Biochimica et Biophysica Acta 937 (1988) 112–126
Elsevier

BBA 73823

Asymmetric orientation of the reconstituted aspartate / glutamate carrier from mitochondria

Thomas Dierks and Reinhard Krämer

Institut für Physikalische Biochemie, Universität München, München (F.R.G.)

(Received 13 July 1987)
(Revised manuscript received 2 October 1987)

Key words: Mitochondrion; Reconstitution; Transmembrane orientation; Carrier-mediated transport; Aspartate/glutamate carrier

A partially purified preparation of the aspartate / glutamate carrier from bovine heart mitochondria was reconstituted into liposomal membranes by chromatography on hydrophobic ion exchange resins. Based on the favorable conditions of this reconstituted system the transmembrane orientation of the inserted carrier protein could be determined by functional analysis. For reliable measurement of the reconstituted aspartate–glutamate exchange activity an optimized inhibitor-stop technique using pyridoxal phosphate was developed. By simultaneous application of both forward and backward exchange experiments the practical usefulness of the reconstituted system could be extended to investigations including variation of internal and external substrate concentrations over a wide range. Thereby a complete set of K_m values for both aspartate and glutamate at both the internal and external side of the proteoliposomes could be established. These experiments led to the following results and conclusions: (i) The observed substrate affinities are clearly different for the two different membrane sides both for aspartate (external 50 μM, internal 3 mM) and glutamate (external about 200 μM, internal 3 mM). (ii) The exclusive presence of only one type of transport affinity for every single substrate at one side of the liposomal membrane clearly demonstrates the asymmetric orientation of the functionally active carrier protein molecules. (iii) When comparing the values of these constants with published data obtained in mitochondria, an inside-out orientation of the aspartate / glutamate carrier after isolation and reinsertion into liposomes is strongly suggested.

Introduction

Transport of anionic metabolites across the inner mitochondrial membrane is mediated by a variety of specific carrier proteins, most of which can be classified as antiporters (for review, see Refs. 1 and 2). Among these the Asp/Glu carrier catalyzes a counter exchange of aspartate and glutamate, which is a central part of the malate/aspartate shuttle. This metabolic shuttle plays an important role in the transfer of reducing equivalents from the cytosol to the mitochondrial respiratory chain. Furthermore, the Asp/Glu carrier connects parts of two other metabolic pathways, which are compartmentalized between the cytosol and the mitochondrial matrix, namely gluconeogenesis from lactate and urea synthesis [3]. Beside its metabolic importance the mechanism of

Abbreviations: Asp/Glu carrier, aspartate/glutamate carrier; $C_{12}E_8$, dodecyl octaoxyethylene ether; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MOPS, 4-morpholinepropanesulfonic acid.

Correspondence (present address): R. Krämer, Institut für Biotechnologie 1, Kernforschungsanlage Jülich, P.O. Box 1913, D-5170 Jülich, F.R.G.

the Asp/Glu carrier is also of fundamental interest, since it is the only mitochondrial transporter to be modulated both by membrane potential and pH gradient [4]. Although aspartate as well as glutamate are monovalent anions at neutral pH, the carrier-mediated exchange becomes electrogenic, because glutamate and not aspartate is cotransported with a proton. Thus in energized mitochondria aspartate is efficiently pumped out of the matrix, driven by the membrane potential.

Transport kinetics of this carrier has been extensively studied in mitochondria by two groups [5,6]. However, there is still disagreement about some basic properties concerning the kinetic mechanism of exchange and proton cotransport. Also the phenomenon of functional asymmetry as observed in uncoupled mitochondria [7] is not understood. Even the reported K_m values are contradictory, especially the K_m value for aspartate at the matrix side of the protein. On the one hand discrepancies between the determination of this constant in intact mitochondria [6] and submitochondrial particles [5] were explained to be caused by the sonication process during preparation of particles [6]. On the other hand it was postulated, that the determination of the K_m value for matrix aspartate leads to erroneous values due to mirocompartmentation of internal substrate in intact mitochondria [5,8]. In this paper a complete set of all four K_m values could be established in the reconstituted system, including the values for the alternate substrate, namely glutamate at the 'matrix' side, i.e. the side analogous to that originally facing the mitochondrial matrix, and aspartate at the 'cytosolic' side, respectively.

In order to elucidate the catalytic mechanism of the Asp/Glu carrier and to clarify the questions concerning carrier function as mentioned above, a well defined reconstituted system has to be established. Isolation and functional reconstitution of the Asp/Glu carrier was reported recently [9]. Furthermore, the kinetic resolution of the transport measurement could be improved significantly by using a newly developed reconstitution method [10]. This procedure is based on hydrophobic chromatography and leads to the formation of large proteoliposomes carrying a relatively high amount of reconstituted protein. Nevertheless, kinetic analysis of aspartate–glutamate exchange is impeded by the fact, that no specific reagent for this carrier is known, that could serve as an efficient stopping reagent. Furthermore, a detailed investigation of the exchange mechanism would require side-specific inhibitors, which unfortunately are not available. Basically a random orientation of the reconstituted protein has to be expected, which, without application of side-specific inhibitors, would drastically limit the significance of kinetic experiments for elucidation of the transport mechanism. Extensive studies were therefore carried out in order to define the orientation of the reconstituted carrier protein. By functional analysis of its kinetic properties it was demonstrated in this paper, that the actively exchanging Asp/Glu carrier is in fact asymmetrically inserted into the liposomal membrane. This oriented reconstitution provides a well suited system not only for the determination of all the different K_m values as shown here, but also for the clarification of open questions concerning the carrier mechanism.

Materials and Methods

Preparation of Asp/Glu carrier was described recently [9]. The protein fraction obtained after chromatography on Sephadex G-25 (about 17-fold enriched exchange activity) was used for reconstitution.

Incorporation of the carrier protein into liposomes was carried out by a hydrophobic chromatography procedure [10]. The exact conditions were as follows: 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 amberlite 'moist beads', 100 mM Mops/KOH (pH 6.5); internal substrate concentrations are given in the text. The reconstitution mixture was passed 15 times through the amberlite columns.

