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Kinetic and energetic characterization of solute flux through the reconstituted aspartate/glutamate carrier from beef heart mitochondria after modification with mercurials

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Abstract

The functional switch from specific, coupled antiport to unspecific unidirectional transport (efflux) of the reconstituted aspartate/glutamate carrier from mitochondria after chemical modification with mersalylic acid was investigated in kinetic and energetic terms. The rate of mercurial-induced efflux was determined for a number of solutes which differ from the physiological substrate aspartate in structure, size and charge, namely oxoglutarate, sulfate, glucose, lysine and arginine. These values were compared to the rates of efflux as well as antiport of aspartate. Measurement of the temperature dependence of all rates led to evaluation of the activation energy of the different substrates. The activation energy was similar for all substrates and for both transport modes, whereas the efflux rates could be ordered in the following sequence: anions > uncharged solutes > cations. When extrapolating to V_{max} conditions, the resulting turnover numbers for uniport substrates become similar and exceed the turnover numbers for aspartate and glutamate antiport. Trans-inhibition of efflux was only observed in the case of externally added aspartate or glutamate and only for internal anionic substrates (at the cis side), thus indicating that after efflux induction the specificity of the external binding site is fully and that of the internal site is partially retained. The consequence of these results for understanding the transport function of the aspartate/glutamate carrier in the slippage mode (uniport) is discussed in energetic and kinetic terms.

Keywords: Transport; Mitochondrion; Aspartate/glutamate carrier; Reconstitution; Energetics; Kinetics; Uncoupled transport; Facilitated transport

1. Introduction

Transport of metabolites across the inner mitochondrial membrane is catalyzed by a number of specific carrier proteins [1,2]. One of these, the aspartate/glutamate carrier (AGC), mediates the exchange of cytosolic glutamate with matrix aspartate. The antiport reaction is electrogenic since glutamate is cotransported with a proton. In fact, the AGC is the only mitochondrial carrier which is modulated both by the membrane potential and the pH gradient. The mechanism of antiport was studied both in intact mitochondria and in reconstituted proteoliposomes with defined orientation of the AGC [3–5]. The AGC acts in a simultaneous type of bisubstrate kinetics, i.e., the two binding sites on the opposite sides of the membrane have both to be occupied by substrate in order to render the AGC catalytically active. Thus, a ternary complex is involved, which is formed in a so-called rapid-equilibrium random mechanism.

Treatment of proteoliposomes containing reconstituted AGC with SH-reagents (mercurials) led to efflux of internal substrate [6–8]. We showed that this efflux resulted from modification of two distinct exofacial cysteines. Thus the 'normal' carrier activity, i.e., the coupled antiport was not only inhibited but was converted into a unidirectional transport reaction. This uniport shows peculiar properties. On the one hand it resembles channel functions, such as non-saturability and drastically reduced substrate specificity, leading to transport of a large variety of molecules. On the other hand, typical properties of a carrier are

Abbreviations: AAC, ADP/ATP-or adenine nucleotide-carrier; AGC, aspartate/glutamate carrier; $C_{12}E_8$, octaethylene glycol monododecyl ether; DTE, dithioerythritol; Mops, 3-(*N*-morpholino)propanesulfonic acid; n_t, turnover number; PLP, pyridoxal phosphate; ts, transition state.

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retained, such as high and unchanged activation energy for mercurial-induced aspartate uniport, as well as retained interaction of the substrate with the external binding site [7]. The mercurial-induced functional conversion of the AGC was fully reversible by addition of dithioerythritol. Since this antiport/uniport interconversion was observed for several mitochondrial carrier proteins, it was taken as further indication for the presence of a functional family of mitochondrial translocators, although its physiological significance is still unknown [6,7,9].

The mercurial-induced efflux activity of the AGC was supposed to be due to uncoupling of the inward-and outward-directed component of antiport. Consequently, the observed pore-like properties were interpreted to indicate the presence of an intrinsic unspecific channel [7]. This functional element presumably takes part also in the physiological antiport activity, where it is hidden by appropriate gates. Generally speaking, these observations were taken as indication, that under particular conditions some common principles of carrier- and channel-mediated transport may become obvious. This concept was so far mainly based on studies of the mitochondrial AGC and the phosphate carrier [10] in monitoring the uniport of their physiological substrates, e.g., aspartate or phosphate. The present paper extends these investigations on the kinetic and energetic properties of solute transfer in the mercurial-induced efflux of 'unphysiological' substrates, i.e., those which are only transported after modification of the carrier protein.

2. Materials and methods

2.1. Materials and their sources

L-[U-^{14}C]Aspartate, L-[U-^{14}C]arginine, [*methyl*-^{14}C]choline, L-[U-^{14}C]glucose L-[U-^{14}C]lysine, [U-^{14}C]oxoglutarate and [^{35}S]sulfate were obtained from Amersham Buchler, [2,3-^{14}C]succinate from Du Pont-Nuclear. Dithioerythritol, mersalylic acid, Mops, and turkey egg-yolk phospholipid were purchased from Sigma. Amberlite XAD-2, Dowex W-X8 and Dowex 1-X8 were from Fluka, Sephadex from Pharmacia, hydroxyapatite (Bio-Gel HTP) from Bio-Rad and pyridoxal 5'-phosphate from Merck. All other chemicals were of analytical grade.

