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Biochimica et Biophysica Acta 1503 (2001) 303–313

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Kinetic mechanism of antiports catalyzed by reconstituted ornithine/citrulline carrier from rat liver mitochondria

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Received 23 June 2000; accepted 31 August 2000

Abstract

The transport mechanism of the reconstituted ornithine/citrulline carrier purified from rat liver mitochondria was investigated kinetically. A complete set of half-saturation constants (K_m) was established for ornithine, citrulline and H^+ on both the external and internal side of the liposomal membrane. The internal affinity for ornithine was much lower than that determined on the external surface. The exclusive presence of a single transport affinity for ornithine on each side of the membrane indicated a unidirectional insertion of the ornithine/citrulline carrier into liposomes, probably right-side-out with respect to mitochondria. Two-reactant initial velocity studies of the homologous (ornithine/ornithine) and heterologous (ornithine/citrulline) exchange reactions resulted in a kinetic pattern which is characteristic of a simultaneous antiport mechanism. This type of mechanism implies that the carrier forms a ternary complex with the substrates before the transport reaction occurs. A quantitative analysis of substrate interaction revealed that rapid-equilibrium random conditions were fulfilled, characterized by a fast and independent binding of internal and external substrates.

Keywords: Mitochondrion; Transport; Reconstitution; Liposome; Kinetics

1. Introduction

The ornithine/citrulline carrier is one of the transport systems of the inner membranes of mitochondria that make these membranes selectively permeable to various metabolites [1]. After preliminary and contradictory studies performed in intact mitochondria on the transport mode of ornithine and citrulline [2–8], the ornithine/citrulline carrier was purified from rat liver mitochondria and its functional properties elucidated in reconstituted liposomes [9–12]. Besides ornithine and citrulline, the carrier also accepts lysine and arginine, although less efficiently. The carrier-catalyzed exchange of ornithine (or lysine) for citrulline is electroneutral, since the positive charge of ornithine or lysine is compensated by a H^+ co-transported with citrulline [11]. Furthermore, the same carrier protein catalyzes an electroneutral exchange of ornithine for H^+ [12].

The ornithine carrier accomplishes an essential step in the urea cycle by exchanging cytosolic ornithine and intramitochondrial citrulline [5,9,11]. In

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0005-2728/01/$ – see front matter
PII: S0005-2728(00)00208-5

addition, through its ornithine/H^+ transport mode it plays an important role in the catabolism of excess arginine and in other metabolic processes such as the biosynthesis of polyamines [12]. Transport of ornithine across the mitochondrial membrane is also important in lower eukaryotic organisms [13–15]. *Saccharomyces cerevisiae* and *Neurospora crassa* contain two related genes, ARG11 and ARG13 respectively; the former was identified as the mitochondrial ornithine carrier by bacterial expression and functional reconstitution into liposomes [16]. The yeast ornithine carrier sequence was then used to identify the orthologous protein in man [17]. The corresponding gene, designated ORNT1, maps to 13q14 and is defective in the hyperornithinemia–hyperammonemia–homocitrullinuria (HHH) syndrome [17]. Both the *S.cerevisiae* and the human ornithine carriers have the characteristic sequence features of the members of the mitochondrial carrier family with known function [18–21].

For a detailed functional characterization of the reconstituted ornithine/citrulline carrier, and for comparison with other members of the mitochondrial carrier family, it is of particular interest to establish the orientation of the inserted protein and investigate the mechanism of transport. In this paper, by using proteoliposomes reconstituted with the purified ornithine/citrulline carrier and by applying two-substrate kinetic analysis, it was possible to determine whether one (ping-pong mechanism) or two binding sites (simultaneous mechanism) have to be occupied before the transport reaction occurs. We show that the exchange reactions catalyzed by the ornithine/citrulline carrier proceed via a simultaneous mechanism. Furthermore, evidence is provided that the reconstituted ornithine/citrulline carrier is oriented unidirectionally in the liposomal membrane, most probably right-side-out compared to mitochondria.

2. Marterials and methods

2.1. Materials

Hydroxyapatite (Bio-Gel HTP) and Bio-Beads SM-2 were purchased from Bio-Rad, Celite 535 was from Roth, DEAE–Sephacel, Sephadex PD-10, Sephadex G-50 and G-75 were from Pharmacia, L-[*ureido*-^{14}C]citrulline was from Du Pont-NEN, L-[2,3-^{3}H]ornithine, egg yolk phospholipids (L-α-phosphatidylcholine from fresh turkey egg yolk), PIPES, Hepes, and Triton X-100 were from Sigma. All other reagents were of analytical grade.

2.2. Purification and reconstitution of the ornithine/citrulline carrier

The ornithine/citrulline carrier was purified from rat liver mitochondria as previously described [9]. The purified protein was reconstituted into liposomes by cyclical removal of the detergent with a hydrophobic column [22]. The composition of the initial mixture used for reconstitution was: 400 μl of purified protein in 3% Triton X-100 (Celite eluate, about 1 μg protein), 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes, L-ornithine or L-citrulline at the concentrations indicated in the legends to tables and figures, and 20 mM PIPES/20 mM Hepes at the indicated pH in a final volume of 700 μl. After vortexing, this mixture was passed 14 times through the same Bio-Beads SM-2 column (0.5×2.5 cm), pre-equilibrated with the same buffer and substrate present in the initial mixture. All the operations were performed at 4°C, except the passages through Bio-Beads SM-2 column that were performed at room temperature.