Measurement of the reconstituted transport activity in the forward exchange mode has been described previously [11]. After generation of a substrate gradient by size exclusion chromatography of the proteoliposomes on Sephadex G-75 (100 mM Mops/KOH (pH 6.5), and 1–60 mM sucrose, which substitutes for the osmolarity of the internal substrate) the assay was started by addition of ^{14}C-labelled substrate in different con-

centrations, that are indicated in the context of each experiment. Composition of buffer during the assay was the same as described for Sephadex chromatography. The exchange was stopped using 25 mM pyridoxal phosphate. In control samples pyridoxal phosphate was added together with the substrate at zero time. Throughout all the experiments valinomycin (500 ng/mg phospholipid) and nigericin (20 ng/mg phospholipid) were added to the liposomes in order to provide deenergized conditions. Finally the radioactivity, that was not taken up by the vesicles, was removed by a passage over anion exchange columns (Dowex 1-X8, acetate form). 130 μl of each sample were applied on 5×45 mm columns, which were preequilibrated with 3 mg phospholipid and 3 mg bovine serum albumin per column in order to reduce loss of proteoliposomes. The vesicles were eluted with 40 mM sodium acetate (800 μl). The exchange velocities were calculated as described for the adenine nucleotide carrier [12]. However, for comprehensive kinetic experiments the data points for every single determination of transport velocity had to be reduced to a minimum number of four. This was achieved by using a computer fitting program, that adjusts the time course of isotope equilibration to the data points according to a first order process, which is expressed by the equation

$$\mathrm{dpm}_{in} = \frac{\mathrm{dpm}_{tot} \cdot S_{in} V_{in}}{S_{ex} V_{ex} + S_{in} V_{in}} (1 - e^{-kt}) \quad (1)$$

where dpm_{in} means radioactive label taken up by the proteoliposomes, dpm_{tot} is the total amount of externally added label at the beginning of the experiment, S = substrate concentration and V = volume of external and internal compartment of the liposomes, respectively; k means the first-order reaction constant and t is the reaction time. The curve of isotope equilibration indeed follows almost exactly this theoretically derived equation in the first five minutes, i.e. until the kinetic equilibration is reached for the majority of the active vesicles.

The exchange activities determined alternatively with high and low kinetic resolution, as described above, are in good agreement (not shown). Throughout this paper the transport activities are expressed as volume activities (μmol/l vesicles per min) and not as specific activities (μmol/mg protein per min), since protein determination in reconstituted liposomes is very inaccurate. If different preparations of liposomes were used within one experiment, the measured transport activities were normalized on the basis of phospholipid determinations [13], thereby cancelling out different dilution factors due to the different column passages.

The backward exchange method [14,15], that is applied for kinetic experiments, requires prelabelling of the internal substrate pool. This was done by loading the proteoliposomes with labelled substrate in the forward exchange mode using external substrate of high specific radioactivity at 5 μM concentration. After a second size exclusion chromatography on Sephadex G-75, which removes the radioactivity not taken up by the proteoliposomes, export of internal label was started by adding unlabelled substrate in different concentrations. Exchange was stopped by 50–80 mM pyridoxal phosphate. Since pyridoxal phosphate acts as a competitive inhibitor, it is applied in higher concentration as compared to forward exchange experiments, which usually are carried out with lower external substrate concentrations (see below). Gel chromatography, loading of vesicles and backward exchange measurement were all carried out in the same buffer described for the forward exchange method. The evaluation of transport activity from backward exchange experiments was carried out graphically as described for measurements of ATP export from mitochondria [14]. For that purpose, the percentage of isotope equilibration, α, is introduced as a measuring parameter (dpm $(0, t, \infty)$ = internal radioactivity at the beginning, at time t and at the end of isotope equilibration, respectively)

$$\alpha = \frac{\mathrm{dpm}(0) - \mathrm{dpm}(t)}{\mathrm{dpm}(0) - \mathrm{dpm}(\infty)} \cdot 100 \quad (2)$$

If the amount of external substrate is large as compared to the internal pool ($S_{ex} V_{ex} \gg S_{in} V_{in}$), the time dependence is described by Eqn. 3

$$\alpha = 100(1 - e^{-kt}) \quad (3)$$

which can be linearized to

$$-\ln[(100-\alpha)/100] = kt = vt/S_{in}V_{in} \quad (4)$$

In a plot of $-\ln[(100-\alpha)/100]$ vs. time the first-order rate constant k is represented by the slope of the resulting straight line. The calculation of exchange velocity v from Eqn. 4 is discussed in connection with the description of the backward exchange method.

Calculations of the active internal volume, i.e. that part of the liposome which is actively participating in the exchange reaction, were carried out on the basis of determinations of the isotope equilibrium [10]. This can be rationalized in the following equation, that describes the balance of specific activities in both compartments; the external volume is set to 100% or 1000 μl/ml

$$V_{in}(\mu l/ml) = 1000 \times \frac{dpm_{in} \cdot S_{ex}}{dpm_{ex} \cdot S_{in}} \quad (5)$$

The chemicals and their sources were as follows: labelled compounds (Amersham Buchler), turkey egg yolk phospholipid, DTNB and nigericin (Sigma), valinomycin (Boehringer Mannheim), pyridoxal phosphate (Merck), Sephadex (Pharmacia), Dowex 1-X8 (Fluka). Turkey egg yolk phospholipid from Sigma proved to be superior to other commercially available phospholipids tested. All other chemicals were of analytical grade.

Results

Optimization of exchange measurement in the reconstituted system

Variation of the stopping reagent. An essential prerequisite for application of an inhibitor-stop technique is an efficient stopping reagent. The response time of its action determines the kinetic resolution. From the inhibitors described for the Asp/Glu carrier [11,16] only pyridoxal phosphate is suitable as a stop-reagent for kinetic measurements in the reconstituted system [11]. Since pyridoxal phosphate acts as a competitive inhibitor, it must be added in relatively high concentrations depending on the external substrate concentration. In order to optimize the stopping efficiency, not only different concentrations of pyridoxal phosphate, but also combinations of pyridoxal phosphate and several SH-reagents were tested.

The efficiency of the inhibitors was evaluated by determining in backward exchange experiments the remaining internal radioactivity at zero time (see Materials and Methods). In a normal backward exchange experiment, the export of labelled substrate, entrapped in the internal volume of the liposomes, is initiated by the addition of external, unlabelled substrate. If an efficient inhibitor is added before the substrate, the internal radioactivity should remain at a fixed level. A decrease of internal label indicates that inhibition is incomplete, or at least, that there is a certain delay in the action of the reagent. If the time between the addition of inhibitor and substrate is varied, the

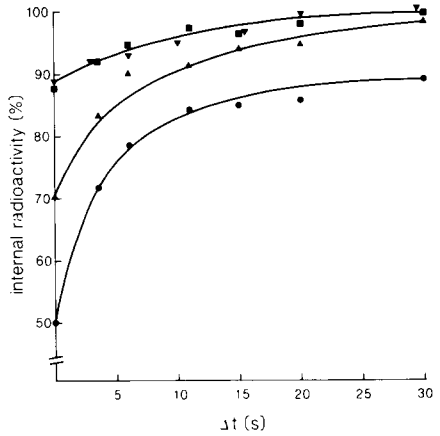

Fig. 1. Inhibition of the reconstituted Asp/Glu carrier. Efficiency and response time of pyridoxal phosphate action on exchange activity. Proteoliposomes were loaded with labelled substrate (20 mM aspartate) and passed through Sephadex G-75 as described for backward exchange measurements (see Methods). Then pyridoxal phosphate was added at different concentrations (20 mM, ●; 40 mM ▲; 80 mM, ■) and in combination with DTNB (80 mM pyridoxal phosphate/0.2 mM DTNB, ▼) followed by addition of high concentration of unlabelled substrate (10 mM aspartate). The remaining internal radioactivity, that is not exchanged against the external substrate, is dependent on the efficiency of carrier inhibition (see text). By variation of the time interval Δt between addition of inhibitor and substrate the response time of inhibition is evaluated. The applicability of the inhibitor as stopping reagent in inhibitor-stop kinetics is revealed by the effectiveness to retain the internal substrate, if inhibitor and substrate are added simultaneously ($\Delta t = 0$). The amount of internal radioactivity is expressed as percentage of the maximum value obtained after 30 s of preincubation with 80 mM pyridoxal phosphate.

