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Chemical modification of the mitochondrial ornithine/citrulline carrier by SH reagents: effects on the transport activity and transition from carrier to pore-like function

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Abstract

The transport activity of the purified and reconstituted ornithine/citrulline carrier from rat liver mitochondria was correlated to modification of its sulfhydryl groups by various reagents. Both the ornithine/ornithine (antiport) and the ornithine/H^+ (unidirectional) transport modes catalysed by the ornithine/citrulline carrier were inhibited by methanethiosulfonates, mercurial reagents, *N*-ethylmaleimide (NEM) and 5,5′-dithiobis(2-nitrobenzoate) (DTNB). The treatment of the ornithine/citrulline carrier with mercurial reagents, at concentrations above 5 μM, caused the induction of an additional (pore-like) transport mode, characterized by loss of substrate specificity and a transport activity higher than that of the unmodified carrier. The S–S forming reagent Cu^{2+}-phenanthroline inhibited the transport catalysed by the carrier, indicating the presence of close sulfhydryl groups. The effect of consecutive addition of the various reagents revealed a peculiar aspect of the ornithine/citrulline carrier, i.e. the presence of three distinct populations of sulfhydryl groups. The first was responsible for the inhibition of the physiological transport modes by methanethiosulfonates, NEM and DTNB and low concentrations (<5 μM) of mercurials; the second population was responsible for the transition to the pore-like activity induced by higher concentrations (>5 μM) of mercurials; the third population was involved in S–S bridge formation.

Keywords: Mitochondrion; Membrane; Transport; Carrier; Liposome; Ornithine

1. Introduction

The ornithine/citrulline carrier is a metabolite transport protein of the inner mitochondrial membrane. After preliminary studies on the ornithine and citrulline transport [1–7], performed with intact mitochondria, the existence of this carrier was definitively demonstrated by the identification of a 33.5-kDa (on SDS-PAGE) protein that catalysed ornithine/ornithine and ornithine/citrulline antiport when reconstituted into liposomes [8]. More recently, the amino acid sequences of the yeast ARG-11 [9] and the human ORNT1 [10] ornithine carriers have been identified. Both the yeast and the mammalian proteins show the structural properties of the mitochondrial carrier family [11].

The mammalian ornithine/citrulline carrier plays an important role in the urea cycle as it was proposed by functional studies in intact mitochondria and in reconstituted liposomes [4,8,12,13] and confirmed by the finding that deficiency of ORNT1 gene lead to HHH syndrome [10] and that the hormonal regulation of the ORNT1 expression in liver is integrated with that of the urea cycle enzymes [14].

The ornithine/citrulline antiport catalysed by this carrier has been extensively characterized in reconstituted liposomes. It is electroneutral since the positive charge of ornithine is compensated by a H^+ co-transported with citrulline [13]. The half-saturation constant of the carrier for

Abbreviations: *p*-OHMB, *p*-hydroxymercuribenzoate; *p*-CMBS, *p*-chloromercuribenzenesulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoate); MTS, methanethiosulfonate reagents; MTSES, sodium(2-sulfonatoethyl)-methanethiosulfonate; MTSEA, (2-aminoethyl)methanethiosulfonate hydrobromide; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate; NEM, *N*-ethylmaleimide; DTE, 1.4-dithioerythritol

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ornithine on the external side is about 20 times lower than that on the internal side [15]; on the contrary, the half-saturation constants for citrulline on the external and internal side are comparable. The asymmetry of the carrier in the mitochondrial membrane should facilitate channeling of the urea cycle substrates among the enzymes and the transport system [15–17]. The substrate antiport (ornithine/ornithine and ornithine/citrulline) catalysed by the ornithine/citrulline carrier follows a simultaneous (sequential) transport mechanism [15] as all but one of the mitochondrial carriers characterized so far [11].

A further role for the ornithine/citrulline carrier has been proposed on the basis of functional studies in intact mitochondria [3] and in reconstituted liposomes [18]: the carrier catalyses unidirectional transport of ornithine which should be important for arginine catabolism and polyamine biosynthesis. This second transport mode is active in the absence of countersubstrate and is electroneutral since a H^+ is transported in exchange with ornithine (ornithine/H^+ exchange). Ornithine/H^+ transport rate under optimal condition is about one third of the maximal ornithine/citrulline antiport rate.

To clarify the relationships between structure and function of this metabolically important transport system, further studies on the role of specific amino acid residues are required. Both the human and the mouse ornithine carriers contain nine Cys residues [10]; nevertheless, the sensitivity of the ornithine/citrulline carrier to SH reagents is still unclear. In studies performed with intact mitochondria, it was reported that *N*-ethylmaleimide (NEM) inhibited ornithine transport whereas mersalyl had no effect [5]. Later on, in studies performed with the reconstituted carrier, it was found that mercurial reagents and maleimides inhibited to different extent the ornithine/ornithine antiport [8].

