The purified and reconstituted ornithine/citrulline carrier from rat liver mitochondria: electrical nature and coupling of the exchange reaction with H^+ translocation

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The mechanism and the electrical nature of ornithine/citrulline exchange has been investigated in proteoliposomes reconstituted with the ornithine/citrulline carrier purified from rat liver mitochondria. The stoichiometry of the exchanging substrates was close to 1:1. The exchange was not affected by inducing electrogenic flux of K^+ with valinomycin. In contrast, the pH gradient generated by the K^+/H^+ exchanger nigericin in the presence of an outwardly directed K^+ gradient stimulated the ornithine$_{out}$/citrulline$_{in}$ exchange, but not the ornithine/ornithine homoexchange. Experiments in which either the internal or the external pH was varied, while keeping constant the pH in the other compartment, indicated that maximal exchange rates are found at pH 6 in the compartment containing citrulline and at pH 8 in the compartment containing ornithine. Changes in fluorescence of the pH indicator pyranine, included inside the proteoliposomes, showed that the exchanges ornithine$_{out}$/citrulline$_{in}$ and citrulline$_{out}$/ornithine$_{in}$ are accompanied by translocation of H^+ in the same direction as citrulline. It is concluded that the mitochondrial ornithine/citrulline carrier catalyses an electroneutral exchange of ornithine$^+$ for citrulline plus an H^+. A reasonable model is one in which ornithine binds to a deprotonated carrier and citrulline to a protonated carrier and both substrate–carrier complexes are neutral. The physiological implications of this transport process are discussed.

INTRODUCTION

The urea cycle is accomplished by the activity of enzymes located partly in the cytosol and partly in the intramitochondrial space. Owing to this compartmentation, ornithine, which is formed in the cytosol, must enter the mitochondrial matrix, where it is carbamoylated to citrulline, and citrulline must leave the mitochondria in order to regenerate ornithine. Early work performed in intact mitochondria provided only preliminary and contradictory results about the mode of transport of ornithine and citrulline across the mitochondrial inner membrane [1–7]. Gamble and Lehninger suggested two independent transport systems, both of which are required for the operation of the urea cycle: one electrophoretic specific for the ornithine cation and the other electroneutral for citrulline ([1], but see [2–4]). Bradford and McGivan, however, postulated the existence of an ornithine/citrulline exchange system ([5,6], but see [7,8]).

We have previously purified the ornithine/citrulline carrier from rat liver mitochondria [9]. In SDS-containing gels, the purified fraction consists of a single band with an apparent molecular mass of 33.5 kDa. Upon incorporation into phospholipid vesicles, the purified carrier catalyses a highly active ornithine/citrulline exchange and a negligible activity of unidirectional transport of ornithine. The carrier has been characterized with respect to substrate specificity, inhibitor-sensitivity and kinetic properties [9,10]. It has a very narrow specificity, since only lysine and arginine, besides ornithine and citrulline, are transported by an antiport mechanism. Its turnover number corresponds to 107 min^{-1}. Both the maximum transport rate and the half-saturation constant of ornithine for the reconstituted carrier are independent of the nature of the countersubstrate. The rate of the ornithine/ornithine exchange reaction exhibits a pH optimum of 8 and is inhibited by cations, thiol-group-containing reagents and pyridoxal 5′-phosphate. On the basis of its functional properties and its apparent molecular mass, the ornithine/citrulline carrier appears to belong to the family of metabolite carrier proteins that reside within the mitochondrial inner membrane, conferring a highly selective permeability to this membrane (for a review, see [11]).

When considering the main function of the ornithine/citrulline carrier, i.e. to connect the cytosolic and the intramitochondrial reactions of the urea cycle by exchanging cytosolic ornithine with matrix citrulline, a question arises regarding the electrical nature of this exchange. Thus, at physiological pH, ornithine carries a positive charge, whereas citrulline is neutral. The present paper addresses this question in liposomes reconstituted with the purified ornithine/citrulline carrier. The data presented show that the carrier catalyses the electroneutral exchange of ornithine for citrulline + H^+. A possible mechanism for this transport process and its physiological implications are described.

EXPERIMENTAL

Materials

Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad, Celite 535 from Roth, Amberlite XAD-2 from Fluka, DEAE-Sephacel, Sephadex PD-10, Sephadex G-50 and G-75 from Pharmacia, L-[U-^{14}C]ornithine and L-[U-^{14}C]lysine from Amersham International, L-[^{3}H]ornithine and L-[*ureido*-^{14}C]citrulline from Du Pont–NEN, egg-yolk phospholipids (L-α-phosphatidyl-

Abbreviation used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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choline from fresh turkey egg yolk), Hepes, Triton X-100, valinomycin and nigericin 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 [9]. The purified protein was reconstituted into liposomes by removing the detergent with a hydrophobic ion-exchange column [9,12]. In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through the same Amberlite XAD-2 column. 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 of protein), 100 μl of 10 % egg-yolk phospholipids in the form of sonicated liposomes prepared as described previously [13], 30 mM L-ornithine or 30 mM L-citrulline (unless otherwise indicated) and 20 mM Hepes, pH 8 (unless otherwise indicated), in a final volume of 680 μl. After vortex-mixing, this mixture was passed 14 times through the same Amberlite column (0.5 cm × 2.5 cm), pre-equilibrated with the same buffer and the same substrate present in the initial mixture. All the operations were performed at 4 °C, except the passages through Amberlite, which were performed at room temperature.