response time for complete blocking of the transport reaction can be evaluated. For this purpose a very high external substrate concentration (10 mM) is chosen, that (without inhibitor) would lead to an export of radioactivity at maximum velocity, thus making the assay as sensitive as possible. In Fig. 1 such an experiment is shown for several representative stopping mixtures. It can be seen that under the applied conditions 20 mM pyridoxal phosphate does not block the carrier totally within 30 s. The substantial amount of internal radioactivity, that is lost when substrate and pyridoxal phosphate are added simultaneously, can be decreased, if the pyridoxal phosphate concentration is raised to 40 mM. Furthermore, at this 4-fold excess of inhibitor over substrate, pyridoxal phosphate completely stops the transport activity, when preincubation is long enough. Since in a normal exchange experiment the stop-inhibitor has to be effective in the presence of substrate, the observed time dependence of inhibitor action must be reduced. This can be achieved by raising the pyridoxal phosphate concentration further up to 80 mM (Fig. 1). Even in the presence of 10 mM external substrate the inhibition of carrier activity is nearly complete from the beginning. In order to take into account remaining inaccuracies resulting from a slight delay in carrier inhibition if very high substrate concentrations are used, correct zero time values have always to be determined: this means adding pyridoxal phosphate and substrate simultaneously, and not preincubating the proteoliposomes with the inhibitor.

Beside pyridoxal phosphate several SH-reagents are capable of inhibiting aspartate–glutamate exchange, as is shown in Fig. 2 for DTNB. However, inhibition by DTNB alone is not complete, and a stopping mixture composed of DTNB and high concentrations of pyridoxal phosphate doesn't improve the inhibitory effect either (Fig. 1). Though DTNB is not a suitable stop-inhibitor, its observed influence on transport activity brings up another phenomenon, which has to be taken into account when measuring the activity of the reconstituted Asp/Glu carrier. In a more detailed analysis of the impact of SH-reagents on the Asp/Glu translocator, a carrier-mediated export of internal substrate could be observed, that occurs in the absence of external substrate. This

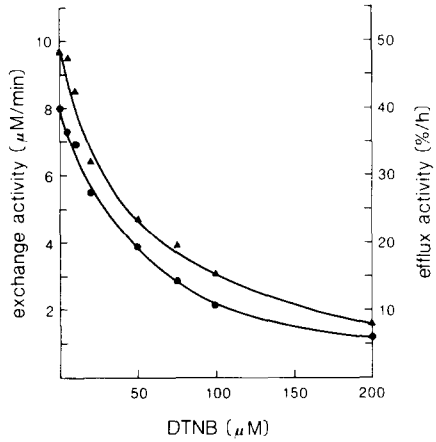

Fig. 2. Inhibition of the reconstituted Asp/Glu carrier. Effect of DTNB on exchange and efflux activity. Different concentrations of DTNB were added to the proteoliposomes immediately after size exclusion chromatography. One minute later exchange and efflux activity were measured (see text). Exchange velocities (●) were calculated from forward exchange kinetics (0.1 mM aspartate at the outside of the liposomes), whereas efflux (▲) is expressed as percentage decrease of labelled internal substrate pool, that is observed in the absence of external substrate within one hour. Internal substrate in both cases was 20 mM aspartate.

activity, which is referred to as efflux, can be stimulated by certain SH-reagents and inhibited by others (results not shown). Experiments to eliminate this efflux of substrate will be described below.

In spite of this observation, Fig. 1 demonstrates, that pyridoxal phosphate has to be used in a concentration that exceeds that of the external substrate several fold in order to achieve sufficient kinetic resolution.

Elimination of efflux. For measuring exchange kinetics in terms of an isotope equilibration the removal of external substrate is an important step, before starting the exchange assay in the 'forward direction' by addition of labelled substrate. This is achieved by size exclusion chromatography as described in Materials and Methods. Under these conditions the antiport reaction stops, since external substrate is lacking. However, an unexpected decrease of the internal substrate pool could be observed showing a half-time in the range of 1 h (not shown). This can be attributed to the efflux activity described above. It can be shown to be

definitely dependent on the presence of active carrier protein, and therefore this unidirectional transport interferes with the determination of exchange activity. Because the observed efflux will change the substrate concentrations in the two compartments, it may lead to erroneous determinations of substrate affinities.

An effective tool to avoid these difficulties could be found by the use of the SH-reagent DTNB. Incubation of the proteoliposomes with submillimolar concentrations of DTNB inhibits both exchange and efflux within a few seconds as demonstrated in Fig. 2. In the experiments shown in Table I DTNB is added before the vesicles are subjected to Sephadex chromatography for removal of external substrate. The efflux inhibition is comparable to that observed when DTNB was added after size exclusion chromatography (Fig. 2). This behaviour could have been expected, since DTNB is known to bind covalently to cysteine residues in disulfid linkage and hence is not removed on Sephadex G-75. Thus efflux of substrate during gel chromatography is efficiently inhibited by DTNB. Furthermore Table I demonstrates that the exchange activity of the reconstituted aspartate/glutamate carrier, inhibited by DTNB as well, can be reactivated by adding 4 mM dithioerythritol. It has to be pointed out that the same restitution can be observed for the efflux activity (Table I). However, the simultaneous reactivation of efflux is not interfering with respect to the following exchange measurement, because these kinetic experiments will take only a few minutes and the catalytic activity of efflux is very low. Dithioerythritol reverts the DTNB-block immediately and can be added just before the exchange is started.

For limitation of efflux activity to the time interval between addition of dithioerythritol and stop-inhibitor, i.e. the exchange time, the antiport stopping reagents have to fulfil also the criteria of efflux inhibition. This is important, since during the final passage of the vesicles over ion exchange columns (see Material and Methods) again efflux promoting conditions are generated, because the external substrate is completely removed. The extent of efflux caused by ion exchange chromatography is shown in Table II. It becomes obvious, that even under conditions of an artificially retarded passage only in the presence of low pyridoxal phosphate concentrations a consider-

TABLE I

INHIBITION OF THE RECONSTITUTED Asp/Glu CARRIER. RESTITUTION OF DTNB-INHIBITED CARRIER ACTIVITY BY DITHIOERYTHRITOL

Proteoliposomes were inhibited by different concentrations of DTNB immediately before they were loaded on Sephadex G-75. During gel chromatography external substrate and DTNB, not bound to the carrier protein, was removed. Then dithioerythritol (DTE) or elution buffer (control samples) was added. After one minute of incubation efflux and exchange activity were determined as described in Fig. 2.