In this paper, we have performed a detailed study on the effect of chemical modification of Cys of the ornithine/citrulline carrier, which provided new information on the role of SH groups on the transport function. Using SH reagents with different properties, we could discriminate three populations of SH groups. Two of them are functionally involved in the translocation pathway of the reconstituted ornithine/citrulline carrier. A further population is responsible for the induction of an additional transport mode that is characterized by loss of specificity for substrate, similarly to the pore-like activity described for the aspartate/glutamate [19,20], the adenine nucleotide [19], the carnitine [21] and the phosphate [22] carriers of mitochondria.

2. Materials and methods

2.1. Materials

Hydroxyapatite (Bio-Gel HTP) and Bio-Beads SM-2 were purchased from Bio-Rad; Celite 535 from Roth; DEAE-Sephacel, Sephadex PD-10, Sephadex G-50 and G-75 from Pharmacia; L-[2,3-^{3}H]ornithine, egg yolk phospholipids (L-α-phosphatidylcholine from fresh turkey egg yolk), HEPES and Triton X-100 from Sigma; (2-aminoethyl)methanethiosulfonate hydrobromide (MTSEA), [2-(trimethylammonium)ethyl]methanethiosulfonate (MTSET) and sodium(2-sulfonatoethyl)methanethiosulfonate (MTSES) were from Toronto Research Chemicals (North York, Ontario, Canada). 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 [8]. The purified protein was reconstituted into liposomes by removing the detergent with a hydrophobic column [23,24]. 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% Triton X-100 (Celite eluate, about 1 μg protein), 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as described previously [25], 30 mM L-ornithine (unless otherwise indicated) and 20 mM HEPES pH 8.0 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 × 3.2 cm), preequilibrated 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 by passing 550 μl of proteoliposomes through a Sephadex G-75 column (0.7× 15 cm) preequilibrated with 10 mM HEPES pH 8.0 (unless otherwise indicated) and 60 mM 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), and readily used for transport measurements by the inhibitor-stop method [26]. For uptake measurements, transport was initiated by adding 10 μl of [^{3}H]ornithine at the final concentration of 0.1 mM. After the required time interval, the reaction was stopped by adding 20 mM pyridoxal 5-phosphate/1 mM NEM. In control samples, the inhibitor was added together with the labeled substrate at time zero. 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 × 8 cm). The liposomes eluted with 1.2 ml of 40 mM NaCl were collected in 4 ml of scintillation mixture, vortexed and counted. The experimental values were corrected by subtracting the respective control values. The transport rate was evaluated from the radioactivity taken

up by the proteoliposomes in 2 min, i.e. within the initial linear range of substrate uptake.

For efflux measurements, the proteoliposomes containing 30 mM ornithine were prelabeled by carrier-mediated exchange equilibration before starting the transport assay [23]. This was achieved by incubating the proteoliposomes (600 μl), 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 μCi/nmol) for 20 min at 25 °C. Then, the external radioactivity was removed by passing again the proteoliposomes through Sephadex G-75 as described above except that this chromatography was performed at 0 °C to minimize the loss of internal substrate during the chromatography. Transport was started by adding unlabeled external ornithine (ornithine/ornithine antiport) or buffer alone (ornithine/H^+ exchange) at the concentration indicated in the legends to tables and figures and stopped, at the appropriate time interval, as described above. In the samples treated with *p*-hydroxymercuribenzoate (*p*-OHMB) or *p*-chloromercuribenzenesulfonate (*p*-CMBS) (>5 μM), where NEM was no more effective, the start time was defined as the time of addition of the reagent or buffer alone; the elution on the Sephadex G-50 columns was performed within 1 min at 0 °C in order to minimize the efflux of labeled substrate during the chromatography. Efflux activity was expressed as intraliposomal cpm. Initial rate of efflux (reported as cpm/min) was obtained from time courses by fitting the experimental data in a single exponential decay equation from which the efflux rate was calculated as the product of k (the first order rate constant) and A (the radioactivity effluxed from proteoliposomes at infinite time) [23].

2.4. Other methods

The protein was determined by the Lowry method, modified for the presence of nonionic detergents [27].

