The purified and reconstituted ornithine/citrulline carrier from rat liver mitochondria catalyses a second transport mode: ornithine⁺/H⁺ exchange

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The mechanism of unidirectional transport of ornithine (i.e. in the absence of a counter-metabolite) has been investigated in proteoliposomes reconstituted with the ornithine carrier purified from rat liver mitochondria. The efflux of [³H]ornithine from proteoliposomes was stimulated by the addition of H⁺ (but not of other cations) to the incubation medium. On keeping the pH in the compartment containing ornithine constant at 8.0, the flux of ornithine into or out of the proteoliposomes increased on decreasing the pH in the opposite compartment from 8.0 to 6.0. Ornithine influx was also stimulated when a higher H⁺ concentration was generated inside the vesicles relative to the outside by the K⁺/H⁺ exchanger nigericin in the presence of an outwardly directed K⁺ gradient. A valinomycin-induced electrogenic flux of K⁺ did not affect ornithine transport in the absence of a counter-

metabolite. Furthermore, changes in fluorescence of the pH indicator pyranine, included inside the proteoliposomes, showed that the flux of ornithine is accompanied by translocation of H⁺ in the opposite direction. It is concluded that the mitochondrial ornithine carrier catalyses an electroneutral exchange of ornithine⁺ for H⁺, in addition to the well-known 1:1 exchange of metabolites. Lysine⁺, but not citrulline, can also be exchanged for H⁺ by the ornithine carrier. The ornithine⁺/H⁺ transport mode of the exchanger is an essential step in the catabolism of excess arginine.

Key words: arginine catabolism, lysine transport, membranes, ornithine aminotransferase, proteoliposomes.

INTRODUCTION

The ornithine carrier, also known as the ornithine/citrulline carrier, is a metabolite transport protein of the mitochondrial inner membrane that has been purified from rat liver mitochondria by chromatography on hydroxyapatite, DEAE-Sephacel and celite [1]. The purified protein shows an apparent molecular mass of 33.5 kDa on SDS/PAGE, a value that falls into the range of molecular masses shown by all mitochondrial metabolite carriers purified so far [2]. The amino acid sequence of the rat liver ornithine carrier is not yet known. However, the recently identified ornithine carrier from *Saccharomyces cerevisiae* [3] shows all the sequence features that are characteristic of the mitochondrial carrier family (see [2,4–7] for reviews). Upon reconstitution into liposomes, the purified rat liver ornithine carrier has been found to catalyse highly active ornithine/citrulline exchange and a low activity of unidirectional transport of ornithine (i.e. uptake by proteoliposomes in the absence of internal substrate) [1].

Because of the high activity of ornithine/citrulline exchange and its importance in the urea cycle [1,8–10], recent investigations with the purified and reconstituted ornithine transport protein have focused on the exchange properties of the carrier. Besides ornithine and citrulline, lysine and arginine are also transported by the antiport mechanism [1,10]. The half-saturation constant of the carrier for external ornithine is much lower than that for the other substrates, and does not depend on the type of counter-substrate under saturating internal concentrations [10]; the maximum exchange transport rate is independent of the nature of the substrate present at the external or internal space of the proteoliposomes [10]. Furthermore, the carrier-catalysed 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 [8].

At variance with the exchange transport mode catalysed by the ornithine carrier, the mechanism of the unidirectional transport of ornithine (in the absence of a counter-metabolite) previously observed in proteoliposomes [1] has not yet been characterized. Unfortunately, the early studies performed in isolated mitochondria provided only preliminary and contradictory results regarding the mode of translocation of ornithine across the mitochondrial membrane. First, Gamble and Lehninger [11] postulated that ornithine is transported into mitochondria electrophoretically. However, Bryla and Harris [12] and McGivan et al. [13] showed that the uptake of ornithine is not dependent on respiratory energy. McGivan et al. [13] found that ornithine uptake was stimulated by acidification of the matrix induced by the addition of dinitrophenol to mitochondria respiring in the presence of valinomycin and K⁺, and suggested that ornithine is taken up in exchange for H⁺ (see also [14]). However, in a subsequent study, Aronson and Diwan [15] could not obtain evidence for ornithine/H⁺ exchange.

In the present paper we have investigated in detail the transport of ornithine in the absence of a counter-metabolite in liposomes reconstituted with the purified rat liver ornithine carrier. The data presented show that the carrier catalyses an electroneutral ornithine⁺/H⁺ exchange, in addition to the 1:1 exchange of metabolites. The ornithine⁺/H⁺ transport mode exhibits a rate that is only 3–4 times lower than that of ornithine/ornithine exchange. The physiological implications of this ornithine⁺/H⁺ exchange are discussed.