2.3. Transport measurements

The external substrate was removed from proteoliposomes by chromatography at 4°C on a Sephadex G-75 column (0.7×15 cm) pre-equilibrated with 20 mM PIPES/20 mM Hepes (unless otherwise specified) at the pH indicated and with an appropriate concentration of sucrose to balance the internal osmolarity. The transport activity was determined by measuring the uptake (forward exchange) or the efflux (backward exchange) of labeled substrate in exchange for unlabeled substrate [22]. For backward exchange measurements the proteoliposomes containing internal ornithine were prelabeled by carrier-mediated exchange equilibration by adding 0.16 mM [^{3}H]ornithine at high specific radioactivity for 15 min. The external substrate was removed from proteoliposomes on a Sephadex G-75 column as de-

scribed above. Transport at 25°C was started by adding [^{3}H]ornithine or [^{14}C]citrulline to the proteoliposomes (forward exchange) or unlabeled substrate (backward exchange) to the prelabeled proteoliposomes. In both cases the carrier-mediated transport was terminated by addition of a mixture of 20 mM pyridoxal 5′-phosphate and 1 mM *N*-ethylmaleimide. In control samples the inhibitors were added at time 0 according to the 'inhibitor-stop' method [22,23]. The external labeled substrate was removed, and the radioactivity in the liposomes was measured [22]. In forward-exchange kinetic measurements, the initial transport rate was calculated in mmol/min per g of protein from the radioactivity taken up by the proteoliposomes within the initial linear range of substrate uptake or from the time course of isotopic equilibration, as has been described previously [22]. In the case of backward exchange, the decrease in radioactivity inside the liposomes was fitted to a single exponential decay equation from which the first-order rate constant (k) of efflux was derived. The rates were expressed as apparent velocities, i.e., the product of k and the substrate concentration inside the liposomes, and they are directly proportional to the actual transport rate [22]. When a ΔpH was imposed across the proteoliposomal membrane, the rates were calculated from the radioactivity effluxed in 2 min incubation time, during which the equilibration of the H^+ gradient was almost negligible [12]. The concentration of substrate in the active liposomes, i.e., the carrier-loaded liposomes, was estimated essentially as published previously [24].

2.4. Other methods

Protein was determined by the Lowry method modified for the presence of non-ionic detergents [25].

3. Results

3.1. Determination of external and internal transport affinities for ornithine, citrulline and H^+

For kinetic analysis of the exchange reactions catalyzed by the ornithine/citrulline carrier, internal and external K_m had to be determined for ornithine, citrulline and H^+. They were derived from double-reciprocal plots of the exchange rate versus substrate concentration. Furthermore, for methodological reasons [22] we applied two different methods measuring either the flux of labeled substrate from outside to inside (forward exchange) or from inside to outside (backward exchange), respectively. For ornithine and citrulline the results of these experiments are reported in Tables 1 and 2. Firstly, the half-saturation constants (K_m) of ornithine and citrulline, both in-

Table 1
External and internal K_m values of ornithine for the reconstituted ornithine/citrulline carrier

Membrane side	Countersubstrate	pH	Ornithine K_m (mM)	Experiments (n)
External	Ornithine (30 mM)	8.0	0.16 ± 0.03	(8)
		7.5	0.19 ± 0.04	(8)
		7.0	0.21 ± 0.04	(8)
		6.5	0.24 ± 0.06	(3)
	Citrulline (30 mM)	8.0	0.16 ± 0.02	(3)
	None (H^+)	8.0	0.24 ± 0.09^{t}	(4)
Internal	Ornithine (1.6 mM)	8.0	3.8 ± 1.0	(9)
	Ornithine (20 mM)	8.0	$4.0^{b\ t}$	(1)
	Ornithine (1.6 mM)	7.0	4.1 ± 0.6	(3)
	Citrulline (30 mM)	8.0	$3.6^{b\ t}$	(1)
	None (H^+)	8.0	$5.2 \pm 0.2^{b\ t}$	(3)
		7.0	$6.5 \pm 1.1^{b\ t}$	(2)

K_m values were determined by the forward-exchange method, except for the values indicated by (^{b}) that were determined by the backward-exchange method. The range of ornithine concentration was 0.05–2 mM for the determination of external K_m and 0.5–20 mM for the determination of internal K_m. The transport rate was evaluated from time course where indicated by (^{t}) or by the radioactivity taken up in 2 min in all the other cases. Both the external and internal compartment were buffered at the indicated pH as described in Section 2. The values are mean ± S.D. (n).