DTNB (mM)	DTE (mM)	Exchange activity (μM/min)	Efflux activity (%/h)
0	0	10.8	24
0.2	0	4.7	5
0.5	0	2.7	6
0	4	9.5	21
0.2	4	9.3	26
0.5	4	8.3	24

TABLE II

INHIBITION OF THE RECONSTITUTED Asp/Glu CARRIER: PROTECTION AGAINST EFFLUX DURING ION EXCHANGE CHROMATOGRAPHY

Proteoliposomes were loaded with labelled substrate, passed through Sephadex G-75 and incubated with different concentrations of pyridoxal phosphate (PLP) as indicated. All samples contained the same amount of radioactivity, until they were subjected to ion exchange chromatography; this value was set to 100%. Ion exchange chromatography is necessary in each assay of reconstituted transport activity in order to remove labelled substrate from the outside of the liposomes (see Methods). The recovery of label entrapped in the internal volume of the liposomes, that are collected in the eluate of the DOWEX-acetate columns, is shown to be dependent on the one hand on the pyridoxal phosphate concentration applied and, on the other hand on the time of exposure to the anion exchange resin, which was experimentally prolonged up to 5 min. Data are expressed in relation to the 100% value, i.e. the radioactivity applied on the columns.

Time on Dowex columns	Recovery of internal label (%); concentration of added PLP		
	10 mM	30 mM	80 mM
1 min	96.3	97.1	98.0
3 min	92.3	93.8	95.2
5 min	89.6	92.7	93.6

able loss of internal radioactivity is observed, thereby proving the reliability of the method used for kinetic analysis of the exchange reaction.

Establishing the backward exchange method in the reconstituted system

The exchange activity of the reconstituted aspartate/glutamate carrier is monitored as a catalyzed isotope equilibration between two compartments. However, the difference between inward and outward flux of radioactivity is measured. In forward exchange experiments the uptake of label after addition of labelled substrate is recorded. The equilibrium, which is reached, resembles the ratio of internal and external substrate pool, i.e. $V_{in}S_{in}/V_{ex}S_{ex}$ (V, S = volume and substrate concentration in internal and external compartment, respectively). This ratio is the limiting factor in experiments involving high external and low internal substrate concentrations, since the external volume usually exceeds the internal volume about 500-fold, as can be estimated from the isotope equilibrium (see Methods). Under these conditions the situation is more favorable for transport measurements, if the internal substrate pool is labelled, so that the export of radioactivity can be measured in a classical backward exchange experiment after addition of unlabelled substrate to the external space (see Methods).

Evaluation of the exchange velocity from the kinetics of substrate export is difficult, because determination of the internal specific radioactivity depends on the size of the active internal volume, the quantitation of which is relatively inaccurate due to methodical reasons (see below). However, it is possible to calculate the rate constant k from the time course of the backward exchange (Ref. 14, see Methods). In this first order process, k is directly proportional to the exchange velocity v, assuming the same internal substrate pool for all active proteoliposomes within one preparation (Eqn. 4). Fig. 3 demonstrates that this calculation can be done by a logarithmic plot of the percentage of equilibration, α, vs. time (14); k is derived as the slope of the linearized curve.

According to Eqn. 4, k equals the exchange velocity divided by the internal substrate pool, which explains a decrease of the slope with increasing internal substrate concentrations, although the absolute transport activity will rise under these conditions (Fig. 4). For determinations of the internal substrate affinity constants the internal substrate concentrations have to be varied. In these cases the rate constants k are multiplied with the corresponding internal substrate concentrations. These relative velocities, v', are expressed in (mmol/l vesicles per min).

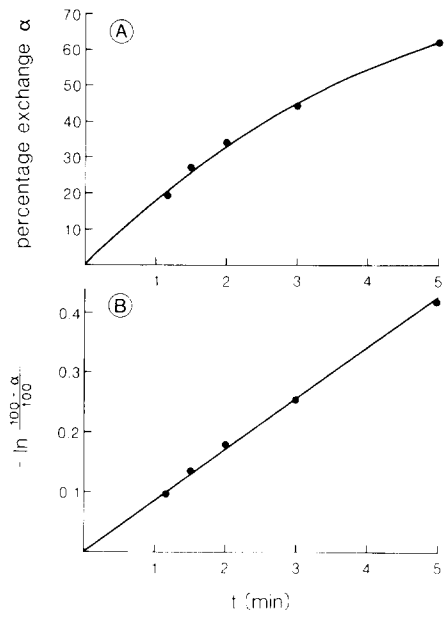

Fig. 3. Evaluating rate constant k of reconstituted aspartate–glutamate exchange in backward exchange measurements. (A) Time course of carrier-mediated isotope equilibration. At zero time, export of labelled substrate from the internal volume (16 mM aspartate) was initiated by addition of 0.2 mM unlabelled aspartate. For definition of percentage exchange (α) see Eqn. 3. (B) Logarithmic presentation of data according to Eqn. 4. The rate constant k is derived as the slope of the resulting straight line.

In order to make possible direct comparison of transport activities obtained from forward and backward exchange experiments, respectively, exchange velocities were determined by both methods with the same liposome preparation, varying the substrate distribution over a wide concentration range. These data are compiled in Table III. The estimation of the absolute backward exchange velocity v_b^* was carried out on the basis of a common active internal volume of 2 μl/ml, which was derived from the exchange equilibrium (see Methods). It can be seen, that in case of equal

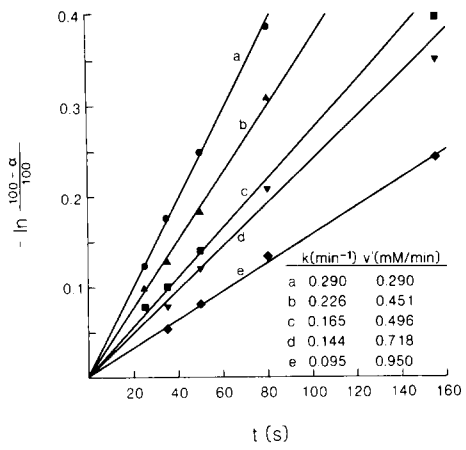

Fig. 4. Determination of transport affinities at the inner side of the liposomal membrane in backward exchange measurements. For determinations of the internal transport affinity constants the internal substrate concentrations have to be varied: 1 (●), 2 (▲), 3 (■), 5 (▼) and 10 mM glutamate (◆); external substrate was saturating (8 mM glutamate). The rate constants k obtained from the slope of the logarithmic plots show decreasing values, as the internal substrate concentration is raised. However, the exchange velocities v', calculated by multiplication of k with the corresponding internal substrate concentration (see text), increase along with the size of the internal substrate pool, as is shown in the inset. The corresponding Eadie-Hofstee plot is shown in Fig. 6. k (min^{-1}): a, 0.290; b, 0.226; c, 0.165; d, 0.144; e, 0.095; v' (mM/min): a, 0.290; b, 0.451; c. 0.495; d, 0.718; e, 0.950.

substrate distributions, the transport rates determined by the two different methods are in the same order of magnitude, but 'forward exchange activities' are higher than 'backward exchange activities' by a constant factor of about 2. This systematic deviation is mainly due to the dilution of vesicles during the additional size exclusion chromatography, which contributes by a factor of 1.5. A further reason is connected to inaccuracies in the estimation of the active internal volume. Since this determination depends on long time measurements of isotope equilibrium (Eqn. 5), it is definitely affected by efflux activity, thereby changing the ratio of substrate pools at the expense of the internal compartment. This would lead to a somewhat underestimated active internal volume. Though it is difficult to quantitate the extent of this effect, it seems to be reasonable to conclude from this comparison of activity data, that there is good agreement between the two different methods of analysis. The fact, that backward exchange activity is underestimated by a small, but constant factor independent of the applied substrate concentrations, promises coinciding and reliable determinations of K_m values. As a matter of fact, this becomes obvious in the context of later experiments (see Table IV).

