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Reaction mechanism of the reconstituted aspartate/glutamate carrier from bovine heart mitochondria

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A functional model for the aspartate/glutamate carrier of the inner mitochondrial membrane was established based on a kinetic evaluation of this transporter. Antiport kinetics were measured in proteoliposomes that contained partially purified carrier protein of definite transmembrane orientation (Dierks, T. and Krämer, R. (1988) Biochim. Biophys. Acta 937, 122–126). Bireactant initial velocity analyses of the counterexchange reaction were carried out varying substrate concentrations both in the internal and the external compartment. The kinetic patterns obtained were inconsistent with a pong-pong mechanism; rather they demonstrated the formation of a ternary complex as a consequence of sequential binding of one internal and one external substrate molecule to the carrier. Studies on transport activity in the presence of aspartate and glutamate in the same compartment (formally treated as substrate inhibition) clearly indicated that during exchange only one form of the carrier at either membrane surface exposes its binding sites, for which the two different substrates compete. In the deenergized state (pH 6.5) both substrates were translocated at about the same rate. Aspartate/glutamate antiport became asymmetric if a membrane potential was imposed, due to the electrogenic nature of the heteroexchange resulting from proton cotransport together with glutamate. Investigation of the electrical properties of aspartate/aspartate homoexchange led to the conclusion that the translocating carrier-substrate intermediate exhibits a transmembrane symmetry with respect to the (negative) charge, which again only is conceivable assuming a ternary complex. Thus, an antiport model is outlined that shows the functional complex of the carrier with two substrate molecules bound, one at either side of the membrane. The conformational change associated with the transition of both substrate molecules across the membrane then occurs in a single step. Furthermore the model implicates a distinct proton binding site, which is derived from the different influence of H^+ concentration observed on transport affinity and transport velocity, respectively, when glutamate is used as a substrate.

Introduction

Among the ensemble of different carrier proteins located in the inner mitochondrial membrane (for review, see Refs. 1 and 2) the Asp/Glu carrier is one of the most interesting systems, because it is the only one to be regulated both by membrane

Abbreviations: Asp, aspartate; Asp/Glu carrier, aspartate/glutamate carrier; $C_{12}E_8$, dodecyl octaoxyethylene ether; Glu, glutamate; Mops, 4-morpholinepropanesulfonic acid; S_{in}, S_{ex}, internal or external substrate, respectively (Asp or Glu).

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potential and pH gradient [3]. While aspartate is translocated as a monovalent anion, the negative charge of glutamate is compensated by a cotransported proton. As a consequence, in energized mitochondria export of aspartate into the cytosol and import of glutamate into the matrix space is strongly favored. Since this countertransport represents a central component in the malate/aspartate shuttle, which transfers reducing equivalents from the cytosol to the mitochondrial respiratory chain, NADH/NAD ratios in the two compartments are modulated by this transport system. Furthermore, the Asp/Glu carrier connects extra- and intramitochondrial steps in gluconeogenesis from lactate and urea synthesis [4].

Mainly two groups have studies the Asp/Glu carrier in mitochondria trying to elucidate its complex mechanism in kinetic terms [5,6]. The reported models derived from classical bireactant initial velocity studies (bisubstrate analyses) as well as from substrate competition experiments show a fundamental contradiction. On the one hand results of bisubstrate kinetics using sonicated particles from rat heart mitochondria were found to be in favor of a ping-pong mechanism [5] indicating the exposure of only one binding site alternating between opposing membrane surfaces. On the other hand using intact rat liver mitochondria, the kinetic pattern obtained was consistent with a sequential type of mechanism, which involves the binding of two substrate molecules during transport [6]. This would lead to the formation of a ternary complex with the carrier, before one of the substrates is released from the protein. However, no conclusive evidence was reported concerning the sequence of binding steps necessary to form this ternary complex or whether it could represent the translocating intermediate.

Moreover, both a competitive [5] and a noncompetitive inhibition mechanism [7] was observed in inhibition studies investigating the influence of the second substrate (aspartate or glutamate) in the same compartment. Likewise the mode of proton cotransport is controversial. LaNoue et al. [5] outlined a model involving a distinct proton binding site, whereas Murphy et al. [6] came to the conclusion that H^+ is bound in association with glutamate. An additional finding that is not well understood is the reported functional asymmetry of Asp/Glu exchange observed in deenergized mitochondria [7].

In the present paper we address these unresolved issues related to the transport mechanism by examining Asp/Glu antiport in a reconstituted system. Enriched Asp/Glu carrier [8] was reconstituted into liposomal membranes by chromatography on hydrophobic ion exchange resins [9,10]. This system has previously been used to establish a complete set of K_m values for both substrates at either membrane side [10]. Thereby it turned out that almost all (80-100%) functionally active proteoliposomes contain Asp/Glu carrier molecules inserted with definite protein orientation. This well suited model system was used in the present paper to elucidate the mechanism of transport. The reconstituted system has several advantages as compared to experiments in intact mitochondria, since no complications have to be considered, which may be caused by interference of mitochondrial metabolism or microcompartmentation of internal substrate. Microcompartmentation was postulated to exist in intact mitochondria [5,11] and would severely restrict the interpretation of kinetic data. In the case of complex studies like bisubstrate analyses, the proteoliposomal system also offers more versatility, because both the external and internal compartments are earily accessible to experimental variation.

Materials and Methods

The isolation of the Asp/Glu carrier from bovine heart mitochondria was done previously [8]. The protein fraction obtained after the second purification step, i.e. chromatography on Sephadex G-25, was used for reconstitution (about 17-fold enriched transport activity). For investigations of the influence of the membrane potential, the size exclusion chromatography was carried out using 100 mM sodium phosphate (pH 6.5) as elution buffer instead of 300 mM ammonium acetate (pH 6.5) in order to avoid interference of NH_4^+ ions.