EXPERIMENTAL

Materials

Hydroxyapatite (Bio-Gel HTP) and Bio-Beads SM-2 were purchased from Bio-Rad; Celite 535 was from Roth; DEAE-

Abbreviation used: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

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Sephacel, Sephadex PD-10, Sephadex G-50 and Sephadex G-75 were from Pharmacia; L-[U- ^{14}C]lysine was from Amersham International, L-[ureido- ^{14}C]citrulline was from Du Pont-NEN; L-[2,3- ^3H]ornithine, egg yolk phospholipids (L- α -phosphatidylcholine from fresh turkey egg yolk), Hepes, Triton X-100, valinomycin, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazine (FCCP) and nigericin were from Sigma; and pyranine was from Molecular Probes. All other reagents were of analytical grade.

Purification and reconstitution of the ornithine/citrulline carrier

The ornithine/citrulline carrier was purified from rat liver mitochondria as previously described [1]. The purified protein was reconstituted into liposomes by cyclical removal of the detergent with a hydrophobic column [16]. In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through a column filled with Bio-Beads SM-2 resin. The composition of the initial mixture used for reconstitution was: 400 μl of purified protein in 3% (w/v) Triton X-100 (Celite eluate; about 1 μg of protein), 100 μl of 10% (w/v) egg yolk phospholipids in the form of sonicated liposomes prepared as previously described [17], 20 mM L-ornithine or 30 mM L-citrulline or 30 mM L-lysine (unless otherwise indicated) and 20 mM Hepes, pH 8.0 (unless otherwise indicated), 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 cm $\times$ 2.5 cm), pre-equilibrated with the same buffer and substrate present in the initial mixture. All the operations were performed at 4 $^{\circ}\text{C}$, except the passages through the Bio-Beads SM-2 column, which were performed at room temperature.

Transport measurements

The external substrate was removed by passing 550 μl of proteoliposomes through a Sephadex G-75 column (0.7 cm $\times$ 15 cm) pre-equilibrated with 10 mM Hepes, pH 8.0 (unless otherwise indicated), and an appropriate concentration of sucrose to balance the internal osmolarity. The first 600 μl of the turbid eluate from the Sephadex column was collected, transferred to reaction vessels (100 μl each), and used for transport measurements by the inhibitor stop method [18]. For uptake measurements, transport was initiated by adding 10 μl of the labelled substrate (^3H]ornithine, ^{14}C]citrulline or ^{14}C]lysine) at the final concentrations indicated in the legends to the Figures. After the desired time interval, the reaction was stopped by adding 10 μl of 330 mM pyridoxal 5'-phosphate [16]. In control samples, the inhibitor was added together with the labelled substrate at time zero. The assay temperature was 25 $^{\circ}\text{C}$. The external radioactivity was removed by passing the samples (100 μl) through a Sephadex G-50 column (0.6 cm $\times$ 8 cm). The liposomes eluted with 1.2 ml of 40 mM NaCl were collected in 4 ml of scintillation mixture and vortexed, and the radioactivity was counted. The experimental values were corrected by subtracting the respective control value. The transport rate was evaluated either from the radioactivity taken up by the proteoliposomes within the initial linear range of substrate uptake, or from time-course experiments fitted according to a first-order equation, as the product of k (first-order rate constant) and A (substrate taken up at infinite time) [16].

For efflux measurements, proteoliposomes containing 20 mM ornithine, 30 mM citrulline or 30 mM lysine were prelabelled by carrier-mediated exchange equilibration before starting the transport assay [16]. This was achieved by incubating the proteoliposomes (600 μl), previously passed through Sephadex

