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Inactivation by Hg^{2+} and methylmercury of the glutamine/amino acid transporter (ASCT2) reconstituted in liposomes: Prediction of the involvement of a CXXC motif by homology modelling

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ABSTRACT

The effect of $HgCl_2$, methylmercury and mersalyl on the glutamine/amino acid (ASCT2) transporter reconstituted in liposomes has been studied. Mercuric compounds externally added to the proteoliposomes, inhibited the glutamine/glutamine antiport catalyzed by the reconstituted transporter. Similar effects were observed by pre-treating the proteoliposomes with the mercurials and then removing unreacted compounds before the transport assay. The inhibition was reversed by DTE, cysteine and N-acetyl-cysteine but not by S-carboxymethyl-cysteine. The data demonstrated that the inhibition was due to covalent reaction of mercuric compounds with Cys residue(s) of the transporter. The IC_{50} of the transporter for $HgCl_2$, methylmercury and mersalyl, were 1.4 ± 0.10, 2.4 ± 0.16 or 3.1 ± 0.19 μM, respectively. Kinetic studies of the inhibition showed that the reagents behaved as non-competitive inhibitor. The presence of glutamine or Na^+ during the incubation of the mercuric compounds with the proteoliposomes did not exerted any protective effect on the inhibition. None of the compounds was transported by the reconstituted transporter. A metal binding motif CXXC has been predicted as possible site of interaction of the mercuric compounds with the transporter on the basis of the homology structural model of ASCT2 obtained using the glutamate transporter homologue from *Pyrococcus horikoshii* as template.

1. Introduction

Mercury causes toxic effects in several tissues and organs. In the environment, Hg^{2+} is the most common cationic form and methylmercury is the most common organic mercury compound. It is well known that both cationic and organic mercuric compounds react with thiol groups with very high affinity constants. However, several aspects of the molecular mechanisms of mercury toxicity remain to be elucidated [1–6]. Membrane proteins containing Cys residues exposed to the extracellular environment represent important sites of interaction with mercuric compounds. Transport systems, among membrane proteins, are particularly interesting since they both could allow the toxic compounds to enter the cells and could be chemically modified by these compounds with consequent alterations of their function. Indeed, it was reported that some transport systems catalyze the transport of mercuric compounds through biological membranes [2,7–9]. Furthermore, interaction of mercuric compounds with transport systems of plasma membrane [1,10–15] and also of mitochondrial membrane [16,17] have been reported to cause alterations of function. In several cases the effects and the sites of interaction of mercurials have been characterized ([14–24] and refs. herein). The study of interaction of mercuric reagents with transport systems for glutamine is still at an initial stage, in spite of the essential role of these transporters in cell homeostasis [25]. It was reported that methylmercury modify the permeability to glutamine of cultured astrocytes by an indirect mechanism based on inhibition of expression of the amino acid transporters ASCT2 and SNAT3; however, this mechanism only partially explained the permeability alteration [26]. The rat glutamine/amino acid transporter ASCT2 which is expressed in kidney, intestine and nervous system besides other tissues [25], was reconstituted in liposomes [27,28]. The transporter was inserted unidirectionally in the proteoliposomal membrane, right-side-out with respect to the cell membrane; thus, the properties of the internal side of the reconstituted transporter correspond to those of the intracellular side and vice versa. The studies in the proteoliposome system, which gives the advantage of reducing the interferences and improving the kinetic resolution in respect to intact cell systems, revealed novel functional properties of this transporter. It was found that the reconstituted transporter is

Abbreviations: $C_{12}E_8$, octaethylene glycol monododecyl ether; DTE, 1,4-dithioerythritol.

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functionally and kinetically asymmetrical, is regulated by intraliposomal (intracellular) ATP and functions by a simultaneous mechanism of transport. In preliminary experiments it was also found that externally added mersalyl, p-hydroxymercurybenzoate and $HgCl_2$ inhibited the transporter [27,28]. Thus, the proteoliposome experimental model is suitable for investigating the effects of mercurial compounds on the ASCT2 transporter. The effects of $HgCl_2$ and methylmercury, together with the prototypical protein reagent mersalyl, on the transporter have been characterized in this work. Some thiolic antioxidant compounds can reverse the effect of the mercuric reagents. The relationships among the inhibition and the predicted structure of the ASCT2 transporter are described.

2. Materials and methods

2.1. Materials

Amberlite XAD-4 and egg yolk phospholipids (3-sn-phosphatidylcoline from egg yolk) were purchased from Fluka; $C_{12}E_8$ from Anatrace (USA 434W, Dussel Drive Maumee, OH 43537); L-[^{3}H]glutamine from GE Healthcare (Milano, Italy); Sephadex G-75, L-glutamine, Adenosine 5′-triphosphate, N-acetyl-L-cysteine, L-cysteine, S-carboxymethyl-L-cysteine, methylmercury, $HgCl_2$, mersalyl and all the other reagents were from Sigma–Aldrich (Milano, Italy).