Incorporation of carrier protein into liposomes was carried out by hydrophobic chromatography on amberlite beads [9]. Mixed micelles of carrier protein, phospholipid and detergent ($C_{12}E_8$) were repeatedly applied on small columns of Amberlite

XAD2. Thereby the solubilizing detergent was slowly removed under formation of liposomes, which carry functionally active Asp/Glu carrier of asymmetric protein orientation. Exact conditions were as described recently [10].

Determination of the reconstituted transport activity was done by flux measurement of radiolabelled substrate. For that purpose an inhibitor-stop technique was applied, which uses pyridoxal phosphate as the stop inhibitor. A detailed description of exchange measurement both in the forward and the backward direction is given in Ref. 10. For the backward exchange experiments reported here the internal substrate pool was first prelabelled in the forward exchange mode (5 min) by adding 0.5 μM substrate of high specific radioactivity.

Forward exchange activities were calculated by a computer fitting program (cf. Ref. 10). As indicated in some experiments, for comprehensive bisubstrate or competition analyses only single time kinetics (20 s values) could be measured in order to obtain a sufficient number of data points. In control experiments it was proven that the initial 30 s of the isotope equilibration kinetics are within the linear range. Statistical certainty is warranted as long as enough activity data are available for a reliable linear regression in reciprocal plots, which are necessary for the evaluation of kinetic constants.

Transport activities are expressed as volume activities (mol/l vesicles per min) and not as specific activities, 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 [12], thereby cancelling out different dilution factors due to the various column passages (cf. Ref. 10). In contrast to activities determined in the forward exchange mode, backward exchange velocities have to be considered as relative rates (v'), since they are calculated on the basis of internal substrate concentrations and not of substrate pool sizes, the determination of which is somewhat inaccurate (see Ref. 10 for further explanation).

In order to eliminate efflux of internal substrate from the liposomes, which occurs to some extent during generation of substrate gradients by gel chromatography [10], 200 μM 5,5′-dithiobis(2-nitrobenzoic acid) was added to the liposomes before applying them to the Sephadex G-75 column. This reagent blocks efflux completely. After the column passage the carrier activity was restituted by adding 4 mM dithioerythritol 30 s before the exchange assay was started (cf. Ref. 10).

Adjustment of $\Delta\psi$. In most experiments valinomycin (500 ng/mg phospholipid) and nigericin (20 ng/mg phospholipid) were added to the proteoliposomes in order to provide deenergized conditions. For investigations concerning the influence of the membrane potential, K^+-diffusion potentials were generated using valinomycin (500 ng/mg phospholipid) and potassium gradients (100 mM internal/1 mM external or vice versa). Potassium gradients were established by substituting Mops-NaOH for Mops-KOH during gel chromatography of liposomes (internal Mops-KOH) or (for opposite polarity) by addition of 50 mM K_2SO_4 (internal Mops-NaOH).

The chemicals and their sources were as follows: labelled compounds (Amersham Buchler, $C_{12}E_8$ (Kouyoh Trading Company, Tokyo), turkey egg yolk phospholipid and 5,5′-ditiniobis(2-nitrobenzoic acid) (Sigma), valinomycin (Boehringer Mannheim), pyridoxal phosphate (Merck) and Amberlite XAD2 (Serva). All other chemicals were of analytical grade.

Results

Bireactant initial velocity studies

The counterexchange of substrates across membranes catalyzed by an antiport carrier can be described in terms of a bisubstrate reaction. In order to gain insight into the sequence of binding and release of substrates during the catalytic cycle of the exchange reaction the method of choice is the determination of antiport velocity as a function of substrate concentrations in both the external and the internal compartment. From the resulting kinetic pattern a clear decision between the two basic types of bisubstrate mechanisms, namely ping-pong or sequential type, should be possible [13].

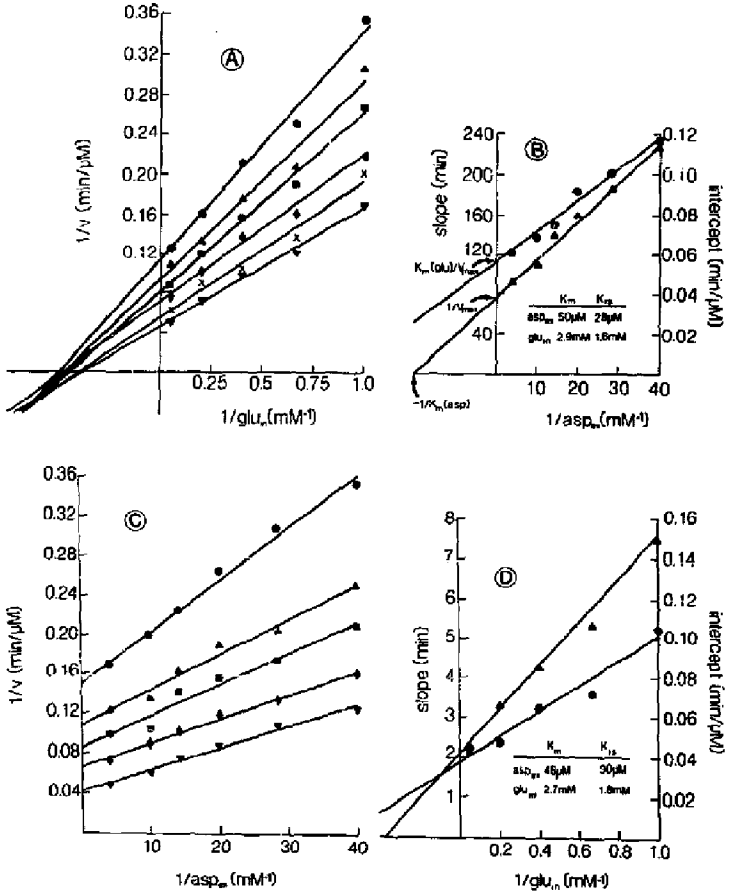

Fig. 1. Bisubstrate analysis of the heteroexchange reaction (Glu_{in}/Asp_{ex}) mediated by the reconstituted Asp/Glu carrier. Transport kinetics were measured under deenergized conditions (pH 6.5). (A,C) Lineweaver-Burk plots showing the dependence of exchange activity on internal glutamate or external aspartate, respectively. The concentrations of the countersubstrate were as follows. A: 25 (●), 35 (▲), 50 (■), 70 (◆), 100 (×) and 250 μM (▼) external aspartate; C: 1 (●), 1.5 (▲), 2.5 (■), 5 (◆) and 20 mM (▼) internal glutamate. Antiport velocities were calculated from the amount of labelled aspartate taken up by the liposomes within 20 s (forward exchange). (B,D) Slope (●) and intercept (▲) replots of primary plots A and C, respectively. The concentration independent K_m values (see text) given in the inset can be extracted graphically from the intercepts of the secondary plots as indicated in B. The meaning of K_{is} values, i.e. K_{iA} and K_{iG}, is explained in the text (Eqn. 1). V_{max} values extrapolated for infinite concentration of internal and external substrate are 26 μM/min (B) and 25 μM/min (D), respectively.