G-75 in order to remove the external substrate (see above), with 10 μl of 0.3 mM ^3H]ornithine (proteoliposomes with internal ornithine), 0.3 mM ^{14}C]citrulline (proteoliposomes with internal citrulline) or 0.3 mM ^{14}C]lysine (proteoliposomes with internal lysine) at high specific radioactivity (3–10 $\mu\text{Ci/nmol}$) for 20 min at 25 $^{\circ}\text{C}$. Then the external radioactivity was removed by again passing the proteoliposomes through Sephadex G-75 as described above, except that this chromatography was performed at 0 $^{\circ}\text{C}$ to minimize the loss of internal substrate. Transport was started by adding unlabelled external substrate or buffer alone at the concentrations indicated in the legends to Tables and Figures, and stopped, after appropriate time intervals, as described above. The initial rate of efflux (reported as c.p.m./min) was obtained from the time course of the efflux by fitting the experimental data to a single-exponential decay equation [16] from which the efflux rate was calculated as the product of k (the first-order rate constant) and A (the radioactivity that had effluxed from proteoliposomes at infinite time). In some experiments the data were expressed as efflux activity. This was calculated as (c.p.m.)₀ – (c.p.m.)_{*t*}, where (c.p.m.)₀ is the intraliposomal radioactivity at time zero and (c.p.m.)_{*t*} is the residual intraliposomal radioactivity at time *t*. Since the efflux of labelled substrate was almost linear for the first 4 min, the efflux activity, when determined within 4 min, was nearly proportional to the efflux rate. Comparisons among efflux activities or efflux rates were done only among samples within a single proteoliposomal preparation.

Other methods

K⁺-diffusion potentials were generated by adding valinomycin (1.5 $\mu\text{g}/\text{mg}$ of phospholipid) to proteoliposomes in the presence of different KCl gradients. For the formation of an artificial ΔpH (acidic inside), nigericin (100 ng/mg of phospholipid) was added to proteoliposomes in the presence of an outwardly directed potassium gradient. Changes in intraliposomal pH were monitored by measuring the fluorescence of pyranine included inside the proteoliposomes. In these experiments, 2 mM pyranine was added to the reconstitution mixture. Then the external dye was removed by passing the proteoliposomes through Sephadex G-75 as described above. Fluorescence was measured using excitation and emission wavelengths of 460 and 520 nm respectively [19]. The active intraliposomal volume (i.e. the volume of the liposomes containing active carrier) was determined as described previously [20] with 0.1 mM ^3H]ornithine. The total internal volume of the liposomes was measured as described in [21]. The protein concentration was determined by the Lowry method modified for the presence of non-ionic detergents [22].

RESULTS

H⁺-induced efflux of ornithine from proteoliposomes reconstituted with ornithine carrier purified from rat liver mitochondria

In liposomes reconstituted with the purified ornithine carrier, we have previously observed a low uptake of ornithine in the absence of internal substrate [1]. In order to obtain information about the mechanism of this ornithine transport, the efflux of ^3H]ornithine from preloaded proteoliposomes was measured (Figure 1A). In the absence of external substrate, at external pH 8.0 (with or without 10 mM buffer) a slow efflux of ^3H]ornithine was detected (Figure 1A); its rate (40 c.p.m./min) was much lower than that measured in the presence of 5 mM external ornithine (ornithine/ornithine exchange; rate 567 c.p.m./min) or 20 mM external citrulline (ornithine/citrulline exchange; rate 394 c.p.m./min). Interestingly, upon addition of 0.1 mM HCl to

Figure 1 Stimulation by external H⁺ of [³H]ornithine and [¹⁴C]lysine efflux from proteoliposomes

Proteoliposomes were reconstituted in the presence of 20 mM ornithine (A) or 30 mM lysine (B). The internal substrate pool was labelled by carrier-mediated exchange equilibration. The proteoliposomes were then passed through Sephadex G-75 columns, pre-equilibrated with 60 mM sucrose and 10 mM Hepes at pH 8.0 (□, ■, ●, × and △) or with 80 mM sucrose at pH 8.0 (▲, ○ and ▽). The efflux of [³H]ornithine or [¹⁴C]lysine was started by the following additions: ▲ and □, none; ■, 5 mM ornithine; ●, 20 mM citrulline; ○, 0.1 mM HCl; ×, 30 mM pyridoxal 5'-phosphate; △, 5 mM ornithine plus 30 mM pyridoxal 5'-phosphate; ▽, 0.1 mM HCl plus 30 mM pyridoxal 5'-phosphate. Similar results were obtained in three independent experiments.

unbuffered external medium, in the absence of counter-substrate the efflux was strongly stimulated and its rate was increased approx. 5-fold (198 c.p.m./min). Both the efflux at pH 8.0 and the HCl-induced efflux were inhibited by 30 mM pyridoxal 5'-phosphate, as was the efflux induced by ornithine (Figure 1A, upper curves). No effect on ornithine efflux in the absence of external substrate was observed upon addition of 0.1 mM Tris/HCl, pH 8.0 (results not shown), indicating that the stimulation of efflux was not due to Cl⁻. As shown in Figure 1(B), similar results were obtained with proteoliposomes containing [¹⁴C]lysine, another positively charged substrate of the ornithine carrier [1,8]. These results suggest that the ornithine carrier can catalyse an exchange of ornithine (or lysine) for protons, besides the previously characterized ornithine/ornithine homo-exchange and the hetero-exchange of ornithine with citrulline (or lysine) [1,8,10].

