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Relationships of Cysteine and Lysine residues with the substrate binding site of the mitochondrial ornithine/citrulline carrier: An inhibition kinetic approach combined with the analysis of the homology structural model

Annamaria Tonazzi [a,c], Nicola Giangregorio [a,c], Ferdinando Palmieri [c], Cesare Indiveri [b,c,*]

[a] *National Research Council Institute of Biomembranes and Bioenergetics (IBBE), via Amendola 165/A, 70126, Bari, Italy*
[b] *Department of Cellular Biology, University of Calabria, Via P.Bucci cubo 4c, 87036 Arcavacata di Rende (CS), Italy*
[c] *Department of Pharmaco-Biology, Laboratory of Biochemistry and Molecular Biology, University of Bari, via Orabona 4, 70125, Bari, Italy*

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Abstract

To gain insights in the relationships of specific amino acid residues with the active site of the mitochondrial ornithine/citrulline carrier, we studied the effect of specific protein modifying reagents on the transport catalysed by the carrier reconstituted into liposomes. It was found that, besides the sulfhydryl reagents NEM, MTSEA, p-hydroxymercuribenzoate, diamide also the lysine reagents PLP, DIDS, SITS, the carboxyl reagents WRK, EDC and the arginine reagent methylglyoxal inhibited the carrier. NEM, MTSEA and PLP inhibited the ornithine/citrulline carrier with a completely competitive type of mechanism. A 1:1 interaction of NEM with the carrier molecule has been demonstrated. The results are in agreement with the localization of one sulfhydryl and at least one amino group in the substrate binding site. On the basis of the interferences between SH reagents and PLP in the transport inhibition, it has been deduced that the distance between the SH and the NH_2 residues of the active site should be comparable to the distance between the γ-NH_2 and COOH residues of the ornithine molecule. The structural model of the ornithine/citrulline carrier has been obtained by homology modelling using as template the ADP/ATP carrier structure. The combined analysis of the experimental data and the structural model allows to deduce that Cys-132 is located in the substrate binding site, flanked by at least one Lys residue.
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Keywords: Mitochondria; Membrane; Transport; Carrier; Liposome; Ornithine; Structure; Reconstitution

1. Introduction

The ornithine/citrulline carrier is a metabolite transport system of the inner mitochondrial membrane. The carrier protein has been identified, purified and functionally characterized in reconstituted liposomes [1]. The amino acid sequences of the yeast ARG-11 [2] and the human ORNT1 [3] ornithine carriers have been identified; they show the structural properties of the mitochondrial carrier family [4].

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 [1,5–7] and confirmed by the finding that deficiency of ORNT1 gene lead to HHH syndrome [3] and that the hormonal regulation of the ORNT1 expression in liver is integrated with that of the urea cycle enzymes [8].

The carrier function has been extensively characterized in reconstituted liposomes. The ornithine/citrulline antiport catalysed by the carrier is electroneutral, since citrulline is cotransported with an H^+ [6]. The antiport reaction follows a simultaneous (sequential) transport mechanism [9] as all but one of the mitochondrial carriers characterized so far [4]. The ornithine/citrulline carrier is functionally asymmetrical; the transport protein is inserted in the liposomal membrane

Abbreviations: p-OHMB, p-hydroxymercuribenzoate; MTSEA, (2-aminoethyl)methanethiosulfonate hydrobromide; NEM, N-ethylmaleimide; DTE, 1.4-dithioerythritol; PLP, pyridoxal 5-phosphate; SITS, 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid; DIDS, 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid; WRK, Woodward's reagent K; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimmide

* Corresponding author. Dipartimento di Biologia Cellulare, Università della Calabria, Via P.Bucci cubo 4c, 87036 Arcavacata di Rende (CS), Italy. Tel.: +39 0984 492939; fax: +39 0984 492911.
E-mail address: indiveri@unical.it (C. Indiveri).

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in the same orientation as in mitochondria; the substrate affinities on the two sides of the carrier are in line with the hypothetical channelling of the urea cycle substrates among the enzymes and the transport system [9–11].

The carrier catalyses also an electroneutral ornithine/H^+ exchange that is active in the absence of countersubstrate and should play the function of exporting ornithine from the mitochondria to be used for the polyamine synthesis [12].

Recently, by chemical modification of thiol groups [13], we have shown that the ornithine/citrulline carrier possesses three populations of SH groups: the first population leads to inhibition of the physiological transport upon chemical modification of one or more SH groups; the second is responsible for the transition from carrier to pore-like function; the third is involved in the formation of disulphides, leading to transport inhibition. The presence of three distinct population of SH groups is a peculiar property of the ornithine/citrulline carrier related with the presence, in the amino acid sequence, of 9 Cys that is the highest number of Cys residues of functionally known mitochondrial carriers [13]. Thus, it would be interesting to investigate the possible relationships of some of the SH residues with the substrate binding site. In this paper, by a strategy based on the kinetic analysis of the transport inhibition, it has been revealed that one SH group is located in the substrate binding site. Furthermore, the involvement of amino group(s) of Lys in the transport function and its spatial relationship with the SH group located in the active site have been evidenced. The results are discussed on the light of the structural homology model of the ornithine/citrulline carrier based on the X-ray structure of the mitochondrial ADP/ATP carrier [14].

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, L-[ureido-^{14}C]citrulline, egg yolk phospholipids (L-α-phosphatidylcholine from fresh turkey egg yolk), HEPES and Triton X-100 from Sigma, MTSEA 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 [1]. The purified protein was reconstituted into liposomes by removing the detergent with a hydrophobic column [15,16]. 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% Triton X-100 (Celite eluate, about 1 μg protein), 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as previously described [16], 30 mM L-ornithine (intraliposomal substrate) 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 × 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 [17]. Transport was initiated by adding either 10 μl of [^{3}H]ornithine (ornithine/ornithine antiport) or 10 μl of [^{14}C]citrulline (ornithine/citrulline antiport) at the final concentration indicated in the legends to Tables and Figures. After the required time interval, the reaction was stopped by adding 5 μl PLP plus 5 μl NEM at the final concentration of 20 mM and 1 mM, respectively. 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 antiport rate was evaluated from the radioactivity taken up by the proteoliposomes in 1 min.