Fig. 1 shows the results of a bireactant initial velocity study (bisubstrate analysis) of the heteroexchange reaction of glutamate (internal) and aspartate (external) under deenergized conditions. Internal glutamate was varied from 1 to 20 mM and external aspartate from 25 to 250 μM. Antiport velocities were calculated from measurements of aspartate uptake within 20 s (see Methods).

When the kinetic data were analyzed in Lineweaver-Burk plots (Figs. 1 A and C) straight lines were obtained. These lines are not parallel, but form a pattern with a common point of intersection. It becomes even more obvious that the lines are not parallel, when the data are further analyzed in secondary plots (Figs. 1 B and D), which demonstrate that a replot of the slope of the primary curves (shown in Figs. 1 A and C) vs. reciprocal concentration of the non-varied substrate is by no means horizontal. The slope in Lineweaver-Burk plots equals the ratio $K_{m,app}/V_{max,app}$. Thus, when changing the substrate concentration in one compartment different effects on (apparent) maximum exchange velocity and transport affinity of the substrate in the opposite compartment are observed. This result can only be explained by a sequential mechanism [13], the characteristic feature of which is the formation of a ternary complex of the carrier protein with (in the case of heteroexchange) one aspartate and one glutamate molecule during the catalytic cycle.

The fact that the same kinetic pattern is obtained whether the internal or the external substrate is chosen as the varied or the non-varied one (Figs. 1 A and C), respectively, indicates the validity of the following bisubstrate rate equation, which in algebraic terms is symmetric with respect to both substrates. The equation was adopted from the more general form [13] and is written indicating external aspartate as the varied substrate.

$$1/v = 1/V_{max} + K_A/(V_{max} \cdot A) + K_G/(V_{max} \cdot G) + K_{iA} \cdot K_G/(V_{max} \cdot A \cdot G) \quad (1)$$

A and G represent concentrations of external aspartate and internal glutamate, respectively. The Michaelis constants K are indexed accordingly. K_{iA} is the dissociation constant for the binary carrier-aspartate complex. K_{iG} can be introduced by simply transcribing the equation for glutamate as the variable substrate. The value of these particular constants in ping-pong mechanisms would be zero, since no ternary complex is involved. However, as clearly seen in Figs. 1 B and D, all parameters used in this equation have finite values, which can be calculated from the intercepts of the secondary plots [13]. Different from K_m values determined under pseudo-first-order conditions (i.e. keeping the second substrate at a fixed saturating level), the Michaelis constants obtained in Fig. 1 are extrapolated to an infinite concentration of the non-varied substrate. These so-called 'concentration-independent' transport affinities of 46–50 μM for external aspartate and 2.7–2.9 mM for internal glutamate are almost identical with those values determined at finite substrate concentrations (cf. Ref. 10).

Bisubstrate analysis of Asp/Asp or Glu/Glu homoexchange led to very similar kinetic patterns as obtained for the heteroexchange reaction (results not shown). Consequently, homo- and heteroexchange must be assumed to follow the same basic mechanism. The observed points of intersection were always located near the abscissa indicating that the corresponding K_m and K_{is} (i.e. K_{iA} or K_{iG}) constants have similar values (see Figs. 1 B and D). Thus, the binding affinity of both internal and external substrate to the carrier molecule is similar, whether the carrier is available in the free or single-substrate occupied form.

Symmetry of the exchange reaction in the de-energized state

Tischler et al. [7] reported functional asymmetry of Asp/Glu antiport in uncoupled mitochondria. Although respiration was inhibited, the Asp_{in}/Glu_{ex} exchange was found to be favored about five times as compared to the reverse direction (i.e. Asp_{ex}/Glu_{in}). Since such phenomena are important in respect to the transport mechanism and its regulation, a comparison of transport velocities of both substrates was carried out in our reconstituted system.

Table I compiles homo- as well as heteroexchange activities of the reconstituted Asp/Glu carrier under de-energized conditions at pH 6.5. All data were determined in one experiment using two preparations of proteoliposomes that were identical in their composition except for the internal substrate. In order to eliminate substrate limitation, forward exchange rates were measured at different external substrate concentrations thus allowing extrapolation of V_{max} values; internal substrate was saturating (24 mM). As can be seen from Table I, all maximum exchange activities

TABLE I

MAXIMUM ANTIPORT VELOCITIES OF THE RECONSTITUTED Asp/Glu CARRIER UNDER DEENERGIZED CONDITIONS (pH 6.5)

V_{max} values at different substrate distributions are compared. They were obtained by extrapolating exchange activities determined at various external substrate concentrations in the forward exchange mode (20 s values). Internal substrate was saturating (24 mM). Electrochemical equilibration of the liposomal membrane was provided by adding valinomycin and nigericin (see Methods). All data come from the same experiment.

Substrate		V_{max}
internal	external	(μM/min)
Aspartate	aspartate	13.5
Aspartate	glutamate	12.9
Glutamate	aspartate	11.6
Glutamate	glutamate	11.9

determined are very similar. This comparison demonstrates, that in the absence of a transmembrane driving force, the antiporter exchanges aspartate and glutamate symmetrically in either direction.