side and outside the proteoliposomes, did not change with changing the type of substrate present on the opposite side of the membrane. Secondly, whereas the internal and external K_m values of citrulline were more or less the same, the internal K_m of ornithine was about 20 times higher than that measured on the external membrane surface. Because of this large difference in the transport affinities of ornithine, in control experiments we looked for a lower K_m on the inside by using internal substrate concentrations as low as 50 μM, by keeping external ornithine constant at 20 mM and by applying the backward-exchange method. A similar internal K_m (3.7 ± 0.8 mM in three experiments) was also extrapolated in this case, thus excluding the presence of a second inward-facing binding site with a higher affinity for ornithine. Similarly, the low-affinity binding site on the external surface of the reconstituted carrier was excluded by applying sufficiently high external concentrations of ornithine. Thirdly, the K_m of ornithine on both sides of the membrane showed very little dependence on the pH, with a slight increase, if any, at lower pH. Also with citrulline, pH dependence of the external and internal K_m was rather low. However, in this case the K_m slightly decreased on lowering the pH. It should be noted that the S.D. calculated for the K_m of the citrulline/citrulline exchange at pH 8.0 is very high. This inaccuracy is due to the very low transport rate of the citrulline/citrulline homoexchange reaction at pH 8.0 (V_{max} of 0.5 ± 0.4 μmol/min per mg protein in three experiments) as compared to that at pH 6.5 (V_{max} of 2.5 ± 1.0 μmol/min per mg protein in three experiments) and to the rate of the ornithine/ornithine exchange at pH 8.0 (V_{max} of 2.8 ± 1.2 μmol/min per mg protein in eight experiments; see also [10]). The usually high S.D. of the V_{max} values is due to a large variation in the amount of active ornithine/citrulline carrier protein in the different preparations [10].

In principle, in the electroneutral ornithine/citrulline+H^+ exchange, citrulline might be transported unprotonated or protonated as citrulline$^+$. The carboxylic group of citrulline has a pK_a value of 2.43 [26] and consequently it is nearly exclusively present as neutral citrulline in the range of pH 6.0–8.0. Furthermore, when the apparent transport affinity was calculated for citrulline$^+$ at pH values from 6.5 to 8.0 it was found that the K_m markedly increased on decreasing the pH (the K_m for external citrulline$^+$ was 10.3, 27.3, 83.8 and 213 nM at pH 8.0, 7.5, 7.0 and 6.5, respectively). On the other hand, the observed K_m for total citrulline and the calculated K_m for unprotonated citrulline vary very little with the pH and in the opposite way (i.e., they slightly decrease on lowering the pH) (Table 2). Calculations of the transport affinities for the different citrulline species at various pH values on the internal surface of the reconstituted ornithine/citrulline carrier lead to similar results. This means that the species transported by the carrier are neutral citrulline and H^+, which bind to the carrier separately.

The K_m of H^+ was determined by varying the pH from 6.0 to 8.0. Above and below these pH values the transport activity was drastically reduced, in agreement with data previously reported [11,12]. The K_m value of H^+ was more or less the same on both membrane sides (Table 3) and was independent of the reaction used for its determination, either the ornithine/citrulline+H^+ exchange or the ornithine/H^+ transport mode. The K_m of H^+ corresponded to a pH range of 7.9–8.1. When the experimental data of the pH dependence of the transport rate on H^+ concentration were fitted according to the Hill equation, a Hill coefficient of 0.93 ± 0.16 on the external membrane side and of 0.88 ± 0.10 on the internal membrane side was obtained from five determinations each. These values are in agreement with the existence of a single binding site for H^+ on each surface of the reconstituted carrier and with the electroneutral nature of the ornithine/citrulline+H^+ and ornithine/H^+ transport modes catalyzed by the ornithine/citrulline carrier [11,12]. As previously reported [10,11], H^+ inhibits the ornithine/ornithine exchange as well as the ornithine/citrulline exchange when it is present on the same side as ornithine. The half-inhibition constant of H^+ as inhibitor of ornithine transport was determined from the dependence of the ornithine/ornithine exchange rate on external ornithine concentration at external pH 7.0 and 8.0 (internal pH, 8.0; internal ornithine, 30 mM). The half-inhibition constant of H^+ on ornithine transport, at the external site, was 13 nM corresponding to pH 7.9.

The different K_m values for ornithine on the two membrane sides clearly indicate that the carrier is oriented asymmetrically in the liposomal membrane, as was previously found for other reconstituted mi-

Table 2
External and internal K_m values of citrulline for the reconstituted ornithine/citrulline carrier

Membrane side	Countersubstrate	pH	Citrulline K_m (mM)	Experiments (*n*)
External	Ornithine (30 mM)	8.0	3.8 ± 0.41	(5)
		7.5	3.2	(1)
		7.0	3.1 ± 0.42	(10)
		6.5	2.5 ± 0.50	(5)
	Citrulline (30 mM)	8.0	4.2 ± 2.81	(3)
		6.5	2.5 ± 0.40	(3)
Internal	Ornithine (1.6 mM)	8.0	2.8 ± 0.58	(3)
		7.0	1.9 ± 0.35	(3)
		6.0	1.5 ± 0.28	(3)

K_m values were determined by the forward-exchange method. The range of citrulline concentration was 0.5–5 mM for the determination of external K_m and 0.5–20 mM for the determination of internal K_m. The transport rate was evaluated by the radioactivity taken up in 2 min. Both the external and internal compartment were buffered at the indicated pH as described in Section 2. The values are mean ± S.D. (*n*).

tochondrial transporters [20]. It could be argued that artifacts, for example physical constraints due to the curvature of the membrane, are responsible for the high internal K_m value, i.e., the marked difference in substrate affinity at the two membrane sides. Although this assumption seems to be extremely hypothetical, to confirm the existence of low-affinity binding sites on the internal surface of the carrier, experiments were carried out to achieve a scrambling of the protein orientation. To this end, the proteoliposomes, after formation, were frozen, thawed and sonicated. After this procedure, a non-linear dependence became evident in an Eadie–Hofstee plot of the transport rate of the ornithine/ornithine homoexchange as a function of external ornithine concentration (see Fig. 1). The two different K_m observed, 0.19 mM and 4.5 mM, corresponded well to the external and internal K_m values reported in Table 1. The two kinetic components contributed to the V_{max} in a ratio of approximately 3:1, indicating that the orientation of the carrier was substantially randomized.