In order to investigate whether other positive ions can substitute for H⁺ in stimulating the transport of ornithine, we measured the influence of various externally added cations on the efflux of [³H]ornithine from proteoliposomes. As shown in Table

Table 1 Dependence of [³H]ornithine or [¹⁴C]citrulline efflux from proteoliposomes on the addition of cations or metabolites

Proteoliposomes were reconstituted in the presence of 20 mM ornithine (Expt. 1) or 30 mM citrulline (Expt. 2). Then the internal substrate pool was labelled by carrier-mediated exchange equilibration. Efflux was started by the addition of cations as Cl⁻ salts or substrates, and stopped after 4 min. In the case of HCl addition, the proteoliposomes had been passed through Sephadex G-75 columns pre-equilibrated with 80 mM sucrose, pH 8.0, rather than with 60 mM sucrose/10 mM Hepes, pH 8.0. The values are means ± S.E.M. for three experiments.

	Internal substrate	External addition (mM)	Efflux activity (c.p.m./4 min)
Expt. 1	Ornithine	None	162 ± 14
	Ornithine	Na ⁺ (10)	153 ± 16
	Ornithine	K ⁺ (10)	148 ± 22
	Ornithine	Li ⁺ (10)	136 ± 14
	Ornithine	NH ₄ ⁺ (10)	136 ± 19
	Ornithine	Ca ²⁺ (5)	80 ± 11
	Ornithine	Mg ²⁺ (5)	92 ± 10
	Ornithine	H ⁺ (0.1)	691 ± 103
	Ornithine	Citrulline (10)	1716 ± 154
	Ornithine	Ornithine (5)	1728 ± 164
Expt. 2	Citrulline	None	< 10
	Citrulline	Na ⁺ (10)	< 10
	Citrulline	Ca ²⁺ (5)	< 10
	Citrulline	H ⁺ (0.1)	< 10
	Citrulline	Ornithine (5)	526 ± 74
	Citrulline	Citrulline (10)	56 ± 10

1, among the cations tested only H⁺ ions were capable of stimulating the efflux of ornithine from proteoliposomes. Na⁺, K⁺, Li⁺ or NH₄⁺ at 10 mM caused little, if any, inhibition, while 5 mM Ca²⁺ or Mg²⁺ led to a clear inhibition of ornithine efflux. In these experiments the activity of the H⁺-induced efflux of ornithine was about 2.5 times lower than the activities of the ornithine/ornithine and ornithine/citrulline exchanges, in good agreement with the ratios that can be calculated from the results of Figure 1(A). Expt. 2 in Table 1 further shows that there was no detectable efflux of [¹⁴C]citrulline from citrulline-loaded proteoliposomes with or without the addition of H⁺. These results suggest that the ornithine carrier is unable to catalyse significant uniport of citrulline or citrulline/H⁺ exchange. As a control, as shown in Table 1, the addition of ornithine to [¹⁴C]citrulline-loaded proteoliposomes caused a considerable efflux of citrulline. The addition of unlabelled citrulline also induced the efflux of labelled citrulline from proteoliposomes. However, the activity of citrulline/citrulline exchange was much lower than that of ornithine/citrulline exchange. It should be noted that, in all the efflux experiments performed with [¹⁴C]citrulline, the amount of radioactivity accumulated into proteoliposomes during the prelabelling procedure, by carrier-mediated exchange equilibration (see Experimental section), was nearly 10 times lower than that accumulated in the experiments with [³H]ornithine. Several factors may contribute to the low uptake of [¹⁴C]citrulline under the experimental conditions used, i.e. the low activity of citrulline/citrulline exchange (in agreement with the results of Table 1), the relatively low specific radioactivity of [¹⁴C]citrulline, and the high K_m of the carrier for citrulline (see [10]).