Influence of the second substrate when present in the same compartment

The transport mechanism was also investigated by means of inhibition kinetics, measured when both substrates were present on the same side of the membrane. Such kinetics can predict the different numbers of carrier-forms involved and/or the existence of rapid interconversion between these forms. In this kind of experiment only one substrate is labelled. The corresponding unlabelled substrate can be treated as an inhibitor, since not its own translocation is monitored but its effect on the transport of the labelled substrate.

Fig. 2 shows the inhibition of aspartate uptake caused by the presence of glutamate at the external side of the liposomes. When data were analyzed according to Eadie and Hofstee, straight lines were obtained which converge on the ordinate. This finding is consistent with a competitive inhibition mechanism indicating that both substrates bind to the same external site(s). The inhibition constant K_i determined in a secondary plot (inset of Fig. 2) was 180 μM, hence showing good coincidence with the external K_m value ob-

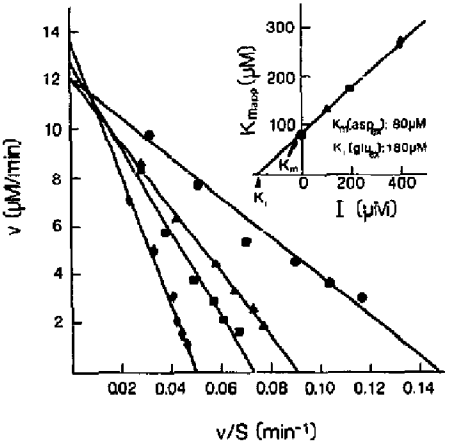

Fig. 2. Influence of external glutamate on the uptake of aspartate into liposomes carrying Asp/Glu carrier. Transport kinetics were measured under deenergized conditions (pH 6.5). The calculation of exchange activities was carried out on the basis of 20 s values (forward exchange). The figure shows an Eadie-Hofstee presentation of inhibition kinetics for different external glutamate concentrations: 0 (●), 100 (▲), 200 (■), 400 μM (◆). Each time external aspartate was varied from 25 to 300 μM. Internal substrate was 10 mM aspartate and 10 mM glutamate. The inset demonstrates the extrapolation of affinity constant (K_m) and competitive inhibition constant (K_i) for external aspartate and glutamate, respectively.

tained for glutamate (about 200 μM [10]). Similar inhibition studies with glutamate as the labelled substrate corroborated the competitive nature of substrate inhibition at the external membrane surface (data not shown).

Further substrate competition studies were carried out for the inner compartment, since divergent inhibition types have been reported for the matrix and cytosolic surfaces of mitochondrial membranes [5,7,14]. The number of kinetic data points which can be obtained for the interior of the liposomes is limited (Fig. 3), because each substrate/inhibitor combination needs a separate vesicle preparation. In order to provide external substrate saturation, inhibition kinetics were measured using the backward exchange technique, which monitors export of labelled substrate from the liposomes. This more elaborate mode of flux measurement needs thorough registration of iso-

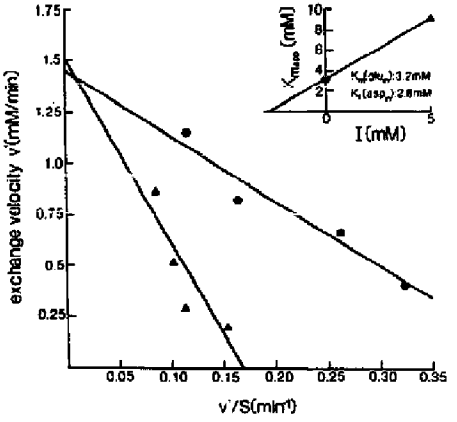

Fig. 3. Influence of internal aspartate on the export of glutamate from liposomes carrying Asp/Glu carrier. Transport kinetics were measured under deenergized conditions (pH 6.5). The rate constant k of Glu/Glu exchange was determined in the absence (●) or presence (▲) of internal aspartate (5 mM) in the backward exchange mode [10]; in both cases internal glutamate was varied from 1.25 to 10 mM. External substrate was 3 mM glutamate. Relative velocities (v') were calculated as described in Methods. For presentation of data and extrapolation of internal K_m(Glu) and K_i(Asp) constants see Fig. 2.

tope equilibration curves [10,15]. The prelabelling of the internal glutamate pool is carried out in the forward exchange mode (see Methods). In this procedure, alterations in the ratio of (internal) substrate and inhibitor concentrations, which are established by the reconstitution step, had to be avoided. Therefore only minute concentrations of labelled glutamate were added to the liposomes during prelabelling. After passing the liposomes over Sephadex G-75 to remove external isotope, backward exchange was initiated by adding saturating substrate concentrations (3 mM glutamate). The resulting inhibition pattern is shown in Fig. 3. Similar to Fig. 2, curves intersect on the ordinate, which is strongly in favor of a competitive behavior of aspartate and glutamate. Accordingly, the K_i value obtained for internal aspartate (2.8 mM) agrees well with the internal affinity constant (about 3 mM, [10]).

Influence of the membrane potential

Krämer and Klingenberg [16] have shown that the influence of a transmembrane electrical gradient ($\Delta\psi$) on the transport activity of the reconstituted ADP/ATP carrier cannot be explained by changing the substrate affinity of the carrier, but represents an electrophoretic modulation of the transport rate constant of a charged carrier-substrate complex. Thus, the charge distribution in the translocating carrier-substrate intermediate can be evaluated from an investigation of the electrical properties of the exchange reaction at different substrate combinations. In the case of nucleotide antiport, the kinetic model, which was theoretically derived and experimentally verified [16], not only accounts for the influence on the electrogenic ADP/ATP heteroexchange, but additionally predicts an inhibition of the non-electrogenic ATP/ATP homoexchange by an imposed membrane potential of either polarity. This prediction applies to translocation intermediates showing asymmetrical charge distribution (with respect to the transmembrane plane), as is supposed to be true for the binary [carrier^{3+}-ATP^{4-}] complex [17]. As a consequence, this complex is shifted to the positive side of the energized bilayer. The resulting imbalance of carrier conformations facing the opposite compartments leads to a pronounced inhibition of the overall transport reaction, which indeed was observed for the ATP^{4-}/ATP^{4-} but not for the ADP^{3-}/ADP^{3-} homoexchange [16].