Determination of substrate affinities

In Fig. 5 Eadie-Hofstee plots are shown, which are obtained from measurements of transport rates in the forward exchange mode under variation of the external aspartate and glutamate concentrations, respectively. The internal substrate con-

TABLE III

COMPARISON OF TRANSPORT ACTIVITIES DETERMINED IN FORWARD AND BACKWARD EXCHANGE EXPERIMENTS

Exchange activities were determined at different combinations of substrate pools inside and outside the proteoliposomes. Thereby a wide range of substrate concentrations was covered on both membrane sides as indicated. Forward exchange activities (v_f) were determined directly from initial velocity measurements, as described in Materials and Methods. Backward exchange activities (v_b^*) were estimated by multiplication of rate constant k_b with the size of the internal substrate pool. This value was calculated on the basis of a uniform internal volume of 2 μl per ml of liposomes in the assay mixture (see text), i.e. about 0.25 μl/mg phospholipid under these conditions. Symbols are indexed only for identification of transport direction.

External aspartate (mM)	0.5 mM internal aspartate			20 mM internal aspartate		
	k_b (1/min)	v_b^* (μM/min)	v_f (μM/min)	k_b (1/min)	v_b^* (μM/min)	v_f (μM/min)
0.25	0.37	0.37	0.74	0.11	4.4	8.9
0.5	0.51	0.51	1.2	0.14	5.6	10.7
20	0.69	0.69	n.d. [a]	0.16	6.4	n.d. [a]

[a] Cannot be determined.

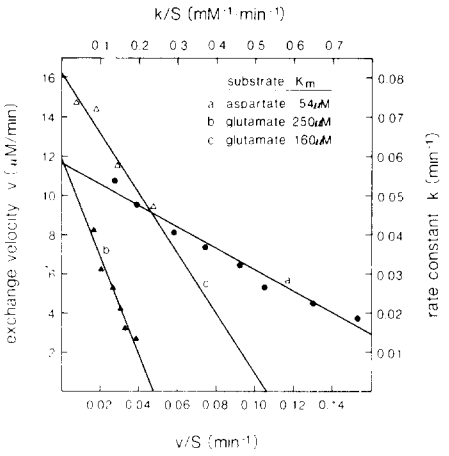

Fig. 5. Transport affinities of the reconstituted Asp/Glu carrier at the external membrane side. Eadie-Hofstee plots were obtained from forward exchange measurements of exchange velocity (●, ▲) and from backward exchange measurement of rate constant k (△), respectively, under variation of external substrate concentration (●, 0.025–0.4 mM aspartate; ▲, 0.07–0.5 mM glutamate; △, 0.2–2.0 mM glutamate). The three determinations were carried out in homo-exchange measurements, i.e. with the same substrate inside and outside the liposomes (16 mM in each case). The corresponding K_m values are (μM): a (aspartate), 54; b (glutamate), 250; c (glutamate), 160.

centration was saturating, providing pseudo first-order conditions. In order to avoid interference by a pH gradient, which presumably will be generated by proton cotransport together with glutamate, care is taken that the membrane is electrochemically equilibrated. This is achieved by the action of the ionophores nigericin and valinomycin (in the presence of the same concentration of potassium ions in both compartments); furthermore, the system is provided with a high buffering capacity (see Methods).

The affinity towards aspartate is significantly higher ($K_m = 50$ μM) than towards glutamate ($K_m = 250$ μM) at pH 6.5. Furthermore Fig. 5 shows, that the K_m value for glutamate could be confirmed by backward exchange measurements (160 μM), although the method is at the barrier of its applicability within this low external concentration range. For aspartate a value below 100 μM results, if determined with the backward exchange method (not shown). The K_m values in general are not dependent on the nature of the second substrate, which in this case is the internal substrate (aspartate or glutamate). Thus, they can be determined both in homo-exchange or in hetero-exchange experiments (not shown).

For determinations of the internal transport affinities the backward exchange technique is preferable, because this method ensures external substrate saturation even in combination with low internal concentrations. In the forward exchange mode a reliable kinetic analysis is only possible with internal concentrations higher than 1 mM, otherwise the external substrate concentrations have to be decreased reaching the range of the external K_m values in order to have an appropriate ratio of substrate pools. Because of its higher affinity it seems reasonable to use aspartate as the external substrate in forward exchange measurements of this kind.

Fig. 6 shows the exchange activity of the reconstituted aspartate/glutamate carrier under variation of the internal substrate concentration in an Eadie-Hofstee plot. In Fig. 6A the results of back-

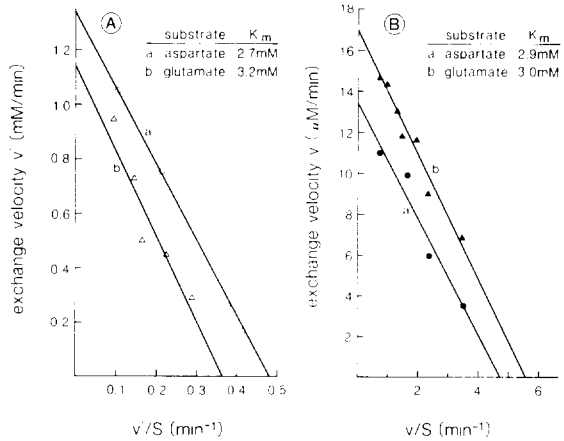

Fig. 6. Transport affinities of the reconstituted Asp/Glu carrier at the internal membrane side. Eadie-Hofstee plots were obtained from backward (A) and forward exchange measurements (B) of transport under variation of internal substrate concentration (○, ●, aspartate; △, ▲, glutamate). For definitions of v' see legend of Fig. 4. Substrate concentrations were as follows. (A) ○, 8 mM external and 0.25–10 mM internal aspartate; △, 8 mM external glutamate and 1–10 mM internal glutamate. (B) 0.4 mM external aspartate in both experiments, 1–15 mM internal aspartate (●) and 2–20 mM internal glutamate (▲), respectively. The K_m values derived are (mM): (A): a (aspartate), 2.7; b (glutamate), 3.2; (B): a (aspartate), 2.9; b (glutamate), 3.0.

ward exchange measurements are given; glutamate transport velocities are calculated from the kinetics shown in Fig. 4. The internal K_m values for the two substrates aspartate and glutamate are both about 3 mM at pH 6.5. As mentioned above, this concentration range can be measured in the forward exchange mode as well (Fig. 6B). Again good agreement is seen between the results obtained by the two alternate methods of kinetic analysis, which is an additional proof of the reliability of the backward exchange method.