Purification and reconstitution of the ADP/ATP translocase and the aspartate/glutamate carrier

The ADP/ATP translocase and the aspartate/glutamate carrier were isolated from bovine heart mitochondria as described by Krämer and Klingenberg [14] and by Bisaccia et al. [15] respectively. The two carrier proteins were reconstituted using the same conditions described above for the ornithine/citrulline carrier, except that octa(ethylene glycol) mono-n-dodecyl ether (instead of Triton X-100) and glutamate or aspartate (instead of citrulline or ornithine) were used for the reconstitution of the aspartate/glutamate carrier, and ATP or ADP (instead of citrulline or ornithine) was used for the reconstitution of the ADP/ATP translocase.

Transport measurements

The external substrate was removed by passing 550 μl of proteoliposomes through a Sephadex G-75 column (0.7 cm × 15 cm) pre-equilibrated with 10 mM Hepes, pH 8 (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 were collected, transferred to reaction vessels (100 μl each), incubated at 25 °C for 3 min, and then used for transport measurements by the inhibitor stop method [12]. Transport was initiated by adding 10 μl of labelled substrate at the final concentrations indicated in the legends to the Tables and Figures. After the desired time interval, the reaction was stopped by adding 10 μl of 330 mM pyridoxal 5′-phosphate in the case of the ornithine/citrulline and the aspartate/glutamate carriers and 10 μl of 0.11 mM carboxyatractyloside in the case of the ADP/ATP translocase. In control samples, the inhibitor was added together with the labelled substrate at zero time. The assay temperature was 25 °C. The external radioactivity was removed by passing the samples (100 μl) through a Sephadex G-50 column (0.6 cm × 8 cm). The liposomes were eluted with 1.2 ml of 40 mM NaCl and were collected in 4 ml of scintillation mixture, vortex-mixed and counted for radioactivity. The experimental values were corrected by subtracting the respective control value. The transport rate was evaluated from the radioactivity taken up by the proteoliposomes within the initial linear range of substrate uptake.

For efflux measurements, the proteoliposomes containing 10 mM ornithine were prelabelled by carrier-mediated exchange equilibration before starting the transport assay [12]. This was achieved by incubating the proteoliposomes (600 μl) that had been passed through Sephadex G-75 in order to remove the external substrate (see above) with 10 μl of 0.3 mM [^{3}H]ornithine at high specific radioactivity (3 mCi/nmol) for 20 min at 25 °C. Then the external radioactivity was removed by again passing the proteoliposomes through Sephadex G-75 as described above. In order to calculate the internal specific radioactivity necessary for the calculation of the amount of substrate effluxed from the prelabelled proteoliposomes, the volume of active proteoliposomes, i.e. the liposomes containing active carrier, was determined, in parallel samples, by using the equation:

$$\text{c.p.m.}_{\text{in}} = \text{c.p.m.}_{\text{out}} \cdot S_{\text{in}}/S_{\text{out}} \cdot V_{\text{in}}(\text{active})/V_{\text{out}}$$

where $\text{c.p.m.}_{\text{in}}$ and $\text{c.p.m.}_{\text{out}}$ are the radioactivities inside and outside at equilibrium, S is the substrate concentration, and V_{in}(active) is the volume of the active proteoliposomes [12]. Three aliquots of the same proteoliposomes used for the prelabelling procedure were treated in triplicate as described above except that, first, unlabelled ornithine was used instead of [^{3}H]ornithine and, secondly, after the second passage through Sephadex G-75, 0.1, 0.2 or 0.4 mM [^{3}H]ornithine was added. After 60 min, the internal radioactivity was measured and the V_{in}(active) was calculated for each sample according to the equation given above. If a single value of V_{in}(active) differed more than 10 % from the mean value the proteoliposomal preparation was discarded.

Other methods

K^+-diffusion potentials were generated by adding valinomycin (1.5 μg/mg of phospholipid) to proteoliposomes in the presence of different KCl gradients. For the formation of an artificial pH gradient (ΔpH) (acidic inside), nigericin (100 ng/mg phospholipid) was added to proteoliposomes in the presence of an outwardly directed K^+ 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. The fluorescence was measured using excitation and emission wavelengths of 460 and 520 nm respectively [16]. The protein was determined by the Lowry method, modified for the presence of non-ionic detergents [17].