3. Results

3.1. Effect of SH reagents on ornithine/ornithine antiport

The inhibition of the ornithine/ornithine antiport catalysed by the reconstituted ornithine/citrulline carrier by SH reagents of different type was analysed as concentration dependence; data are reported as semilogarithmic plots (Fig. 1). All the reagents tested lead to complete inhibition of transport, even though with different efficiency. Among the methanethiosolfonates (MTS), the most effective inhibitor was MTSEA (IC_{50}, 0.17 ± 0.03 μM) followed by MTSET (IC_{50}, 0.42 ± 0.08 μM) and (not shown) MTSES (IC_{50}, 200 ± 39 μM). The most effective mercurial reagents were *p*-OHMB (IC_{50}, 0.59 ± 0.06 μM) and *p*-CMBS (IC_{50}, 0.61 ± 0.05 μM) followed by mersalyl (IC_{50}, 1.1 ± 0.11 μM) and (not shown) $HgCl_2$ (IC_{50}, 1.4 ± 0.21 μM). NEM

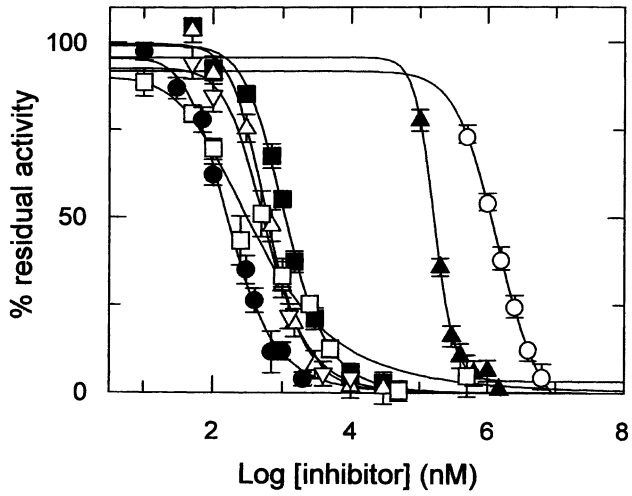

Fig. 1. Dose–response curves for the inhibition of the reconstituted ornithine/ornithine antiport by SH reagents. Proteoliposomes were incubated with the inhibitors for 2 min. Then, ornithine/ornithine antiport was measured by adding 0.1 mM [^{3}H]ornithine and stopped after 2 min, as described in Materials and methods. (▲) NEM, (○) DTNB, (■) mersalyl, (▽) *p*-OHMB, (△) *p*-CMBS, (●) MTSEA, (□) MTSET. Percent residual activity with respect to the control are reported. The control activity (uninhibited ornithine/ornithine antiport) was 0.53 ± 0.15 mmol/2 min × g protein; the values are means ± S.D. from three experiments.

and 5,5′-dithiobis(2-nitrobenzoate) (DTNB) exhibited IC_{50} of 0.16 ± 0.02 and 1.45 ± 0.06 mM, respectively. After complete inactivation by MTSEA, *p*-OHMB, *p*-CMBS and DTNB, the protein could be reactivated by treatment with 1.4-dithioerythritol (DTE). On the contrary, after treatment with NEM, the addition of DTE did not restore the transport activity (Table 1). Taking advantage by the difference in reactivity of NEM (irreversible) and other reagents (reversible), we have investigated whether the various reagents bind to the same or to different SH groups. Proteoliposomes were first incubated with reversible reagents and then NEM was added; after incubation, aliquots of the samples were treated with DTE. As shown by Table 1, in the samples treated with reversible inhibitors before NEM, a substantial recovery of activity was observed upon addition of DTE, indicating a more or less extensive protection of NEM inhibition by the reagents. Recovery (protection) ranged from about 50% to 80% depending on the reagent used for preincubation. This means that reversible reagents and NEM react mainly with the same SH groups; however, a fraction of about 20%, 30% or 50% of the protein can still bind NEM, in the presence of MTSEA, DTNB or mercurials, respectively.

3.2. Effect of SH reagents on the ornithine/H^+ transport mode and induction of a pore-like transport mode

In order to investigate the effect of SH reagents on the alternative (ornithine/H^+) transport mode of the ornithine/citrulline carrier [18], we used the efflux procedure, which is more effective for the detection of unidirectional flux of

Table 1
Protection by sulfhydryl reagents against NEM inhibition of the reconstituted ornithine/citrulline carrier

Additions at			
0 min	2 min	10 min	Percent residual activity
–	–	–	100
–	–	DTE	108 ± 4.0
MTSEA	–	–	5 ± 3
MTSEA	–	DTE	100 ± 4.7
p-OHMB	–	–	6 ± 4
p-OHMB	–	DTE	106 ± 16
p-CMBS	–	–	7 ± 4
p-CMBS	–	DTE	103 ± 14
DTNB	–	–	8 ± 7
DTNB	–	DTE	101 ± 3.4
NEM	–	–	2 ± 1
NEM	–	DTE	11 ± 3
MTSEA	NEM	–	5 ± 3
MTSEA	NEM	DTE	81 ± 12
p-OHMB	NEM	–	2 ± 1
p-OHMB	NEM	DTE	47 ± 5
p-CMBS	NEM	–	4 ± 2
p-CMBS	NEM	DTE	53 ± 7
DTNB	NEM	–	7 ± 4
DTNB	NEM	DTE	69 ± 14