3.2. Kinetic transport mechanism

The counterexchange of substrates catalyzed by an antiport carrier follows one of the two basically different two-substrate mechanisms, the ping-pong or the simultaneous (sequential) type [27–29]. In the ping-pong mechanism, the first substrate (transported from side a to side b) is released before the second substrate binds to the carrier at side b. Thus, only one binding site exists which is alternately ex-

Table 3
External and internal K_m values of H^+ for the reconstituted ornithine/citrulline carrier

Membrane side	Cosubstrate	Countersubstrate	H^+ K_m (nM)	Experiments (*n*)
External	Citrulline (30 mM)	Ornithine (30 mM)	8.0 ± 1.4^b	(3)
Internal	None	Ornithine (1.6 mM)	13^f	(1)
External	None	Ornithine (30 mM)	11.6 ± 1.6^b	(6)
Internal	Citrulline (30 mM)	Ornithine (1.6 mM)	8.6 ± 1.8^f	(4)

K_m were determined by the forward (^{f}) or by the backward (^{b}) exchange method. For the determination of internal K_m of H^+, 20 mM PIPES/20 mM Hepes at pH ranging from 6.0 to 8.0 was present in different reconstitution mixtures. After removal of external substrate and buffer by Sephadex G-75 equilibrated and eluted with 60 mM sucrose and 0.2 mM PIPES/0.2 mM Hepes at the same pH present inside, transport was started by the addition of 1.6 mM [^{3}H]ornithine in 20 mM PIPES/20 mM Hepes (pH 8.0) and stopped after 1 min. For the determination of external K_m of H^+, proteoliposomes, reconstituted in the presence of 20 mM PIPES/20 mM Hepes (pH 8.0), were prelabeled by carrier-mediated exchange equilibration. After removal of external substrate and buffer by Sephadex G-75 equilibrated and eluted with 60 mM sucrose and 0.2 mM PIPES/0.2 mM Hepes (pH 8.0), the efflux of [^{3}H]ornithine was started by adding 20 mM PIPES/20 mM Hepes at pH ranging from pH 6.0 to 8.0 and stopped after 2 min. The values are mean ± S.D. (*n*).

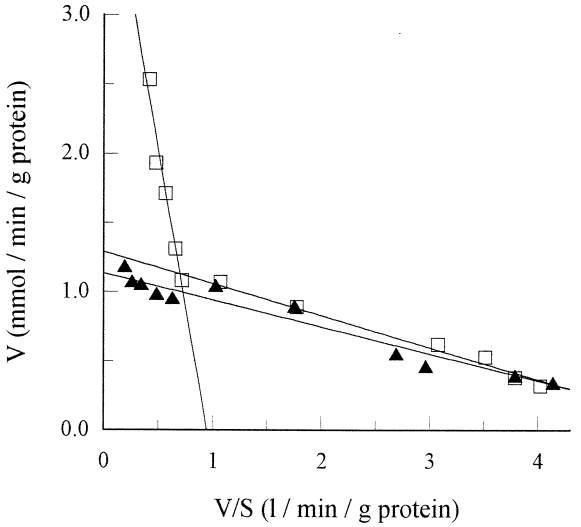

Fig. 1. Reshuffling of internal and external binding sites of the reconstituted ornithine/citrulline carrier after freeze/thaw/sonication of proteoliposomes. K_m values for ornithine were measured at the external membrane side by the forward-exchange method before (▲) and after (□) freeze/thaw/sonication of the proteoliposomes. The internal ornithine concentration was 30 mM. The transport reaction was started by adding [^{3}H]ornithine at concentrations ranging from 0.08 to 6 mM and stopped after 2 min as described in Section 2. The pH was 8.0 in the internal and external proteoliposomal compartment. The K_m values were calculated from the slope of the Eadie–Hofstee plots. S, concentration of external ornithine. In this figure and the subsequent ones, data from representative experiments are reported. Similar results were obtained in at least three independent experiments.

