Kinetic study of the aspartate/glutamate carrier in intact rat heart mitochondria and comparison with a reconstituted system

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The homologous exchange of external [^{14}C]aspartate/internal aspartate catalyzed by the aspartate/gl carrier of rat heart mitochondria was investigated using aspartate-loaded, glutamate-depleted mitochon inhibitor-stop technique was developed for kinetic studies by applying pyridoxal phosphate. Direct ini determinations from the linear phase of [^{14}C]aspartate uptake were insufficiently accurate at high external low internal substrate concentrations. Therefore, the full time-course of [^{14}C]aspartate uptake until i isotope equilibrium was fitted by a single exponential function and was used to calculate reliable initial stea rates. This method was applied in bisubstrate analyses of the antiport reaction for different external and aspartate concentrations. The kinetic patterns obtained in double reciprocal plots showed straight lines co on the abscissa. This result is consistent with a sequential antiport mechanism. It implies the existence of a ternary complex that is formed by the translocator and substrate molecules bound from both sides membrane. The K_m values for aspartate were clearly different for the external and the internal side membrane, 216 ± 23 μM and 2.4 ± 0.5 mM, respectively. These values indicated a definite transm asymmetry of the carrier. The same asymmetry became evident when investigating the isolated protein fro heart mitochondria after reconstitution into liposomes. In this case the K_m values for external and aspartate were determined to be 123 ± 11 μM and 2.8 ± 0.6 mM, respectively. This comparison demon right-side out orientation of the carrier after insertion into liposomal membranes. The sequential t mechanism of the aspartate/glutamate carrier, elucidated both in proteoliposomes and in mitochond seems to be a common characteristic of other mitochondrial antiport carriers.

Introduction

The molecular mechanism of any biomembrane carrier is far from being elucidated from structure-function relationships because the structural information available cannot be unequivocally interpreted in terms of transport mechanism. Therefore, information about carrier function obtained by kinetics, the most powerful approach to discriminate between mechanisms, imposes strict limitations on the interpretation of structural and molecular data.

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Few of the specific carriers catalyzing anti cesses in the inner mitochondrial membrane h extensively investigated in order to determ mechanism in kinetic terms. The first antipor carefully studied as a classic bireactant syste oxoglutarate carrier of rat heart mitochondri investigated via bisubstrate analyses of initial as well as substrate competition experiments results led to the conclusion that the oxo carrier follows a sequential mechanism in ternary complex of the protein and two subs ands. This catalytic complex is formed by ra librium random binding of substrate molecu independent internal and external binding si over, detailed kinetic studies have shown tha

haviour of the translocator is intrinsically asymmetric regarding the two sides of the membrane and the two exchanged substrates, oxoglutarate and malate (for review see Ref. 3 and 4).

Further attempts at classic kinetic studies in mitochondria were undertaken on the aspartate/glutamate carrier [5,6] and on the ADP/ATP carrier [7–9]. These studies led to fundamental contradictions in the conclusions drawn by different groups. Mainly technical problems impeded initial rate measurements in intact mitochondria at various concentrations of both external and internal substrate. A set of prerequisites must be fulfilled for initial rate analysis under these conditions (as discussed in Ref. 9).

Another way to elucidate catalytic mechanisms of carriers which avoids several methodological difficulties, is to establish a well-defined reconstituted system and to use it for kinetic studies, provided the carrier is not randomly oriented in the liposomal membrane. The kinetic mechanism of the aspartate/glutamate carrier was successfully determined [10] for such a system [11–13] and was demonstrated to be the same type of sequential mechanism as described for the oxoglutarate carrier. The substrate affinities were clearly different for the two different membrane sides for both aspartate and glutamate. When the affinity constants were compared with published data obtained in mitochondria and submitochondrial particles [5,6] an inside out orientation of the carrier protein in the liposomes was suggested [13].

The controversy surrounding the results, obtained for the antiport mechanism of the aspartate/glutamate carrier and its transport affinity constants in mitochondria, emphasizes the need to revisit this carrier. In the present paper these problems have been addressed on the basis of experiments using both intact mitochondria and a reconstituted system which led to concordant results. Part of these results has been presented briefly elsewhere [14–16].

