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Structure/function relationships of the human mitochondrial ornithine/citrulline carrier by Cys site-directed mutagenesis. Relevance to mercury toxicity

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ABSTRACT

The effect of SH reagents on the human mitochondrial ornithine/citrulline carrier (hORC) was studied. Site-directed Cys mutants were employed to gain information on structure/function relationships. The substitutions of each Cys by Ala did not alter the hORC activity measured as [^{3}H]ornithine/ornithine antiport in proteoliposomes. *N* ethylmaleimide inhibited the transport of WT with IC50 of 149 μM. C51A, C50A and C132A showed a much higher IC50. MTSEA and MTSET also inhibited the WT with IC50 of 0.40 μM and 1.60 μM, respectively. C51A and C132A showed much higher IC50 values for both reagents. The triple mutant C50/51/132A showed an IC50 for the three reagents that was higher than that of the single mutants. The data strongly suggests that C132, C50 and C51 are involved in inhibition of hORC. Inhibition of WT and mutants by CuPhenanthroline, an S-S forming reagent, suggested that C132 may form disulfides with C50 or C51, impairing the transporter function. The structure/function relationships information deriving from the inhibition studies, were corroborated by the homology structural model of the transporter. The effect of $HgCl_2$ and methyl mercury was also tested on hORC in the light of their capacity to bind thiol residues. Both reagents potently inhibit the transporter.

1. Introduction

The ornithine/citrulline carrier is a membrane transport protein belonging to the mitochondrial carrier family, SLC25 [1], which catalyzes a specific antiport of ornithine, citrulline and few other cationic substrates [2,3]. The physiological function of ORC in mammals consists in mediating ornithine transport from cytosol to mitochondrial matrix and citrulline in the opposite direction, thus allowing connection of the cytosolic and mitochondrial enzyme pathways operating in the urea cycle [4,5]. The antiport reaction is coupled to a proton which is exported from mitochondria together with citrulline. The proton, which is released in the matrix by the Ornithine Carbamoyl Transferase reaction, compensates the positive charge of ornithine thus leading to an electroneutral transport reaction ornithine$^+$/citrulline + H^+. This mechanism prevents matrix acidification and dissipation of the electrochemical gradient across the inner mitochondrial membrane [2]. The role of ORC in the urea cycle completion was unequivocally demonstrated by the finding that defects of the ORC coding gene is a cause of the HHH syndrome [6]. A second isoform of ORC, SLC25A2, also called ORNT2, was identified that may have a marginal role, indeed it is less expressed than the major isoform SLC25A15. In more recent studies, the amino acid residues of the ORC responsible for substrate binding were identified by site directed mutagenesis [3,7]. Some of these residues correspond to those identified as responsible of substrate binding in the mitochondrial carnitine carrier, which is involved in the β-oxidation pathway [8,9]. The mouse and human ORC possess nine Cys residues each. These residues have been targeted in previous studies with the rat protein, using SH specific chemical reagents. A prediction of the Cys residues involved in the inhibition of transport by SH reagents was previously attempted on the basis of a first homology model of ORC, built using the structure of the bovine ANC, as template [10]. In this model, some of the Cys residues are located in the vicinity of the hypothetical substrate binding site. However, no conclusive information on the specific Cys residues responsible for inhibition could be derived

Abbreviations: MTSEA, 2 aminoethyl methanethiosulfonate; MTSET, [2 (trimethylammonium)ethyl]methanethiosulfonate; NEM, *N* ethylmaleimide; ANC, adenine nucleotide translocator; OGC, Oxoglutarate carrier; CACT, Carnitine carrier; PLP, pyridoxal phosphate; DTE, 1,4 Dithioerythritol.

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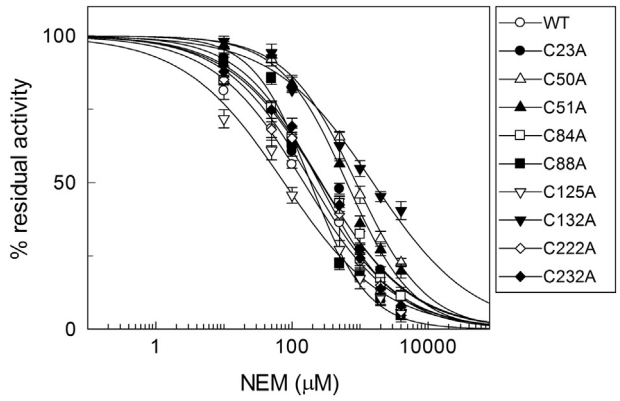

Fig. 1. Dose-response analysis of the inhibition of ornithine transport by NEM. Transport was measured as described in Materials and methods on hORC WT or Cys mutants. The indicated concentrations of NEM were added 2 min before the [^{3}H]ornithine. Percent of residual activity with respect to the control (without NEM) is reported. The values are means ± SD from three independent experiments.

from the previous studies. Interestingly, mercury compounds were also predicted to interact with SH of ORC. These compounds are known pollutants and, hence, might underlie toxicity mechanisms [11]. Therefore, the structure/function relationships of Cys residues of the human ORC were an important issue to be clarified. This issue is dealt with in the present work using a combined experimental strategy based on site-directed mutagenesis, chemical targeting and bioinformatics. The results point out the redox state of the thiol residues of the human ORC is critical for function.