Proteoliposomes were incubated with different sulfhydryl reagents and DTE was added at the time indicated. After a total incubation time of 20 min, ornithine/ornithine antiport was measured in all the samples by adding 0.1 mM [^{3}H]ornithine and stopping the reaction at 10 min, as described in Materials and methods. Reagents were used at the following concentrations: 5 μM *p*-OHMB, 5 μM *p*-CMBS, 10 mM DTNB, 2 μM MTSEA, 1 mM NEM, 5 mM DTE. Percent residual activity was calculated for each experiment with respect to its control sample (referred as 100%); the values are means ± S.D. of the percentages for three experiments. The average specific activity of the control samples of the three experiments analysed was 1.80 ± 0.54 mmol/10 min/g protein.

substrate [23]. Fig. 2 describes the effect of SH reagents on [^{3}H]ornithine efflux from preloaded proteoliposomes in the absence of external substrate (ornithine/H^+ transport mode) and, as control, in the presence of external ornithine (ornithine/ornithine transport mode). In agreement with the data reported in Fig. 1, 4 μM MTSEA, 4 μM *p*-OHMB or 1 mM NEM strongly inhibited the ornithine/ornithine antiport. Ornithine/H^+ transport mode was also inhibited by MTSEA and NEM, but it was not affected by the presence of 4 μM *p*-OHMB or (not shown) 4 μM *p*-CMBS or 4 μM mersalyl. DTNB (6.5 mM) showed a behaviour similar to that of MTSEA (not shown). In order to gain further insight on the lack of inhibition of the ornithine/H^+ transport mode by mercurials, the effect of various concentrations of *p*-OHMB (0.1–100 μM) was studied (Fig. 3). The ornithine/H^+ efflux was inhibited in a concentration-dependent manner at 0.1 and 0.3 μM. At higher concentration of *p*-OHMB, the efflux of labeled ornithine increased again, being clearly stimulated at concentrations above 5 μM. At 100 μM *p*-OHMB, the efflux rate of [^{3}H]ornithine (first-order rate constant, *k*, 0.28) was much higher than the basic efflux (ornithine/H^+ transport mode; *k*, 0.006) and it was also higher than the rate of the ornithine/ornithine antiport

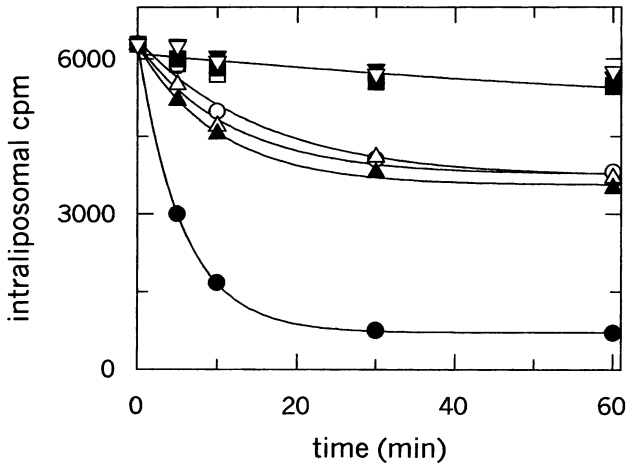

Fig. 2. Effect of SH reagents on the ornithine/H^+ transport mode. Efflux of 30 mM [^{3}H]ornithine from prelabeled proteoliposomes was monitored, as described in Materials and methods, in the presence of external buffer alone (10 mM HEPES pH 8.0, 60 mM sucrose; ornithine/H^+ exchange, open symbols) or in the presence of 10 mM external ornithine (in a buffer containing 10 mM HEPES pH 8.0, 40 mM sucrose; ornithine/ornithine antiport, solid symbols). Inhibitors were added to the proteoliposomes 2 min before the start of the efflux: (△ and ▲) 4 μM *p*-OHMB; (□ and ■) 1 mM NEM; (▽ and ▼) 4 μM MTSEA; (○ and ●) controls without inhibitor. Similar results were obtained in four different experiments.

(Fig. 2; *k*, 0.17). After the inactivation of the carrier by incubation of the proteoliposomes with trypsin, the *p*-OHMB-induced efflux was virtually abolished (Fig. 3), indicating that the process is protein mediated. *p*-OHMB induced a fast efflux of labeled ornithine with a mechanism different from both the ornithine/ornithine antiport and ornithine/H^+ exchange presumably due to a transition of the reconstituted translocator to a pore-like function. A similar phenomenon was previously observed for other