posed to each side of the membrane. The simultaneous mechanism, on the other hand, involves the binding of the two substrates at the same time, leading to the formation of a ternary complex with the carrier protein, before translocation takes place. In order to discriminate between these two possibilities for the reconstituted ornithine/citrulline carrier, the exchange activity had to be analyzed by varying in a single experiment both external and internal substrate concentrations in the K_m range. Fig. 2 shows the results of such a bi-reactant initial-velocity study (two-substrate analysis) of the ornithine/ornithine homoexchange reaction. When the kinetic data were analyzed in Lineweaver–Burk plots, showing the dependence of the transport rate on external ornithine at four different internal concentrations, a pattern of straight lines was obtained converging close to the abscissa axis (Fig. 2A). A similar pattern was observed when plotting the same data as a function of the internal ornithine concentration (Fig. 2B). This intersecting pattern, in contrast to the parallel pattern of a ping-pong reaction, demonstrates that the ornithine/ornithine homoexchange reaction follows a simultaneous mechanism. The intersection point close to the abscissa shows that there was no significant influence of the first, i.e., internal (or external) substrate on the apparent K_m for the second, i.e., external (or internal) substrate. Thus, the binding of the internal and external substrate occurred largely independently of each other. This is indicative of a special case of simultaneous mechanism, i.e., the random reaction mechanism, different from the ordered one characterized by the intersection point on the ordinate [28,30]. The transport rate was stimulated when raising the internal or the external ornithine concentration, as can be recognized from the effect on the apparent V_{max} (ordinate intercept and slope effect). These interrelations could be quantitatively analyzed in secondary plots of the slopes and ordinate intercepts of Fig. 2A and B versus the reciprocal concentration of the respective opposite substrate, as carried out in Fig. 2C and D, respectively (cf. [27,28]). The linear relations obtained allowed us to extrapolate K_m values which were independent of the concentration of the respective countersubstrate. These 'concentration-independent' half-saturation constants (K_m), 0.14 mM and 3.9 mM for external and internal ornithine, respectively, were very similar to the values determined at finite substrate concentrations (see Table 1). From the secondary plots the K_{is} values, representing the dissociation constants of the binary carrier–substrate complexes in a simultaneous mechanism [27,28], were also derived. These constants have a finite value only in the case of simultaneous mechanism. The K_{is} values obtained from Fig. 2C and D, 0.14 mM for external ornithine and 3.9 mM for internal ornithine, were identical to the respective K_m values. 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 in the single-substrate occupied form. Furthermore, the constant ratio between K_m and K_{is} values for external ornithine and for internal ornithine is characteristic of a rapid-equilibrium random mechanism [27,28].

In order to establish whether simultaneous (se-

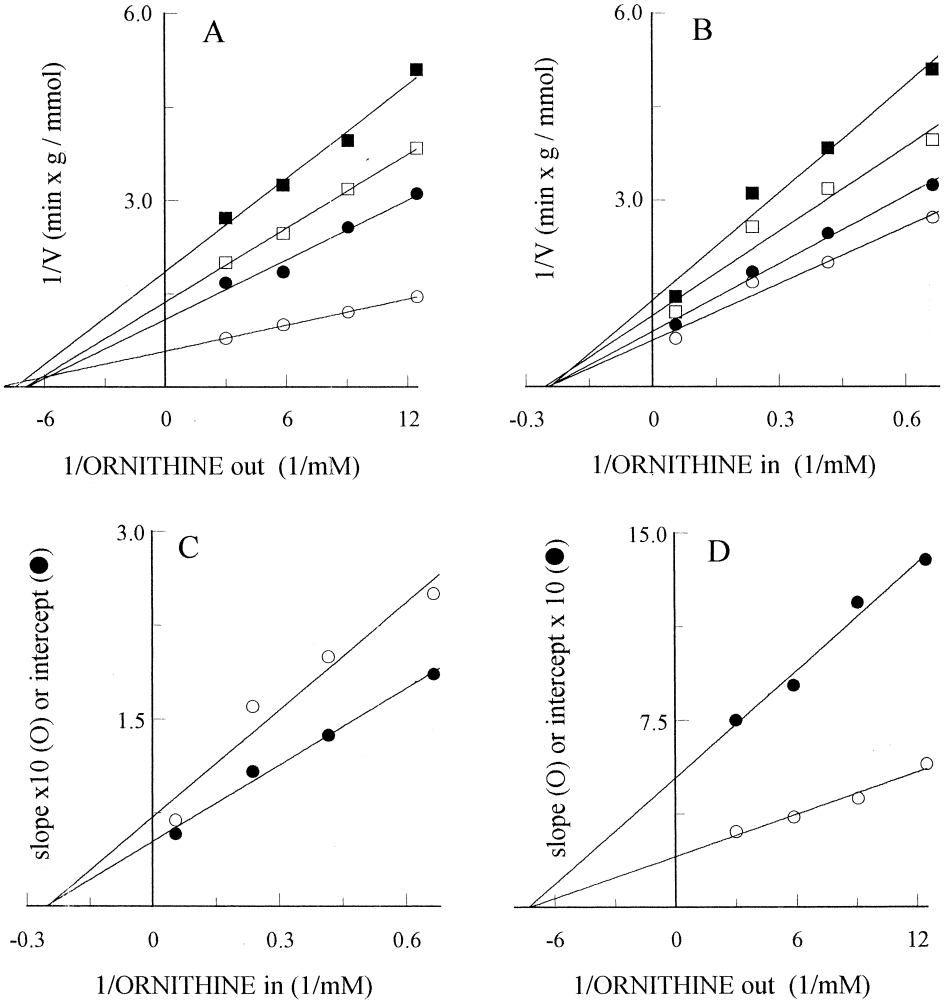

Fig. 2. Two-substrate analysis by the forward-exchange method of the ornithine/ornithine exchange reaction catalyzed by the reconstituted ornithine/citrulline carrier. Lineweaver–Burk plots showing the dependence of exchange rate, measured as [^{3}H]ornithine uptake in 2 min, on external (A) and internal (B) ornithine. The pH was 8.0 both in the internal and external proteoliposomal compartment. The concentrations of the countersubstrates were as follows: (A) 18 (○), 3.9 (●) 2.3 (□) and 1.5 (■) mM internal ornithine; (B) 0.33 (○), 0.17 (●), 0.11 (□) and 0.08 (■) mM external [^{3}H]ornithine. (C,D) Slope (○) and intercept (●) replots of the primary plots A and B, respectively.