Material and Methods

Preparation and loading of mitochondria

Rats were 200–250 g fed males from
Mitochondria from heart ventricles wer
cording to Tyler and Gonze [17], the iso
contained 225 mM mannitol, 75 mM su
mM EDTA neutralized with 1 mM Tris
mitochondria contained approx. 6 mM
2.5 mM glutamate. The study of the
$aspartate_{out}/aspartate_{in}$ required glutar
aspartate-loaded mitochondria with diff
aspartate:

(a) The internal level of glutamate was
30-min incubation at 0°C in isolation me
ing 20 mM Tris-HCl buffer (pH 7.4) and
(Mal-incubation). The matricial oxaloace
by oxidation of the entered malate was t
by glutamate leading to aspartate and oxo
latter being metabolized. The Mal-incuba
a mitochondrial preparation with a high ir
tate concentration and a low level of glut
I, line 1). The presence of internal mala
does not interfere with this, since neithe
oxoglutarate influence the activity of the
partate/glutamate carrier reincorporated
liposomes (Dierks, T., unpublished data);

(b) A mitochondrial preparation containi
level of aspartate was obtained by perform
incubation in isolation medium containing
HCl (pH 7.4), 5 mM oxoglutarate and 1
(OG-incubation) before the Mal-incubati
placement of the equilibrium of the reacti
by the glutamate-oxaloacetate transamin
glutarate transformed aspartate to oxalo
finally to malate. There was a parallel
glutamate released into the incubation me
glutamate/OH^- carrier. The OG-incubat
the total pool of aspartate plus glutamate.

TABLE 1

Loading of mitochondria

Aspartate and glutamate concentrations of the mitochondrial matrix as determined after different incubations of mitochondria
and Methods). D.M., depletion medium; (6–8), number of measurements.

	Incubations				Aspartate (mM)	Glutamate (mM)
	OG	Mal	D.M.	Asp		
1	–	+	–	–	5.0	0.29
2	+	+	–	–	2.6	0.62
3	–	+	+	–	0.18	0.13
4	–	+	–	+	5.0 ±1.2 (7)	0.36±0.08 (7)
5	+	+	–	+	3.3 ±1.0 (8)	0.18±0.07 (8)
6	–	+	+	+		

of glutamate with a parallel increase in aspartate (Table I, line 2); and

(c) A low internal aspartate concentration was achieved by a 10-min incubation at 25°C of the mitochondrial preparation obtained in (a) in the medium usually applied to obtain malate-depleted mitochondria (depletion medium) [18] leading to a concomitant decrease of aspartate (Table I, line 3).

In order to improve the glutamate depletion by an exchange aspartate$_{out}$/(glutamate-H$^+$)$_{in}$, the three mitochondrial preparations were incubated for 30 min at 0°C in isolation medium containing 20 mM Tris-HCl (pH 7.4) and 10 mM aspartate (Asp-incubation). During this step 2 mM aminooxyacetate, an inhibitor of transaminases but not of the aspartate/glutamate carrier as tested in the reconstituted system, was present. Under these conditions the glutamate level in preparations (b) and (c) decreased and did not exceed 8% of the internal aspartate level for the high, medium or low concentrations (Table I, lines 4–6).

All mitochondrial preparations were finally washed in a large volume of the isolation medium (containing 2 mM aminooxyacetate) to eliminate external aspartate contamination and resuspended in a small volume of the medium (10 mg mitochondrial protein/ml).

The aspartate and glutamate content of the mitochondrial matrix was determined after perchlorate extraction of sedimented mitochondria. An aliquot (1 μl) of the extract supernatant, after neutralization with carbonate/KOH, was applied to a reversed-phase column (Merck Licrospher RP-18, 150 × 3 mm). Separation of amino acids was carried out according to the method of Jones and Gilligan [19] using a gradient HPLC system (HP 1090M, Hewlett Packard). The amino acids were detected fluorometrically after precolumn derivatization with *o*-phthaldialdehyde. Intramitochondrial concentrations could be calculated on the basis of matrix volumes, which were determined from the [^{3}H]water content of the mitochondrial pellet taking into account the [^{14}C]sucrose-accessible space [20].

Kinetic measurements with mitochondria: improvement of incubation conditions and stop-technique

The stock suspension of glutamate-depleted and aspartate-loaded mitochondria (0.5 mg protein) was diluted in incubation medium which contained 22.5 mM mannitol, 7.5 mM sucrose, 15 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA and 50 mM Tris-HCl (pH 7.4). Aspartate/aspartate exchange was measured at 2°C by following the uptake of [^{14}C]aspartate. The inhibitor-stop technique applied was as described previously [18] but using pyridoxal phosphate as inhibitor (see below). Labelled aspartate dissolved in the incubation medium and inhibitor were added with Hamilton syringes (CR-700) allowing instantaneous mixing.