2.4. Other methods

The protein was determined by the Lowry method, modified for the presence of non-ionic detergents [18]. The size of the substrate and reagent molecules were calculated by using the ACD/ChemSketch 5.12 software. The structural model of the ornithine/citrulline carrier was analysed by the Swiss-Pdb Viewer 3.7; the same software was used to deduce the distances among amino acid residues of the protein.

3. Results

3.1. Effect of specific reagents on the mitochondrial ornithine/citrulline carrier

It was previously found that a number of SH reagents are very effective inhibitors of the ornithine/citrulline carrier in a concentration-dependent manner [13]. Here, the analysis of modification of amino acid residues was extended by using reagents selected among the most common inhibitors that react with lysine (PLP, SITS, DIDS [19,20]), cysteine (NEM, pOHMB, MTSEA, diamide [13,21]), arginine (methylglyoxal [22]) and glutamate or aspartate (WRK [23], EDC [20]) functional groups (Table 1). To obtain IC_{50} values for each inhibitor, the [^{3}H]ornithine uptake rate into proteoliposomes containing internal ornithine (ornithine/ornithine antiport) was measured as dependence of the reagent concentration; in the case of NH_2-reagents the [^{14}C]citrulline uptake (ornithine/citrulline antiport) was assayed, since these reagents may react with the δ-amino group of ornithine, thus reducing the concentration of free ornithine. The IC_{50} values are listed in Table 1. The carrier showed an IC_{50} in the millimolar range for all the reagents with the exception of pOHMB and MTSEA; the IC_{50} for these inhibitors was in the micromolar range (Table 1 and see Ref. [13]). These data indicated that, besides sulfhydryl groups also amino groups of lysine, carboxyl groups of glutamate/aspartate and guanidino groups of arginine are related with the transport function.

Table 1
Inhibitors of the ornithine/citrulline carrier

Inhibitor	Target functional group	IC_{50}
NEM	SH	0.16 mM ± 0.02 (from Ref. [13])
p-OHMB	SH	0.59 μM ± 0.06 (from Ref. [13])
MTSEA	SH	0.17 μM ± 0.03 (from Ref. [13])
Diamide	SH	1.1 mM ± 0.4
PLP	NH_2	6.2 mM ± 1.05
SITS	NH_2	1.5 mM ± 0.3
DIDS	NH_2	0.15 mM ± 0.04
Methylglyoxal	guanidino	7.3 mM ± 0.4
WRK	COOH	1.8 mM ± 0.1
EDC	COOH	4.3 mM ± 1.1

The IC_{50} values were derived from semilogarithmic plots of dose–response curves for the inhibition of the reconstituted ornithine/citrulline carrier. Inhibitors were added to the proteoliposomes 2 min before the addition of 0.1 mM [^{3}H]ornithine or [^{14}C]citrulline (in the case of the analysis of the inhibition by PLP, SITS and DIDS). The transport was stopped after 1 min as described in Materials and methods. The IC50 values are means ± S.D. from three experiments.

3.2. Relationships of amino acid functional groups with the active site

To verify whether the functional groups of the ornithine/citrulline carrier modified by the specific inhibitors have some relationships with the active site, the influence of the substrate on the extent of the inhibition was tested. We found that 10 mM ornithine or 20 mM citrulline, added to the proteoliposomes before the reagents, exerted a protection of the transport inhibition. A protection of about 70% was found towards NEM, p-OHMB, MTSEA and PLP; whereas a protection lower than 40% was found towards diamide and WRK; no protection was observed on the inhibition by methylglyoxal (experiments not shown). For a more reliable characterization of the relationships among the functional groups sensitive to the reagents and the substrate binding site, an inhibition kinetic study was performed. This was possible since a competitive behaviour can be found also in the case of covalent inhibitors, when the inhibitors interact with the substrate binding site. However, in the case of covalent inhibitors, the competitive effect will disappear, in favour of the expected non-competitive inhibition, after incubation of the inhibitors with the protein for longer times [24–26]. Thus, the dependence of the antiport rate on the substrate concentration, was studied in the absence and presence of the inhibitor added simultaneously with the substrate or added before the substrate (longer incubation times). The analysis was performed with the SH reagents NEM and MTSEA, with the disulphide inducer diamide, with the NH_2 reagent PLP that is highly specific for lysine (differently from DIDS or SITS, that may react also with SH groups [19,20,27]) and with WRK that is specific for COOH groups. The experimental data, analysed according to Lineweaver–Burk, gave competitive inhibition patterns for NEM (Fig. 1A), MTSEA (Fig. 1B), PLP (Fig. 1C) added together with the substrate; differently, non-competitive patterns were obtained when the same inhibitors were added 4 min before the substrate. The same inhibition pattern was found for p-OHMB (not shown). The data indicate that, under initial velocity condition, the inhibitors interfere with the substrate binding to the active site. However, a problem may arise in the case of partially competitive inhibition, i.e., the inhibitors bind to a site different from that of the substrate causing an enzyme conformational change which alters the binding of the substrates. This situation cannot be