RESULTS

Stoichiometry of the ornithine/citrulline exchange

To investigate the stoichiometry of the ornithine/citrulline exchange in proteoliposomes, we measured the efflux of [^{3}H]ornithine and the uptake of [^{14}C]citrulline simultaneously. For this the intraliposomal substrate pool (10 mM ornithine) was labelled by carrier-mediated exchange equilibration with [^{3}H]ornithine. After removal of the external labelled ornithine, 4 mM [^{14}C]citrulline was added to the proteoliposomes to start the heteroexchange. Figure 1 shows the time course of [^{3}H]ornithine efflux and [^{14}C]citrulline uptake. The data demonstrate that, at each point during incubation of the proteoliposomes with added citrulline, the amount of ornithine leaving the proteoliposomes is approximately equal to that of citrulline taken up, indicating a

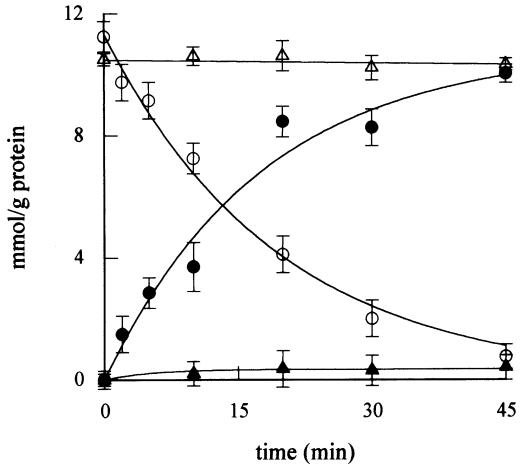

Figure 1 Stoichiometry of the reconstituted ornithine/citrulline exchange

The exchange was started by the addition of 4 mM [^{14}C]citrulline to proteoliposomes containing 10 mM [^{3}H]ornithine (see the Experimental section). ▲, △, Proteoliposomes had been treated with trypsin (0.02 mg/ml) for 30 min before the second passage through Sephadex G-75. The reaction was stopped at the indicated times and the intraliposomal ^{14}C and ^{3}H radioactivities were measured. ○, △, Ornithine; ●, ▲, citrulline. Data represent means ± S.E.M. for four experiments.

stoichiometry close to 1:1. In a series of four experiments the ratio Δornithine/Δcitrulline after 60 min incubation ranged from 0.9 to 1.2. In order to eliminate the possibility that the exchange between ornithine and citrulline occurs purely through the membrane, controls were performed by using proteoliposomes which had been treated with trypsin for 30 min before measuring transport. Under these conditions, which inactivate the reconstituted ornithine carrier protein (C. Indiveri, A. Tonazzi and F. Palmieri, unpublished work), the uptake of citrulline and the efflux of ornithine through the proteoliposomal membrane were negligible (see Table 1).

Influence of the membrane potential on the ornithine/citrulline exchange

Since, at physiological pH, ornithine carries a positive charge whereas citrulline is neutral, their exchange should be electrophoretic. We therefore investigated the influence of the membrane potential on ornithine/citrulline exchange. In these experiments the membrane potential was generated across the proteoliposomal membrane as a K^+ diffusion potential using valinomycin. In Table 1 the activities of the ornithine/citrulline heteroexchange and, as a control, of the ornithine/ornithine homoexchange, are reported in the presence and absence of valinomycin under different conditions of K^+ gradient. The rate of the [^{14}C]ornithine/citrulline exchange was not influenced by the addition of valinomycin in the absence of K^+ gradient and also in the presence of K^+ gradients of 20:1, 50:1 or 1:50 (mM/mM, in/out) corresponding to membrane potentials of about 75, 100 mV positive outside or 100 mV positive inside respectively. Table 1 further shows that different results were obtained when liposomes were reconstituted with two other carriers (i.e., the ADP/ATP translocase and the aspartate/glutamate carrier), which are known to catalyse electrophoretic heteroexchanges between ADP^{3-} and ATP^{4-} and between aspartate$^-$ and glutamate$^-$ + H^+ respectively [18–21]. Thus the ADP_{out}/ATP_{in} exchange was stimulated by the addition of valinomycin to proteoliposomes in the presence of a K^+ gradient of 50:1, and the aspartate$_{out}$/glutamate$_{in}$ exchange was stimulated by valinomycin in the presence of a K^+ gradient of 1:50. In both cases very little effect was observed in the absence of potential (without K^+ gradient) or when the ADP/ADP and aspartate/aspartate homoexchanges were measured (Table 1). The very little increase in the rate of transport observed in the presence of valinomycin was due to an unspecific effect of the ionophore, since this effect disappeared by decreasing the valinomycin concentration to 0.7 μg/mg of phospholipid (results not shown). All these results indicate that the ornithine/citrulline heteroexchange is not electrophoretic. We therefore became interested in the question as to whether the positive charge of ornithine is compensated for by a positive ion co-transported with citrulline.

Table 1 influence of the membrane potential on the activity of the reconstituted ornithine/citrulline, ADP/ATP and aspartate/glutamate carriers

The exchange was started by the addition of 0.1 mM [^{14}C]ornithine (for the ornithine/citrulline carrier), 0.1 mM [^{32}P]ADP (for the ADP/ATP translocase) or 0.1 mM [^{14}C]aspartate (for the aspartate/glutamate carrier) to proteoliposomes which contained 30 mM of the indicated internal substrate. 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 for by the addition of appropriate concentrations of sucrose in the opposite compartment. Valinomycin (1.5 μg/mg of phospholipid) was added in 10 μl ethanol/ml proteoliposomes. In the samples without valinomycin the solvent alone was added. The exchange reactions were stopped after 1 min. The data are from representative experiments. Similar results were obtained in four different experiments for each carrier investigated.