Influence of the pH gradient on the transport of ornithine in reconstituted liposomes

If the reconstituted ornithine carrier catalyses ornithine/H⁺ exchange, it should be expected that the flux of labelled ornithine in reconstituted liposomes is dependent on the proton gradient

Figure 2 Influence of external pH on ornithine⁺_{in}/H⁺_{out} exchange and of internal pH on H⁺_{in}/ornithine_{out} exchange

(A) The proteoliposomes contained 20 mM ornithine and 20 mM Hepes, pH 8.0. After removal of external substrate and buffer by Sephadex G-75 columns (see the Experimental section), equilibrated and eluted with 0.5 mM Hepes, pH 8.0, and 40 mM sucrose, efflux was started by adding 20 mM Pipes/20 mM Hepes at the indicated pH and terminated after 2 min. (B) The reconstitution mixture contained 20 mM Pipes/20 mM Hepes at the pH values indicated. After removal of external buffer by Sephadex G-75 columns, equilibrated and eluted with 40 mM sucrose and 0.5 mM Hepes at the same pH as used in the reconstitution, transport was started by adding 0.1 mM [³H]ornithine together with 20 mM Hepes, pH 8.0. The uptake was stopped after 1 min. The data are from a representative experiment; similar results were obtained in three independent experiments.

across the proteoliposomal membrane. In Figure 2(A) the effect of the external pH at fixed internal pH on the efflux of ornithine from prelabelled proteoliposomes is shown. With an intraliposomal pH of 8.0, ornithine efflux increased on decreasing the external pH from 8.0 to 6.0. Below an external pH of 6, the efflux was diminished due to inactivation of the carrier protein, as previously observed for reconstituted ornithine/ornithine exchange [1]. Similarly, variation of the intraliposomal pH at fixed external pH had a strong influence on the uptake of ornithine by proteoliposomes. Ornithine uptake increased on decreasing the internal pH from 8.0 to 6.0 (Figure 2B). It should be noted that, during the incubation period (1–2 min) used in the experiments of Figures 2(A) and 2(B), the equilibration of the H⁺ gradient was almost negligible, since our proteoliposomal preparations equilibrated a pH gradient of 1 unit in 9–12 min [8]. By showing that an imposed proton gradient (alkaline in the compartment containing ornithine) increases ornithine transport, these experiments support the idea that the ornithine carrier catalyses ornithine/H⁺ exchange.

In another set of experiments planned to verify this hypothesis, a different approach was employed to generate a pH gradient. A higher proton concentration inside the vesicles relative to the outside was created by the addition of the K⁺/H⁺ exchanger nigericin to proteoliposomes in the presence of a K⁺ gradient of 50:1 (in/out; mM/mM). Without nigericin the uptake of labelled

Figure 3 Stimulation of [³H]ornithine entry into proteoliposomes by the pH gradient generated by the addition of nigericin

Proteoliposomes contained 50 mM KCl (□, ■, △, ▲) or 50 mM NaCl (○, ●) and no buffer. The Sephadex G-75 equilibration and elution buffer consisted of 80 mM sucrose and 10 mM Hepes, pH 8.0. Transport was started by the addition of 0.1 mM [³H]ornithine (□, ○) or 0.1 mM [¹⁴C]citrulline (△) and terminated at the indicated times. Nigericin (100 ng/mg of phospholipids) in ethanol (10 µl/ml of proteoliposomes) (■, ●, ▲) or ethanol alone (□, ○, △) was added at 15 min. The data are from a representative experiment; similar results were obtained in three independent experiments.

ornithine increased with time up to 0.25 mmol/g of protein, approaching equilibrium after about 10 min (Figure 3). When nigericin was added at 15 min, a further uptake of labelled ornithine was observed, up to 0.46 mmol/g of protein, which is about twice the value measured in the absence of nigericin. It should be noted that, upon addition of nigericin, a very low stimulation of ornithine uptake was observed in proteoliposomes containing NaCl instead of KCl (Figure 3), and no stimulation at all was detected in proteoliposomes containing sucrose instead of KCl (results not shown). In the same experiment we measured the uptake of [¹⁴C]citrulline instead of [³H]ornithine. In this case, uptake was negligible even upon addition of nigericin (Figure 3). Furthermore, we determined the initial rate of ornithine uptake by proteoliposomes (measured as ornithine/H⁺ exchange) in the presence of nigericin and a K⁺ gradient of 50:1 (in/out; mM/mM) at 25 °C and with 0.1 mM external [³H]ornithine. The value obtained of 0.29 ± 0.06 mmol/min per g of protein (mean $\pm$ S.E.M. of four experiments) should be compared with that of 1.12 ± 0.28 mmol/min per g measured for the rate of ornithine/ornithine exchange in four experiments at 25 °C and with 0.1 mM external ornithine under optimal conditions (see [10]).