quential) kinetics is in fact the general transport mechanism of the reconstituted ornithine carrier, it was important to determine whether this particular mechanism also holds true for the ornithine/citrulline+H^+ heteroexchange reaction. In view of the likely existence of two different binding sites for citrulline and H^+ on the carrier protein (see above), the ornithine/citrulline+H^+ heteroexchange is a three-substrate transport reaction. This reaction can be analyzed by ter-reactant initial velocity study [28] by varying the concentration of two substrates and keeping the third substrate constant and close to saturation. When the concentrations of ornithine (out) and citrulline (in) were varied at constant internal and external pH, the same intersecting kinetic pattern was obtained (Fig. 3), very similar to that shown in Fig. 2 for the ornithine/ornithine homoexchange reaction. This indicates that homo- and heteroexchange reactions follow the same basic mechanism. The concentration-independent K_m values for internal citrulline and for external ornithine, extrapolated from the replots (not shown) of Fig. 3A and B, were 3.5 mM and 0.19 mM, respectively. The K_{is} values, that in this case, i.e., the ornithine/citrulline+H^+ ex-

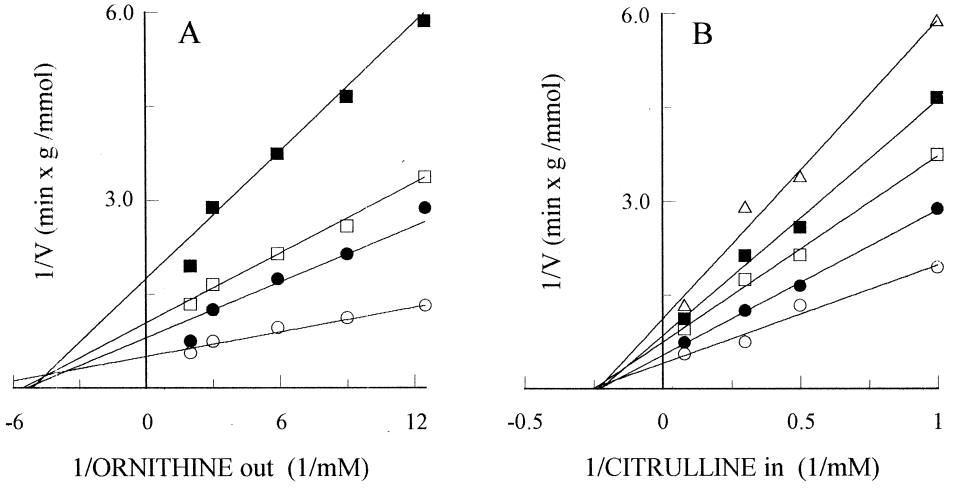

Fig. 3. Two-substrate analysis by the forward-exchange method of the ornithine/citrulline exchange reaction catalyzed by the reconstituted ornithine/citrulline carrier. Lineweaver–Burk plot showing the dependence of exchange rate, measured as [^{3}H]ornithine uptake in 2 min, on external ornithine (A) and internal citrulline (B). The pH was 7.5 in the internal and external proteoliposomal compartment. The concentrations of internal citrulline (A) were 12 (○), 3.3 (●), 2 (□) and 1 (■) mM; the concentrations of external [^{3}H]ornithine (B) were 0.5 (○), 0.33 (●), 0.17 (□), 0.11 (■) and 0.08 (△) mM.

change, represent the dissociation constants of a pseudo-binary complex between one of the variable substrates and the carrier (to which the third, invariable substrate, i.e., the H^+, is bound), were 0.21 mM and 4.2 mM for external ornithine and internal citrulline, respectively.

In another set of experiments, the initial velocity study of the ornithine (out)/citrulline (in)+H^+ (in) exchange was carried out by varying the concentrations of H^+ and ornithine or, alternatively, the concentrations of H^+ and citrulline in the same experiment. The data from typical experiments are shown