Carrier	Uptake of:	Internal substrate	K^+_{in}/K^+_{out} (mM/mM)	Exchange activity (nmol · min^{-1} · mg^{-1})	
				− Valinomycin	+ Valinomycin
Ornithine/citrulline	[^{14}C]Ornithine	Citrulline	1/1	211	232
		Citrulline	20/1	217	243
		Citrulline	50/1	190	218
		Citrulline	1/50	112	125
		Ornithine	1/1	340	345
		Ornithine	50/1	285	307
		Ornithine	1/50	173	195
ADP/ATP	[^{32}P]ADP	ATP	1/1	341	375
		ATP	50/1	408	1650
		ADP	50/1	512	614
Aspartate/glutamate	[^{14}C]Aspartate	Glutamate	1/1	113	129
		Glutamate	1/50	145	382
		Aspartate	1/50	136	154

Table 2 Effect of cations on the reconstituted citrulline/ornithine exchange

Transport was started by the addition of 0.1 mM [^{14}C]citrulline, followed by incubation for 10 min. The indicated cations were added as Cl^- salts together with the labelled substrate. The transport values given in the Table are the means for three experiments.

Addition	Concentration	Citrulline uptake (nmol/10 min per mg of protein)
None	–	1450
Na^+	10 mM	1189
	20 mM	870
K^+	10 mM	1145
	20 mM	852
Li^+	20 MM	750
NH_4^+	20 mM	680
Ca^{2+}	10 mM	449
Mg^{2+}	10 mM	319
H^+	10 μM	1856

Effect of cations on the ornithine/citrulline exchange

To investigate the influence of cations on [^{14}C]citrulline uptake in ornithine-loaded proteoliposomes, different cations were added together with the labelled substrate as Cl^- salts. As shown in Table 2, Na^+, K^+, Li^+, NH_4^+, Ca^{2+} and Mg^{2+} inhibited the [^{14}C]citrulline/ornithine exchange activity, although to a different extent. In contrast, the addition of 10 μM H^+ stimulated the uptake of citrulline in exchange for internal ornithine. Interestingly, H^+, as well as the other cations tested here, have previously been found to inhibit the ornithine/ornithine homoexchange [10].

Influence of the pH gradient on the ornithine/citrulline exchange

Under a first set of experimental conditions the proton gradient across the proteoliposomal membrane was imposed by the addition of labelled substrate and buffer at different pH values to proteoliposomes with the same internal pH. The exchange activity was measured after 1 min. During this incubation time the equilibration of the H^+ gradient was almost negligible, since we found that our proteoliposomal preparations equilibrated a pH gradient of 1 unit in 9–12 min (results not shown). Figure 2 shows that variation of the external pH at fixed internal pH had a strong effect on the rate of the exchange reactions catalysed by the ornithine carrier. With an intraliposomal pH of 8, the uptake of citrulline in exchange for internal ornithine was maximal at an external pH of 6.0–6.5 (Figure 2A). In the reverse experiment, with the same intraliposomal pH, the uptake of ornithine in exchange for internal citrulline was maximal at an external pH of 8.0–8.5 (Figure 2B). Figure 2(B) further shows that the activity of the exchange between external ornithine and internal citrulline was higher at each external pH tested when the internal pH was 7.0 instead of 8.0. As a control, in Figure 2(C) we show the dependence of the [^{14}C]ornithine/ornithine homoexchange and of the [^{14}C]lysine/ornithine heteroexchange on the external pH (at internal pH 8). In both cases the activity was maximal at an external pH of 8.0–8.5.

In further experiments the influence of the intraliposomal pH (at a fixed external pH) on the rate of the exchange between ornithine and citrulline was analysed. To this end proteoliposomes were reconstituted at various pH values in the presence of either citrulline or ornithine. After removal of external substrate and buffer by passage through Sephadex G-75, [^{14}C]ornithine (buffered at pH 8) or [^{14}C]citrulline (buffered at pH 7)

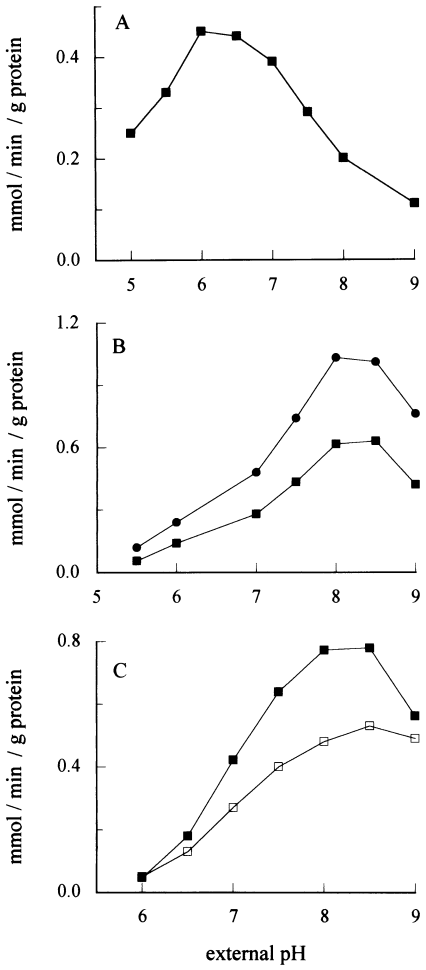

Figure 2 Influence of the external pH (at fixed internal pH) on the rate of the reconstituted exchanges between different couples of substrates