2.2. Preparation of aspartate / glutamate carrier (AGC) and ADP / ATP carrier (AAC)

The preparation of the AGC from bovine heart mitochondria was performed as described previously [11]. The protein fraction obtained after hydroxyapatite centrifugation chromatography and desalting on Sephadex G-25 was used for reconstitution of the AGC. This fraction contained both the AGC and the AAC. The AAC from bovine heart mitochondria was isolated by using a modification of the method described in [12]. For purification, wet HTP in a ratio of 70 mg per mg mitochondrial protein was used (without protease inhibitors). In addition, a final chromatographic step using 3 g tightly packed HTP was carried out at 4°C. A volume of 500 μl of HTP-centrifugation supernatant was passed through the HTP-column (diameter 1 cm). The AAC was eluated with a buffer containing 1 M ammonium acetate, 0.75% $C_{12}E_8$ and 0.25 mM EDTA. The first fraction of 500 μl was collected and incorporated immediately into liposomes. The protein concentration was determined according to a modified Lowry method after precipitation with deoxycholate and trichloroacetic acid and extraction of detergent and lipid with organic solvents [7].

2.3. Reconstitution procedure

Incorporation of different protein preparations into liposomes was carried out by a hydrophobic chromatography procedure [13]. The exact reconstitution conditions were as follows: (i) AGC: 12 mg/ml phospholipid, 1.75 mg $C_{12}E_8$/mg phospholipid, 0.04 mg protein/mg phospholipid, 20 mg $C_{12}E_8$/g washed amberlite; (ii) AAC: 12 mg/ml phospholipid, 1.5 mg $C_{12}E_8$/mg phospholipid, 0.01 mg protein/mg phospholipid, 20 mg $C_{12}E_8$/g washed amberlite. The reconstitution mixture was passed 15 times through the amberlite columns.

2.4. Determination of transport activities

The transport activities of the reconstituted carrier proteins were determined by measuring the flux of ^{14}C-labeled substrate. For monitoring the antiport activity of the AGC or AAC, the assay was started by the addition of 50 μM labeled aspartate or ATP, respectively, to proteoliposomes containing 16 mM aspartate or 20 mM ATP, respectively. For both carrier fractions, the transport reaction was stopped using 40 mM pyridoxal phosphate.

The efflux activities of the AGC or AAC were measured by detecting the export of internal ^{14}C-labeled substrate after induction by mersalyl. Two distinct procedures were used to preload the liposomes with labeled substrate: (i) in the case of the physiological substrates aspartate or ATP, respectively, the proteoliposomes were preloaded with ^{14}C-labeled substrate by using the antiport function of the translocators. After removal of external substrate by size-exclusion chromatography on Sephadex G75 columns, 0.5 μM aspartate or ATP of high specific radioactivity was added to the proteoliposomes containing 16 or 20 mM internal unlabeled countersubstrate. After 10 min incubation at room temperature the external substrate was again removed by size-exclusion chromatography. (ii) In the case of unphysiological substrates of the carrier proteins the proteoliposomes were prepared in the presence of 16 or 20 mM labeled substrate. External substrate was removed and

replaced by sucrose using size-exclusion chromatography on Sephadex G-75 [5]. For control measurements liposomes were prepared in the absence of protein; all other conditions were identical.

The efflux was induced by the addition of 100 or 200 μM mersalyl to the proteoliposomes in the absence of external substrate. Uniport activity was inhibited by the addition of a stop mixture containing 10 mM dithioerythritol and 40 mM PLP [6]. After treatment with the stop mixture each sample was passed through an anion-exchange column (Dowex 1-X8, acetate form) in the case of anionic substrates, through a cation-exchange column (Dowex W-X8, lithium form) in the case of cationic solutes and through a size exclusion column (Sephadex G-75, equilibrated with buffer and sucrose) in the case of glucose in order to remove external radioactivity. The proteoliposomes of each sample were collected in vials and the remaining entrapped radioactivity was determined by liquid scintillation counting. Further details of the evaluation of transport activities in the reconstituted system are described elsewhere [5,6]. The efflux of anionic substrates was measured in a time range of 30 min, the efflux of glucose in a time range of 60 min and the efflux of cationic solutes in a time range of 120 min [6]. In both antiport and efflux assays, the time course of the flux of isotope was fitted according to a first order reaction [5,6].