Proton translocation accompanying the transport of ornithine in proteoliposomes

The movement of protons across the proteoliposomal membrane was investigated by measuring the fluorescence changes of the pH indicator pyranine, which was included inside the vesicles. It is known that pyranine fluorescence increases with pH [19]. The addition of 20 mM ornithine to proteoliposomes without internal substrate induced alkalization of the internal compartment (Figure 4, curve a), indicating an efflux of protons from the vesicles in exchange with ornithine. A similar, but less pronounced, increase in fluorescence was observed when 20 mM

Figure 4 Fluorescence changes of pyranine-loaded proteoliposomes caused by operation of ornithine⁺/H⁺ exchange

The proteoliposomes without internal substrate and buffer contained the pH indicator pyranine (2 mM) and 20 mM NaCl. After the passage through Sephadex G-75, 150 µl of proteoliposomes was added to 1.5 ml of 10 mM sucrose/10 mM Hepes, pH 8.0. Where indicated by the arrow, the reaction was started by the addition of 20 mM ornithine (trace a), 20 mM lysine (trace b), 20 mM citrulline (trace c) or 20 mM ornithine plus 5 µg of FCCP (trace d). The upward deflection of the trace indicates alkalinization of the intraliposomal space.

Figure 5 Influence of membrane potential on reconstituted ornithine/H⁺ exchange

(A) Efflux of 20 mM internal [³H]ornithine from prelabeled proteoliposomes was measured at external pH 8.0 (○, ●) or at external pH 6.0 (△, ▲) in the presence of a KCl_{in}/KCl_{out} ratio of 1:50 (mM/mM). (B) Uptake of 0.1 mM [³H]ornithine (○, ●) or [¹⁴C]lysine (□, ■) into proteoliposomes was measured at external pH 8.0 in the presence of a KCl_{in}/KCl_{out} ratio of 50:1 (mM/mM). In some cases (●, ▲, ■), valinomycin (1.5 µg/mg of phospholipid) was added in 10 µl of ethanol/ml of proteoliposomes. In the samples without valinomycin, the solvent alone was added. K⁺_{in} was included as KCl in the reconstitution mixture, whereas K⁺_{out} was added as KCl in the Sephadex G-75 equilibration and elution buffer. The differences in osmolarity were compensated by the addition of appropriate concentrations of sucrose in the opposite compartment.

lysine, instead of ornithine, was added to proteoliposomes (Figure 4, curve b). In contrast, when 20 mM citrulline was added to the same proteoliposomal preparation, no variation in internal pH was observed (Figure 4, curve c). Furthermore, no change in fluorescence was detected when ornithine was added to proteoliposomes together with the protonophore FCCP (Figure 4, curve d), indicating transmembrane H⁺ movement. These results demonstrate that the transport of ornithine (or lysine) is accompanied by translocation of H⁺ in the opposite direction.

Electrical nature of ornithine/H⁺ exchange

The reconstituted ornithine/H⁺ exchange is expected to be electroneutral. To verify this hypothesis, the influence of the membrane potential on [³H]ornithine efflux from proteoliposomes was tested. To this aim, a membrane potential (positive inside) was generated by adding valinomycin to proteoliposomes in the presence of an inwardly directed KCl gradient of 1:50 (in/out; mM/mM). Adding valinomycin did not significantly affect the efflux of [³H]ornithine, at external pH 8.0 or pH 6.0 (Figure 5A).

In further experiments the influence of the membrane potential on the uptake of [³H]ornithine by proteoliposomes was tested without internal substrate. Under conditions where a membrane potential (positive outside) was generated by the addition of valinomycin to proteoliposomes in the presence of an outwardly directed KCl gradient of 50:1 (in/out; mM/mM), the uptake of ornithine and of lysine was not influenced by the addition of valinomycin (Figure 5B). Furthermore, the uptake of ornithine or lysine was also not influenced by valinomycin in the presence of an inwardly directed KCl gradient of 1:50 (in/out; mM/mM) or in the absence of KCl (results not shown). It should be noted that the electrophoretic hetero-exchanges between ADP and ATP and between aspartate and glutamate [23–26], catalysed by the purified and reconstituted mitochondrial ADP/ATP and aspartate/glutamate carriers respectively [27,28], were found to be influenced by the membrane potential under the same experimental conditions as for Figures 5(A) and 5(B) (see also [8]). These data are consistent with an electroneutral exchange of ornithine with H⁺.

The ornithine carrier catalyses both ornithine/citrulline and ornithine/H⁺ exchange

The experiments described here were performed with electrophoretically homogeneous ornithine carrier protein [1]. However, in order to obtain further evidence that the same transport protein can catalyse both transport modes, i.e. the ornithine/citrulline and ornithine/H⁺ exchanges, other experiments were carried out.