The proteoliposomes contained 30 mM ornithine (**A** and **C**) or 30 mM citrulline (**B**). The intraliposomal pH was 8.0 except in ● of **B**, where it was 7.0. After reconstitution of the carrier into liposomes, 40 mM unbuffered sucrose was used to equilibrate and elute the Sephadex G-75 columns (see the Experimental section). Transport was started by adding 0.1 mM [^{14}C]citrulline (**A**), [^{14}C]ornithine (**B**, and ■4 of **C**) or [^{14}C]lysine (□ of **C**) together with 30 mM Pipes/30 mM Hepes at the pH values indicated in the Figure. The exchange reactions were stopped after 1 min. In this Figure and the subsequent ones, data from representative experiments are reported. Similar results were obtained in at least three independent experiments.

was added to the proteoliposomes and the exchange was measured after 1 min incubation. The results, shown in Figure 3, demonstrate that when citrulline was present inside (labelled ornithine outside) the activity was maximal at internal pH 6 (Figure 3A), whereas when ornithine was present inside (labelled citrulline outside) the activity was maximal at internal pH 8 (Figure 3B). These experiments, showing that acidification of the compartment where citrulline is present stimulates the citrulline/ornithine exchange, suggest that the positive charge of ornithine is compensated by a H^+ transported in the same direction of citrulline.

In order to support this hypothesis a different approach was employed to generate a pH gradient. In these experiments, 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

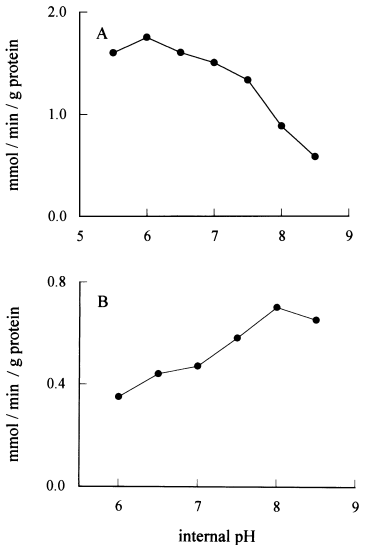

Figure 3 Influence of the internal pH (at fixed external pH) on the rate of the ornithine$_{out}$/citrulline$_{in}$ and citrulline$_{out}$/ornithine$_{in}$ exchanges

The reconstitution mixture contained 20 mM Pipes/20 mM Hepes at the pH values indicated in the Figure, and 30 mM citrulline (**A**) or ornithine (**B**). After removal of external substrate and buffer by Sephadex G-75 columns, equilibrated and eluted with 40 mM unbuffered sucrose, transport was started by adding 0.1 mM of [^{14}C]ornithine (**A**) or [^{14}C]citrulline (**B**), together with 30 mM Pipes/30 mM Hepes at pH 8 (**A**) or at pH 7.0 (**B**). The exchange reactions were stopped after 1 min. The data are from representative experiments. Similar results were obtained in three different experiments.

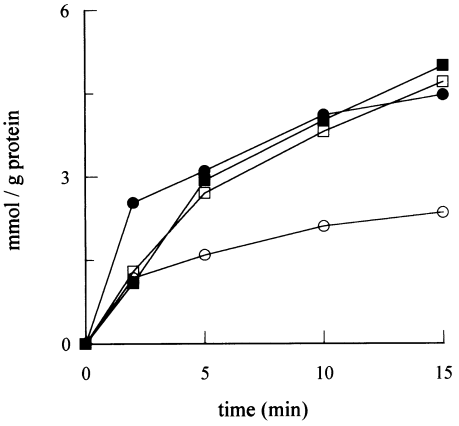

Figure 4 Stimulation of the reconstituted ornithine/citrulline exchange by the pH gradient generated by the addition of nigericin

The proteoliposomes contained 30 mM citrulline (○ and ●) or ornithine (□ and ■), 50 mM KCl and no buffer. The Sephadex G-75 equilibration and elution buffer consisted of 80 mM sucrose and 20 mM Hepes at pH 8.0. The exchange was started by the addition of 0.1 mM [^{14}C]ornithine and terminated at the indicated times. Nigericin (100 ng/mg of phospholipids) in ethanol (10 μl/ml proteoliposomes) (● and ■) or ethanol alone (○ and □) was present in the reaction mixture. The data are from a representative experiment. Similar results were obtained in three independent experiments.

50:1. Under these conditions the uptake of [^{14}C]ornithine in exchange for internal citrulline was stimulated (Figure 4). In contrast, no stimulation of [^{14}C]ornithine uptake was observed upon addition of nigericin to proteoliposomes containing ornithine (ornithine/ornithine homoexchange, Figure 4). Furthermore, the ornithine/citrulline exchange was not affected by nigericin in the absence of a K^+ gradient (results not shown).