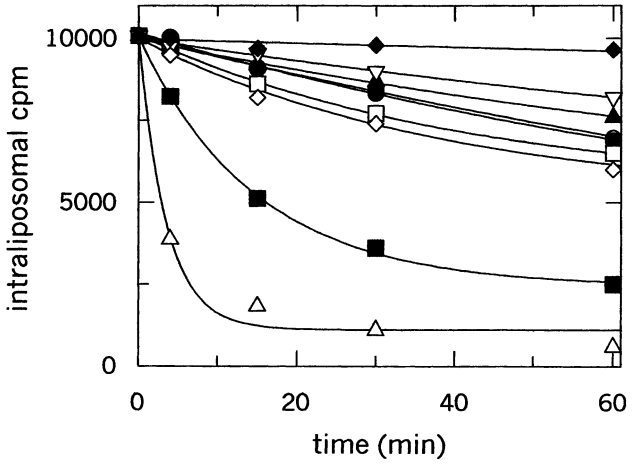

Fig. 3. Dependence of [^{3}H]ornithine efflux from proteoliposomes on *p*-OHMB concentration. Efflux of 30 mM [^{3}H]ornithine was measured in the presence of external 10 mM HEPES pH 8, 60 mM sucrose (ornithine/H^+ exchange): (□) no addition; (●) 0.1 μM, (▽) 0.3 μM, (▲) 0.7 μM, (○) 1 μM, (◇) 5 μM, (■) 10 μM and (△) 100 μM *p*-OHMB. (♦) 100 μM *p*-OHMB was added to proteoliposomes preincubated with 0.3 mg/ml trypsin for 15 min. Similar results were obtained in four different experiments.

mitochondrial carriers [19–22]; it was accompanied by loss of specificity for substrate. Therefore, we have tested the specificity of the ornithine/citrulline carrier after reaction with 0.1 mM *p*-OHMB. For methodological reasons [21], these experiments were carried out by measuring the uptake of labeled substances into proteoliposomes without internal substrate. Furthermore, since pyridoxal 5-phosphate and NEM were not more effective as stop inhibitors on the modified protein, we measured the amount of substrate taken up after 30 min. The results in Table 2 clearly demonstrate that the substrate specificity of the ornithine/citrulline carrier was significantly reduced after treatment with mercurials. The carrier, which in the unmodified condition catalysed unidirectional transport of only ornithine, lysine and arginine (see also Ref. [18]), after reaction with *p*-OHMB, catalysed transport of glutamine, choline, 2-oxoglutarate, malate and carnitine. ATP, citrate and sucrose were not transported by the modified carrier. In control experiments, it was tested that the specificity of the protein was restored after the addition of DTE to proteoliposomes preincubated with *p*-OHMB (experiments not shown). To achieve the same effect of 100 μM *p*-OHMB, 150 μM *p*-CMBS or 1 mM $HgCl_2$ was required. NEM, DTNB and MTS compounds were ineffective (not shown).

3.3. Differentiation of SH group populations

We have investigated whether the inhibition of the physiological transport (ornithine/ornithine and ornithine/H^+ transport modes) and the induction of the pore-like activity can be correlated to different classes of SH groups of the carrier protein. To this aim, NEM, which reacts irreversibly with the carrier and does not induce uniport, was first added to proteoliposomes, thus blocking physiological activity in ornithine-loaded vesicles (Fig. 4); *p*-OHMB, subsequently added to the same pool of proteoliposomes, was still able to induce efflux of ornithine. The following addition (45 min) of DTE stopped the mercurial induced efflux, i.e. although the effect of *p*-OHMB was abolished, the previous inhibition by NEM was still retained. Thus, the effect of *p*-OHMB is reversible and the two reagents interact with different SH groups. In agreement, the addition of DTE after incubation of the proteoliposomes with *p*-OHMB slowed down the efflux at a rate overlapping that of the ornithine/H^+ transport mode. Further addition of external ornithine stimulated again the efflux by inducing the ornithine/ornithine antiport. The data described indicate the existence of at least two different SH group populations involved in the inhibition of the physiological transport modes or in the induction of the pore-like activity, respectively.

In order to verify whether different SH groups of the protein may be in close proximity, we have tested the influence of S–S forming reagents on the activity of the ornithine/citrulline carrier. The incubation of proteoliposomes with 20 μM Cu^{2+}-phenanthroline for 2 min resulted in less than 20% inhibition of the ornithine/citrulline carrier. Thus, we studied the time dependence of the inhibition on the ornithine/ornithine antiport measured as uptake of labeled ornithine into reconstituted proteoliposomes. As

Table 2
Substrate specificity of the unidirectional transport catalysed by the reconstituted ornithine/citrulline carrier in the presence and absence of *p*-OHMB

Substrate	Activity (mmol/30 min/g protein)	
	−*p*-OHMB	+*p*-OHMB
Ornithine (liposomes)	0.01 ± 0.01	0.02 ± 0.01
Ornithine (proteoliposomes, boiled carrier)	0.02 ± 0.01	0.02 ± 0.02
Ornithine	0.23 ± 0.06	0.31 ± 0.07
Citrulline	0.02 ± 0.01	0.45 ± 0.20
Lysine	0.16 ± 0.06	0.39 ± 0.06
Arginine	0.20 ± 0.06	0.40 ± 0.08
Carnitine	0.04 ± 0.11	0.28 ± 0.06
Glutamine	0.06 ± 0.03	0.30 ± 0.15
Choline	0.01 ± 0.01	0.28 ± 0.06
Malate	0.01 ± 0.01	0.16 ± 0.12
ATP	n.d.	n.d.
Citrate	n.d.	n.d.
2-Oxoglutarate	n.d.	0.54 ± 0.20
Sucrose	n.d.	0.05 ± 0.05