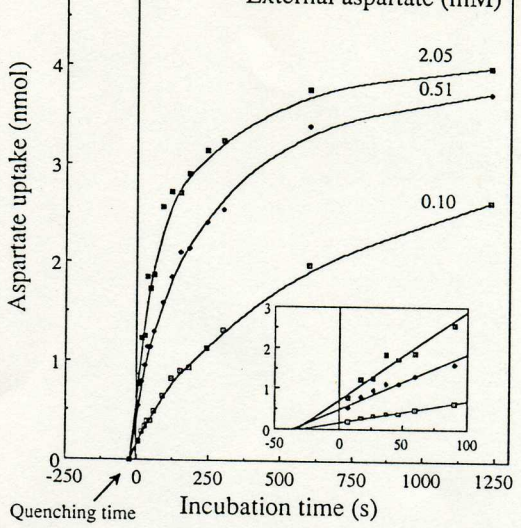

Fig. 1. Time-course of [^{14}C]aspartate$_{out}$/aspartate$_{in}$ exchange aspartate-loaded, glutamate-unloaded mitochondria (centrifugat stop). The exchange was started by injection of [^{14}C]aspartate stopped by centrifugation (2.5 μl mitochondrial pellet correspon to 0.5 mg protein). The [^{14}C]aspartate in the mitochondrial p was corrected for contamination in the sucrose-accessible sp [Asp]$_{in}$ = 4.8 mM (5 nmol), [Glu]$_{in}$ = 0.13 mM.

In a first attempt, the time-course was followed [^{14}C]aspartate uptake up to 30 min using a rapid c trifugation-stop technique. The transport was m sured at 2°C in the presence of 2 mM aminooxyaceta an inhibitor of glutamate-oxaloacetate transamina both in the stock suspension of mitochondria and the incubation medium. The internal aspartate conc tration was 3.25 mM and three external [^{14}C]aspart concentrations were tested between 0.02 and 1.8 m Under these conditions the uptake of labelled asp tate exceeded the theoretical value of isotope equ bration which can be calculated on the basis of t amount of unlabelled aspartate available inside t matrix (not shown). This observation suggested a ra metabolization of the substrate which entered and accumulation of radioactive metabolites in the mat

If the same time-course was recorded in the pr ence of aminooxyacetate with, additionally, 2 mM closerine, rotenone (4 μg/mg protein) and antimy (6 μg/mg protein), the uptake of labelled aspart remained lower than the available internal aspartate nmol) for all three external substrate concentratio tested, as shown in Fig. 1. This means that [^{14}C]asp tate taken up was not metabolized under these con tions. The equilibrium was approached at incubati times higher than 30 min (not shown). The inset of F 1, magnifying the initial phase, shows that the quenc ing (sedimentation) time was not negligible compar with the linear part of the time-course. These obser

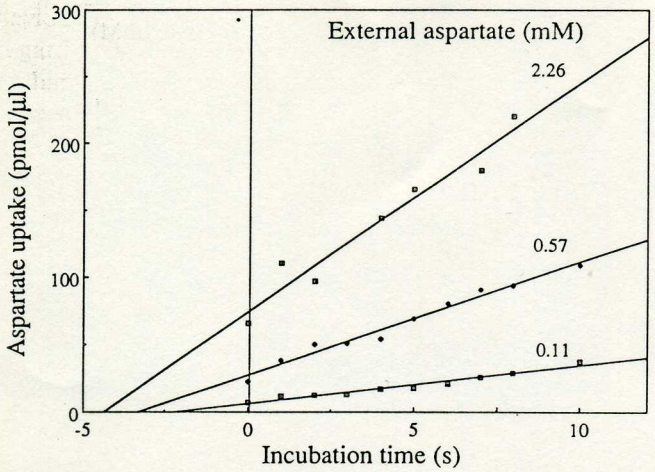

Fig. 2. Inhibitor-stop measurement of [^{14}C]aspartate uptake by aspartate-loaded mitochondria. Incubation time represents the interval between the injections of labelled aspartate and inhibitor (23 mM pyridoxal phosphate); $[Asp]_{in}$ = 5.8 mM, $[Glu]_{in}$ = 0.30 mM. The uptake was corrected for the amount of [^{14}C]aspartate accumulated in the mitochondrial pellet when the inhibitor was injected 5 s before the substrate and is given in pmol/µl of mitochondrial pellet.

tions led to two conclusions: (a) the matricial metabolism of aspartate can be frozen by the inhibitor cocktail, and (b) the exchange had to be blocked by an inhibitor-stop technique in order to reduce the quenching time.

Pyridoxal phosphate was used for this purpose. It has been described as an effective, though unspecific inhibitor of reconstituted aspartate/glutamate transport [13] acting on the cytosolic surface of the protein (unpublished data). When the inhibitor was added 5 s before [^{14}C]aspartate, the radioactivity in the mitochondrial pellet was equal to that of the aspartate in the sucrose-accessible space indicating a complete block of the substrate uptake. Thus, the amount of [^{14}C]aspartate in the pellet, determined when the inhibitor was added 5 s before the labelled substrate, was subtracted from all other values. Fig. 2 shows the data, corrected accordingly, for the first 10 s of uptake (0.11, 0.57 and 2.26 mM [^{14}C]aspartate). A linear phase of uptake (about 10 s) existed even at high external substrate concentrations. The intercepts on the ordinate indicate that the uptake was not zero when inhibitor and substrate were added simultaneously; however, the observed quench delay was shorter than 5 s even at the highest external aspartate concentration. After this delay the inhibition was complete, since no additional uptake was measured. The initial rates are properly determined despite the quench delay because this delay is much lower than the characteristic time of relaxation towards equilibrium (134, 270 and 741 s at 2.26, 0.57 and 0.11 mM external aspartate, respectively).