Transferring this model to our examinations concerning the mechanism of Asp/Glu counterexchange, the binary [carrier-Asp$^-$] complex should be influenced by $\Delta\psi$, and not the [carrier/H$^+$-Glu$^-$) complex. Therefore the following analysis focussed on the Asp/Asp homoexchange. The membrane potential (120 mV as calculated by the Nernst equation, negative inside or outside, respectively) was generated as a potassium diffusion potential using the K$^+$-ionophore valinomycin (see Methods). The results obtained at all possible substrate distributions are compiled in Table II. It has to be pointed out that these transport activities were determined in two separate sets of experiments carried out at different pH values, thereby taking into account that the charge of carrier-substrate intermediates might be very sensitive to changes of pH. Consequently, the absolute values can only be compared within one set of data. Table II clearly shows that no significant

TABLE II

INFLUENCE OF THE MEMBRANE POTENTIAL ON THE EXCHANGE ACTIVITY OF THE RECONSTITUTED Asp/Glu CARRIER AT DIFFERENT SUBSTRATE DISTRIBUTIONS

Transport activities were calculated from initial velocity measurements in the forward exchange mode (see Methods). Data are compiled from two experiments carried out under different conditions: (a) external 50 μM aspartate/internal 16 mM aspartate or glutamate, respectively (pH 6.5); (b) external 100 μM glutamate/internal 24 mM aspartate or glutamate, respectively (pH 7.0). Preparation of protein and generation of $\Delta\psi$ as K^+-diffusion potentials see Methods. The asymmetry factor denotes the ratio of exchange activities at membrane potentials of opposite polarity (inside positive : inside negative).

Substrate		Exchange activity (μM/min) at K^+_{in} (mM)/K^+_{ex} (mM)				Asymmetry factor
internal	external	1/100 [a]	1/1	100/100	100/1 [a]	
Asp	Asp (a)	4.4	4.0	4.6	3.6	1.2
Glu	Asp (a)	7.5	2.3	2.6	0.9	8.3
Asp	Glu (b)	0.8	n.d. [b]	2.1	3.1	0.26
Glu	Glu (b)	2.0	2.1	2.1	2.2	1.1

[a] Theoretically corresponding to a value of 120 mV, inside positive or negative, respectively, if calculated according to the Nernst equation.
[b] Not determined.

inhibition of either Glu/Glu or of Asp/Asp homoexchange was seen upon energization of membranes. In contrast, the two possible heteroexchange arrangements show pronounced asymmetry factors (8.3 and 0.26, respectively), i.e. the ratio of exchange activities varies at membrane potentials of opposite polarity. No fundamental difference was observed when the pH is changed from 6.5 to 7.

Influence of pH

The intrinsic mechanism of H^+ cotransport together with glutamate is considered controversially [5,6]. On the one hand a distinct binding site for a proton is favored [5], whereas on the other hand glutamate is supposed to bind in the protonated, zwitterionic form [6]. Investigating the influence of the third substrate of the Asp/Glu carrier, the proton, on antiport activity is rather complicated, especially because reversible and irreversible pH effects on the carrier protein are difficult to discriminate. Experiments were carried out trying to elucidate which of the kinetic parameters are mainly affected upon changes of H^+ concentration. In order to avoid further complication by pH gradients, the pH of internal and external compartment was varied equally. Because of the mutual dependence of glutamate and H^+ transport, the analysis was carried out in terms of bisubstrate kinetics varying H^+ and external glutamate (or aspartate) concentrations, while keeping internal substrate at a saturating level. In these studies the proton was treated as a substrate of the Asp/Glu carrier.

Table III demonstrates that increasing pH from 6.2 to 6.9 reduced the affinity of the carrier towards glutamate by 5-fold, whereas the K_m value for aspartate is only slightly affected in this pH range. In contrast to affinity constants, which can be derived from relative transport activities, determinations of absolute V_{max} values depend on the amount of active carrier protein ($V_{max} = k_3 \cdot C_{active}$) and hence can not be compared directly if determined at different pH values. In Table III it is shown that upon variation of pH the apparent V_{max} values do not vary to the same extent as is observed for the K_m constant for external glutamate. Instead, the data fit into an optimum curve. Thus the reduced maximum transport velocities at pH 6.2 and 7.4 may primarily be caused by protein inactivation during titration of protein and proteoliposomes to lower or higher pH. This view was substantiated when the activity data were normalized on the basis of exhange equilibrium determinations. This correction is based on the assumption that the exchange equilibrium, i.e. the steady state of uptake and efflux of label, more directly resembles the number of active proteoliposomes [8]. Applying this normalization, the differences of V_{max} values become much smaller

TABLE III

INFLUENCE OF pH ON KINETIC CONSTANTS OF THE RECONSTITUTED Asp/Glu CARRIER

Asp/Asp and Glu/Glu homoexchange were analyzed in terms of bisubstrate kinetics varying external substrate and proton concentration ($pH_{in} = pH_{ex}$); internal substrate was saturating (30 mM). Like in most other experiments the ionophores valinomycin and nigericin provided deenergized conditions (see Methods). Transport activities were calculated on the basis of 20 s kinetics (forward exchange). K_m and apparent V_{max} values were extrapolated from reciprocal plots. Results are not shown in the more instructive Lineweaver-Burk presentation (cf. Fig. 1), since activity data determined at different pH cannot be compared directly in the same plot. For the same reason maximum velocities have to be considered as relative values. In order to correct for irreversible pH effects, a normalization of data ($V_{max,norm}$) was carried out on the basis of the exchange equilibrium at pH 6.5 (see text). The significance of changes in transport affinities is not restricted, since determination of K_m values is not dependent on absolute number of active carrier molecules (see text).