For determination of the activation energies, the proteoliposomes containing labeled substrate were distributed into aliquots that were incubated for 5 min at temperatures between 15 and 37°C. The efflux activity was started by the addition of 100 μM mersalyl from a 15-fold concentrated stock solution. For measuring the effect of external substrate on the unidirectional transport, efflux was induced by 200 μM mersalyl as described above. 2.5 min after induction, either buffer, 2.5 mM aspartate, glutamate or 2.5 mM of the corresponding internal substrate was added to the proteoliposomes [7]. For determination of the internal K_M value of lysine uniport, proteoliposomes were prepared containing lysine in concentrations from 0.25 mM up to 256 mM. Uniport was induced by the addition of 200 μM mersalyl as described above. The apparent uniport velocity was calculated as described in [7].

3. Results

3.1. Quantification of solute fluxes mediated by the aspartate / glutamate (AGC) and ADP / ATP-carrier (AAC), respectively

The AGC fraction used for the kinetic experiments contains, as the only transport protein contamination, significant amounts of the highly active AAC [6,13]. It was previously shown that the mercurial-induced ATP efflux mediated by the AAC is relatively small in comparison to the aspartate efflux by the AGC [6]. For quantitating the fluxes of unspecific solutes used in the present study we compared the activity of highly purified AAC and of AAC-containing AGC fractions. The rate of ATP antiport for both protein fractions is a direct measure for the amount of AAC, whether pure or copurified in the AGC fraction. In addition, the activity of aspartate antiport as well as the first order rate constants of ATP, aspartate and lysine efflux were determined (Table 1). The aspartate antiport activity in the purified AAC fraction was negligible, which indicates a lack of cross-contamination by the AGC. On the other hand the ATP exchange activity in the AGC fraction was 14% of the value of the purified ATP/ADP-carrier. The lysine efflux rates were similar in both reconstituted fractions. Thus, assuming a 14% cross-contamination of AAC activity in the AGC fraction, the portion of efflux in the AGC fraction mediated by the AAC is less than 10% for the three solutes measured. All experiments were carried out using this AGC fraction. The pattern of efflux activity observed for the AAC and the AGC fraction were similar, however, the absolute values of the rate constants of mercurial-induced efflux in comparison to antiport were very different for the two carriers. The efflux mediated by the modified AGC was found to be more then ten times faster as compared to the AAC when normalized to the antiport activity.

3.2. Permeability of proteoliposomes and influence of mercurials

In this study, we deal with four possible fluxes, three of which have to be compared in order to evaluate correctly

Table 1
Exchange and uniport activity of the reconstituted ADP/ATP-carrier (AAC) and aspartate/glutamate carrier (AGC)

Transport activity	AAC	AGC
ATP exchange rate (mmol min^{-1} (g protein)$^{-1}$)	1.117	0.161
Aspartate exchange rate (mmol min^{-1} (g protein)$^{-1}$)	0.001	0.031
Lysine efflux rate constant (min^{-1})	0.011	0.019
Aspartate efflux rate constant (min^{-1})	0.026	0.084
ATP efflux rate constant (min^{-1})	0.013	0.025

All internal solutes were 20 mM. Exchange activity (transport rate) was determined by adding 50 μM labeled aspartate or ATP, respectively. Uniport activity (first order rate constant of efflux) was measured after induction by 0.1 mM mersalyl. The internal volume of the proteoliposomes was determined to about 1 ml/g phospholipid.

the activity of the mercurial-induced efflux. Entrapped solutes may leave the proteoliposomes via (a) direct membrane leakage, or leakage through membrane-inserted carrier proteins either (b) in their native state (e.g., by kinetic slippage mechanisms) or (c) after efflux induction by mercurials. Finally, (d) transfer according to the 'physiological' antiport activity has to be considered. For a successful discrimination, the fluxes (a) and (b) have to be relatively small in comparison to fluxes (c) and (d).

Table 2 compares the fluxes (a) to (c) at two different temperatures after addition of buffer or 0.1 mM mersalyl, respectively. The flux in pure liposomes in dependence of addition of mersalyl was not differentiated, since only in the case of glucose at 37°C, a significant influence of mersalyl on the lipid membrane could be deduced (Table 2). With the exception of glucose, the basic membrane leakage was low in all cases, even after treatment with mersalyl. The same holds true for the discrimination of the mercurial-induced efflux from the other fluxes. Only in the case of glucose and lysine at 37°C, this background value amounted to about 20% of the observed mercurial-induced activity and was thus subtracted in the further experiments (see Fig. 1).

In order to determine the influence of the applied mercurial concentration on the observed efflux activity, aspartate efflux was induced by mersalyl in different concentrations up to 0.8 mM (data not shown). A plot of the mersalyl concentration versus efflux rate constant led to a correlation which could be fitted by a Michaelis-Menten equation. The mersalyl concentration leading to half maximum induction was about 0.1 mM (not shown). For determination of activation energies a concentration of 0.1 mM mersalyl was chosen to avoid possible unspecific reactions of mersalyl besides the modification of the two cysteine residues. Only in the trans-inhibition experiments 0.2 mM mersalyl was applied, since it was advantageous in this case to measure the relatively small effects under conditions of rate constants as high as possible.