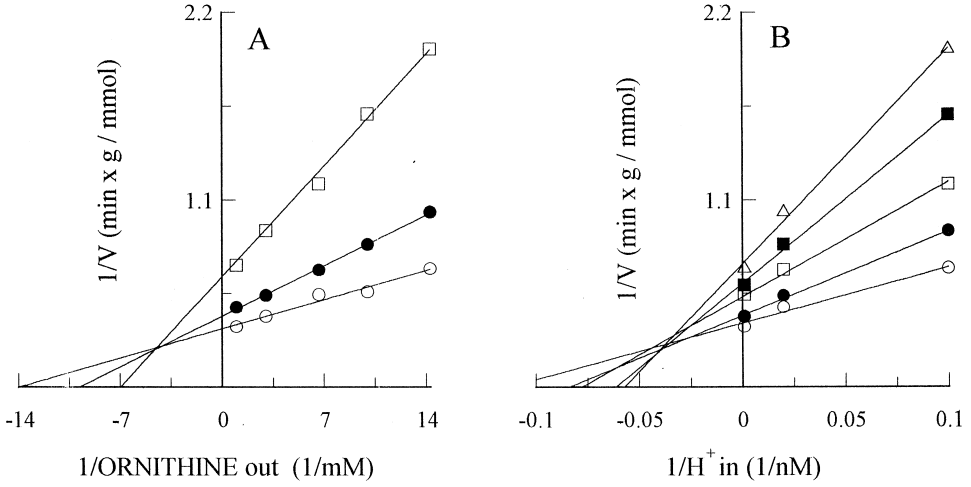

Fig. 4. Two-substrate analysis by the forward-exchange method of the ornithine/H^+ exchange in the presence of internal citrulline catalyzed by the reconstituted ornithine/citrulline carrier. Lineweaver–Burk plot showing the dependence of exchange rate, on external ornithine (A) and internal H^+ (B). After removal of external substrate and buffer by Sephadex G-75 column, equilibrated and eluted with 0.2 mM PIPES/0.2 mM Hepes at the same pH present inside and 40 mM sucrose, the transport reaction was started by adding [^{3}H]ornithine at the indicated concentration in 20 mM PIPES/20 mM Hepes (pH 8.0) and stopped after 2 min. The concentrations of internal H^+ (A) were 1000 (○), 50 (●) and 10 (□) nM. The concentrations of external ornithine (B) were 1 (○), 0.33 (●), 0.15 (□), 0.1 (■) and 0.07 (△) mM. The concentration of internal citrulline was 40 mM in both A and B.

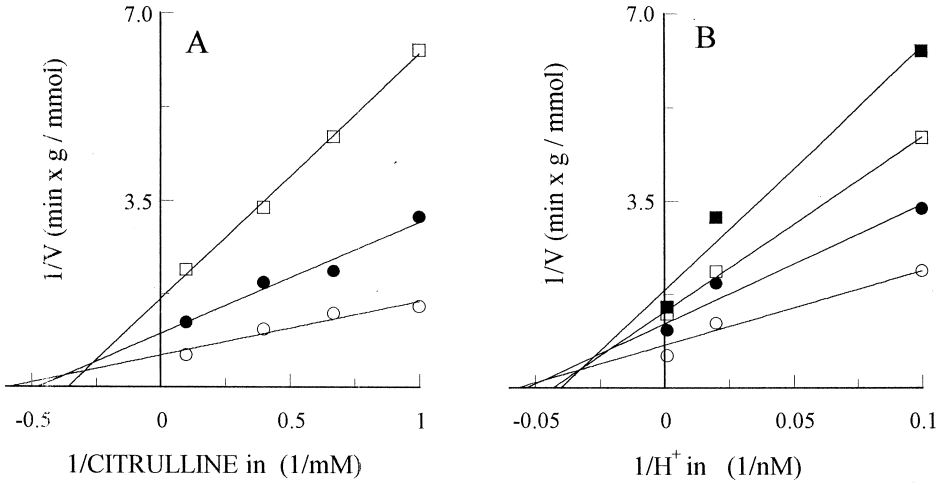

Fig. 5. Two-substrate analysis by the forward-exchange method of the citrulline+H^+ cotransport in exchange for ornithine catalyzed by the reconstituted ornithine/citrulline carrier. Lineweaver–Burk plot showing the dependence of exchange rate on internal citrulline (A) and H^+ (B). After removal of external substrate and buffer by Sephadex G-75 column, equilibrated and eluted with 0.2 mM PIPES/0.2 mM Hepes at the same pH present inside and an appropriate sucrose concentration to balance the internal osmolarity, the transport reaction was started by adding 1.6 mM [^{3}H]ornithine in 20 mM Hepes/20 mM PIPES, pH 8.0. The concentrations of internal H^+ (A) were 1000 (○), 50 (●) and 10 (□) nM. The concentrations of internal citrulline (B) were 10 (○), 2.5 (●), 1.5 (□) and 1 (■) mM.

in Figs. 4 and 5, respectively. All kinetic patterns obtained were of the same, i.e., intersecting, type and the point of intersection was usually located close to the abscissa axis and, in any case, far from the ordinate. The K_m/K_{is} ratios in all experiments were almost constant for each pair of substrates tested. This ratio was sometimes a little higher than 1, indicating some influence of the second varied substrate on the binding affinity of the first; for example, K_m/K_{is} derived from the secondary plots of Fig. 4A and B was 2.2 for both external ornithine and internal H^+. Despite these variations, all data shown are in agreement with a rapid-equilibrium random mechanism and clearly rule out the occurrence of an ordered reaction mechanism for the reconstituted ornithine/citrulline carrier.