Transported substrate	pH	External K_m (μM)	$V_{max,app}$ (μM/min)	$V_{max,norm}$ (μM/min)
Glutamate	6.2	120	8.3	13
	6.5	185	15.4	15
	6.9	605	17.3	19
	7.4	1810	7.3	19
Aspartate	6.2	80	6.8	9
	6.5	70	13.9	14
	6.9	95	20.2	19
	7.4	170	16.8	21

(Table III). Taken together these results demonstrate that the affinity of the Asp/Glu carrier towards glutamate is very sensitive to changes of pH, whereas the rate constant k_3 obviously remains more or less constant.

Discussion

The basic disagreement about the kinetic mechanism of Asp/Glu antiport was controversially discussed in the literature [5,6] to be possibly due to the different systems used for kinetic investigation. On the one hand LaNoue et al. [5,18] argued that intact mitochondria show microcompartmentation of internal substrate and therefore used submitochondrial particles, if internal substrate concentrations had to be varied. On the other hand Williamson and co-workers [6,19] referred to the sonication process during preparation of particles to cause divergent results. The reconstituted system avoids these complications related to the complexity of mitochondrial metabolism, transmembrane transport and energy status, since for purpose of transport measurements it is abstracted to the essential components. By the reconstitution method applied in the present paper, functionally active Asp/Glu carrier molecules were inserted into a model membrane with highly preferential protein orientation [10]. These well-defined conditions allow unambiguous interpretation of antiport kinetics. The inverted transmembrane arrangement of the carrier as compared to the physiological situation [10] is unlikely to change the intrinsic catalytic mechanism of transport.

Bireactant mechanism of Asp / Glu antiport

The theoretical basis of bisubstrate kinetics [13] can also be applied to antiport carriers, as was done in previous investigations of the Asp/Glu carrier [5,6] and of the adenine nucleotide carrier [20,21]. In kinetic terms, bisubstrate reactions of catalytically active proteins can be divided into ping-pong and sequential mechanisms. Enzymes following the ping-pong type of mechanism expose only one substrate binding site at the same time. When transferred to antiport carriers this single binding site has to be assumed to appear in alternating order at the internal or external membrane surface, thereby translocating substrates into opposite directions. This basic mechanism was described for the mitochondrial adenine nucleotide carrier by Klingenberg [17] and also for the Asp/Glu carrier by LaNoue et al. [5]. In a kinetic analysis of bisubstrate reactions, the ping-pong type can be identified by a parallel pattern of straight lines, if the transport data are plotted according to Lineweaver and Burk [13]. The slope of the lines, i.e. the ratio $K_{m,app}/V_{max,app}$, is not influenced by the second, so-called non-varied substrate, because an increase of V_{max} due to a rise of substrate concentration in one compartment is accompanied by a proportional decrease of apparent transport affinity of the substrate in the other compartment. This can be visualized considering that an increased translocation of the substrate, the concentration of which has been raised, brings about a greater availability of carrier bind-

ing sites in that compartment the substrate is transported to. Such interrelation always is observed in mechanisms, which comprise no reversible connection between association steps of the two substrates with the carrier. In case of the ping-pong type the first (transported) substrate leaves the protein before the second one is bound. The results presented in this paper clearly exclude a model of this kind for the Asp/Glu carrier (Fig. 1). Instead, the intersecting pattern obtained is consistent with a sequential type of mechanism. Characteristic for this mechanism is the formation of a ternary complex of the carrier with two substrate molecules, which both have to bind before one of the transport 'products' is released from the protein. Thus a reversible connection between the two consecutive binding steps exists as can be seen from a slope effect. The binary complex intermediates with only one occupied binding site can dissociate before the second substrate is bound. The corresponding dissociation constant K_{ss}, which appears in the last term of Eqn. 1, is an additional characteristic of a sequential mechanism. The values found in Fig. 1, if compared to the respective affinity constants, indicate that external aspartate as well as internal glutamate bind to the free carrier or to the binary carrier-substrate complexes with similar affinity. Thus it is conveivable that there is no obligatory order in the binding steps of internal and external substrate (random mechanism).

Comparing our results with the bisubstrate analysis of LaNoue et al. [5] it should be noted that their measurements using submitochondrial particles were carried out at only two different internal glutamate concentrations (50 and 150 mM), which both were chosen highly above the corresponding K_m value (3 mM, [5,10]). For this reason the published data [5], which moreover show considerable scattering, are insufficient to prove that the proposed parallel curves indicate a ping-pong mechanism. On the other hand, detailed bisubstrate kinetics published by Murphy et al. [6], who measured aspartate export from intact mitochondria varying medium glutamate and matrix aspartate, basically agree with our results. This group also found a sequential kinetic pattern with the point of intersection located near the abscissa. However, the K_m value for matrix aspartate derived from this analysis was 100-times higher than the corresponding constant found in our liposomal system (cf. Ref. 10).

Formation of the ternary complex

When considering a sequential model, the question arises as to how the ternary complex is formed. Several alternatives with respect to the order of substrate binding and to the distribution of binding sites between the two membrane sides can be envisaged. One binding site at either membrane side may be exposed at the same time or two binding sites at one side or even two sites at both sides. All three possibilities have been formulated for the nucleotide carrier [20–22] in addition to the ping-pong model mentioned above.

First the alternatives involving two binding sites at one or both membrane sides shall be discussed. When analyzing transport kinetics by variation of aspartate or glutamate concentrations in either compartment, two binding sites can only be identified if they show different affinities towards the varied substrate. This would become apparent from biphasic reciprocal plots leading to a mixed type bisubstrate pattern. However, as can be seen from Fig. 1 only binding sites showing higher affinity could be detected on the outside of the liposomal membrane, whereas only low-affinity binding sites were found on the inside. The linearity of reciprocal plots was substantiated earlier in backward exchange experiments encompassing an even larger range of substrate concentrations in both compartments [10].