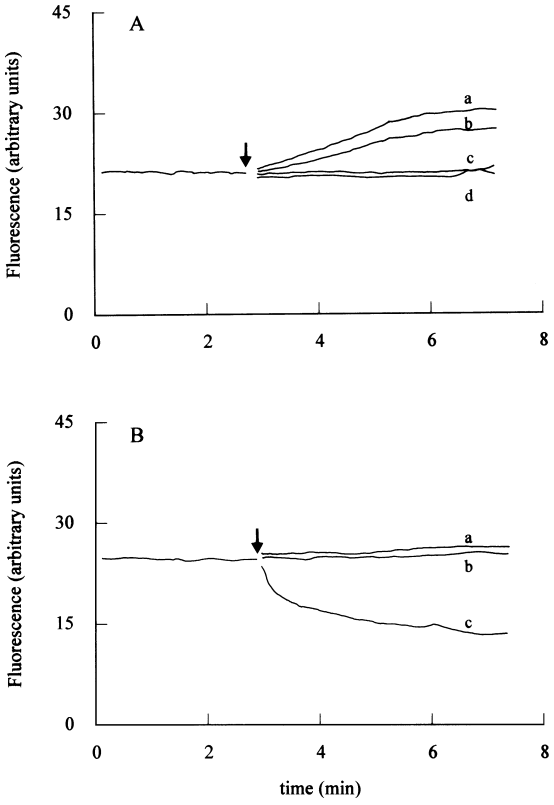

Figure 5 Fluorescence changes of pyranine-loaded proteoliposomes caused by the operation of the citrulline$_{in}$/ornithine$_{out}$ and ornithine$_{in}$/citrulline$_{out}$ exchanges

The proteoliposomes contained the pH indicator pyranine (2 mM), either 30 mM citrulline (**A**) or ornithine (**B**), and no buffer. After the passage through Sephadex G-75, 150 μl of proteoliposomes were added to 1.5 ml of 40 mM sucrose and 10 mM Hepes, pH 8. Where indicated by the arrows, the reaction was started by the addition of: (**A**) 10 mM ornithine (trace a), 10 mM lysine (trace b), 10 mM citrulline (trace c) or 10 mM ornithine plus 5 μg of FCCP (trace d); and of: (**B**) 10 mM ornithine (trace a), 10 mM citrulline (trace c), or 10 mM citrulline to proteoliposomes which had been treated with trypsin (0.02 mg/ml) for 30 min before the passage through Sephadex G-75 (trace b). A downward deflection of the trace indicates acidification of the intraliposomal space.

Proton translocation accompanying the ornithine/citrulline exchange

The movement of H^+ across the proteoliposomal membrane was investigated by measuring the fluorescence of the pH indicator pyranine, which was included inside the proteoliposomes. It is known that pyranine fluorescence increases with pH [16]. As shown in Figure 5(A), the addition of ornithine (curve a) or lysine (curve b) to citrulline-loaded proteoliposomes caused an increase in the fluorescence of pyranine, i.e. an alkalinization of the internal compartment, indicating an efflux of H^+ from the vesicles together with citrulline. No increase in fluorescence was elicited by the addition of citrulline (citrulline/citrulline homoexchange) (Figure 5A, curve c). Furthermore, the intraliposomal alkalinization induced by ornithine was not observed in the presence of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (Figure 5A, curve d), indicating a transmembrane H^+ movement. These results further support the idea that the ornithine carrier catalyses an electro-

neutral exchange of ornithine (or lysine) against citrulline + H^+. If this were the case, a H^+ translocation in a direction opposite to that observed in Figure 5(A), curve a, should be predicted upon addition of citrulline to ornithine-loaded proteoliposomes. Figure 5(B), curve c demonstrates that the addition of citrulline to proteoliposomes containing ornithine caused a decrease in the fluorescence of pyranine, i.e. an acidification of the internal compartment, indicating an influx of H^+ into the vesicles together with citrulline. No change in fluorescence was observed upon addition of ornithine (Figure 5B, curve a) nor when the proteoliposomes had been treated with trypsin (Figure 5B, curve b), which inactivates the reconstituted ornithine carrier (C. Indiveri, A. Tonazzi and F. Palmieri, unpublished work).