Kinetic analysis of the reconstituted aspartat carrier

The isolation of the aspartate/glutar from bovine heart mitochondria and its fu constitution was described in [11–13]. T tuted transport activity was determined a perature by the forward exchange metho uptake of labelled aspartate into the prot [13]. The time-course (first 4 min) of the is bration was fitted by a first-order rate equa computer program that enabled initial ra port to be derived [21].

The dependence of the transport aff reconstituted carrier for external and int tate on pH was determined by varying the neously inside and outside the proteoli pH = 0). The pH was adjusted by passing th of proteoliposomes, prepared at pH 6.5 Mops/KOH) and supplemented with valin ng/mg phospholipid) and nigericin (40 n pholipid), through Sephadex G-75 columns equilibrated with 100 mM Mops/KOH at or 7.4. This buffer contained sucrose to osmolarity of the internal aspartate.

Chemicals

Special reagents were obtained from th sources: [U-^{14}C]aspartic acid, [U-^{14}C]sucros (Amersham); rotenone, aminooxyacetate a ine (Sigma); antimycin (Boehringer Man pyridoxal phosphate (Merck).

Results

In order to study the antiport activity o tate/glutamate carrier in intact mitochon netic experiments had to be made as possible. To choose the simplest condition troneutral homologous exchange [^{14}C]as $aspartate_{in}$ was investigated. By using aspar and glutamate-depleted mitochondria no net-import of aspartate from the incubati (in exchange for matricial glutamate) could thus avoiding changes of substrate concent ing the transport assay. The metabolism could efficiently be blocked by working at applying several inhibitors of transaminatio ration (see Materials and Methods).

Initial rate measurements

The activity of aspartate/aspartate ex investigated at various internal and extern concentrations using the inhibitor-stop te scribed in Materials and Methods. Initial be evaluated directly from the slope of the phase of [^{14}C]aspartate uptake. Such initial

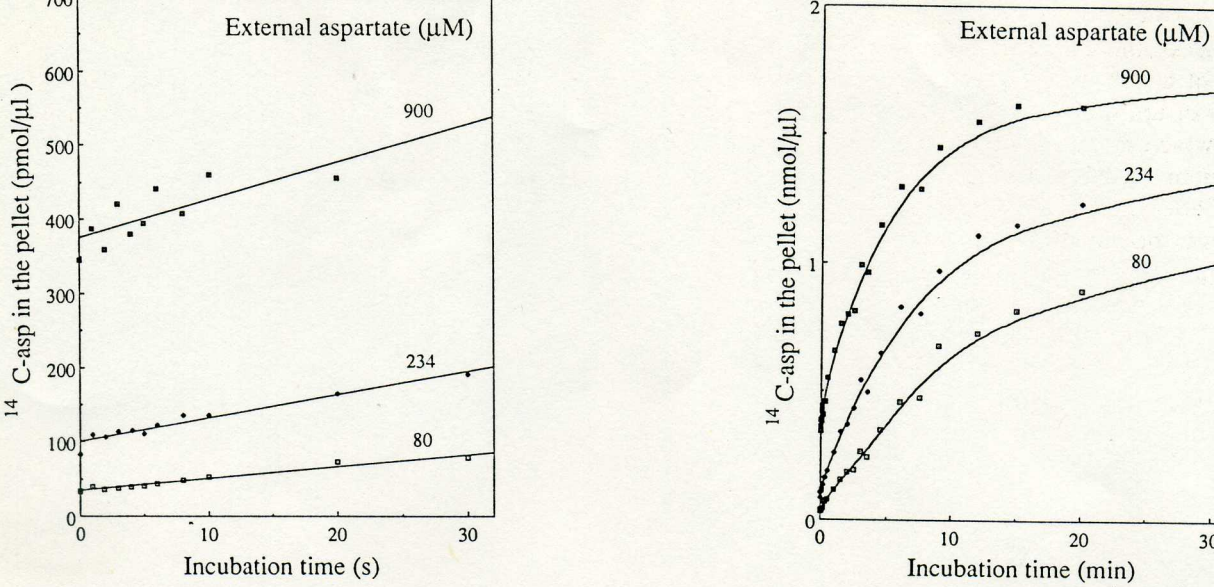

Fig. 3. Initial rates and full progress-curves of [^{14}C]aspartate$_{out}$/aspartate$_{in}$ exchange. (A) Initial rates of the [^{14}C]aspartate$_{out}$/asp exchange; [Asp]$_{in}$ = 4.92 mM, [Glu]$_{in}$ = 0.47 mM. The aspartate uptake for incubation time = 0 (simultaneous injection of aspartate and pyridoxal phosphate) corresponds to the residual uptake after the injection of the inhibitor plus the aspartate content in the sucrose a space (not corrected). (B) Same experiment as in (A), data shown for longer incubation times (full progress-curve).