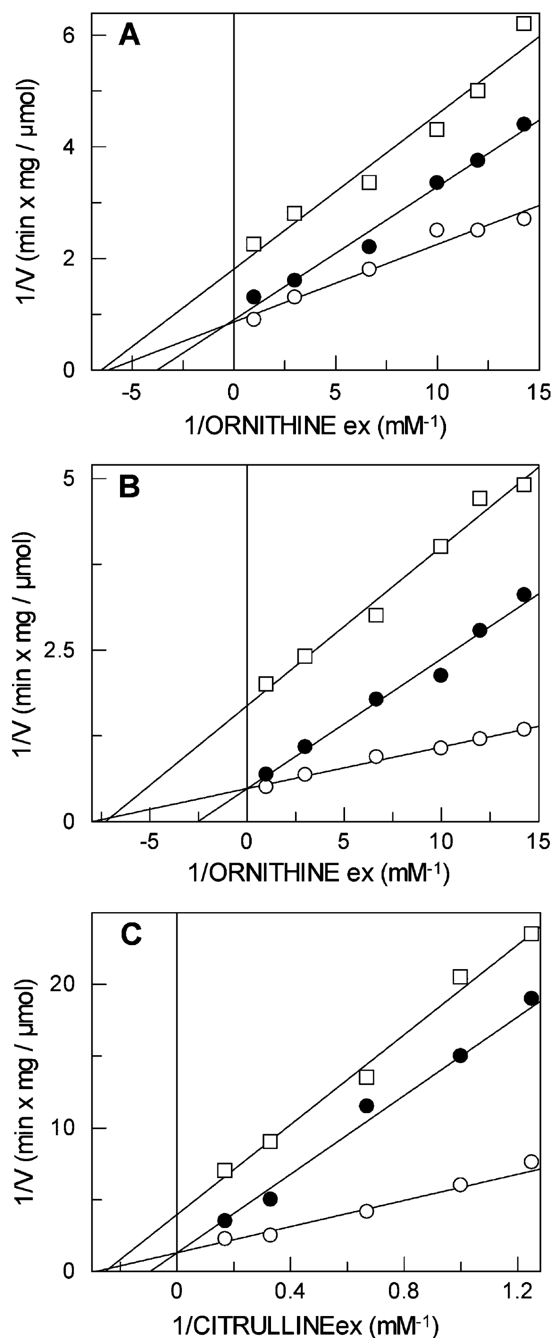

Fig. 1. Kinetic analysis of the inhibition of the reconstituted ornithine/citrulline carrier by NEM, MTSEA, PLP, using the Lineweaver–Burk plot. The dependence of antiport rate was measured as [^{3}H]ornithine or [^{14}C]citrulline uptake into proteoliposomes as described in Materials and methods. 0.1 mM NEM (A), 0.3 μM MTSEA (B), 12 mM PLP (C) were added to the proteoliposomes simultaneously with (●) or 4 min before (□) the substrate; (○) control without inhibitor. Similar results were obtained in three different experiments.

easily discriminated by the Lineweaver–Burk analysis; the problem can be resolved by varying the inhibitor concentration at fixed substrate concentrations and plotting the results according with the method of Dixon [26,28,29]. The data analysed with this method give a non-linear dependence in the case of partial inhibition. The Dixon plots obtained using either NEM (Fig. 2A), MTSEA (Fig. 2B) or PLP (Fig. 2C) showed a linear dependence indicating that the inhibition is completely competitive. These results show that the binding of the inhibitors occurs in or near the substrate binding site. In the case of WRK (Fig. 3A) and diamide (that induces the oxidation of two vicinal cysteines to form a S–S bridge; Fig. 3B) added together with the labelled substrate, the common interception of the straight lines in Lineweaver–Burk plots fell between the axis, indicating a mixed type of inhibition; when the same inhibitors were added before the labelled substrate, non-competitive inhibition was found for WRK and again mixed inhibition for diamide.

3.3. Titration of the SH groups in the active site

Due to the chemical properties of NEM, it was possible to study the inactivation kinetics [26], i.e., the time dependence of the inhibition of the carrier with NEM. This reagent, in fact, differently from the other SH reagents here tested, leads to an irreversible alkylation of the SH group(s); thus, its reaction with the protein can be promptly stopped by DTE, that inactivates the unreacted NEM molecules, without affecting the formed NEM-protein adduct. This approach can give information on the number of SH residues that must be derivatized to inactivate the protein [26,30,31]. The dependence of the residual carrier activity on the time of incubation with various NEM concentrations was analysed in a semi logarithmic plot: non-linear dependence of the Log of residual activity vs. time was found at each NEM concentration (not shown), indicating a complex interaction between the reagent and the protein. However, by performing the same experiment at pH 7.0 instead of pH 8.0, a pattern of straight lines was obtained (Fig. 4A); from the slope of the lines, the apparent first-order rate constants of the inactivation reaction of the protein with NEM were derived. From the slope of the double-logarithmic plot (Fig. 4B) a reaction order of 0.75 was calculated. This result indicated that the modification of a single sulfhydryl group, per functional carrier molecule, is sufficient for the complete inhibition of the transport catalysed by the ornithine/citrulline carrier. By performing the experiment described in Fig. 1A at pH 7.0 instead of pH 8.0, the same competitive pattern was obtained in the case of the inhibitor added together with the labelled substrate (not shown).

3.4. Interaction between PLP and sulfhydryl reagents

On the basis of the results above described, one SH and at least one NH_2 functional groups must be located into the substrate binding site of the ornithine/citrulline carrier. It may be hypothesized that the two groups are located at an