2.2. Solubilization of the glutamine/amino acid transporter

Brush-border membranes were prepared from rat kidney [29] and stored as previously described [14]. The glutamine/amino acid transporter was solubilized by treating the membrane preparation (50 μl, about 0.15 mg protein) with 1.3% $C_{12}E_8$ in a final volume of 150 μl and centrifuged at 13,000 × *g* for 4 min at 4 °C. The supernatant (extract) was used for the reconstitution.

2.3. Reconstitution of the glutamine/amino acid transporter into liposomes

The glutamine/amino acid transporter was reconstituted by removing the detergent with a hydrophobic chromatography column [16,30]. In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through the same Amberlite XAD-4 column. The composition of the initial mixture used for reconstitution was: 25 μl of the solubilized protein (25–35 μg protein in 1.3% $C_{12}E_8$), 75 μl of 10% $C_{12}E_8$, 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as previously described [30], 30 mM L-glutamine (except where differently specified), 4 mM ATP, 20 mM Hepes/Tris pH 7.0 in a final volume of 700 μl. All the operations were performed at 4 °C. After vortexing, this mixture was passed 16 times at room temperature through the same Amberlite column (0.5 cm diameter × 2.5 cm height) preequilibrated with a buffer of the same composition of the initial mixture with the exception of protein, detergent and phospholipid.

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) preequilibrated with 20 mM Hepes/Tris pH 7.0 and sucrose at an appropriate concentration to balance the internal osmolarity (“unlabeled” proteoliposomes). For efflux measurements, the “unlabeled” proteoliposomes (600 μl), containing 30 mM glutamine, were “prelabeled” by transporter-mediated exchange equilibration [16] by incubation with 10 μl of 0.6 mM [^{3}H]glutamine at high specific radioactivity (2 μCi/nmol) for 60 min at 25 °C; then, the external radioactivity was removed by passing again the proteoliposomes through Sephadex G-75 as described above. Transport was started, in the case of uptake, by adding [^{3}H]glutamine at the indicated concentrations and 50 mM Na-gluconate to the “unlabeled” proteoliposomes, or, in the case of efflux, by adding non-radioactive substrates to the “prelabeled” proteoliposomes. In both cases, transport was stopped by adding 20 μM mersalyl at the desired time interval. In control samples the inhibitor was added at time zero according to the inhibitor stop method [31]. The assay temperature was 25 °C. Finally, each sample of proteoliposomes (100 μl) was passed through a Sephadex G-75 column (0.6 cm diameter × 8 cm height) to separate the external from the internal radioactivity. Liposomes were eluted with 1 ml 50 mM NaCl and collected in 4 ml of scintillation mixture, vortexed and counted. For the determination of the specific activity of [^{3}H]glutamine uptake, the experimental values were corrected by subtracting the respective controls (samples inhibited at time zero); the initial rate of transport was measured by stopping the reaction after 10 min, i.e., within the initial linear range of [^{3}H]glutamine uptake into the proteoliposomes. [^{3}H]glutamine efflux activity cannot be calculated as specific activity for methodological reasons [16]. It was expressed as % residual intraliposomal radioactivity with respect to the radioactivity present in the proteoliposomes at time zero.

2.5. Other methods

The protein concentration was determined by the modified Lowry procedure [32].

Mercuric conjugates of cysteine or N-acetylcysteine were formed by incubating $HgCl_2$, methylmercury or mersalyl with cysteine or N-acetylcysteine with 1:1.2 ratio as described in Ref. [7]. The homology structural model of the ASCT2 was built using the glutamate transporter homologue from *Pyrococcus horikoshii* crystal structure (1XFH) as the template. The amino acid sequence of the rat ASCT2 (NP_786934) and the glutamate transporter (NP_143181) were aligned using ClustalW and the alignment was manually adjusted as described in Ref. [33] for ASCT1, a member as ASCT2, of the family of glutamate and neutral amino acid transporters. The optimized alignment was used to run the program Modeller 9v7 [34].

3. Results

3.1. Inhibition of the transporter by mercuric reagents

The effect of $HgCl_2$, methylmercury and mersalyl on the glutamine/amino acid (ASCT2) transporter reconstituted in liposomes was investigated. The transport activity was measured as 0.1 mM [^{3}H]glutamine uptake into proteoliposomes containing 30 mM glutamine (glutamine/glutamine homologous antiport), in the absence or presence of externally added mercuric reagents. The time courses of the glutamine transport are shown in Fig. 1. As previously described [27], the accumulation of labeled glutamine into the proteoliposomes depended on the time. It reached the equilibrium at 60 min with a transport activity of 140–170 nmol/mg protein in the different experiments (○). The addition during the transport assay of each of the reagents strongly inhibited the transport (△). In the presence of $HgCl_2$ (Fig. 1A), methylmercury (Fig. 1B) or mersalyl (Fig. 1C) 75, 69 or 43% inhibition at 15 min was found; at 90 min the transport was still inhibited by 67, 47 or 34%. The experimental data was interpolated in a first order rate equation. The initial transport rate, calculated as the product of *k* and transport at equilibrium, was 11.8 ± 1.4 nmol min^{-1} mg protein^{-1} in absence of reagents and 1.7 ± 0.7, 2.3 ± 0.2 or 5.5 ± 0.2 in the presence of $HgCl_2$,