DISCUSSION

When considering the electrical nature of an exchange reaction which occurs between two substrates across the osmotic barrier of a membrane, their ionization state has first to be taken into account. From pK data [22] it can be calculated that, at physiological pH values, and for an ionic strength (I) of about 0.04, ornithine is essentially present as a singly charged cation, whereas citrulline is present as a zwitterion. On this basis, given that the stoichiometry of the exchange between ornithine and citrulline is 1:1 (Figure 1), as reported for most mitochondrial metabolite carriers [23], one possibility is that the ornithine carrier catalyses an electrophoretic transport between the positively charged ornithine and the neutral citrulline. This type of exchange would give rise to a membrane potential and should, consequently, be stimulated by promoting a compensatory flux of cations. Our experimental results, however, show that the ornithine/citrulline exchange is not stimulated by inducing electrogenic flux of K^+ with valinomycin, thus indicating that the carrier-mediated ornithine/citrulline exchange is not electrophoretic. This conclusion is supported by the finding that the electrophoretic ADP/ATP and aspartate/glutamate exchanges, catalysed by the well-known $\Delta\psi$-dependent adenine nucleotide translocase and aspartate/glutamate carrier respectively [18–21], were markedly stimulated by valinomycin under the same conditions in which the ornithine/citrulline exchange was tested. The alternative possibility that the positive charge of ornithine is compensated by the movement of a positive ion in the opposite direction, co-transported with citrulline (thereby making the exchange electroneutral) is substantiated by several findings, which are all consistent with the conclusion that the charge imbalance is compensated by H^+. The first indication that H^+ ions are involved came from the observation that externally added H^+ (at variance with all the other cations tested) increased the entry of [^{14}C]citrulline into ornithine-loaded proteoliposomes. Among other experimental evidence, the following can be quoted. Independently of the side of the membrane where citrulline or ornithine was present, the ornithine (or lysine) 'compartment' showed a pH-dependence with maximal activity at pH 8.0–8.5, whereas the citrulline 'compartment' showed a different pH-dependence with maximal activity at pH 6.0–6.5. Furthermore, the pH gradient created by the K^+/H^+ exchanger nigericin in the presence of an outwardly directed K^+ gradient stimulated the exchange of intraliposomal citrulline with external ornithine. A more conclusive proof for the occurrence of a co-transport of citrulline and H^+ in exchange for ornithine is the demonstration that the exchange of intraliposomal citrulline with external ornithine was accompained by efflux of H^+ from the vesicles, whereas the reverse reaction between intraliposomal ornithine and external citrulline caused H^+ movement in the opposite direction. That the H^+ translocation is a direct consequence of the carrier-mediated ornithine/citrulline exchange reaction is demonstrated by the observation that the entry of citrulline into ornithine-loaded proteoliposomes and the accompanying H^+ influx into the vesicles were both inhibited by externally added ornithine (or lysine) as well as by treatment of the proteoliposomes with trypsin. A reasonable model for the transport process catalysed by the ornithine/citrulline carrier might involve binding of ornithine to a deprotonated carrier and of citrulline to a protonated carrier. Both carrier–substrate complexes would exist as neutral species and would therefore not respond to an electrical potential gradient. On the other hand, the pK of the carrier group which is protonated and deprotonated could determine the pH profile of the transport system. Since the transport of ornithine is inhibited at pH 6.0–6.5 and the transport of citrulline at pH 8.0–8.5, our data tend to support the proposed model assuming that the functional pK group of the carrier is relatively alkaline.

The physiological consequences of the occurrence of the electroneutral ornithine$^+$/(citrulline + H^+) exchange in relation to the urea cycle require some comment. The ornithine transcarbamylase reaction occurs with liberation of a H^+ in the mitochondrial matrix:

$$\text{Ornithine}^+ + \text{carbamoyl phosphate}^{2-} \rightarrow \text{citrulline} + \text{phosphate}^{2-} + H^+$$

Therefore the H^+ transported out on the ornithine/citrulline exchange is essentially the proton which is produced by the ornithine transcarbamylase reaction in the mitochondrial matrix (see also [6,24]). On the basis of this consideration, a major function of the ornithine$^+$/(citrulline + H^+) exchange is to remove this proton from the matrix, preventing matrix acidification. Furthermore, it is apparent from the data of this paper that, during the physiological exchange between the cytosolic ornithine and the intramitochondrial citrulline, required for the operation of the urea cycle, H^+ ions have to move against the ΔpH component of the protonmotive force generated by electron transport [25]. It could be argued, therefore, that, under physiological conditions, the operation of the ornithine/citrulline exchange is unlikely in the direction required for the functioning of the urea cycle. However, re-entry of citrulline into the mitochondria via the ornithine/citrulline carrier is prevented by competition with ornithine, since the external K_m value of the carrier for ornithine is more than one order of magnitude lower than that for citrulline (0.16 mM and 3.6 mM respectively) [10]. In principle, the driving force for the electroneutral ornithine$_{out}$/citrulline$_{in}$ exchange could be provided by appropriate gradients of the two substrates across the membrane. To our knowledge, the intramitochondrial and cytosolic concentrations of citrulline have not yet been determined. There is, on the other hand, a report showing that the concentration of ornithine in isolated hepatocytes is 1.6 mM in the mitochondrial matrix and 0.6 mM in the cytosol [26]. If these measurements are correct, since the ΔpH across the mitochondrial membrane in perfused liver is about 0.33 unit [27], a concentration gradient of citrulline (in/out) of 5.3 should be sufficient to drive entry of ornithine in exchange for intramitochondrial citrulline. A comparable citrulline gradient is not unlikely to exist in hepatocytes. It is known that, in the presence of saturating concentrations of ammonia, ornithine and lactate, citrulline and argininosuccinate accumulate in perfused liver or isolated hepatocytes (see [24] for references). The possibility that a relatively high mitochondrial/cytosolic gradient of citrulline occurs *in vivo* is supported by the studies of Cheung et al. [28]. These authors provided evidence that the three extramitochondrial enzymes of the urea cycle do not move freely in the cytosol but are grouped around the mitochondria in such

a way that channelling of mitochondrially exported citrulline to argininosuccinate synthase becomes possible. Similarly, it has been suggested that ornithine transcarbamylase and carbamoylphosphate synthetase form a complex within the mitochondria that is bound to the inner mitochondrial membrane, to allow channeling of extramitochondrial ornithine from its carrier to ornithine transcarbamylase [29]. This would make the cytosolic/mitochondrial concentration gradient of ornithine more favourable for driving the ornithine/citrulline exchange in the physiological direction than indicated by the measured concentration gradient of 0.4 reported above [26].