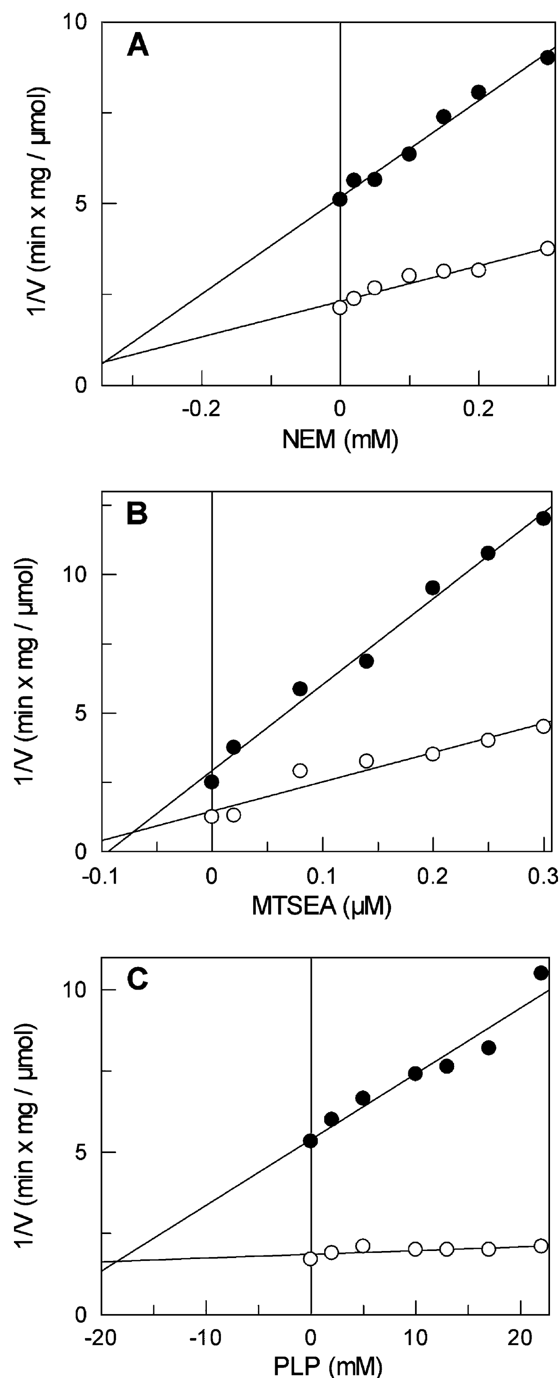

Fig. 2. Kinetic analysis of the inhibition of the reconstituted ornithine/citrulline carrier by NEM, MTSEA, PLP, using the Dixon plot. NEM (A) or MTSEA (B) was added at the indicated concentrations to proteoliposomes simultaneously with 0.1 (●) or 0.3 (○) mM ^{3}H-ornithine; PLP (C) was added at the indicated concentrations to proteoliposomes simultaneously with 0.8 mM (●) or 3.0 mM (○) [^{14}C]citrulline. The antiport rate was measured as described in Materials and methods. Similar results were obtained in three different experiments.

appropriate distance to interact with the functional groups of the substrate ornithine (or citrulline). Since the maximal distance of the ornithine functional groups (δ-amino and carboxyl groups) is about 7.5 Å, it can be expected a steric hindrance between the amino and the sulfhydryl reagents if both of them interact with the residues of the active site. The experimental approach to verify this hypothesis was

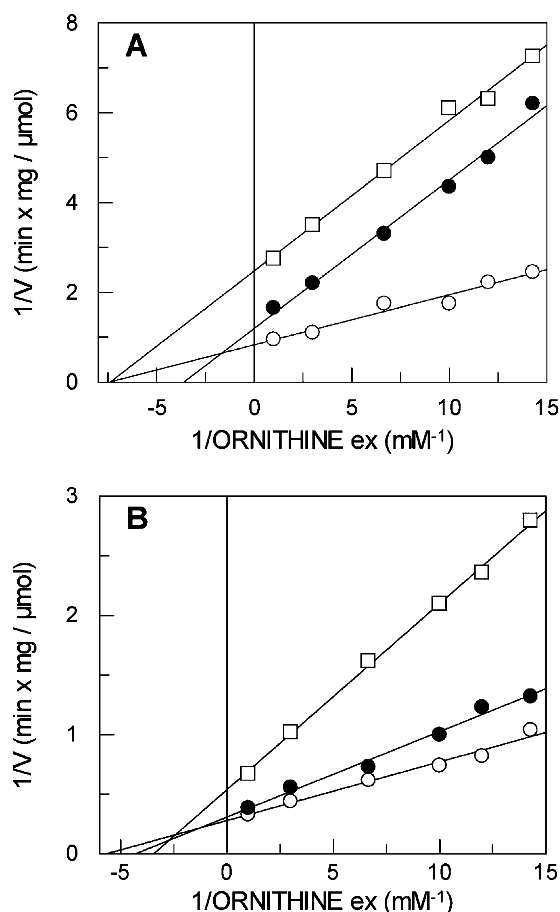

Fig. 3. Kinetic analysis of the inhibition of the reconstituted ornithine/citrulline carrier by WRK and diamide, using the Lineweaver–Burk plot. The dependence of antiport rate was measured as [^{3}H]ornithine uptake into proteoliposomes as described in Materials and methods. 1 mM WRK (A) and 0.5 mM diamide (B) were added to the proteoliposomes simultaneously with (●) or 4 min before (□) the substrate; (○) control without inhibitor. Similar results were obtained in three different experiments.

based on the possibility to displace the aldimine linkage of PLP, but not the linkage of the SH reagents with the reconstituted protein by size exclusion chromatography. The virtually complete inhibition caused by 20 mM PLP (see legend to the Table 2) could be reversed by Sephadex chromatography (Table 2); on the contrary, the inhibition caused by treatment of the proteoliposomes with $HgCl_2$ NEM, pOHMB, MTSEA and diamide could not be reversed. To test the possible interference among PLP and the SH reagents, proteoliposomes were incubated with 20 mM PLP, and then with $HgCl_2$, NEM, pOHMB, MTSEA or diamide. After the treatment with both the reagents, the proteoliposomes were passed through Sephadex G-75 to remove the non-reacted reagents and reverse the PLP–protein linkage. The treatment with PLP before the SH reagents, prevented the inhibition by $HgCl_2$, NEM, pOHMB, MTSEA; this effect was proportional to the size of the SH inhibitor: the prevention was lower with the smaller reagents $HgCl_2$ and NEM and higher with the larger reagents MTSEA and pOHMB. These data clearly indicated the occurrence of interference among the SH reagents and PLP, i.e., the amino and the sulfhydryl groups of the active site have some spatial relationships. The inhibition by diamide was not influenced by the preincubation of the protein with PLP, indicating that the vicinal SH groups are not located in the same environment of the NH_2 group of the active site.