however, occurred only for a short time at high external aspartate concentrations. This is illustrated in Fig. 3, where the [^{14}C]aspartate content of sedimented mitochondria was plotted vs. time, as recorded for three external aspartate concentrations (80, 234 and 900 μM) at constant internal aspartate (4.9 mM). The initial phase (Fig. 3A) revealed that linearity lasted for 20–30 s. Within this period the amount of isotope exchanged was less than 6% for the internal substrate and negligible (< 1%) for the external substrate. The slopes of the straight lines can thus be considered as initial rates of the exchange amounting to 1.61 ± 0.11, 3.16 ± 0.27 and 5.11 ± 1.53 pmol/s per μl of mitochondrial pellet for 80, 234 and 900 μM external aspartate, respectively. It became obvious, however, that the accuracy of the slopes was unsatisfactory and that the error increased with the external substrate concentration. This was due to the fact that the signal to be detected, namely the uptake of [^{14}C]aspartate into the matrix, is only part of the overall radioactivity in the mitochondrial pellet and this part decreased when the external concentration was increased. At 900 μM external aspartate the uptake of label into the matrix within 20 s was only 22% of the pellet content. This can be seen in Fig. 3A from the high radioactivity in the pellet at $t = 0$, which was mainly due to the [^{14}C]aspartate in the sucrose-accessible space, i.e., the extra-matrix space of the pellet and, to a lesser extent (less than 13%), due to the residual uptake during the quenching time of the inhibitor (Fig. 2). These problems of method became even more severe in experiments with lower internal aspartate concentrations, because the isotope equilibration with the small nal substrate pool is faster and, as a consequen initial linear phase is shorter.

From these initial rate experiments (Fig. 3 following conclusions could be drawn: (a) the act the carrier at 2°C was relatively low with isotope bration occurring on a time scale of minutes (Fi (b) the external K_m for aspartate calculated fro 3A was in the range of 200–300 μM; and (accuracy obtained in these experiments was insuf for a bisubstrate kinetic analysis of the antiport r nism, which requires reliable measurements initial rate at a sufficiently large range of sul concentrations on both sides of the membrane led us to consider the full time course of [^{14}C]asp uptake.

Full progress-curve experiments

Under homoexchange conditions the substrat centrations in both compartments remain co Thus, full progress-curves describing [^{14}C]asparta take till equilibrium can be used to calculate the initial steady-state rate without any particular as tion, except that a steady-state is reached. Knowi [^{14}C]aspartate uptake at equilibrium (Y_{eq} progress-curve can be linearized according to:

$$Z = Y_{eq} \ln \frac{Y_{eq}}{Y_{eq} - Y_t} = vt$$

where Y_t is the uptake after incubation time t (at $t = 0$ subtracted). Z can be rationalized

uptake corrected for the variation (during isotope equilibration) of the external and internal specific radioactivities of aspartate. The initial rate, v, represents the velocity of unidirectional aspartate uptake (not only of label), which in fact is constant throughout the whole experiment (see below, Fig. 4). The quench delay of the inhibitor in a given time-course is a constant time that has to be added to t (Eqn. 1) and does not modify the slope. Furthermore, it is small compared with the incubation times considered.

Fig. 3B shows the progress-curves for the same mitochondrial preparation as used in Fig. 3A and for the same internal and external aspartate concentrations. Equilibrium was reached after an incubation for 30 to 60 min. Y_{eq} was 1.25 nmol/μl which was in good agreement with the theoretical value calculated from the external and internal substrate pools. In Fig. 3B, as in Fig. 3A, the labelled aspartate in the pellet at $t = 0$ was not subtracted in order to show that these values were small compared with Y_{eq}, thereby demonstrating the much better signal to noise ratio compared with that for Fig. 3A.

The linearization of the progress-curves according to eqn. 1 is presented in Fig. 4, where Z was plotted vs. time. A perfect linearity was observed indicating that the progress-curves were well-fitted by a single exponential equation. Due to this methodological improvement the initial rates obtained from the Fig. 4 were much more precise (1.48 ± 0.03, 2 4.65 ± 0.06 pmol/s per μl for 80, 234 and external aspartate, respectively) and showed standard deviation. Moreover, the number mental data points (26 between zero time ar rium) led to a high statistical weight of the i values.