Orientation of the reconstituted carrier protein

In the following the two presumably different values for the substrate affinities (K_m values) at the two different sides of the Asp/Glu carrier were used as indicators for its orientation. If one imagines that the protein is inserted in the membrane with random orientation, the two side-specific K_m values for both aspartate and glutamate should be detectable at both sides of the liposomal membrane at about equal proportions. Hence the experimental question was, whether at the external side an additional K_m value in the millimolar range can be found, corresponding to the affinity as determined at the inner side, and, consequently, whether at the internal membrane surface additional binding sites do exist, that show considerable higher affinity than 3 mM. The concentration ranges, that were used for determination of external and internal substrate affinity constants (Figs. 5 and 6), respectively, show some overlap, and indeed only one K_m value at each membrane side could be detected. This would indicate a preferential direction of protein incorporation into the artificial membrane. However, the concentration range covered by each of these experiments is restricted, when compared to the large discrepancy between the internal and the external K_m value. In order to quantitate the distribution of binding sites with higher and lower affinities to the two membrane sides, the substrate concentrations have to be varied covering both K_m values in one experiment.

This demand could only be met by reliable backward exchange kinetics under extreme substrate distributions. The analysis focussed on the glutamate–glutamate homoexchange, since the (compared to aspartate) lower external substrate affinity for glutamate is better suited for experiments with the intention to detect the expected additional internal binding sites showing similar affinity. Two representative experiments of this kind are shown in Fig. 7. In the Eadie-Hofstee plots two straight lines result from determinations of the internal and external K_m value for glutamate down to a concentration of 0.3 and 0.25 mM, respectively, which is the lower limit of the backward exchange method. In both cases monophasic curves are obtained up to a concentration of 10 and 20 mM, respectively, with the slope of the corresponding K_m value. These constants are in agreement with the values determined in Figs. 5 and 6. Thus, they have to be considered as specific and exclusive for the corresponding side of the reconstituted protein. As can be seen in Fig. 7 an

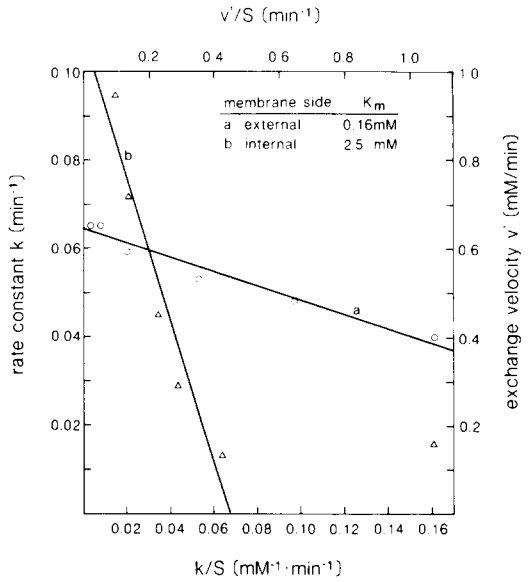

Fig. 7. Functional analysis of protein orientation. In order to elucidate the sidedness of high- and low-affinity binding sites, as found at the external and internal membrane side, respectively (Figs. 5 and 6), the transport affinity constants were determined varying external and internal substrate concentration over a range that covers both K_m values within one experiment (see text). The Eadie-Hofstee plots were obtained from backward exchange measurements of rate constant k in dependence of external (○) and internal (△) glutamate. For definition of v' see legend of Fig. 4. Glutamate concentrations were as follows: ○, 16 mM at the inside and 0.25–20 mM at the outside of the liposomes, △, 0.15–10 mM inside and 5 mM outside, respectively. The calculated K_m values are (mM): a (external), 0.16; b (internal), 2.5.

increase of the external glutamate concentration from 1 to 20 mM caused only a slight enhancement of transport activity, which definitely excludes an external K_m value in the millimolar region. On the other hand all efforts failed to recognize a low K_m value at the internal side, though in this respect there may be some residual uncertainty, because the crucial concentration range (below 0.3 mM internal substrate) is not accessible to exact measurements by the available methods. This circumstance is demonstrated in Fig. 7 with the example of a strongly deviating data point obtained at 0.15 mM internal glutamate, which is evidently outside the range of reliability for this method. However, it seems to be obvious, that no reasonable protein arrangement can be envisaged showing mixed affinities at the inner compartment. Therefore these results indicate the asymmetric membrane insertion of the Asp/Glu carrier by the applied reconstitution method.

In Table IV the complete set of K_m values determined in the reconstituted system is compiled. These data are compared with the K_m values found in the literature for intact mitochondria or sonicated (inverted) particles. On the basis of the affinities towards glutamate at the cytosolic side and towards aspartate at the matrix side, respectively, determined by LaNoue et al. [5] in rat heart mitochondria (3 mM) and sonication particles (55 μM), respectively, a comparison strongly suggests that the aspartate/glutamate carrier in proteoliposomes is oriented inside-out with respect to the direction observed in mitochondria.

TABLE IV

TRANSPORT AFFINITIES OF THE MITOCHONDRIAL Asp/Glu CARRIER. COMPARISON OF NATIVE AND RECONSTITUTED PROTEIN

Membrane side	Substrate	K_m (mM)			
		reconstituted Asp/Glu exchange (pH 6.5)		mitochondria	
		forward	backward		
Internal	aspartate	3	3	0.055	(pH 6.0) [a]
				5.0	(pH 7.2) [b]
	glutamate	3	3	0.5	(pH 7.4) [c]
External	aspartate	0.05	n.d. [d]	3.5	(pH 7.2) [f]
	glutamate	0.25	0.16	3.0	(pH 6.4) [e]
				5.8	(pH 7.2) [g]

[a] Determined in submitochondrial particles from rat heart mitochondria, uptake of labelled aspartate [5].

[b] Determined in rat liver mitochondria, aspartate efflux in dependence of internal aspartate [6].

[c] See note (a), but determination of K_i (competitive inhibition) [5].

[d] Cannot be determined.

[e] Determined in rat heart mitochondria, respiration in dependence of added glutamate [5].

[f] See note (e), but determination of K_i (noncompetitive inhibition) [17].

[g] Determined in rat liver mitochondria, aspartate efflux in dependence of added glutamate [6].