3.5. Homology model of the ornithine/citrulline carrier based on the X-ray structure of the ADP/ATP carrier

The mitochondrial ornithine/citrulline carrier is homologous with the bovine mitochondrial ADP/ATP carrier, for which a crystal structure is available [14]. The two carriers share 20% identical amino acids (not shown). We therefore built a homology model of the mouse ornithine/citrulline carrier (Fig. 5) based on the three-dimensional structure of the carboxyatractyloside-ADP/ATP carrier complex. The figure shows a view of the structure from the cytosolic side, as deduced by the exposure of the N- and C-terminal ends of the protein, typical of the carrier family [4,14]. In Fig. 5B, the accessibility of the highlighted residues from the external (cytosolic) side of the carrier is evidenced by the space-filled

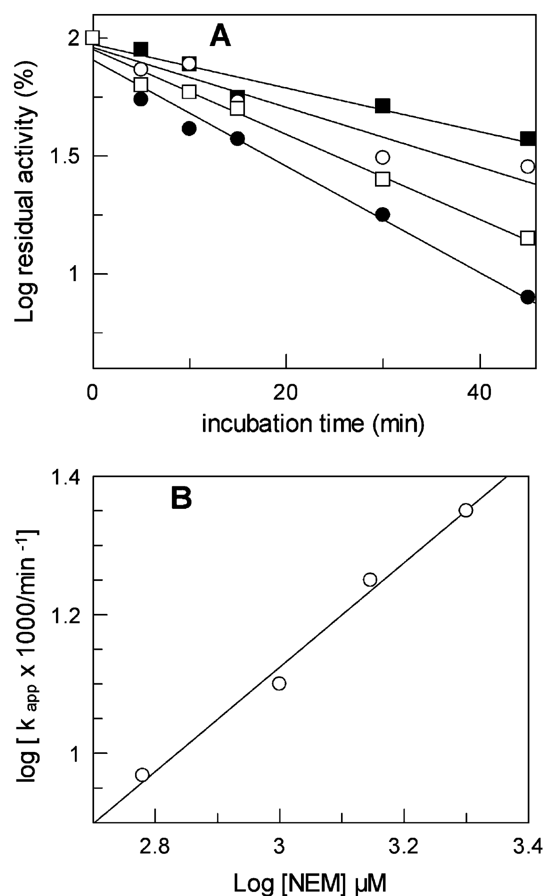

Fig. 4. Kinetic of inactivation of the reconstituted ornithine/citrulline carrier by NEM. (A) Proteoliposomes were incubated with different NEM concentrations (■, 0.6 mM; ○, 1 mM; □, 1.4 mM; ●, 2 mM) for the indicated times. The reaction of the inhibitor with the protein was stopped by the addition of 3 mM DTE. The incubation with NEM and the transport measurement were performed at pH 7.0 (see Results). (B) Double-logarithmic secondary plot of the apparent inactivation constants K_{app} plotted versus the applied concentrations of NEM. Similar results were obtained in three different experiments.

Table 2
Interaction of PLP with sulfhydryl reagents in the inhibition of the reconstituted ornithine/citrulline carrier

Additions at		Residual activity after Sephadex (% of the control)
0 min	5 min	
PLP (20 mM)		110±3
$HgCl_2$ (2 μM)		9±5
NEM (1 mM)		3±1
pOHMB (2 μM)		7±4
MTSEA (2 μM)		8±5
Diamide (0.5 mM)		43±10
PLP	$HgCl_2$	54±9
PLP	NEM	69±6
PLP	pOHMB	93±10
PLP	MTSEA	85±8
PLP	Diamide	48±12

Proteoliposomes (400 μl aliquots) were incubated, at time zero, with the reagents, at the indicated concentrations. At 5 min, aliquots of PLP incubated proteoliposomes were added with the different sulfhydryl reagents. At 10 min, all the proteoliposome samples were passed onto G-75 Sephadex columns (0.7 × 15 cm) and than assayed for the transport measurements as described in Materials and methods. Transport was started by adding 0.1 mM [^{3}H]ornithine and stopped after 10 min. Percent residual activity was calculated for each sample with respect to the control (referred as 100%), i.e., proteoliposomes not treated with any inhibitor; the values are means±S.D. of the percentage of three experiments. In the three experiments analysed: the residual transport activity before G-75 Sephadex chromatography, of the sample treated with 20 mM PLP, was 7±5% of the control; the average specific activity of the control samples was 2.05±0.95 mmol/10 min/g protein.

representation. In this model Cys-132 is located in the middle of the water filled cone-shaped cavity of the carrier and is fully accessible to the outside (cytosolic side).

4. Discussion

In order to gain information on the role of specific amino acid residues in the translocation pathway of the mitochondrial ornithine/citrulline carrier, we have analysed the effect of reagents specific for arginine, lysine, cysteine, aspartate/glutamate. The substrate antiport catalysed by the carrier was completely inhibited by each of the compounds tested, externally added to the proteoliposomes, even though with different efficiency. Thus, there are arginine, lysine, cysteine, aspartate and/or glutamate responsible for the inhibition of the transport activity. The differences in IC_{50} among the various reagents may be determined by two main factors: (i) the mechanism of reaction of the compounds with the protein; (ii) the more or less favourable interaction of each reagent with the surroundings of the functional group specifically derivatized. Also, the alternative (ornithine/H^+) transport mode of the ornithine/citrulline carrier was inhibited by the reagents indicating that similar transport pathways are involved in the two modes of functioning of the protein; differently, none of the inhibitors tested, was able to block the unphysiological pore-like function of the ornithine/citrulline carrier (experiments not shown). When studying the relationships between specific amino acid residues and the function of a protein, it is important to asses whether the residue responsible for the inhibition is located into the substrate binding site. This condition may be evidenced by substrate protection of the inhibition, even though, this experimental approach can be ambiguous due to possible conformational changes caused by the inhibitor reaction with the protein, that can modify the relationships among amino acid residues in the substrate binding site. A more accurate procedure that indicate the interaction of a molecule with the substrate binding site, is the kinetic analysis of the inhibition. This type of analysis leads to reliable results in the case of reversible inhibitors. However, when the inhibitor combines with a substrate binding group in the active centre, a competitive type of inhibition can be observed also with covalent inhibitors. This happens if the irreversible reaction of the inhibitors with the protein is slower than the binding of the substrate; in this case the interaction of the substrate with the active site slows down the velocity constant of the inhibition and the competitive effect is evident [24–26]. Therefore, a competitive inhibition found with