Figure 6 shows the time-dependence of the effects of trypsin on both ornithine/H⁺ and ornithine/citrulline exchanges. Clearly the kinetics of inactivation of the two different transport modes were coincident (Figure 6). In both cases the half-time of inactivation was 38 min, and the activity was completely abolished after a 5 h incubation with trypsin.

Based on measurements of active and total intraliposomal volume, we determined that, under the reconstitution conditions used in this study, only 0.5–1.5% of the reconstituted vesicles contained an active carrier molecule in their membrane. Under these conditions the possibility that a hypothetical ornithine/H⁺ carrier was incorporated in the same vesicles that contained an ornithine/citrulline carrier is extremely low. For this reason we investigated whether the ornithine taken up by proteoliposomes in the absence of internal substrate (i.e. by ornithine/H⁺ exchange) can be released by adding external unlabelled ornithine

Figure 6 Inactivation of ornithine⁺/H⁺ and ornithine/citrulline exchange by trypsin

Proteoliposomes containing 20 mM [³H]ornithine had been treated with trypsin (5 µg/ml) for the times indicated before the second passage through Sephadex G-75. The residual efflux activity was measured in the presence of external 0.1 mM HCl (●) or 20 mM citrulline (○). The activity of the control (without trypsin) was 680 ± 72 c.p.m./4 min in the presence of external 0.1 mM HCl, and 1380 ± 150 c.p.m./4 min in the presence of 20 mM citrulline. The data are means ± S.E.M. for three experiments.

Figure 7 Time course of [³H]ornithine uptake into proteoliposomes without internal substrate and of its efflux after addition of an excess of ornithine

[³H]Ornithine (1 mM) was added to proteoliposomes reconstituted without substrate. At 27 min (indicated by the arrow), 20 mM unlabelled ornithine was added. Transport was stopped at the indicated times, as described in the Experimental section. The data are from a representative experiment; similar results were obtained in three independent experiments.

(i.e. by ornithine/ornithine exchange). The results of this type of experiment reported in Figure 7 demonstrate that virtually all the radioactivity accumulated in the vesicles was released by the addition of external ornithine, thus indicating that the same transport protein is responsible for both activities.

In further experiments we measured the ratio between the ornithine/citrulline and ornithine/H⁺ exchange activities during the course of carrier purification [1]. The ratio between the ornithine/citrulline and ornithine/H⁺ exchange activities was 3.5 ± 0.6, 2.9 ± 0.6 and 3.4 ± 0.7 after the chromatographic steps on hydroxyapatite, DEAE-Sephacel and Celite respectively (means ± S.E.M. of three independent experiments). The finding that the ratio between the two activities was practically constant during purification of the ornithine carrier further validates the conclusion that a single protein catalyses both transport modes.

DISCUSSION

The experiments described in the present paper were designed to clarify the mechanism of unidirectional transport of ornithine (in the absence of a counter-metabolite) that has been previously observed in liposomes reconstituted with the ornithine carrier purified from rat liver mitochondria [1].

At physiological pH values, ornithine is essentially present as a single charged cation. One possibility is that the ornithine carrier catalyses an electrophoretic transport of ornithine across the proteoliposomal membrane. Our experimental results, however, show that ornithine flux into or from proteoliposomes was not stimulated by inducing a compensatory flux of K⁺ with valinomycin, thus indicating that the carrier-mediated transport of ornithine is not electrophoretic. The alternative possibility that the positive charge of ornithine is compensated by the movement of a H⁺ in the opposite direction is substantiated by several experimental observations. For example, the transport of ornithine into or from proteoliposomes was stimulated by creating a pH difference across the membrane (acidic in the compartment opposite that where ornithine was present). In addition, the trans-stimulation of ornithine flux by H⁺ was sensitive to inhibitors of the ornithine carrier protein and was specific. Furthermore, the uptake of ornithine by proteoliposomes was accompanied by efflux of H⁺ from the vesicles.

It could be argued that the observed ornithine⁺/H⁺ exchange is catalysed by a protein present as a contaminant in the purified ornithine/citrulline carrier preparation. However, several findings reported here contradict this hypothesis, and are all consistent with the conclusion that the same transport protein catalyses both the ornithine⁺/H⁺ and ornithine⁺/citrulline + H⁺ exchange processes. For example, the ratio between the two activities remained virtually the same during purification of the ornithine carrier.

Our results also show that the rate of the ornithine⁺/H⁺ exchange catalysed by the ornithine carrier in the reconstituted system is significant as compared with the rates of the ornithine/citrulline and ornithine/ornithine exchanges. Furthermore, the ornithine⁺/H⁺ exchange rate is of the same order of magnitude as that found for other reconstituted mitochondrial carriers [29,30].

