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Large scale production of the active human ASCT2 (SLC1A5) transporter in *Pichia pastoris* — functional and kinetic asymmetry revealed in proteoliposomes ☆

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ABSTRACT

The human glutamine/neutral amino acid transporter ASCT2 (hASCT2) was over-expressed in *Pichia pastoris* and purified by Ni^{2+}-chelating and gel filtration chromatography. The purified protein was reconstituted in liposomes by detergent removal with a batch-wise procedure. Time dependent [^{3}H]glutamine/glutamine antiport was measured in proteoliposomes which was active only in the presence of external Na^+. Internal Na^+ slightly stimulated the antiport. Optimal activity was found at pH 7.0. A substantial inhibition of the transport was observed by Cys, Thr, Ser, Ala, Asn and Met (≥70%) and by mercurials and methanethiosulfonates (≥80%). Heterologous antiport of [^{3}H]glutamine with other neutral amino acids was also studied. The transporter showed asymmetric specificity for amino acids: Ala, Cys, Val, Met were only inwardly transported, while Gln, Ser, Asn, and Thr were transported bi-directionally. From kinetic analysis of [^{3}H]glutamine/glutamine antiport Km values of 0.097 and 1.8 mM were measured on the external and internal sides of proteoliposomes, respectively. The Km for Na^+ on the external side was 32 mM. The homology structural model of the hASCT2 protein was built using the GltPh of *Pyrococcus horikoshii* as template. Cys395 was the only Cys residue externally exposed, thus being the potential target of SH reagents inhibition and, hence, potentially involved in the transport mechanism.

1. Introduction

Amino acid transport systems play the pivotal role of maintaining the amino acid homeostasis in mammalian cells. Among many transporters involved in this function there is a group which shares specificity for glutamine, thus playing the role of mediating glutamine trafficking in different tissues and intestinal and renal (re)absorption [1–3]. A lot of functional data have been obtained by studying the transporters in cell systems such as cancer cell lines or *Xenopus laevis* oocytes. Thus the glutamine-specific transporters have been firstly classified on the functional basis. More recently the various transporters have been assigned to different protein families (SLC) on the basis of gene and primary structure analysis. Taking into account both types of classifications, the glutamine-specific transporters can be divided in sodium-dependent systems: system ASC/ATB0 (SLC1), system $B^{0,+}$ (SLC6), system y + L (SLC7), system N and A (SLC38) and sodium-independent systems: system L (SLC7) and system $b^{0,+}$ (SLC6). Some transporters can be further distinguished in tolerant (N and y + L) or not tolerant (ASCT2) for the substitution of Na^+ by Li^+ or sensitivity towards inhibitors such as MeAIB (system A) or BCH (System L, LAT1) [1–3]. However, several functional properties of the amino acid transporters remain unknown or controversial, due to some limitations of the cell experimental models given by the contemporary presence of similar transport systems and/or enzymes which could affect the transport assays and by the difficult access to the internal side. Most of these problems can be overcome using simpler models for studying transport, such as the proteoliposome experimental system which revealed suitable for studying functional and kinetic properties of transporters [4,5]. Concerning the structure of mammalian amino acid transporters no crystallographic data is available so far. High resolution structures have only been achieved for some

Abbreviations: MeAIB, a-(methylamino)isobutyric acid; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; $C_{12}E_8$, octaethylene glycol monododecyl ether; YPDS, Yeast Extract Peptone Dextrose Sorbitol; BMGY, Buffered Glycerol-complex Medium; DDM, n-dodecyl-beta-D-maltoside; LDAO, n-dodecyl-N,N-dimethylamine-N-oxide; CHAPS, 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate; p-OHMB, p-hydroxymercuribenzoate; MTSET, 2-(trimethylammonium)ethyl methanethiosulfonate, Bromide; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; NEM, N-ethylmaleimide; PEM, N-phenylmaleimide; PLP, pyridoxal-5-phosphate; DEPC, diethyl pyrocarbonate

