our case [5]. Possibly, the gating of the 'channel' is disturbed upon SH-modification of the carrier protein. Due to a reduction in the specificity and affinity of the internal binding site, the protein-immanent channel is opened transiently for various solutes. This flux, nevertheless, shows properties of carrier-mediated transport, since the channel also functions in the normal catalytic cycle of antiport.

It should be emphasized that like the erythrocyte anion exchanger and, in addition, the mitochondrial carnitine carrier [19] also the unmodified Asp/Glu carrier catalyzed net flux, albeit very slowly (Fig. 2). This basic efflux activity was clearly dependent on reconstituted Asp/Glu carrier protein, needed high activation energy and could be inhibited by proteinases, DTNB [7] and other inhibitors (not shown). Although this net flux possibly is not important for the physiological function of the carrier, the efflux properties described here give insights into the mechanism of transmembrane transport in general. Efflux clearly is a simplified mode of transport as compared to antiport. Some steps of the overall translocation process are eliminated, such as the specific binding of two substrate molecules under formation of a catalytic ternary complex and the concerted transport of these molecules into opposite directions [5]. We conclude that, due to this reduced complexity, new aspects of the channeling of a substrate molecule through the carrier protein were revealed. Whatever model correctly describes the reversible switching of transport mechanisms on the molecular level, our results most consistently can be interpreted assuming a preformed channel as a structural requirement for substrate translocation, antiport as well as efflux, instead of a mobile binding site traversing the lipid bilayer upon a conformational change of the whole protein. Also from comprehensive theoretical considerations on the dynamics and energy barriers of transport by Läuger [34,35] a channel-like structure becomes reasonable for several classes of transport proteins. As derived in these studies, many transport systems may function by an intermediate mechanism between a pure carrier and a pure channel. Our experimental data lead to similar conclusions for the Asp/Glu carrier, which most obviously are relevant also for the ADP/ATP carrier and possibly can be extended to further transport systems.

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