The possibility, however, remains that at each membrane surface two types of binding sites exist, one being specific for aspartate, the other for glutamate. This model can be tested in a study of transport inhibition by the second substrate in the same compartment. The kinetic inhibition patterns obtained in the present paper demonstrate competitive behavior of aspartate and glutamate as well on the inside as on the outside of the liposomes (Figs. 2 and 3) indicating that both substrates bind to the same carrier form. These observations agree with results published by LaNoue et al. [5], which were measured at the outer surface of submitochondrial particles prepared from rat heart mitochondria. A similar analysis was carried out previously on the outside of

intact rat heart mitochondria and published in form of a Dixon plot [14]. These data were interpreted to be representative both for a non-competitive [14] and also for a competitive mechanism of inhibition (cf. Ref. 5). In fact primary Dixon plots are not suitable to discriminate between competitive and non-competitive inhibition. Additionally, a non-competitive aspartate inhibition of glutamate uptake into rat liver mitochondria was reported by the same authors [7]. However, neither of the models in question [5,6] adopted this latter finding. Our results demonstrating direct substrate competition in both compartments most easily can be explained, if the ternary complex is formed as a transmembrane unit by substrate occupation of one binding site at either membrane surface.

Concerted transport of internal and external substrate in a single step

The involvement of only one single conformational form of the carrier cannot be derived unambiguously from the observed substrate competition. Competitive inhibition would also be expected if two carrier forms showing specificity for aspartate or glutamate, respectively, were in equilibrium with each other. Thus two alternative models with completely different ways of formation of the ternary complex have to be considered. On the one hand an exchange cycle can be envisaged starting with the binding of one substrate molecule at either side of the membrane. It would require a conformational change of this transmembrane ternary complex to channel both substrates through the membrane in a concerted manner. On the other hand the single-substrate occupied carrier may translocate aspartate or glutamate. In this case the ternary complex has to be formed at one side of the membrane-inserted carrier. This means that for removal of the first substrate following translocation, the second substrate must bind. The model proposed by Murphy et al. [6] in principle fits into such a scheme.

The experimental strategy for a decision between these two reaction mechanisms is based on model calculations concerning the electrical properties of the reconstituted nucleotide carrier [16]. The influence of the membrane potential on this second electrogenic carrier of the inner mitochondrial membrane could be described in terms of rate equations assuming electrophoretic attraction or repulsion of charged carrier-substrate intermediates during translocation. For transferring this so-called 'velocity-type model' of electrophoretic control to the Asp/Glu carrier, one important control experiment had to be carried out. According to the energetic principle of secondary active transport no substrate gradient can be generated in the absence of membrane energization (facilitated diffusion). However, Tischler et al. [7] found a considerable (about 5-fold) stimulation of the (physiological) Glu_{ex}/Asp_{in} counterexchange as compared to the opposite direction even in rotenone-inhibited and in uncoupled mitochondria. Our data obtained in proteoliposomes do not agree with this observation. Table I clearly demonstrates that under deenergized conditions the reconstituted Asp/Glu carrier translocates both substrates with comparable velocity. Most important, the two heteroexchange rates (Asp_{in}/Glu_{ex}, Glu_{in}/Asp_{ex}) are about the same, which disproves an intrinsic functional asymmetry. The contrast of our results to those obtained in mitochondria [7] cannot be explained by a randomization of carrier orientation upon reconstitution (see above, Ref. 10), but may in fact reflect the difficulty of complete uncoupling of mitochondria. Furthermore, some kinetic limitations may contribute to the observed difference in the transport of the two substrate species (high pH, non-saturating substrate concentrations, asymmetric lipid and protein composition of the inner mitochondrial membrane).

It may be concluded from these studies in the deenergized state that the Asp/Glu carrier tends to equilibrate transmembrane gradients of the chemical potentials of transported substrates. According to the kinetic model established for the nucleotide carrier [16] this equilibration is influenced by the electrical potential affecting charged translocation intermediates. The kinetic data shown in Table II confirm the electrophoretic control of the heteroexchange reaction. Membrane potential, when applied in opposite polarity, has a qualitatively reciprocal effect, which is in accord with the conception outlined above. On the contrary, neither Glu/Glu nor Asp/Asp homoexchange is significantly influenced upon energization of the liposomal membrane. This means that

glutamate must be transported together with a proton in either direction conferring electroneutrality to Glu/Glu antiport. Furthermore, the observation that the Asp/Asp homoexchange is not affected by $\Delta\psi$ helps to discriminate between the two functional mechanisms discussed above. The 'velocity type model' predicts an inhibition of Asp/Asp antiport, if binary [carrier-Asp-] complexes represent the translocation intermediates, due to an unsymmetrical distribution of carrier binding sites between the two membrane sides. As a consequence, any stimulating influence on the translocation rate constant (k_3) for Asp^- should be accompanied by a decreased probability to occupy binding sites at the positive membrane surface. This was shown to be the case for the nucleotide carrier (ATP/ATP homoexchange) in terms of a higher apparent K_m value for ATP at the positive side of the membrane [16], which can simply be explained by a rise of k_3 ($K_m = (k_2 + k_3)/k_1$). However, contrary to one observation made in mitochondria [7], no influence of $\Delta\psi$ on Asp/Asp homoexchange was found for the reconstituted Asp/Glu carrier (Table II), which argues against binary complexes as translocation intermediates. The only conceivable alternative, that would account for the obviously symmetrical distribution of binding sites between the two compartments, is a model in which the ternary complex itself is the catalytic intermediate of the transport cycle responsible for the translocation step. Electrophoretic modulation of this integral unit only is possible in the case of heteroexchange, where the charge contributed by aspartate necessarily is localized asymmetrically, thus providing a target for the membrane potential. However, a ternary [Asp^--carrier-Asp^-] complex can be assumed to show approximately symmetrical charge distribution. This model implies that the two substrate molecules are translocated in a single step.

A minimal model for this synchronous antiport of the two substrates is outlined in Fig. 4. Only the formation and translocation of the ternary complex is shown omitting free or single-substrate occupied carrier intermediates. The model is symmetrical, first with respect to the binding of aspartate and glutamate, which both can bind to the same carrier form at either side of the membrane, and second with respect to the translocation of the two substrate species, as is predicted by data obtained from substrate inhibition studies (Figs. 2 and 3 in combination with Table I).