The transport mode for the ornithine carrier described here, i.e. ornithine⁺/H⁺ exchange, may play an important role in cell metabolism under various physiological conditions. The net import of ornithine into the mitochondria is necessary when the dietary content of arginine is high. Under these conditions the ornithine formed by arginine hydrolysis which is in excess of that required for citrulline formation is removed by ornithine aminotransferase, an enzyme that is localized in the mitochondrial matrix [13,31], is abundant in liver and kidney [32,33] and the expression of which is highly regulated in response to the protein level in the diet [33–36]. The ability of this enzyme to catabolize ornithine in isolated rat liver mitochondria has been demonstrated by McGivan et al. [13]. These authors have also shown that the catabolism of ornithine by ornithine aminotransferase is rate-limited by the transport of ornithine into the matrix space. Hepatic ornithine aminotransferase is localized in the pericentral, glutamine synthetase-containing hepatocytes, and not in the hepatocytes containing the urea cycle enzymes [37]. This means that the ornithine/citrulline exchange activity of the transporter is carried out in mitochondria of periportal hepatocytes, whereas ornithine/H⁺ exchange is the function of the same transporter in mitochondria of the pericentral hepatocytes. It should be noted that the conversion of ornithine into glutamate (via glutamate

semialdehyde) occurs with liberation of an H⁺ ion in the mitochondrial matrix:

Therefore the H⁺ transported out by the ornithine carrier via the ornithine⁺/H⁺ exchanger is essentially the proton that is produced by the metabolism of ornithine to glutamate in the mitochondrial matrix. Thus the overall conversion of ornithine (out) into glutamate (in) does not require the net movement of H⁺ across the membrane and would be unaffected by the ΔpH component of the protonmotive force [38], which in liver is about 0.33 pH unit (acidic outside) [39].

The operation of the ornithine⁺/H⁺ exchange in the direction of ornithine efflux from the mitochondria may be involved in other metabolic processes, such as the biosynthesis of polyamines that are produced from ornithine in the cytosol. Under conditions of low arginine content in the diet and/or in tissues such as heart, brain and skeletal muscle where the activity of arginase is negligible [40], ornithine that is produced intramitochondrially from glutamate [33–36] has to be exported to the cytosol to accomplish polyamine biosynthesis. It should be noted that mitochondria from rat heart possess the ornithine carrier, although in smaller amounts than in those from liver (C. Indiveri, unpublished work). In addition, ornithine is also produced in intestinal mitochondria from glutamine, and may supply extra urea cycle intermediates for the liver [41]. Obviously, the ornithine⁺_{in}/H⁺_{out} exchange is favoured by the H⁺ gradient existing across the mitochondrial membrane.

A flux of ornithine from the mitochondria to the cytosol has been shown to be important in lower eukaryotic organisms [42–45]: in *Neurospora crassa* for polyamine biosynthesis and in *Saccharomyces cerevisiae* for arginine biosynthesis. These two organisms contain genes, *arg-11* in *S. cerevisiae* and *arg-13* in *Neurospora crassa*, that encode very similar proteins belonging to the mitochondrial carrier family [2,4–7]. The *S. cerevisiae* protein has been identified as the mitochondrial ornithine carrier by bacterial expression and functional reconstitution into liposomes [3]. The recombinant and reconstituted yeast carrier transports ornithine (and lysine) in exchange for H⁺, as well as exchange of substrates, and differs from the rat ornithine carrier by its inability to accept citrulline. Since we have shown that the ornithine carrier isolated from rat liver mitochondria transports ornithine, lysine and citrulline by an electroneutral antiport mechanism [8] and, in addition, carries out ornithine⁺ (or lysine⁺)/H⁺ exchange (the present paper), the earlier suggestion that rat liver mitochondria have two transport systems for ornithine [46], one for ornithine (and lysine) flux via ornithine⁺/H⁺ antiport and the second for ornithine import in exchange for citrulline, is clearly superfluous. It is likely that the mammalian transporter has evolved in order to retain the ability to catalyse ornithine⁺/H⁺ exchange and to accept citrulline as a substrate. In this way the mammalian carrier can fulfil, besides the functions mentioned above, that of exchanging cytosolic ornithine and intramitochondrial citrulline, which is an essential step in the urea cycle.

This work was supported by the C.N.R. Target Project on Biotechnology, by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), and by the European Social Fund.

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