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bacterial homologues of amino acid transporters [6,7]. On the basis of these structures, homology models have been obtained for mammalian amino acid transporters, some of which have been in part validated by chemical targeting [8–10]. Very interestingly the expression of some of the transporters responsible for glutamine trafficking is up-regulated in tumors [2]. Cancer cells, in fact, require high amounts of glutamine for energy and growth purposes [2,11]. In this scenario it becomes clear that the study of glutamine transporters is a hot research topic and strategies for over-expressing the transporters in large scale and studying their structure/function relationships are very welcome. Among the most interesting transporters in human physiology and pathology there is the Na^+-dependent glutamine/neutral amino acid transporter ASCT2 (SLC1A5) previously known in humans as ATB0. This transport system has been identified in human cell systems even though the kinetic properties and substrate specificity are not fully understood [2,3,12] while the rodent isoform, besides being studied in cell systems [13–16], has been also functionally and kinetically characterized in proteoliposomes [9,17,18]. Basic functional and kinetic parameters of the kidney rat protein determined in both experimental models correlated well. Novel functional properties such as the ATP regulation, the internal side Km, the reaction mechanism, and the pH dependence of glutamate transport were revealed using proteoliposomes [17,18]. Due to its over-expression in cancer cells, ASCT2 has been proposed as a potential target for antitumor drugs [2]. Very recently, a molecular screening of ditiazoles, potent inhibitors of the rat ASCT2, has been carried out in proteoliposomes [19]. These results highlighted the importance of obtaining the recombinant human ASCT2 for performing structural, functional and inhibition studies. In this work, the high level production of the human ASCT2 in *Pichia pastoris* is described. The function of the transporter extracted from yeast membranes has been assayed in proteoliposomes where the protein has been inserted in a right-side-out orientation (see Discussion) with respect to the cell membrane, thus constituting a suitable tool for unequivocal functional characterization and interaction studies with potential drugs.

2. Materials and methods

2.1. Materials

The *P. pastoris* wild type strain (X-33), the pPICZB vector, NuPAGE® 4–12% Bis-Tris Gels were purchased from Invitrogen; restriction endonucleases and other cloning reagents from Fermentas; PD-10 columns, Superdex 200 10/300 GL, ÄKTA FPLC system, ECL plus, Hybond ECL membranes and L-[^{3}H]glutamine from GE Healthcare; Ni-NTA agarose and polypropylene columns from Qiagen, anti His_6 antibody from Clontech; the anti mouse IgG HRP conjugate from Promega; $C_{12}E_8$ from Anatrace; Amberlite XAD-4, egg yolk phospholipids (3-sn-phosphatidylcoline from egg yolk), Sephadex G-75, L- glutamine and all the other reagents were from Sigma-Aldrich.

2.2. Cloning of hASCT2

The wild type gene was isolated from total RNA of primary human fibroblasts by reverse transcription. Initially, the 1623 bp cDNA encoding hASCT2 (GenBank NM_005628.2, SLC1A5) was amplified using the forward primer *Nde*I-hASCT2: 5′-G GAA TTC CAT ATG GTG GCC GAT CCT CCT CG-3′ and the reverse primer *Hind*III-hASCT2: 5′-CCC AAG CTT TTA CAT GAC TGA TTC CTT CTC-3′, respectively. The amplified cDNA sequence was verified by sequencing using the ABI 310 automated sequencer Applied Biosystems. For subsequent cloning to *P. pastoris*, the full length cDNA coding for hASCT2 was amplified using the forward primer *Eco*RI-hASCT2: 5′-ATA CCG GAA TTC **AAA ATG G**TT GCC GAT CCT CCT CGA GAC TCC-3′ and the reverse primer *Xba*I-hASCT2: 5′-A TAC TAG TCT AGA TCA ATG ATG ATG ATG ATG ATG CAT GAC TGA TTC CTT CTC AGA GGC-3′, coding a C-terminal His_6 tag. Restriction sites are underlined and the Kozak consensus sequence is shown in bold.