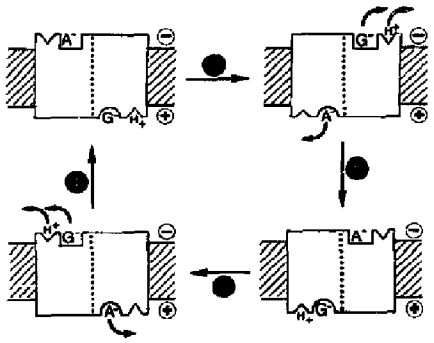

Fig. 4. Functional model of Asp/Glu antiport. The carrier molecule is schematically depicted as an integral unit in the mitochondrial membrane. Two functional 'subunits' are tentatively indicated. Either 'subunit' has one binding site for aspartate (A^-) or glutamate (G^-) and one for a proton. Four states of a complete heteroexchange cycle are shown connected either by translocation steps (1 and 3) or by substrate release/binding steps (2 and 4). The free carrier intermediates resulting after dissociation of transported substrates are omitted for simplicity. The translocation steps take place in a concerted manner between 'subunits' and are under electrophoretic control of the membrane potential

In view of the concerted action of the carrier one might expect a positive cooperativity between binding of internal and external substrate to ensure efficient antiport function. Yet no sigmoidal dependence of transport activity on substrate concentration could be observed in either compartment. Even without an allosteric influence on transport affinities, however, a conformational coupling of inward and outward directed translocation may be envisaged, that is analogous to the enzymatic turnover of two substrates in a sequential bisubstrate reaction. In the line of these arguments concerning the lack of cooperativity, one must also take into consideration the occurrence of substrate efflux (uniport) from liposomes in the absence of external substrate (see Methods).

H^+, a third substrate

Counterexchange of aspartate and glutamate together with a proton is not only regulated by the

electrical component of the proton motive force, but also the pH gradient may modulate the heteroexchange activity [3]. Experiments carried out varying the pH in both compartments ($\Delta pH = 0$) led to the conclusion that the absolute H^+ concentration in the two compartments plays a regulatory role. The affinity of the carrier for glutamate increased drastically at higher H^+ concentrations (Table III), as was previously reported [5]. However, under saturating glutamate concentrations the influence of pH on carrier activity was comparatively low, confirming results obtained in mitochondria [6]. In the case of the experiments presented here the observed alterations may partially be attributed to carrier inactivation during adjustment of pH. This becomes apparent when V_{max} values are normalized on the basis of exchange equilibrium determinations (Table III). Indeed from these corrected data a dependence of Glu^-/H^+ cotransport on H^+ concentration according to Michaelis-Menten can be found. In Lineweaver-Burk plots straight lines are obtained, which with increasing glutamate concentration tend to become parallel to the abscissa, indicating that the influence of pH vanishes under glutamate saturation (results not shown). This kinetic pattern suggests a rapid-equilibrium-ordered mechanism of H^+ and glutamate binding [13] implicating a protonation/deprotonation of the free carrier, that is much faster than the overall transport reaction.

$$\text{carrier} + H^+ \overset{K_a}{\rightleftarrows} \text{carrier}/H^+ \xrightarrow{Glu^-} [\text{carrier}/H^+\text{-}Glu^-]$$

$$\rightarrow \rightarrow \text{(translocation)} \qquad (2)$$

The dissociation constant of the carrier-proton complex was estimated from secondary plots (cf. analysis of the bireactant mechanism) yielding a pK_a equivalent of about 6.5. These findings strongly argue in favor of a distinct proton binding site possibly involving a histidine residue of the carrier protein. According to the rapid-equilibrium mechanism, the binding of glutamate to the protonated carrier shifts the protonation equilibrium to the protonated form (Eqn. 2). Thus, infinite glutamate concentration would transfer all carrier molecules to the ternary $[carrier/H^+\text{-}Glu^-]$ complex (regarding only one membrane side) independent of pH.

Binding of aspartate and glutamate, respectively, to two different carrier forms was already proposed by Tischler et al. [7]. However, this conclusion was drawn from a non-competitive behavior of the two substrate species, which could not be confirmed in our studies. In contrast, a rapid equilibration of two carrier forms, i.e. protonated and unprotonated carrier molecules, consequently explains the observed competitive nature of substrate inhibition. This interpretation of substrate competition based on glutamate/proton cotransport measurements is an alternative to that of LaNoue et al. [5], who assumed that both substrates can bind to both the protonated and unprotonated carrier. This, however, was integrated into a ping-pong model. Using a completely different point of view, Murphy et al. [6] postulated that the proton binds associated to the substrate glutamate, although this seems highly improbable, since the corresponding pK_a of glutamate is very low (about 4.2).

It has to be pointed out that the suggested proton cotransport as described in this publication (Eqn. 2) is partly based on data that had to be corrected for irreversible pH effects. However, this view perfectly fits into the functional model outlined above (Fig. 4) explaining substrate competition, though different carrier forms are involved in the transport of the two substrates. Glutamate/H^+ cotransport depends on glutamate and H^+ concentration, thus providing a means to regulate translocation of glutamate and not of aspartate. The modulation of transport affinity of the carrier towards glutamate is not due to variation of the ratio of protonated to unprotonated carrier (Eqn. 2), since in view of the rapid-equilibrium mechanism of substrate binding the concentration of carrier/H^+ can be neglected in comparison to that of the functional $[carrier/H^+\text{-}Glu^-]$ complex. The physiological significance of this regulation may lie in the possibility of discriminating between aspartate and glutamate in order to avoid futile homoexchange cycles. Glutamate binding would be favored at the (acidic) cytosolic surface, but impeded at the (alkaline) matrix side. Detailed measurements of mitochondrial and cytosolic pH values in dependence of physiological conditions

[23] indicate that the matrix pH may vary by about 0.5 units, which would be sufficient for a marked regulation by pH alone.

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