Discussion

For the conclusions drawn in this paper results from both forward and backward exchange experiments were important. Therefore the compatibility of these two different ways of flux measurement was tested. Using a computer fitting program it was shown, that the time course of the measured isotope equilibration follows a first-order process (see Methods) independent of the direction, in which the flux of radiolabel is monitored, i.e. forward or backward measurement. This is in total agreement with theoretical considerations (Eqns. 1 and 3, respectively). However, in backward exchange experiments only the first-order rate constant k of isotope equilibration can directly be derived from such measurement (Fig. 3). The evaluation of absolute transport activity further needs knowledge of the internal substrate pool (Eqn. 4). By comparison of data obtained by the two different methods of exchange measurement (Table III), it turned out that the apparent 'backward exchange activities' were slightly too low by a constant factor of about 1.3. This factor was found to be independent of the substrate concentrations applied. Therefore it can be attributed to systematic underestimation of the internal substrate pool, or (more precisely) of the internal volume, that is participating in the exchange reaction. The determination of this small volume is somewhat inaccurate, because it is based on long time measurements of the isotope equi-

librium (Eqn. 5), which are affected by efflux activity leading to a decrease of the internal substrate pool. These measurements took about 30 min to make sure that even those liposomes carrying only one carrier molecule per liposome reached equilibrium, thereby eliminating the influence of different amounts of carrier protein per liposome. Nevertheless, the knowledge of exact pool sizes is not necessary for determining transport affinities, since this can be carried out with data of relative transport activities (v'). These v' values (Fig. 4) were calculated by multiplication of rate constant k with the internal substrate concentration. Table IV shows, that in fact there is good agreement between K_m values determined in forward and backward exchange measurement.

A comparison of the two classical methods of measuring carrier-mediated substrate exchange across membranes (mitochondrial or liposomal) in any way means a comparison between theoretical comprehension and practical elucidation of antiport function. At least for liposomal systems this is the first time, that the two methods are confronted with each other in such detail. Though absolute values of outward directed transport activity are difficult to obtain, basic agreement and mutual supplementation of techniques could be demonstrated.

Previous studies carried out in mitochondria by two laboratories [5,6] led to conflicting results and contradictory interpretations of Asp/Glu exchange. Beside different aspects of the overall mechanism, there is disagreement between reported transport affinities of the carrier towards aspartate at the matrix side of the protein. The corresponding Michaelis constant in the one case [6] was determined in intact rat liver mitochondria yielding a value of 5 mM, whereas LaNoue et al. [5] used sonication particles prepared from rat heart mitochondria and obtained a much lower value (55 μM). The suitability of these inverted mitochondrial vesicles for kinetic analysis was discussed controversially [6]. However, on the other hand the objection was put forward, that K_m values referring to the matrix side of the inner mitochondrial membrane cannot be determined in intact mitochondria, since their cristae structure causes microcompartmentation of internal substrate [5] revealed by the difficulty to saturate the corresponding binding sites [8].

In the reconstituted system the substrate binding sites of the Asp/Glu carrier could be saturated at both sides of the liposomal membrane, as can be seen from kinetic measurements of exchange activity shown in this paper. No complication could be observed, which might be caused by the formation of microcompartments with different substrate concentrations. The carrier-mediated transport of substrate was characterized in kinetic terms: The maximum rate of exchange was about 20 μmol/min per liter of vesicles containing roughly 10 mg of reconstituted Asp/Glu carrier. The transport affinities turned out to be significantly higher at the external membrane side both for aspartate and glutamate (50 and 250 μM, respectively) as compared to the inner surface of the proteoliposomes (3 mM for both substrates). Control experiments with external glutamate concentrations as high as 20 mM definitely excluded the existence of a second external K_m value in the millimolar range. Accordingly, at the opposite membrane side only low-affinity binding sites could be detected. Therefore, the obtained K_m values have to be considered as distinctive markers of the two membrane sides.

It is hardly conceivable, that the carrier protein drastically changes substrate affinities in dependence of the direction of its insertion into the liposomal membrane. Thus the exclusive presence of only low- or high-affinity binding sites at each membrane side indicates, that actually the reconstituted carrier molecule is situated in the lipid bilayer with highly preferential orientation.

Interestingly, the K_m values obtained in proteoliposomes seem to be the very opposite (with respect to the sidedness) of data from mitochondria reported by LaNoue et al. [5], as is shown in Table IV. The affinity of the carrier in mitochondria towards externally added glutamate (3.0 mM, pH 6.4) agrees quite nicely with the value found at the internal side of our model membranes (3 mM, pH 6.5). In the same way the affinity of submitochondrial particles and proteoliposomes towards external aspartate correspond to each other (55 μM, pH 6.0 and 50 μM, pH 6.5, respectively). Since the membranes of sonication particles are turned inside-out, these binding sites showing higher affinity have to be attributed to the matrix

side of intact mitochondria. Further support to this view comes from the K_m values for the alternate substrate at each membrane side, which for the first time are presented in this paper. Table IV demonstrates, that there is good agreement on the one hand between the K_m value for internal aspartate measured in the reconstituted system (3 mM) and the inhibitory constant K_i (3.5 mM), which was attributed to external aspartate as a noncompetitive inhibitor of glutamate-stimulated respiration in mitochondria [17]; on the other hand the K_m value for external glutamate, as determined in proteoliposomes (250 μM, pH 6.5), is not very different from the K_i constant (500 μM, pH 7.4) for glutamate, which competitively inhibits uptake of aspartate into submitochondrial particles [5]. The factor of 2 between these values is certainly due to the difference in the concentration of H^+, which is cotransported together with glutamate. Indeed, a pronounced increase of the affinity towards glutamate with decreasing pH values was observed in mitochondria [5] and in proteoliposomes (unpublished results).

However, the transport affinities reported here are at odds with some data published by Murphy et al. [6] for the native translocator. This group could only find low-affinity binding sites both at the matrix (5.0 mM for aspartate, pH 7.2) and the cytosolic side of the inner mitochondrial membrane (5.8 mM for glutamate, pH 7.2). Since the latter K_m value referring to external glutamate was determined at a considerable higher pH-value as compared to the determinations of LaNoue and of the present paper (see above), the factor of 2 between these values is easily explained. On the contrary, a marked change in the affinity towards aspartate due to a shift in pH was not observed, neither in submitochondrial particles [5] nor in proteoliposomes (unpublished results). In our opinion the reason for the large discrepancy between the K_m values for matrix aspartate reported by the two laboratories mentioned above (5.0 and 0.055 mM, respectively) may be due to differences between mitochondria and submitochondrial particles. It has to be pointed out, that more than 90% of the specific transport activity is lost upon preparation of particles. However, a change in substrate affinity by a factor of 100 can hardly be attributed to distortions of protein conformation due to cavitation forces during sonication or due to the inverted topology of the membrane, particularly because this should be assumed to decrease the affinity; instead an increase is observed. On the other hand, as was already mentioned, the group of LaNoue [5,8] raised the argument, that the highly folded arrangement of the inner membrane in intact mitochondria may lead to a decreased accessibility of the binding sites facing the matrix. Surely, this phenomenon is hardly to prove. But, if true, an underestimation of transport affinity can be realized.

Although we cannot solve this discrepancy of published results, the comparison of data obtained in proteoliposomes, as described here, and data obtained with mitochondrial membranes, as reported by LaNoue et al. [5], consequently suggests, that after isolation and reconstitution by hydrophobic chromatography the transmembrane orientation of the Asp/Glu carrier is turned inside-out with respect to the physiological situation.