4. Discussion

When studying a reconstituted carrier, it is important to know whether the protein is oriented unidirectionally within the liposomal membrane. In the absence of side-specific inhibitors, unidirectionality of membrane insertion has previously been elucidated for reconstituted transporters by determining the sidedness of external and internal binding sites characterized by different affinity constants (see [20] for Refs. and [31–33]). In the present study we used the same approach for the reconstituted ornithine/citrulline carrier. The results of Tables 1–3 show that the apparent K_m values on the inside are not very significantly different from those on the outside in the case of citrulline and H^+. However, for internal and external ornithine, the difference is so great (external K_m about 20-fold lower than the internal one) and the statistical error is low enough for reliable discrimination. Furthermore, in the kinetic analysis no significant amount of a carrier population with high K_m ('internal K_m') was found at the outside and vice versa. As well as this result, two independent lines of evidence are in favor of distinct K_m values for ornithine on the two opposite sides. When applying two independent methods of analysis, the same ratio of internal and external K_m for ornithine was obtained in forward-exchange (23.7) and backward-exchange (25.0) determinations at pH 8.0. Furthermore, when scrambling the orientation of the carrier by sonication of the proteoliposomes, in fact both expected K_m values were found at the external

side. These results indicate that the reconstituted functionally active ornithine/citrulline carrier molecules are inserted in the lipid bilayer with highly preferential orientation. Since the K_m value for H^+ is very close to the pKa of the side chain of cysteine [34], it is likely that the binding site for H^+ is a thiolate ($-S^-$). In this respect, it should be mentioned that the recently identified mammalian mitochondrial ornithine carrier contains 9 cysteines [17] and sulfhydryl reagents are known to inhibit the activity of the ornithine/citrulline carrier [9].

To ascertain whether the orientation of the carrier observed in the liposomes is the same as in the mitochondrial membrane, a comparison between the K_m values found in the reconstituted system and those obtained in intact mitochondria had to be performed. In mitochondria, however, only the K_m for external ornithine has been reported [4]. This value (1 mM) is higher than the external and lower than the internal K_m for ornithine as measured in proteoliposomes. However, we previously found that the external K_m of ornithine in proteoliposomes depends on the concentration of cations in the incubation medium [10]. For example, it increased 2.5 times (from 0.16 to 0.4 mM) by increasing the Na^+ concentration from 20 to 50 mM [10]. Since the K_m value determined using mitochondria [4] was obtained in the presence of 120 mM KCl, it is likely to correspond to the K_m of the external side of proteoliposomes. In addition, some metabolic considerations have to be taken into account. An important function of the ornithine/citrulline carrier is to catalyze the exchange of cytosolic ornithine for intramitochondrial citrulline. Thus, in the urea cycle ornithine has to enter mitochondria, whereas citrulline has to leave the mitochondria. In isolated hepatocytes, the concentration of ornithine is 1.6 mM in the matrix and 0.6 mM in the cytosol [35]. The concentration of citrulline is not known, though the existence of a high mitochondrial/cytosolic gradient of citrulline is supported by the studies of Cheung et al. [36]. For all these reasons it is conceivable that the high affinity binding site (K_m, 0.16 mM) for ornithine is located on the cytosolic face of the carrier. The opposite orientation would limit the binding of cytosolic ornithine to the carrier as well as the binding of intramitochondrial ornithine to the ornithine transcarbamilase, whose K_m for ornithine is about 0.4 mM or higher [37,38]. In summary, although we cannot decide unequivocally whether the orientation of the reconstituted protein is right-side-out or inside-out compared to mitochondria, on the basis of the K_m found in mitochondria for external ornithine and of metabolic considerations we suggest a right-side-out orientation.

Fortunately, with respect to the kinetic mechanism, the situation is not at all ambiguous. The two-reactant initial-velocity studies reported in Figs. 2–5 clearly revealed a simultaneous (sequential) type of mechanism. The intersecting lines in the reciprocal plots and the definite slopes obtained in secondary plots can only be interpreted in terms of this kinetic mechanism [27,28]. The same kinetic pattern was observed not only for the ornithine/ornithine homologous exchange but also for the ornithine/citrulline+H^+ heterologous exchange. Further observations are in line with this conclusion. Firstly, the concentration-independent K_m values, obtained by extrapolation from secondary plots, are similar to the apparent K_m values at finite substrate concentration. Secondly, the K_{is} values, representing substrate interaction with the unloaded carrier (in the case of the ornithine/ornithine exchange) or with the carrier to which the third, invariable substrate is bound (in the case of the ornithine/citrulline+H^+ exchange), are close to the K_m values for each substrate tested. Thus, the affinity is almost unchanged whether binding occurs to the free carrier, the single-substrate-occupied carrier (ornithine/ornithine exchange) or the double-substrate-occupied carrier (ornithine/citrulline+H^+ exchange). Thirdly, the intersection point in the reciprocal plots is close to the abscissa and in any case far from the ordinate. All these findings are in agreement with a special case of a simultaneous mechanism, i.e., a rapid-equilibrium random mechanism [27–30]. This means that there is no obligatory order in which the two (or three) substrates bind to the carrier. The simultaneous mechanism shown here for the reconstituted ornithine/citrulline carrier implies the occurrence of a complex between the substrates and the carrier molecule during the catalytic cycle of antiport before the translocation step occurs. With respect to its kinetic mechanism, the ornithine/citrulline transporter resembles the mitochondrial carrier proteins that have so far been kinetically characterized in the reconstituted

system [20] and differs from the mitochondrial carnitine/acylcarnitine carrier that functions according to a ping-pong type of mechanism.

Acknowledgements

This work was supported by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), by the C.N.R. Target Project on Biotechnology, by Piano Biomedicina, progetto n.1 cluster CO4 legge 488/92 and by the European Social Fund.

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