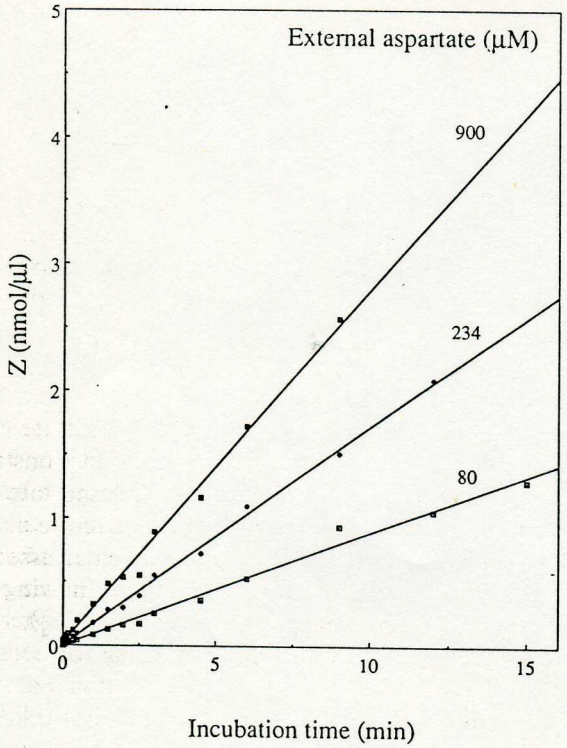

Fig. 4. Initial rate measurements from full progress-curves. The Z values (for explanation see text) were calculated from the full progress-curves of Fig. 3B. The initial rates derived from the slope of

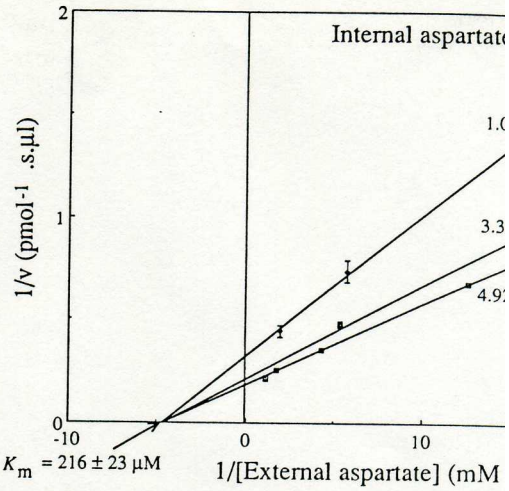

Fig. 5. Influence of the external aspartate concentration rate of [^{14}C]aspartate$_{out}$/aspartate$_{in}$ exchange for tl aspartate concentrations. Full progress-curves were rec ferent concentrations of internal and external aspartate (2°C, pH 7.4). Initial rates (± S.D.) were calculated fro of Z vs. time (see Fig. 4). The straight lines were fitte common intersection point on the abscissa axis (unkno

Bisubstrate kinetic analysis

In order to elucidate the transport mec the aspartate/glutamate carrier we had to i the mutual interaction of internal and ext strate with the carrier. For this purpose the e described in Fig. 4, where only the external was varied, was reproduced at different inter tate concentrations. The incubation times justed to obtain proper equilibrium values (valuable kinetic resolution. Three sets of initi values were determined from the full progr for three internal aspartate concentrations and 4.9 mM) which are given in the double plots of Fig. 5. The straight lines obtained regression showed convergence very close t scissa which indicates a sequential type of kin port mechanism (see Discussion). If a comn section point was placed on the abscissa (Fig. lations by a least square method [2] led to a of 216 ± 23 μM for external aspartate.

The convergence on the abscissa as observ 5 indicated that the value of the external independent of the internal aspartate conc

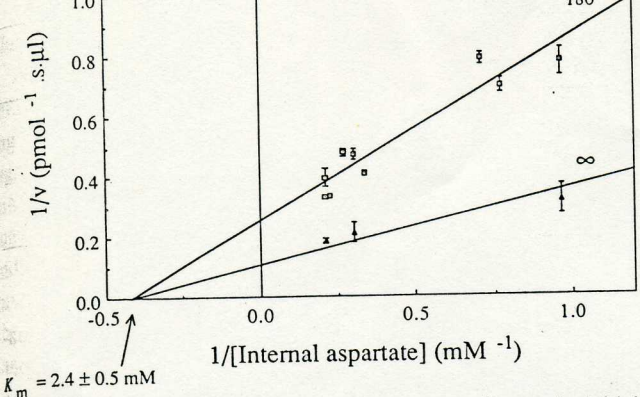

Fig. 6. Influence of the internal aspartate concentration on the initial rate of [^{14}C]aspartate$_{out}$/aspartate$_{in}$ exchange for limiting and infinite external aspartate. Full progress-curves were recorded at different internal aspartate concentrations and 180 µM external [^{14}C]aspartate (2°C, pH 7.4). The data for infinite external aspartate concentration were taken from Fig. 5 (V_{max} values). The internal glutamate was less than 7% of the internal aspartate for each condition.