2. Materials and methods

2.1. Chemicals

Amberlite XAD-4, egg yolk phospholipids (3 *sn* phosphatidylcoline from egg yolk), *N* dodecanoylsarcosine (sarkosyl), Sephadex G-50 and G-75, L ornithine, *N* ethylmaleimide (NEM), Cardiolipin, Mercury chloride (Hg^{2+}), CH_3Hg^+ (as CH_3HgCl), Cu^{2+}-phenanthroline, Triton X-100, pyridoxal 5 phosphate (PLP), 1,4 Dithioerythritol (DTE) and NiNTA Resin were from Sigma; L [2,3 ^{3}H]ornithine from Scopus Research BV Costerweg; MTSEA, MTSET from Fluorescent Dyes Inc. All other reagents were of analytical grade.

2.2. Cloning, over-expression and isolation of the Wild Type protein and Cys-Ala mutants of hORC1

The 903 bp cDNA coding for the hORC1 Wild Type (WT) protein was amplified from the Mega Man Human Transcriptome Library with the forward and reverse primers 5′-GGAATTCCATATGAAATCCAATCCTG CTATCCAG-3′ and 5′-CCCAAGCTTGTATGCTTCCAACTGGTTCATC-3′, containing the *Nde*I and *Hind*III sites, respectively. The amplified cDNA was then cloned in the *Nde*I/*Hind*III sites of the pET-21a(+) expression vector. The resulting recombinant plasmid, defined as pET-21a(+)-hORC1, encodes a full length hORC1 protein with a His6 tag before the termination codon.

The nine Cys residues of hORC1 have been mutated to Ala by PCR and Dpn1 method [12] using the primers designed in Supplementary table (Table S1). Bacterial over-expression of WT and mutant proteins has been performed, in *E. coli* C0214, as described previously for the carnitine/acylcarnitine carrier [13,14]. Wild Type and mutant hORC1 proteins, as inclusion body fraction, were solubilized and then purified by Ni-chelating chromatography (NiNTA Resin) as described previously [15].

2.3. Reconstitution of WT and mutants hORC1 transporters into liposomes

hORC1 WT and Cys mutants purified by affinity chromatography were reconstituted by cyclic removing of the detergent from mixed micelles containing detergent, protein and phospholipids with a hydrophobic column of Amberlite resin (Amberlite XAD-4) [16]. The initial mixture used for the reconstitution was composed of 6 μg purified His-tag protein, 90 μl of 10% Triton X-100, 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as previously described [17], 30 mM L ornithine, 20 mM HEPES pH 8.0 in a final volume of 680 μl. This mixture was recycled 18 times, at room temperature, through the XAD-4 column (0.5 cm diameter × 2.5 cm height) pre-equilibrated with the same buffer (20 mM HEPES pH 8.0) and the same substrate (30 mM L ornithine).

2.4. Transport measurements

To remove the external substrate, 550 μl of proteoliposomes were passed through a Sephadex G-75 column (0.7 cm diameter, 15 cm height) pre-equilibrated with 10 mM HEPES pH 8.0 and 60 mM sucrose. The first 600 μl of the turbid eluate were collected, transferred to vials (100 μl each), and used for transport measurements by the inhibitor stop method [16]. Transport was started by adding to proteoliposomes 0.1 mM [^{3}H]ornithine (homo-exchange). The catalytic reaction was stopped at appropriate time 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 and the radioactive substrate were added together at time zero. The assay temperature was 25 °C. The activity assay of proteoliposomes, when indicated, was performed in presence or not of various concentrations of cysteine inhibitors (see Results). The external radioactivity was removed by passing the samples (100 μl) through a Sephadex G-50 column (0.6 cm diameter, 8.0 cm height). The proteoliposomes eluted with 50 mM NaCl were collected in 4 ml

Table 1
IC_{50} values for NEM, MTSEA, MTSET, $HgCl_2$ and MeHgCl of ORC WT and mutants. Transport was measured as described in materials and methods. The values are means ± SD from three independent experiments.

Protein	IC_{50} (μM)				
	NEM	MTSEA	MTSET	$HgCl_2$	MeHgCl
WT	149 ± 22	0.40 ± 0.020	1.6 ± 0.30	0.21 ± 0.020	0.38 ± 0.034
C23A	270 ± 18	0.64 ± 0.012	1.9 ± 0.21	0.31 ± 0.016	0.40 ± 0.014
C50A	870 ± 89	0.69 ± 0.031	6.9 ± 0.38	0.37 ± 0.029	0.64 ± 0.034
C51A	637 ± 35	1.23 ± 0.068	5.6 ± 0.51	0.36 ± 0.028	0.92 ± 0.12
C84A	263 ± 43	0.33 ± 0.025	1.6 ± 0.23	0.22 ± 0.011	0.32 ± 0.097
C88A	187 ± 11	0.79 ± 0.031	2.7 ± 0.16	0.36 ± 0.035	0.35 ± 0.015
C125A	76 ± 15	0.27 ± 0.047	2.7 ± 0.15	0.50 ± 0.057	0.96 ± 0.026
C132A	1540 ± 26	1.46 ± 0.36	4.4 ± 0.31	0.57 ± 0.032	1.3 ± 0.033
C222A	195 ± 8.7	0.59 ± 0.045	2.0 ± 0.17	0.41 ± 0.046	0.31 ± 0.012
C232A	249 ± 21	0.66 ± 0.056	1.8 ± 0.40	0.27 ± 0.098	0.36 ± 0.023
C50/51/132A	4900 ± 300	4.9 ± 1.0	9.5 ± 0.48	–	–