The oriented reconstitution of a membrane protein like the Asp/Glu carrier, that is relatively small as compared to other asymmetrically reconstituted proteins (e.g. the complexes of the respiratory chain), is quite extraordinary. It demonstrates the usefulness of the applied reconstitution method. Using techniques, which include freeze-thaw cycles and/or sonication treatment, neither carrier proteins like the hexose carrier of erythrocytes [18] or the lactose carrier of *Escherichia coli* [19] nor larger proteins like cytochrome-*c* oxidase [20] could be reconstituted with significant preference of orientation. In the case of the mitochondrial adenine nucleotide carrier the two possible orientations seem to be present with different frequencies after freezing, thawing and sonication, as was identified by correlating binding of carboxyatractylate and bongkrekate with nucleotide transport [21]. However, due to the high hydrophobicity of bongkrekic acid, partly unspecific binding presumably gave reason to overestimating the degree of asymmetry (unpublished results).

On the other hand, functional reconstitution by slow removal of detergent in several cases succeeded in the formation of proteoliposomes with definite protein orientation. Beside the Asp/Glu

carrier, the uncoupling protein from hamster kidney mitochondria was incorporated into liposomes unidirectionally (rightside-out) by amberlite treatment, as was described recently [22]. However, the lactose carrier of *E. coli* could not be reconstituted asymmetrically by hydrophobic chromatography on Bio-Beads [19]. Removal of detergent by dialysis led to a vectorial membrane insertion in the case of several proteins; beside others different complexes of the mitochondrial respiratory chain can be oriented in this way. For instance reconstituted cytochrome-*c* oxidase has been shown to exhibit a strong bias (about 85%) towards its native orientation [20] *. Both methods, chromatography on amberlite beads and dialysis, obviously ensure mild conditions during reconstitution, which allow oriented organization of the amphiphilic protein molecule into the membrane.In contrast to sonication procedures, a reshuffling of the protein between the two opposite orientations is avoided, which would lead to bimodal membrane insertion.

The investigations on the transmembrane protein orientation of the reconstituted Asp/Glu carrier described in the present paper are exclusively based on functional properties of the translocator, namely its side-specific substrate affinities. The argument can be put forward, that physical constraints due to membrane curvature are responsible for the marked difference in substrate affinity at the two membrane sides. Although this assumption seems to be extremely hypothetical, experiments were carried out trying to achieve a scrambling of protein orientation by various treatments of proteoliposomes. However, even after freezing and thawing the liposomes for two times, followed by short sonication (10 s in total) no significant randomization of membrane topology was detectable, as could have been deduced from the appearance of low-affinity binding sites at the external membrane surface. In the view of these observations an alternative, albeit hypothetical explanation for the determined functional asymmetry of the reconstituted Asp/Glu carrier at least has to be taken into consideration. It may be concluded, that one of the two possible orientations of the membrane-inserted carrier is almost completely inactive and hence remains silent in our experiments based on transport function. Indeed, observations in this direction were made in the case of the reconstituted adenine nucleotide carrier [21].

For the Asp/Glu carrier further side-specific criteria based on structural parameters are currently not available, which would help to test the alternate point of view discussed above. Nevertheless, such circumstance, if true, wouldn't mean any restriction to the suitability of the described analytic system for kinetic analysis on the basis of carrier function. The kinetic data clearly indicate an oriented insertion of the active antiporter into the liposomal membrane. With this particular quality a model system was established, that is well suited to clarify the problems related to the mechanism of aspartate/glutamate transport in mitochondria.

Acknowledgements

We thank Miss Etta Riemer for her technical assistance. Thomas A. Link kindly provided the computer fitting program. The support of Professor M. Klingenberg (Munich) and Professor H. Sahm (Jülich) is gratefully appreciated. This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

References

1 LaNoue, K.F. and Schoolwerth, A.C. (1984) in Bioenergetics (Ernster, L., ed.), pp. 221–268, Elsevier, Amsterdam.
2 Meijer, A.J. and Van Dam, K. (1981) in Membrane transport (Bonting, S.L. and De Pont, J.J.H.H.M., eds.), pp. 235–255, Elsevier, Amsterdam.
3 Williamson, J.R. (1976) in Gluconeogenesis (Mehlman, M.A., ed.), pp. 165–220, Wiley, New York.
4 LaNoue, K.F. and Tischler, M.E. (1974) J. Biol. Chem. 249, 7522–7528.
5 LaNoue, K.F., Duszynski, J., Watts, J.A. and McKee, E. (1979) Arch. Biochem. Biophys. 195, 578–590.
6 Murphy, E., Coll, K.E., Viale, R.O., Tischler, M.E. and Williamson, J.R. (1979) J. Biol. Chem. 254, 8369–8376.
7 Tischler, M.E., Pachence, J., Williamson, J.R. and LaNoue, K.F. (1976) Arch. Biochem. Biophys. 173, 448–462.

* *Note added in proof*: (Received 16 November 1987) Furthermore, the anion transport system of erythrocytes (97 kDa) could be reconstituted unidirectionally (right side-out) by a special dialysis technique [23].

8 Duszynski, J., Mueller, G. and LaNoue, K.F. (1978) J. Biol. Chem. 253, 6149–6157.
9 Krämer, R., Kürzinger, G. and Heberger, C. (1986) Arch. Biochem. Biophys. 251, 166–174.
10 Krämer, R. and Heberger, C. (1986) Biochim. Biophys. Acta 863, 289–296.
11 Krämer, R. (1984) FEBS Lett. 176, 351–354.
12 Krämer, R. and Klingenberg, M. (1982) Biochemistry 21, 1082–1089.
13 Chen, P.S., Jr., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756.
14 Pfaff, E., Heldt, H.W. and Klingenberg, M. (1969) Eur. J. Biochem. 10, 484–493.
15 Krämer, R. and Klingenberg, M. (1980) Biochemistry 19, 556–560.
16 LaNoue, K.F., Bryla, J. and Bassett, D.J.P. (1974) J. Biol. Chem. 249, 7514–7521.
17 LaNoue, K.F. and Tischler, M.E. (1976) in Regulation of Metabolism in Isolated Liver Cells (Tager, J.M., Söling, H.D. and Williamson, J.R., eds.), pp. 106–109, North-Holland, Amsterdam.
18 Wheeler, T.J. and Hinkle, P.C. (1981) J. Biol. Chem. 256, 8907–8914.
19 Seckler, R. and Wright, K. (1984) Eur. J. Biochem. 142, 269–279.
20 Casey, R.P., Ariano, B.H. and Azzi, A. (1982) Eur. J. Biochem. 122, 313–318.
21 Krämer, R. and Klingenberg, M. (1979) Biochemistry 18, 4209–4215.
22 Klingenberg, M. and Winkler, E. (1986) Methods Enzymol. 127, 772–779.
23 Scheuring, V., Kollewe, K., Haase, W. and Schubert, D. (1986) J. Membr. Biol. 90, 123–135.