determined at limiting external aspartate concentrations. In the experiments of Fig. 6 initial rates were derived from full progress-curves obtained at different internal aspartate concentrations in the range of 1–5 mM with the external [^{14}C]aspartate kept constant at 180 µM. The scattering of data points in the double reciprocal plot may be attributed to the fact that every single rate necessarily had to be determined in different mitochondrial preparations. Nevertheless, a linear relationship was obtained leading to an internal K_m of about 3 mM (not shown). The K_m for internal aspartate could also be evaluated by replotting the ordinate intercepts of Fig. 5, i.e., the reciprocal exchange velocities at infinite external aspartate, vs. (internal aspartate)$^{-1}$. This secondary plot was included in Fig. 6. Again a common intersection point of the two straight lines was observed close to the abscissa representing a more reliable value of the internal K_m (2.4 ± 0.5 mM). The maximum exchange rate extrapolated for infinite concentration of both internal and external aspartate was 9.4 ± 1.6 pmol s^{-1} µl^{-1} which was equal to 2.8 nmol min^{-1} mg protein^{-1}.

Comparison with the reconstituted system

Detailed kinetic studies of the aspartate/glutamate carrier from beef heart mitochondria have been carried out in the reconstituted system [10,13]. As observed in intact mitochondria (Fig. 5), bisubstrate kinetics led to a pattern of intersecting straight lines converging very close to the abscissa. In order to substantiate this basic agreement of results from intact mitochondria and proteoliposomes, it was considered interesting to find out whether the interaction of substrate with carrier, as

The exchange velocity was determined by the forward e method measuring uptake of [^{14}C]aspartate supplied in concentrations (external K_m determinations: 25–260 µM, K_m determinations: 50 µM) to proteoliposomes containing or, in the case of internal K_m determinations, 1–9 mM a The K_m values were derived from reciprocal plots (Linewea and Eadie-Hofstee) and are given as mean values from four nations. For adjustment of pH see Materials and Methods.

pH	External K_m (mM)	Internal K_m (mM)
6.5	0.044 ± 0.003	2.6 ± 0.3
6.9	0.084 ± 0.008	3.7 ± 0.6
7.4	0.123 ± 0.011	2.8 ± 0.6

characterized by internal and external K_m valu quantitatively comparable in the two systems. already been shown [13] that the affinity of the stituted aspartate/glutamate carrier for aspa much higher on the outside of the proteolip (K_m: 50 µM) than on the inside (K_m: 3 mM) K_m values were obviously affected by the p which was 7.4 in the experiments presented ab 6.5 in the studies using proteoliposomes. Th the affinity of the reconstituted aspartate/gl carrier for aspartate was redetermined on both the liposomal membrane raising the pH from 7.4. In order to avoid difficulties resulting fr gradients, the pH was adjusted in parallel compartments (see Materials and Metho demonstrated in Table II, a clear increase in the pH from 44 (pH 6.5) to 123 µM (pH observed at the external side, whereas the values calculated at pH 6.5, 6.9 and 7.4 were a range of 3 mM. Consequently, at pH 7.4 a clos lation with the transport affinities determined rat heart mitochondria became evident altho species of mammal differed.

Discussion

Two basic aspects appeared to be importa reinvestigating the kinetic properties of the as glutamate carrier in intact mitochondria. F clarify the controversy over results obtained m 10 years ago concerning the kinetic mechanis carrier in intact mitochondria [6] and in chondrial particles [5]. Secondly, to elucida comparative studies carried out in intact mito and in the reconstituted system how these two systems can supplement and reinforce each so, this new approach would enable the re tained from kinetic experiments in liposome mitochondria to be combined in order to dra

TABLE III

Michaelis constants for aspartate of the mitochondrial aspartate / glutamate carrier at pH 7.4

System	Temperature (°C)	K_m for aspartate (mM)	
		matrix side	cytosolic si
Intact rat-heart mitochondria (this paper)	2	2.4 ± 0.5	0.216 ± 0.0
Proteoliposomes, carrier from bovine-heart mitochondria (this paper)	Room temperature	2.8 ± 0.6	0.123 ± 0.0
Inverted rat-heart submitochondrial particles [5]	17	0.042	–
Intact rat-liver mitochondria (pH 7.2) [6]	10	5.0	–

sions that are meaningful for the in vivo situation of the carrier.