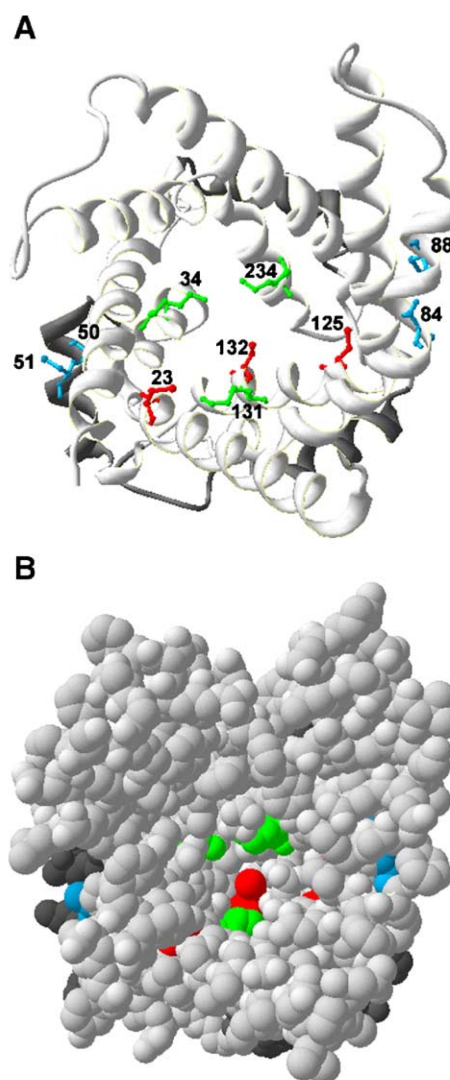

Fig. 5. Structural model of the ornithine/citrulline carrier. (A) Ribbon diagram viewing the carrier from the outside (cytosolic face); the following residues are highlighted: Cys-132, Cys-23 and Cys 125 (red); Cys-50, Cys-51 and Cys-84, Cys-88 couples (light blu); Lys -34, Lys-131, Lys-234 (green); the matrix hydrophilic loops are colored in dark gray. (B) Space-fill diagram of the same picture of panel A.

covalent inhibitors indicates the interaction of the inhibitor with the substrate binding site. Most of the molecules here used required more than 2 min for maximal inhibition under the experimental conditions used (not shown and see Fig. 4). Thus, the reaction of the inhibitors with the carrier is slower than the binding of the substrate. The inhibition by sulfhydryl and amino group reagents of the substrate antiport, analysed under initial velocity condition, was clearly competitive. The occurrence of a complete competitive inhibition was confirmed by the linear Dixon-plots which exclude that the inhibitors may react far from the binding site inducing conformational changes in the active site [26,28,29]. The inhibitory effects observed are determined by the reagent externally added to the proteoliposomes; thus, the specific amino acid residues implicated in the inhibition must be exposed towards the external compartment of the vesicles that corresponds to the cytosolic side of the carrier, since the reconstituted protein has the same orientation as in the mitochondrial membrane [9].The inactivation kinetic analysis showed that the inhibition of the transport is related to a 1:1 reaction of NEM with the protein, i.e., a single SH group in the active site is responsible of the inhibition. This stoichiometry was clearly measurable at pH 7.0; a non-linear dependence was found at more alkaline pH; this may be determined by the reaction of NEM with more than one SH, under the condition of the experiment, i.e., prolonged time of incubation. This is in agreement with previous data showing that the ornithine/citrulline carrier possesses a population of SH groups, related with the transport inhibition, constituted by at least two Cys: the first of which with higher affinity for NEM and the second with a lower affinity [13]. As previously hypothesized [9], the SH of the active site can be involved as thiolate, in the binding with H^+, that is co-transported with citrulline, the second substrate of the carrier that binds to the same site of ornithine [9]. The same thiolate may be implicated in the interaction with the δ-amino group of the ornithine$^+$ cation; this interaction will facilitate the dissociation of an H^+ from ornithine$^+$, thus forming uncharged ornithine that is the substrate form required by ornithine transcarbamoylase in the mitochondrial matrix [6,32]. No information about the number of NH_2 functional groups present in the active site can be obtained by the inactivation kinetic approach used for the SH groups, due to the reversible nature of the aldimine linkage between PLP and the protein (see Table 2). However, it is likely that a single NH_2 group of Lys may be related to the interaction with the carboxyl group of the substrate ornithine (or citrulline). If the thiolate and the NH_2 present in the active site interact, respectively, with the δ-amino and the carboxyl groups of ornithine (see above), the distance between the two functional groups of the protein, should be similar to the distance between the functional groups of ornithine (about 7.5 Å). On this basis, we have hypothesized to find sterical hindrance between two reagents which bind specifically to the amino or sulfhydryl group. Indeed, we have found an interference among PLP and sulfhydryl reagents, evidenced as prevention of the binding of the SH reagent by PLP (see Table 2). Interestingly, the extent of prevention depends on the size of the sulfhydryl reagents. After PLP binding, nearly no reaction of the larger pOHMB and MTSEA (6.9 and 6.6 Å) with the protein occurred; whereas about 30% or 50% of reaction (inhibition) was detected for NEM (4.1 Å) and $HgCl_2$ (from 1 Å, Hg^{++}, to 2.4 Å, Hg Cl_2), respectively. On the basis of these observations, we can hypothesize that the distance between the two functional groups in the active site may be comparable to the size of PLP (about 7.5 Å). Few information could be deduced by the inhibition of WRK and diamide (mixed). It cannot be excluded that WRK could react, besides other COOH, with a carboxyl group in the active site (see also below). Regarding the mixed inhibition found with the disulphide inducing reagent diamide, it may be hypothesized that the formation of the disulphide causes a reduction of mobility of the protein that impedes the conformational changes of the active site responsible for the catalytic cycle of the transport. On the basis of the absence of interference between diamide and PLP (Table 2) and of results previously reported (the S–S forming Cys and the SH derivatized by NEM belong to different populations) [13], it is unlikely that even one of the SH groups of the S–S forming Cys is located in the active centre as the other Cys residue. This is also supported by the structural model discussed below.