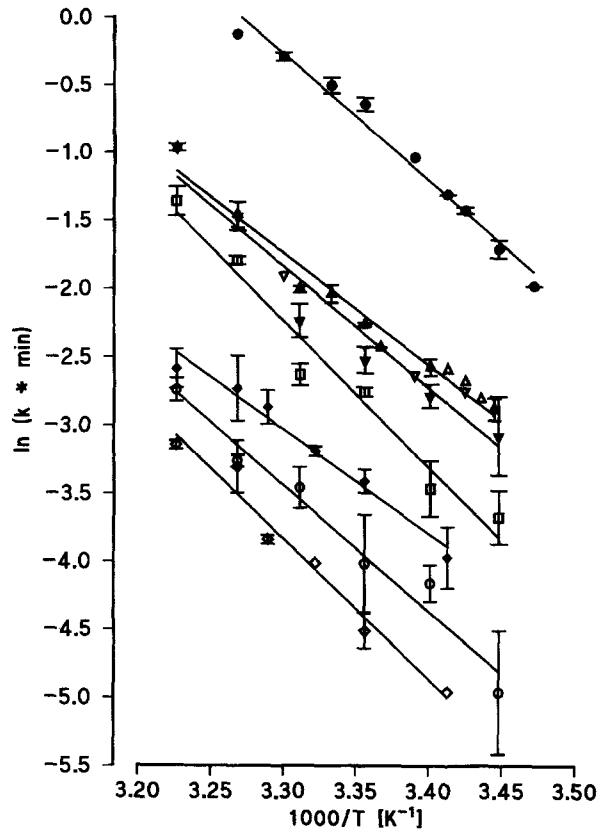

Fig. 1. Temperature dependence of the mercurial-induced uniport activity (Arrhenius plot). The measured first order rate constants for efflux of arginine (◇), lysine (○), glucose (◆), sulfate (△), oxoglutarate (□), and aspartate (▽), as well as the rate constant calculated from aspartate/aspartate exchange (●) are given. The data obtained for glucose efflux were corrected for diffusion (see text).

Table 2
Rate constants of diffusion and uniport (efflux) of various solutes at 25°C and 37°C

Solute	Rate constant (min^{-1})					
	25°C			37°C		
	liposomes	proteoliposomes		liposomes	proteoliposomes	
	+ mers	+ buff	+ mers	+ mers	+ buff	+ mers
Aspartate	< 0.001	0.003	0.080	n.d.	0.020	0.347
Sulfate	< 0.001	n.d.	n.d.	n.d.	0.030	0.289
Arginine	< 0.001	0.001	0.011	< 0.001	n.d.	0.042
Lysine	n.d.	0.001	0.019	n.d.	0.011	0.063
Choline	n.d.	0.001	0.033 [a]	< 0.001	0.014	n.d.
Glucose	0.002 [b]	0.005	0.036	0.029 [c]	0.025	0.108

Liposomes containing 16 mM of the different solutes were treated with 0.1 mM mersalyl (+mers) for determination of the basic diffusion through the phospholipid membrane. Proteoliposomes with the same substrate concentration were treated either with buffer (+buff) for measurement of the basic efflux activity, or with 0.1 mM mersalyl (+mers) in order to quantify the mercurial-induced uniport. In all experiments, the internal volume of the liposomes was close to 1 ml/g phospholipid. (n.d. = not determined).

[a] Choline efflux in this experiment was measured after addition of 0.2 mM mersalyl.

[b] Glucose efflux rate constant from buffer treated liposomes is 0.002 min^{-1}.

[c] Glucose efflux rate constant from buffer treated liposomes is 0.021 min^{-1}.

Table 3
Activation energy and rate constant of antiport and efflux of various solutes

Transport substrate	Transport mode	Rate constant (min^{-1}) at		E_a (kJ/mol)	r^2
		25°C	33°C		
Aspartate	AP	0.536 ± 0.028	0.880	84	0.98
Aspartate	UP	0.080 ± 0.012	0.228 ± 0.030	74	0.94
Oxoglutarate	UP	0.064 ± 0.002	0.166 ± 0.004	89	0.97
Sulfate	UP	0.087 ± 0.016	0.179 ± 0.015	68	0.97
Glucose	UP	0.036 ± 0.002	0.080 ± 0.011	63	0.98
Arginine	UP	0.011 ± 0.001	0.037 ± 0.006	85	0.98
Lysine	UP	0.019 ± 0.007	0.039 ± 0.002	77	0.97

UP, AP means uniport (efflux) and antiport, respectively. Activation energies (E_a) were obtained from the Arrhenius plots (cf. Fig. 1), r^2 represents the squared regression coefficient. The rate constants of glucose were corrected for diffusion. Antiport was started by addition of 50 μM labeled aspartate.