Each of the indicated substrates (0.1 mM) was added in radioactive form to proteoliposomes without internal substrate, in the presence or absence of 0.1 mM *p*-OHMB. After 30-min incubation, the reaction was stopped by passing the samples through cold (0 °C) Sephadex G-50 columns within 1 min as described in Materials and methods. The controls were performed by adding labeled ornithine to liposomes or to proteoliposomes reconstituted with boiled carrier protein.
The values are means ± S.D. for three experiments; n.d., not detectable.

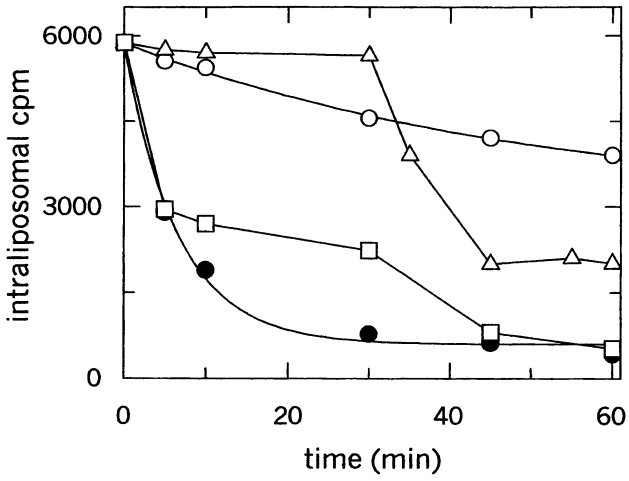

Fig. 4. Induction by *p*-OHMB of [^{3}H]ornithine efflux from proteoliposomes preincubated with NEM and subsequent effect of DTE. Efflux of 30 mM [^{3}H]ornithine from prelabeled proteoliposomes was monitored as described in Materials and methods, in the presence of external 10 mM HEPES pH 8.0, 60 mM sucrose (ornithine/H^+ exchange). Additions were the following: (○) control, no additions; (●) 0.1 mM *p*-OHMB; (△) 1 mM NEM 1 min before the start, 0.1 mM *p*-OHMB at 30 min and 2 mM DTE at 45 min; (□) 0.1 mM *p*-OHMB at the start, 2 mM DTE at 5 min and 10 mM ornithine at 30 min. Similar results were obtained in four different experiments.

shown by Fig. 5, the activity decreased with the incubation time being close to zero after 40-min incubation with the reagent. The activity was reversed by the addition of DTE, with a time dependence not very different from that of inactivation. Complete recovery of activity was achieved about 60 min after the addition of the reducing reagent. After this time, about 110% activity (with respect to the initial situation) was recovered, indicating that a fraction of about 10% of the carrier was spontaneously oxidized before reconstitution (see also Table 1). Therefore, it is clear that at least two SH groups are in close proximity to form an S–S bridge and that the oxidized protein is not active. A similar time dependence of inhibition was observed with diamide (not shown). Samples treated neither with Cu^{2+}-phenanthroline nor with DTE showed no appreciable variation of activity within 100 min.

Now a further question arises, whether these groups correspond to those reacting with NEM or *p*-OHMB. To answer this question, we performed the experiment of Fig. 6. Proteoliposomes pretreated with Cu^{2+}-phenanthroline, under conditions leading maximal inhibition of the carrier, were then used to follow the efflux of labeled ornithine. The negligible efflux in the absence (ornithine/H^+ transport mode, ×) or presence (ornithine/ornithine transport mode, ○) of external ornithine, with respect to the control (proteoliposomes not pretreated with Cu^{2+}-phenanthroline), confirmed that pretreatment with Cu^{2+}-phenanthroline lead to inhibition of both the transport modes catalysed by the ornthine/citrulline carrier. The addition of 0.1 mM *p*-OHMB to the Cu^{2+}-phenanthroline-treated protein determined a fast