To gain further information about structure/function relationships of the ornithine/citrulline carrier we have modelled [33–35] the structure of the carrier protein, using as template the mitochondrial ADP/ATP carrier [14]. On the basis of the structural model of the ornithine/citrulline carrier, Cys-132, located on the 3rd transmembrane α-helix facing to the centre of the aqueous cavity, can be identified as the Cys residue of the active site, for the following reasons: (i) its SH group is fully accessible towards the external (citosolic) side of the carrier (Fig. 5B); thus, it can be reached by NEM, but also by the hydrophilic reagents MTSEA and p-OHMB; (ii) Glu-128 residue is located at about 5 Å from Cys-132. This distance corresponds to the size of the reacted MTSEA, which positive charge can be stabilized by the negative charge of Glu; this is in agreement with the highest affinity of the carrier for MTSEA (Table 1 and see Ref. [13]); (iii) on the basis of the structural model, at least one Lys residue (Lys-34, 131 or 234) lies in the aqueous cavity at a distance of about 7 Å to the SH group of Cys-132, in agreement with the size of the ornithine and PLP molecules (see above); (iv) Cys-132 is homologous to the Cys-136 residue located in the active site of the carnitine/acylcarnitine carrier [36,37]. At least one of the three Lys residues mentioned above is responsible for the competition with the substrate (Fig. 1C) and the interference with the SH reagents (Table 2). The alignment of the ornithine/citrulline carrier with the yeast ornithine carrier ARG-11 [2] (not shown) reveals that the residues corresponding to Cys-132, Lys-34, 131 and 234 are conserved; this finding is in agreement with the role of some of these residues in the interaction with the substrate. The further Cys residue(s) of the first population of the ornithine/citrulline carrier that can react only with NEM at higher concentrations and longer times of incubation but not with MTSEA [13], are, most probably, Cys-23 and Cys-125; these residues are located at the edge of the water cavity and, as shown by the model (Fig. 5B), have a lower accessibility than

Cys-132. Regarding the S–S forming Cys, that belong to a different SH population [13], they could be identified with the Cys couples Cys-84/Cys-88 and Cys-50/Cys-51 (Fig. 5A) that are close enough to form S–S. However, when looking to the space-fill model, these residues are not easily accessible from the outside; in fact, Cys-84 and Cys-88 lies towards the membrane; Cys-50 and Cys-51 are on an internal loop that, however, is not far from the hypothetical substrate binding site. These observations explain the very slow reaction of diamide (see above and Ref. [13]) and, possibly, the mixed type of inhibition found with this reagent.

References

[1] C. Indiveri, A. Tonazzi, F. Palmieri, Identification and purification of the ornithine/citrulline carrier from rat liver mitochondria, Eur. J. Biochem. 207 (1992) 449–454.

[2] L. Palmieri, V. De Marco, V. Iacobazzi, F. Palmieri, M.J. Runswick, J.E. Walker, Identification of the yeast ARG-11 gene as a mitochondrial ornithine carrier involved in arginine biosynthesis, FEBS Lett. 410 (1997) 447–451.

[3] J.A. Camacho, C. Obie, B. Biery, B.K. Goodman, C. Hu, S. Almashanu, G. Steel, R. Casey, M. Lambert, G.A. Mitchell, D. Valle, Hyperornithinaemia–hyperammonaemia–homocitrullinuria syndrome is caused by mutations in a gene encoding a mitochondrial ornithine transporter, Nat. Genet. 22 (1999) 151–158.

[4] F. Palmieri, The mitochondrial transporter family (SLC25): physiological and pathological implications, in: M.A. Hediger (Ed.), The ABC of Solute Carriers, Pflugers Arch.-Eur. J. Physiol., vol. 447, 2004, pp. 689–709.

[5] N.M. Bradford, J.D. McGivan, Evidence for the existence of an ornithine/citrulline antiporter in rat liver mitochondria, FEBS Lett. 113 (1980) 294–298.

[6] C. Indiveri, A. Tonazzi, I. Stipani, F. Palmieri, The purified and reconstituted ornithine/citrulline carrier from rat liver mitochondria: electrical nature and coupling of the exchange reaction with H^+ translocation, Biochem. J. 327 (1997) 349–356.

[7] C. Indiveri, L. Palmieri, F. Palmieri, Kinetic characterization of the reconstituted ornithine carrier from rat liver mitochondria, Biochim. Biophys. Acta 1188 (1994) 293–301.

[8] S.M. Morris Jr., D. Kepka-Lenhart, Hormonal induction of hepatic mitochondrial ornithine/citrulline transporter mRNA, Biochem. Biophys. Res. Commun. 294 (2002) 749–752.

[9] C. Indiveri, A. Tonazzi, A. De Palma, F. Palmieri, Kinetic mechanism of antiports catalyzed by reconstituted ornithine/citrulline carrier from rat liver mitochondria, Biochim. Biophys. Acta 1503 (2001) 303–313.