3.3. Determination of activation energies and estimation of turnover numbers

The activation energy E_a for efflux of different substrates was calculated by using the logarithmic Arrhenius equation:

$$\ln k_n = \ln k_0 - E_a/R \cdot 1/T$$

In this equation, k_n is the calculated rate constant in (min^{-1}) at a defined temperature, k_0 the so-called frequency factor and also the intercept of the resulting graph. Fig. 1 summarizes the data for a number of transported solutes in an Arrhenius plot.

For this comparison, we chose solutes that differ from aspartate and glutamate, the physiological substrates of the AGC, in several respects. Structurally related substances with different charge were used, such as the 2-fold negatively charged oxoglutarate and the cationic lysine, as well as structurally unrelated solutes which are negatively charged (sulfate), positively charged (arginine), as well as uncharged (glucose). The values for the activation energy, as calculated from the slopes in the Arrhenius plot are summarized in Table 3. For comparison, the activation energy for aspartate antiport under the same conditions was also determined. All observed values for E_a are within a range of 63 to 89 kJ mol^{-1}. Furthermore, the corresponding efflux rate constants at two distinct temperatures are presented. These rate constants can be splitted into three groups dependent on the charge the of translocated substrate (Table 3 and Fig. 1). Efflux constants of anionic solutes were about twofold higher than that of glucose, which was again about twofold higher than the rates of positively charged substrates. Aspartate antiport was significantly faster than all observed mersalyl-induced efflux activities. It should be noted, however, that the V_{max} values of uniport were in fact much higher, since the actual rates had to be measured at internal substrate concentrations significantly below the extrapolated high internal K_m value of aspartate efflux ($K_m \geq 200$ mM) [7]. To compare the turnover numbers of antiport and uniport of different substrates, as an example the internal K_m of lysine efflux was determined by varying the internal lysine concentration from 0.25 mM to 256 mM. The apparent efflux velocity was calculated and the K_m was determined to be 823 ± 40 mM (5 independent experiments, not shown). The extremely high K_m is above the range of internal substrate concentrations which could be applied, thus the calculated K_m-value should be rather taken as an estimation. Based

Table 4
Trans-inhibition of solute efflux by the reconstituted AGC

Solute	Mean first order rate constant after addition of			
	buffer (min^{-1})	aspartate (%)	glutamate (%)	substrate (%)
Aspartate	0.099 (5)	49 (3)	55 (2)	49 (3)
Sulfate	0.098 (3)	39 (3)	n.d.	94 (3)
Succinate	0.065 (5)	49 (4)	43 (2)	97 (4)
Glucose	0.059 (3)	100 (3)	n.d.	94 (3)
Arginine	0.030 (1)	127 (1)	n.d.	90 (1)
Choline	0.033 (4)	100 (4)	n.d.	94 (4)
Lysine	0.044 (6)	84 (6)	112 (2)	95 (6)

The proteoliposomes contained different labeled solutes. At time zero, efflux was induced by addition of 0.2 mM mersalyl. After 2.5 min the solute to be tested for trans-inhibition was added (buffer, 2.5 mM of aspartate, glutamate or the respective solute present in the interior of the liposomes, as indicated in the first column). The resulting rate constants are expressed as percent of the rate constant after addition of buffer (= 100%). The numbers in brackets mean number of experiments; n.d. = not determined. All other conditions as in Table 2.

on this values, the turnover numbers of uniport of different solutes can be compared to the turnover number of the AGC in the antiport mode, $n_t = 10\ s^{-1}$ at 25°C [14] and $n_t = 16\ s^{-1}$ at 33°C. Taking into account the internal K_m of aspartate efflux of about 200 mM [7], the turnover number of aspartate uniport can be calculated to $n_t = 17\ s^{-1}$ at 25°C and $n_t = 48\ s^{-1}$ at 33°C. By using the internal $K_m = 823$ mM of lysine efflux, the turnover number of this solute is extrapolated to $n_t = 16\ s^{-1}$ at 25°C and $n_t = 32\ s^{-1}$ at 33°C.

3.4. Trans-inhibition of efflux by external substrate

Trans-stimulation and trans-inhibition are well-known kinetic properties indicative of solute transfer reactions under particular circumstances. The situation is especially interesting in the present case, since in an antiport (bisubstrate) mechanism of the simultaneous type, both the internal and external binding site is involved in formation of the catalytically active ternary complex [3,4]. In contrast, in a simple uniport mechanism only one binding site is necessary to form the active binary complex of translocation. It is therefore interesting to investigate the properties of the exofacial binding site of the AGC during unidirectional transport. We have previously shown that the external binding site is in fact still available to the substrate aspartate during the mercurial-induced unidirectional transport mode (efflux), since we observed trans-inhibition of the efflux of aspartate [7]. Here we investigated whether efflux of unphysiological solutes can be inhibited by substrates at the trans side after induction of uniport by mersalyl (Table 4). This refers to the physiological substrates aspartate and glutamate as well as the various tested unphysiological substrates. In these experiments we included further solutes such as succinate and choline, which also differ from the native substrate in charge and structure. The results shown in Table 4. can be summarized in the following two statements. (i) Only efflux of anionic solutes (aspartate, sulfate and succinate) could be inhibited by the presence of external aspartate or glutamate, and (ii) only aspartate and glutamate, i.e., the physiological substrates of the AGC are able to inhibit efflux when added at the trans side.