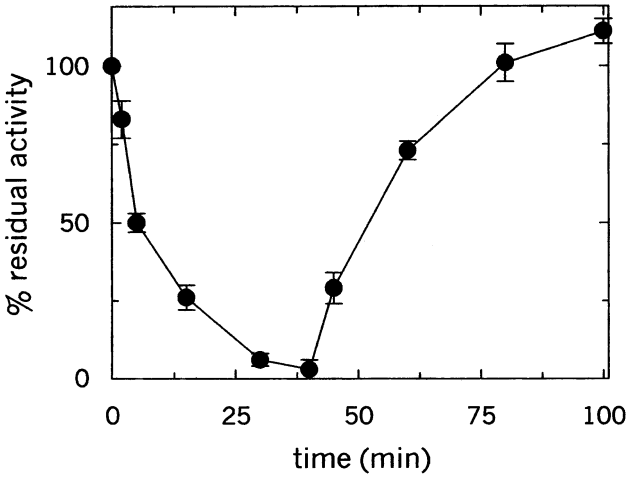

Fig. 5. Time dependence of the inhibition of the ornithine/ornithine antiport by Cu^{2+}-phenanthroline and effect of DTE. Proteoliposomes containing 30 mM ornithine were incubated with 20 μM Cu^{2+}-phenanthroline, and after 40 min, 2 mM DTE was added. At the indicated times, aliquots of proteoliposomes were used for transport assay. Ornithine/ornithine antiport was measured by adding 0.1 mM [^{3}H]ornithine and stopped after 10 min, as described in Materials and methods. Percent residual activity with respect to the control are reported. The control activity (ornithine/ornithine antiport before incubations) was 1.92 ± 0.50 mmol/10 min/g protein; the values are means ± S.D. from three experiments.

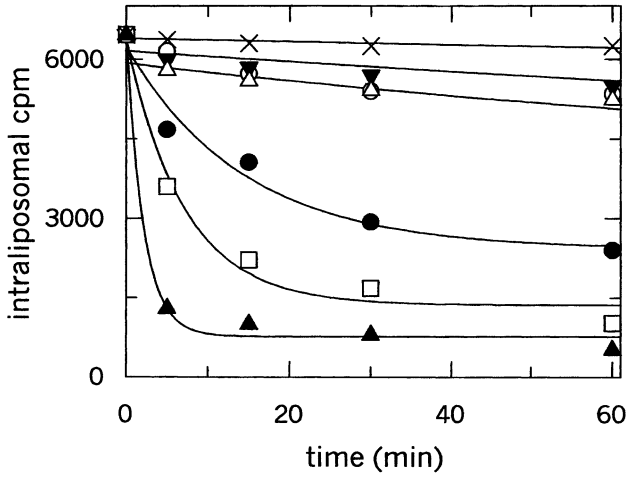

Fig. 6. Inhibition of [^{3}H]ornithine efflux from proteoliposomes by Cu^{2+}-phenanthroline and effect of NEM. Efflux of 30 mM [^{3}H]ornithine from prelabeled proteoliposomes was measured as described in Materials and methods, except that an aliquot of prelabeled proteoliposomes was incubated with 20 μM Cu^{2+}-phenanthroline for 30 min (△, ○, ▲, ● and ×) before the passage through the second Sephadex G-75 (see Materials and methods). In the samples preincubated with Cu^{2+}-phenanthroline, the additions were the following: (×) none (buffer alone: 10 mM HEPES pH 8.0, 60 mM sucrose); (○) 10 mM external ornithine (in a buffer containing 10 mM HEPES pH 8.0, 40 mM sucrose); (●) 10 mM external ornithine plus 10 mM DTE; (△) 1 mM NEM 1 min before the start and 10 mM DTE plus 10 mM ornithine at the start; (▲) 0.1 mM *p*-OHMB. In the samples not preincubated with Cu^{2+}-phenanthroline, the additions were the following: (□) 10 mM external ornithine; (▼) 1 mM NEM 1 min before the start and 10 mM DTE plus 10 mM external ornithine at the start. Similar results were obtained in four different experiments.

efflux of [^{3}H]ornithine, indicating that the SH group(s) reacting with the mercurial reagent (at high concentration) is different from those forming S–S bridge(s). To verify whether SH groups reacting with NEM (MTS and mercurials at low concentration) were involved in the S–S bridge formation, DTE, as an S–S reducing agent, has been used. As shown by Fig. 6, DTE restored the activity of the carrier pretreated with Cu^{2+}-phenanthroline, but not that of the carrier treated with Cu^{2+}-phenanthroline and then by NEM. This indicated that NEM also irreversibly bound to the oxidized protein, i.e. in the presence of S–S bridge(s). Therefore, the SH group population reacting with NEM is different from that involved in the S–S formation. Overlapping results were obtained by using diamide instead of Cu^{2+}-phenanthroline (not shown).

4. Discussion

In order to gain information on the role of SH groups in the transport activity of the mitochondrial ornithine/citrulline carrier, we have carried out a detailed characterization of the effect of several SH reagents on the two transport modes catalysed by the reconstituted carrier.