[10] N.S. Cohen, C.W. Cheung, L. Raijman, Channeling of extramitochondrial ornithine to matrix ornithine transcarbamylase, J. Biol. Chem. 262 (1987) 203–208.

[11] C.W. Cheung, N.S. Cohen, L. Raijman, Channeling of urea cycle intermediates in situ in permeabilized hepatocytes, J. Biol. Chem. 264 (1989) 4038–4044.

[12] C. Indiveri, A. Tonazzi, I. Stipani, F. Palmieri, The purified and reconstituted ornithine/citrulline carrier from rat liver mitochondria catalyses a second transport mode: ornithine$^+$/H^+ exchange, Biochem. J. 341 (1999) 707–711.

[13] A. Tonazzi, C. Indiveri, Chemical modification of the mitochondrial ornithine/citrulline carrier by SH reagents: effects on the transport activity and transition from carrier to pore-like function, Biochim. Biophys. Acta 1611 (2003) 123–130.

[14] E. Pebay-Peyroula, C. Dahout-Gonzalez, R. Kahn, V. Trezeguet, G.J. Lauquin, G. Brandolin, Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside, Nature 426 (2003) 39–44.

[15] R. Krämer, C. Heberger, Functional reconstitution of carrier proteins by removal of detergent with a hydrophobic ion exchange column, Biochim. Biophys. Acta 863 (1986) 289–296.

[16] F. Palmieri, C. Indiveri, F. Bisaccia, V. Iacobazzi, Mitochondrial metabolite carrier proteins: purification, reconstitution, and transport studies, Methods Enzymol. 260 (1995) 349–369.

[17] F. Palmieri, M. Klingenberg, Direct methods for measuring metabolite transport and distribution in mitochondria, Methods Enzymol. 56 (1979) 279–301.

[18] J.R. Dulley, P.A. Grieve, A simple technique for eliminating interference by detergents in the Lowry method of protein determination, Anal. Biochem. 64 (1975) 136–141.

[19] R. Stappen, T. Dierks, A. Broer, R. Kramer, Probing the active site of the reconstituted aspartate/glutamate carrier from mitochondria. Structure/function relationship involving one lysine and two cysteine residues, Eur. J. Biochem. 210 (1992) 269–277.

[20] T. Dierks, R. Stappen, A. Salentin, R. Kramer, Probing the active site of the reconstituted aspartate/glutamate carrier from bovine heart mitochondria: carbodiimide-catalyzed acylation of a functional lysine residue, Biochim. Biophys. Acta 1103 (1992) 13–24.

[21] P.R. van Iwaarden, A.J. Driessen, W.N. Konings, What we can learn from the effects of thiol reagents on transport proteins, Biochim. Biophys. Acta 1113 (1992) 161–170.

[22] T. Oya, N. Hattori, Y. Mizuno, S. Miyata, S. Maeda, T. Osawa, K. Uchida, Methylglyoxal modification of protein. Chemical and immunochemical characterization of methylglyoxal-arginine adducts, J. Biol. Chem. 274 (1999) 18492–18502.

[23] A.J. Brake, B.H. Weber, Evidence for an essential carboxyl group for yeast phosphoglycerate kinase. Reaction with Woodward's reagent K, J. Biol. Chem. 249 (1974) 5452–5457.

[24] M. Dixon, E.C. Webb, Enzymes, Longmans, 1964, pp. 316–317.

[25] J.L. Webb, Enzyme and Metabolic Inhibitors, vol. II, Academic Press, New York, 1966, p. 771, Chapt. 7.

[26] C. Indiveri, A. Tonazzi, N. Giangregorio, F. Palmieri, Probing the active site of the reconstituted carnitine carrier from rat liver mitochondria with sulfhydryl reagents. A cysteine residue is localized in or near the substrate binding site, Eur. J. Biochem. 228 (1995) 271–278.

[27] F.C. Hartman, I.C. Norton, Active-site-directed reagents of glycolytic enzymes, Methods Enzymol. 47 (1977) 479–498.

[28] M. Dixon, The determination of enzyme inhibitor constants, Biochem. J. 55 (1953) 170–171.

[29] J.L. Webb, Enzyme and Metabolic Inhibitors, vol. I, Academic Press, New York, 1963, pp. 157–163, Chapt. 5.

[30] H.M. Levy, P.D. Leber, E.M. Ryan, Inactivation of myosin by 2,4-dinitrophenol and protection by adenosine triphosphate and other phosphate compounds, J. Biol. Chem. 238 (1963) 3654–3659.

[31] M.C. Scrutton, M.F. Utter, Pyruvate carboxylase: V. Interaction of the enzyme with adenosine triphosphate, J Biol. Chem. 240 (1965) 3714–3723.

[32] A.J. Meijer, W.H. Lamers, R.A.F.M. Chamuleau, Nitrogen metabolism and ornithine cycle function, Physiol. Rev. 70 (1990) 701–748.

[33] T. Schwede, J. Kopp, N. Guex, M.C. Peitsch, SWISS-MODEL: an automated protein homology-modeling server, Nucleic Acids Res. 31 (2003) 3381–3385.

[34] N. Guex, M.C. Peitsch, SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling, Electrophoresis 18 (1997) 2714–2723.

[35] M.C. Peitsch, Protein modeling by E-mail, Bio/Technology 13 (1995) 658–660.

[36] C. Indiveri, N. Giangregorio, V. Iacobazzi, F. Palmieri, Site-directed mutagenesis and chemical modification of the six native cysteine residues of the rat mitochondrial carnitine carrier: implications for the role of cysteine-136, Biochemistry 41 (2002) 8649–8656.

[37] A. Tonazzi, N. Giangregorio, C. Indiveri, F. Palmieri, Identification by site-directed mutagenesis and chemical modification of three vicinal cysteine residues in rat mitochondrial carnitine/acylcarnitine transporter, J. Biol. Chem. 280 (2005) 19607–19612.