The kinetic mechanism

The usefulness of the kinetic approach for studying mechanisms of mitochondrial translocators is often limited by technical problems; in fact initial rates may be difficult to obtain. It is thus important to carry out direct and careful kinetic measurements and, if possible, to apply different experimental systems. The kinetic mechanism of the mitochondrial aspartate/glutamate carrier analyzed here in intact rat heart mitochondria is in complete accord with that observed in the reconstituted system [10]. Moreover, the measured K_m values on both sides of the membrane show striking numerical coincidence (see Table III), although the aspartate/glutamate carrier from different sources was investigated, i.e., from rat and bovine heart, respectively. Such a comparison has been made for the first time and provides strong evidence that this translocator does not follow a ping-pong mechanism but a sequential one, as shown in double reciprocal plots by the presence of intersecting straight lines converging near the abscissa. This type of kinetic pattern indicates that considerably raising the substrate concentration in one compartment increases the transport rate (V_{max}) without much effect on the affinity of the carrier for the substrate in the opposite compartment. On the contrary, in a ping-pong mechanism both V_{max} and K_m are affected to the same degree leading to parallel straight lines in the double reciprocal plots. Consequently, for the sequential mechanism, the association of internal and external substrate with the carrier cannot be separated by translocation steps. This means that one internal and one external binding site of the carrier protein must be occupied forming a transport-competent ternary complex. The position of the common intersection point on the abscissa suggests that the binding of the internal and the external substrate is fast and independent (rapid-equilibrium random).

Previous publications on the kinetic mechanism of the mitochondrial aspartate/glutamate carrier [5,6] (for review see Ref. 13) give conflicting results. The significant methodological improvements for cor tion of initial exchange rates presented in enabled these to be resolved, at least for st intact mitochondria. On the one hand, dat obtained for submitochondrial particles fro mitochondria could be rejected. Although in precise, these were interpreted to be consis ping-pong mechanism [5]. On the other ha netic pattern reported here agrees, in pri that obtained for intact rat liver mitochond

In view of the existence of a so-call super-family' of mitochondrial carriers it is to find out whether its members can be ch in terms of a common kinetic mechanism a their structural similarities [22,23]. The mechanism involving two substrate bindin on each membrane side (see above), was dated for the oxoglutarate carrier in earl tions carried out in rat heart mitochondria precisely, the mechanism of the oxoglutar could be described as a rapid-equilibriu mechanism with fast and independent bin internal and one external substrate mole results were convincingly confirmed for th tuted carrier from beef heart (Indiveri, C., Krämer, R. and Palmieri, F., unpublished shown here, this particular mechanism also for the aspartate/glutamate carrier. It shou that the transport mechanism of the ADP rier, the most intensively studied mitochond was described as a gated pore mechanism single binding center [24,25], which in ki would be classified as a ping-pong mecha model was mainly based on studies mea accessibility of binding sites in the two differ mational states of the carrier which can be side-specific inhibitors. However, binding s led to alternative models involving at least site on either membrane side ([26] and therein). Kinetic investigations of the exch tion also provided direct evidence for a mechanism [7–9]. Thus, a general concep evolve of a functional carrier family, so far c three of the main mitochondrial antiporte

The orientation of the carrier in the reconstituted system

As determined in the present paper for both intact mitochondria and proteoliposomes, the external binding site of the aspartate/glutamate carrier showed a significantly lower K_m for aspartate (0.22 and 0.12 mM, respectively) than the internal site (2.4 and 2.8 mM, respectively) (Table III). This correlation clearly indicates a right-side out orientation of the reconstituted protein. The excellent agreement suggests that the K_m values derived from transport studies are similar for the carrier protein from different organisms and are unaffected by isolation or by the different temperatures applied in the experiments (Table III). The influence of pH on K_m values was taken into account (Table II), thereby improving the reliability of this comparison. It appears that intenal K_m for aspartate is least one order of magnitude higher than the external K_m. Such an asymmetry has been already observed with the oxoglutarate carrier of rat-heart mitochondria for oxoglutarate [3,9]: close to 2 orders of magnitude.

The observed right-side out transmembrane topology in proteoliposomes is in contrast to the previous interpretation of an inside out orientation of the reconstituted carrier protein [13]. This conclusion was based mainly on the matrix K_m value for aspartate (0.042 mM) found by LaNoue and co-workers [5] on the outside of inverted submitochondrial particles (Table III). However, in this study the degree of inversion of the particles was not indicated. Other K_m (or K_i) values reported for aspartate, sometimes based on indirect methods, were all in the range of 3–5 mM on both the matrix (Table III) and the cytosolic side, as discussed in [13]. Hence, they could not be used to confirm the correct definition of the orientation of the reconstituted protein. This definition was only made ble by the results of the present paper giving the K_m values for the same substrate (aspartate) on the two different sides of the membrane for both the native and the reconstituted systems, thereby demonstrating the strength of comparative kinetic studies of this type. It should be pointed out that the matrix K_m for aspartate (5.0 mM) and the cytosolic K_m for glutamate at high pH (5.8 mM) found by Murphy et al. [6] in liver mitochondria was of the same order of magnitude as our data (2.8 mM and 1.8 mM, Table III and [10], respectively).

The evaluation of this right-side out transmembrane arrangement of the carrier in proteoliposomes is of importance for further studies of the malate-aspartate shuttle reconstituted in liposomes. It is also of interest in relation to the recent observation of unidirectional export activity of the reconstituted aspartate/glutamate carrier induced by cysteine-modifying function can be related to the efflux phenomena ge ally observed in mitochondria after treatment SH-reagents.

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