An influx of externally added unphysiological solutes has not been observed so far. On the one hand it is possible that the induced efflux activity may to some extent be asymmetric, in accordance with the function of the carrier, which is still asymmetric as indicated by the trans-inhibition. On the other hand, there are technical reasons, which may in part explain this lack of observing reversibility. Transport of unphysiological substrates to the inside of proteoliposomes is very difficult to detect. The volume ratio (internal/external) is extremely unfavourable for these measurements. In view of the large surplus of external label, unspecific effects (binding) are clearly more significant than in the efflux direction. Furthermore, labeled substrate at the internal side of the membrane will be transported back to the outside by efflux activity during separation of the proteoliposomes.

4. Discussion

Mercurial-induced unidirectional transport by the mitochondrial aspartate/glutamate carrier (AGC) as well as other mitochondrial carriers is characterized by both channel- and carrier-like properties [7,9,10]. For a more detailed description of this function of the AGC, we determined energetic and kinetic properties for a number of substrates, which are not natural ligands of the AGC, but only accepted in the efflux mode. They differ from aspartate and glutamate, the only antiport ligands of the AGC, in structure, size and charge. Nevertheless, for all tested solutes, we found activation energies (E_a) in a range between 63 and 89 kJ mol^{-1}. These values are very similar to those of other mitochondrial carriers in the antiport mode [15] and clearly different from E_a values for typical channels, e.g., 16 to 20 kJ mol^{-1} for the mitochondrial porin (R. Benz, personal communication). No obvious correlation of the differences in E_a, however, to structure or charge of the respective solute could be deduced. Most importantly, besides indicating a carrier-type of translocation, the similarity in E_a within the various tested solutes and the different transport modes argues for the fact that the rate-limiting step in solute transfer by the AGC is independent of the kind of substrate, and possibly also independent of whether antiport or uniport is observed.

On the other hand, we observed significantly different apparent efflux rate constants for the different solutes in the efflux mode. Anions like aspartate, sulfate, succinate and oxoglutarate were transported faster than uncharged glucose, which in turn exceeded the cations arginine, lysine or choline in efflux rate. Obviously, the structural aspects are of minor influence, even the basic feature of being an amino acid is no prerequisite for uniport. The existence of an appropriate charge seems to be no indispensable requirement for translocation, however, the substrate's charge significantly influences the apparent transfer rate. Some steps within the translocation cycle must clearly be changed in uniport, as, for example, indicated by the loss of specificity. Nevertheless, a preferential interaction of the substrate with a positive charge in the translocation pathway of the AGC seems to be retained under efflux conditions. This is in line with the results of chemical modification of the AGC, indicating functionally essential lysine and histidine residues [15]. Taking into account the high internal K_m values of solute efflux, which is about 200 mM for anionic aspartate and about 800 mM for cationic lysine, the calculated turnover numbers calculated become roughly similar, indicating a similar rate limiting step in the translocation cycle for all kind of substrates.

We previously showed that the exofacial binding site of

the AGC is still present in the efflux mode [7]. External aspartate in concentrations identical to its K_M in the antiport mode inhibits efflux of internal aspartate. In view of the variety of efflux substrates tested, it was interesting to investigate their potency to cause this trans-inhibition. We found that the physiological substrate aspartate and glutamate are the only ligands active in trans-inhibition, which again indicates the retained specificity of the exofacial binding site after the functional conversion. Interestingly, we observed a specificity of trans-inhibition also with respect to the kind of internal (efflux) substrate. Only efflux of anionic solutes could be inhibited by external aspartate or glutamate. In agreement with the arguments concerning the order of efflux rate constants (see above), we thus conclude that the observed functional specificity indicates the preferential interaction of anions with cationic residues within the efflux pathway. We also tested the effect of the non-specific substrates in higher concentrations, when added to the external side. There was no effect even in concentrations up to 16 mM (not shown, see Table 4 for values of 2.5 mM). Thus, it seems to be unlikely that the unphysiological substrates fail to inhibit simply because their concentrations at the external side is too low. These findings prove the presence of a functional exofacial binding site and the presence of some residual specificity also at the internal site of ligand interaction in the uniport mode of the AGC.