The substrate antiport, monitored as [^{3}H]ornithine uptake into proteoliposomes containing unlabeled ornithine, was

strongly inhibited by several compounds with different efficiency. Thus, there is an SH population responsible for the inhibition of the transport activity. The difference in IC_{50} among inhibitors of the same type may be due to different interaction of side groups of the reagents with specific hydrophilic/hydrophobic residues of the protein. In this respect, the differences among the reagents belonging to the MTS group [28,29] may give some information about the environment in which the SH group responsible of the transport inhibition is located. The finding that the positively charged MTSEA and MTSET show higher affinity for the carrier (lower IC_{50}) than the negatively charged MTSES suggest that the Cys involved in the binding with the reagents is located in an hydrophilic environment, close to an acidic residue (Glu or Asp). Furthermore, since the SH group readily reacts with the membrane-impermeable reagents *p*-CMBS, MTSET and MTSES, it is expected to be exposed to the extraliposomal face of the carrier that corresponds, as previously reported [15], to the cytosolic side. By experiments based on competition among the various reagents, we found that reversible SH reagents prevent binding of NEM to the protein; however, a fraction of the protein ranging from about 20% to 50% can still be derivatized by NEM even after treatment with MTSEA, DTNB or mercurials. These data indicate that the reagents interact mostly with the same SH group. The residual availability of SH for NEM binding, in the presence of the reversible reagents, may be explained by the presence of (at least) two SH groups: the first SH with higher affinity for the reagents, the second with lower affinity. The reagent added before binds completely to the high-affinity SH leading to total inactivation of the protein and it binds only partially to the second SH; thus, a fraction of the protein (depending on the affinity of the reagent added before, for the second SH) is available for the reaction with NEM. On the basis of these considerations, the SH group population related with the transport inhibition should be constituted by more than one Cys residue.

The second mode of action, i.e. the ornithine/H^+ transport mode, measured as the efflux of [^{3}H]ornithine from proteoliposomes, was inhibited by NEM, MTSEA, DTNB and mercurial reagents at low concentrations (<5 μM). At higher concentrations, *p*-OHMB and *p*-CMBS induced an increase of the efflux that masked the inhibition. We explained the experimental results of Figs. 2 and 3 and Table 2 as a transition of the activity from a carrier (ornithine/ornithine and ornithine/H^+ transport modes) to a pore-like function, characterized by loss of specificity for substrate. This transition of transport function was previously described for other mitochondrial carriers as induced by modification of Cys residues [19–22,30], deletion of specific amino acid sequences [31] or treatment with Ca^{2+} [32]. The pore-like transport function of the ornithine/citrulline carrier, similarly to the aspartate/glutamate, carnitine and phosphate carriers [19–22], was induced by mercurials and it was reverted by DTE. Since *p*-OHMB, instead of $HgCl_2$ [19–22], was the most effective inducing reagent in the case of the ornithine/citrulline carrier, a different environment of the active SH group of this carrier with respect to the others is expected. A peculiar property of the ornithine/citrulline carrier upon transition is the increase in the transport rate with respect to the unmodified protein. A possible physiopathological implication of the switch of this carrier to the pore-like function will be matter of further investigation. It should be mentioned that the conversion of the ADP/ATP carrier to a pore has been found to be related with programmed cell death [33–35].

An additional effect, related to Cys residues of the carrier, has been found upon treatment with SH-oxidizing reagents. Experiments performed with Cu^{2+} phenanthroline demonstrate the presence of at least two close SH groups that may form an S–S bridge leading to inactivation of the protein. This probably indicates that the domain(s) of the protein, in which these SH are located, is involved in conformational changes leading to the substrate permeation through the membrane. The cross-link of two Cys residues, which lead to higher degree of rigidity, inhibits the transport activity. The oxidation reaction, leading to the S–S bridge formation, seems to be at least one order of magnitude slower than the reaction of the protein with the other reagents (NEM, MTS, mercurials and DTNB); this may be explained by a low accessibility of the S–S forming Cys, i.e. a location of these residues either in the transmembrane region or on the intraliposomal (matrix [15]) side of the protein.

The effects induced by the different SH reagents: (i) the inhibition of physiological transport modes (substrate antiport and ornithine/H^+ transport mode) due to the modification of SH residues, (ii) the induction of the pore-like activity by mercurials and (iii) the inhibition of physiological transport modes due to S–S formation unraveled the existence of three functionally different SH group populations in the protein. This is a peculiar aspect of the ornithine/citrulline carrier, and it is in agreement with the finding that both the human and the mouse ornithine carrier sequences [10] contain nine Cys, the highest number of Cys residues of functionally known mitochondrial carriers [for a database of mitochondrial carrier sequences, see http://drnelson.utmem.edu/nelsonhomepage.html]. Moreover, the first and the third Cys populations are constituted by at least two residues each, with those of the third population close enough to form S–S bridge. Thus, the ornithine/citrulline carrier possesses at least five independent SH groups involved in the modifications of the activity, some of them being potential sites of regulation of the carrier function.

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