Finally, it should be stressed that the transport mode for the ornithine/citrulline exchange described here does not use the electrochemical proton potential gradient across the mitochondrial membrane produced by electron transport. The ornithine/citrulline exchange does not therefore reduce the driving force for the synthesis of ATP that is required in large amounts for the operation of the urea cycle in the reactions catalysed by carbamoylphosphate synthetase and by argininesuccinate synthetase. It was reported a long time ago that citrulline production in isolated liver mitochondria from added ammonia, bicarbonate and ornithine proceeds perfectly well in fully uncoupled mitochondria at the expense of external ATP (plus oligomycin) [30]. In the light of our results, this early finding can now be interpreted as indicating that citrulline transport is not energy-dependent and thus does not use the mitochondrial proton potential gradient.

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REFERENCES

1 Gamble, J. G. and Lehninger, A. L. (1973) J. Biol. Chem. **248**, 610–618
2 Bryla, J. and Harris, E. J. (1976) FEBS Lett. **72**, 331–336
3 McGivan, J. D., Bradford, N. M. and Beavis, A. D. (1977) Biochem. J. **162**, 147–156
4 Aronson, D. L. and Diwan, J. J. (1981) Biochemistry **20**, 7064–7068
5 Bradford, N. M. and McGivan, J. D. (1980) FEBS Lett. **113**, 294–298
6 McGivan, J. D. (1992) in Mammalian Amino Acid Transport, (Kilberg, M. S. and Häussinger, D., eds.), pp. 101–111, Plenum Press, New York
7 Hommes, F. A., Kitchings, L. and Eller, A. G. (1983) Biochem. Med. **30**, 313–321
8 Passarella, S., Atlante, A. and Quagliariello, E. (1990) Eur. J. Biochem. **193**, 221–227
9 Indiveri, C., Tonazzi, A. and Palmieri, F. (1992) Eur. J. Biochem. **207**, 449–454
10 Indiveri, C., Palmieri, L. and Palmieri, F. (1994) Biochim. Biophys. Acta **1188**, 293–301
11 Palmieri, F. (1994) FEBS Lett. **346**, 48–54
12 Palmieri, F., Indiveri, C., Bisaccia, F. and Iacobazzi, V. (1995) Methods Enzymol. **260**, 349–369
13 Bisaccia, F., Indiveri, C. and Palmieri, F. (1985) Biochim. Biophys. Acta **810**, 362–369
14 Krämer, R. and Klingenberg, M. (1979) Biochemistry **18**, 4209–4215
15 Bisaccia, F., De Palma, A. and Palmieri, F. (1992) Biochim. Biophys. Acta **1106**, 291–296
16 Wrigglesworth, J. M., Cooper, C. E., Sharpe, M. A. and Nicholls, P. (1990) Biochem. J. **270**, 109–118
17 Dulley, J. R. and Greeve, P. A. (1975) Anal. Biochem. **64**, 136–141
18 Pfaff, E. and Klingenberg, M. (1968) Eur. J. Biochem. **6**, 66–79
19 Krämer, R. and Klingenberg, M. (1980) Biochemistry **19**, 556–560
20 LaNoue, K. F. and Tischler, M. E. (1974) J. Biol. Chem. **249**, 7522–7528
21 Palmieri, F., Stipani, I. and Iacobazzi, V. (1979) Biochim. Biophys. Acta **555**, 531–546
22 Fasman, G. D. (1976) in Handbook of Biochemistry and Molecular Biology, 3rd edn., vol. 1, CRC Press/The Chemical Rubber Co., Cleveland, OH
23 LaNoue, K. F. and Schoolwerth, A. C. (1979) Annu. Rev. Biochem. **48**, 871–922
24 Meijer, A. J., Lamers, W. H. and Chamuleau, R. A. F. M. (1990) Physiol. Rev. **70**, 701–748
25 Mitchell, P. (1961) Nature (London) **191**, 144–148
26 Zollner, H. (1984) Int. J. Biochem. **16**, 681–685
27 Soboll, S. and Scholz, R. (1986) FEBS Lett. **205**, 109–112
28 Cheung, C. W., Cohen, N. S. and Raijman, L. (1989) J. Biol. Chem. **264**, 4038–4044
29 Cohen, N. S., Cheung, C. W. and Raijman, L. (1987) J. Biol. Chem. **262**, 203–208
30 Graafmans, W. D. J., Charles, R. and Tager, J. M. (1968) Biochim. Biophys. Acta **153**, 916–919

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