Kinetic trans-stimulation is taken as a proof for carrier-mediated transport [16], whereas the situation is more difficult in the case of trans-inhibition. In general, channels show the effect of trans-inhibition because a high substrate concentration at the trans side inhibits the flow of the substrate to this side [17]. Consequently, increasing the substrate concentration at the trans side of channels will decrease the solute flux, in contrast to the trans-stimulation observed for carriers. However, trans-inhibition is frequently also observed for carrier systems with particular kinetic mechanisms [17], e.g., for the lactose permease from *Escherichia coli* [18] and for other systems [19,20]. In the case of the AGC, three facts with respect to the observed trans-inhibition argue for the involvement of a carrier-catalyzed vectorial process. (i) The inhibition is specific, only aspartate and glutamate are effective at the trans side. (ii) The trans-inhibition is a saturable process, and (iii) the half-maximum saturation occurs at exactly the same concentration as observed for the binding of aspartate to the AGC in its unmodified state.

On the basis of the kinetic data we estimated some thermodynamical parameters of the AGC according to Eyring's theory of the transition state [21]. For this calculation, we used the estimated turnover numbers based upon a molecular activity of the reconstituted AGC in the antiport mode of $n_t = 10\ s^{-1}$ at 25°C (see above). To make sure that small changes in this basical value $n_t = 10\ s^{-1}$ are not critical for the extrapolation of these thermodynamic data, we varied n_t from 2 to 20 s^{-1}, which did not lead to

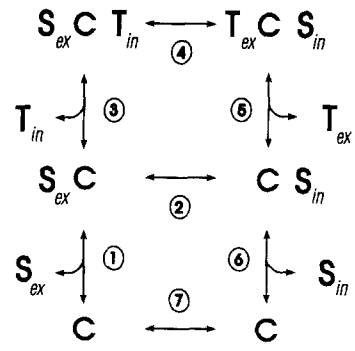

Fig. 2. Hypothetical scheme for the reaction mechanism of the aspartate/glutamate carrier (C) in antiport and uniport. For simplicity, an ordered mechanism is shown, binding of the substrates T and S in antiport is actually a random process [3]. The significance of the different reaction steps (1–7) for the different transport modes is explained in the text.

significant changes. The changes of free energy ΔG^{ts}, enthalpy ΔH^{ts} and entropy ΔS^{ts} of activation were determined. In the antiport mode ΔG^{ts} mainly consisted of an activation enthalpy term ΔH^{ts}, which was largely (about 80%) compensated by the positive activation entropy. In the efflux mode, considering the high turnover numbers when extrapolated to V_{max} values (see Results), ΔG^{ts} is of about same size as in the antiport mode. The entropy term of uniport of all substrates decreased to 30 to 50% in comparison to the corresponding values for antiport. Both antiport and, to a larger extent, uniport thus seem to be mainly driven by the enthalpy term of activation. In a detailed investigation of these data for the erythrocyte anion carrier, a high positive activation entropy was found [22]. Therefore, it was concluded in this case that the energy used for conversion into the transition state is largely compensated by an increase of randomness in the anion carrier protein and/or its aqueous environment.

4.1. Uniport activity of the AGC: a slippage mechanism?

Unidirectional transport of an antiport carrier can be discussed in terms of slippage. This means the use of normally forbidden pathways in the translocation cycle of a coupled carrier. Fig. 2 shows a scheme of an antiport carrier functioning in a simultaneous type of action, i.e., involving a ternary catalytic complex. In the antiport mode, mediated by steps (1) and (3)–(6), reactions (2) and (7) are forbidden. Significant activity of these modes would result in uniport (slippage). A slippage cycle involves reactions (1), (2), (6) and (7). Slippage based on a cycle within the upper part of Fig. 2 is unlikely because of the observed trans-inhibition by external substrate. In the slippage cycle, reorientation of the unloaded carrier (7) is generally assumed to be the rate-limiting step. Since step (7) does not involve substrate binding, it is independent of the kind of the transported solute. This view is consistent with the estimated turnover numbers for induced uniport, which are

roughly similar, independent of the transported substrate. Also the closely similar activation energy can be interpreted in terms of a common step independent of the respective ligand.

It is in principle also possible to interpret the antiport/uniport conversion of the AGC in terms of inducing a facilitated transport mode, since the turnover numbers of uniport of differently charged substrates are roughly the same. In facilitated transport, both the unloaded carrier and the carrier-substrate-complex should be mobile, as shown, e.g., for the choline and glucose transport systems of human erythrocytes [23]. Thus substrate catalysis of carrier reorientation is not required. The transport site fits to the substrate closely and retains its binding configuration during translocation [23]. Consequently, unphysiological solutes should be translocated as fast as physiological substrates. However, the observed specificity of the trans-effect of external substrate does not fit in this concept of facilitated transport. Cationic and uncharged substrates are transported in the uniport mode independent of the presence of external physiological substrate, whereas efflux of anionic solutes is inhibited. This leads to the assumption that for this kind of substrate-carrier interaction an increase in binding energy during translocation is necessary. Furthermore increasing external substrate concentrations is expected to stimulate facilitated transport, since movement of the loaded carrier is in general thought to be faster than reorientation of the unloaded carrier [16,23], which contradicts the observed uniport direction of the AGC. In view of the clear-cut influence of external aspartate or glutamate, we assume that substrate catalysis is still necessary for carrier movement in the uniport mode. In the following, these concepts of slippage and facilitated transport of the AGC are elaborated in terms of both an energetic and a kinetic approach.

4.2. Uniport activity of the AGC: an energetic approach

Similar to enzyme reactions, the catalytic cycle of a carrier has been described by free energy profiles [24,25]. A basic principle is the utilization of binding energy. The intrinsic binding energy (a favorable carrier-substrate interaction) is not expressed, but is stored as conformational energy in the carrier protein, thus lowering the energy of the transition state. The substrate therefore only fits poorly to the active site in the ground state, but strongly binds in the transition state. This model of vectorial catalysis can be applied to the situation observed for the AGC. Modification of two cysteines distorts the protein conformation and thus the energy profile of the system. Thereby, (i) antiport is inhibited by increasing the free energy of the ternary transition complex (Fig. 2, step 4). Simultaneously, the energy barriers of the pathways involving the binary complex (step 2) and the free carrier (step 7) are lowered. (ii) Because of more or less unspecific binding in the efflux mode, the extent of utilisable binding energy is small. The combination of decreased binding energy and a lowered activation energy barrier enables an increased turnover in comparison to the antiport mode. In other words, this is in fact a more channel-like description of solute translocation by the modified AGC. (iii) The exofacial binding site didn't change its specificity after modification of the AGC. Thus a ternary complex with external aspartate or glutamate and internal (unspecific) substrate occurs. We assume that its formation leads to an energy trap, if internal substrate is anionic, and consequently trans-inhibition is observed.

4.3. Uniport activity of the AGC: a kinetic approach

In a series of publications a model for carrier function has been developed combining the concept of a change in binding interaction of the carrier in different conformations with its ligands and the concept of a changing mobility of different carrier conformations [26,27]. Thus, control of carrier translocation is assumed to take place on two levels: (i) The substrate binds more strongly in the transition state and therefore increases the rate of conformational changes. Furthermore, the substrate binding forces are used to couple vectorial processes. The ratio of coupled (antiport) to uncoupled (uniport) rates therefore is governed by the ratio of substrate dissociation constants in the ternary and binary complexes, respectively [26]. (ii) Additionally, the substrate binds more strongly to one conformation and therefore shifts the equilibrium between mobile and immobile conformations. As applied to the formalism described in Fig. 2, all the different species may exist in two possible states, either mobile or immobile. In antiport systems the free carrier and the binary carrier complex are mainly in the immobile form, thus unidirectional transport (slippage) is avoided.

By applying these ideas to the AGC, it may be assumed that the cysteine modification changes the equilibrium between mobile and immobile forms of the unloaded carrier and the binary complex (with internal substrate) in the direction of the mobile species (R. Krupka, personal communication). Thus, the carrier is able to undergo a transport cycle and to mediate efflux. Furthermore, the affinity in forming the binary complex from carrier and internal substrate decreases, as indicated by the high internal K_M. This kinetic model is able to explain the phenomenon of trans-inhibition, but could not explain its specificity. Since we found that the interaction of the substrate at the external side was not affected, formation of an immobile binary complex has to be assumed, which inhibits efflux from the trans side. In contrast, the observation of specificity in trans-inhibition argues for the formation of a ternary carrier complex with external and internal substrate.

Nevertheless, if the conformational change of the mobile form, i.e., the translocation event, is the same whatever substrate is bound, a similar activation energy in

uniport for all substrates will be observed. On the other hand, it may be assumed that the equilibrium between the mobile and immobile binary form of the carrier (in uniport) is differently shifted with different substrates bound. Thus different amounts of the mobile form are available and different rates are observed (R.M. Krupka, personal communication).

4.4. Conclusions

The central aspects of the mercurial-induced uniport of the AGC were (i) the loss of substrate specificity at the internal but not the external active site, (ii) similar activation energy for both modes and all substrates transported, (iii) similar turnover numbers of non-specific solutes, which exceeds that of the exchange transport, and finally, (iv) substrate-specific trans-inhibition. These aspects could to a large extent be explained by different conceptual approaches, either from the energetic or the kinetic point of view. Consistently, the similarity of activation energies of transport of different substrates seemed to be a consequence of the respective concepts. The agreement of the activation energies in antiport and uniport turned out to be more or less fortuitous, since formation of different rate-limiting carrier species has to be assumed. All models are more or less fully based on a carrier concept, i.e., the apparently channel-like properties of the AGC in the mercurial-modified form could be explained by substantially changed functional aspects of a carrier system. This was most convincingly indicated by the unchanged activation energy for the two modes and for all transported solutes. The observed specific trans-inhibition, however, is difficult to explain in terms of these models as discussed above. This seems to be due to a specific characteristic of the aspartate/glutamate carrier. It also has to be mentioned that in contrast to usual models for explaining slippage (e.g., for the erythrocyte anion carrier [28]) or facilitated transport (e.g., for the erythrocyte choline or glucose carriers [23]), differently charged and unrelated transport substrates are studied here.

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