The hASCT2 gene was codon optimized for *P. pastoris* by GenScript and the artificial cDNA included a 5′ *Eco*RI restriction site plus the Kozak consensus sequence and a 3′ *Xba*I restriction site plus a C-terminal His_6 fusion tag. In the optimized gene, the Codon Adaptation Index (CAI) [20] was upgraded from 0.51 (wild type) to 0.82 (optimized) and the GC content was decreased from 63.01% to 45.43%. For cloning in *P. pastoris* both the wild type gene (wt-hASCT2) and the optimized gene (Opt-hASCT2) were inserted in the *Eco*RI/*Xba*I sites of the pPICZB expression vector, resulting in two different recombinant constructs, defined as pPICZB-(wt)hASCT2-His_6 and pPICZB-(Opt)hASCT2-His_6. Both constructs were verified by sequencing.

2.3. Recombinant production of hASCT2

To obtain the recombinant hASCT2-His_6 protein, the resulting plasmids were linearized with *Pme*I and the transformation into the *P. pastoris* wild type strain X-33 was performed by electroporation [21]. To select putative multi-copy recombinants a total of 52 transformants for each construct were tested for growth on YPDS plates containing 2000 μg/mL Zeocin and analyzed after 3 days. Small-scale production was performed in triplicates in shake flask cultures as previously described [22]. For large scale protein production, *P. pastoris* strains producing recombinant hASCT2 (X33/pPICZB-(wt)hASCT2-His_6 and X33/pPICZB-(Opt)hASCT2-His_6) were grown at 30 °C in a 3 L fermentor (Infors HT) having an Initial Fermentation Volume (IFV) of 1.5 L basal salt medium [23] containing 6.53 mL PTM1 trace salts [24]. An overnight pre-culture of 75 mL in BMGY having an OD_{600} of about 4 was used to inoculate the fermentor. The initial glycerol volume was consumed after approximately 24 h and the culture was fed with 150 mL 50% glycerol (v/v) for 24 h to increase biomass. To induce production of recombinant hASCT2, the culture was fed with 150 mL methanol for 48 h. To obtain the membrane fraction, *P. pastoris* cells overproducing hASCT2 were resuspended in buffer A (50 mM Tris, pH 7.4, 150 mM NaCl, 6 mM β-mercaptoethanol and 0.5 mM PMSF) at a concentration of about 1 g/mL. Droplets of the cell suspension were frozen in liquid nitrogen and cells were broken by an X-Press (four passages). The suspension was centrifuged at 6000 *g* for 30 min and the supernatant containing membrane and cytosolic fractions (crude extract) was collected. This supernatant was ultracentrifuged in a Ti45 rotor at 140,000 *g* for 1 h. The resulting membrane pellet was washed with urea buffer (5 mM Tris pH 7.4, 2 mM EDTA, 2 mM EGTA and 4 M urea) and then again ultracentrifuged as above. The washed membrane fractions (pellet) containing (wt)hASCT2 or (Opt)hASCT2 were resuspended in buffer B (25 mM Tris, pH 7.4, 250 mM NaCl, 6 mM β-mercaptoethanol and 10% glycerol) at a final concentration of about 300 mg/mL and homogenized using a handheld electric homogenizer. Aliquots of 6 mL of the membrane fraction were stored at −80 °C. Various stages from the protein purification procedure were analyzed by SDS-PAGE and immunoblot.

2.4. Solubilization and purification of hASCT2

For large-scale solubilization and purification (Opt)-hASCT2, about 1.5 g of washed membranes (300 mg/mL) was resuspended in buffer B containing 1% $C_{12}E_8$ (w/w) to a concentration of 150 mg/mL and gently mixed by agitation for 3 h at 4 °C. After solubilization, the solubilized material was centrifuged at 120,000 *g* for 1 h, imidazole (50 mM) was added to the supernatant which was mixed with 3 mL Ni-nitrilotriacetic acid (NTA) agarose resin equilibrated with the equilibration buffer (20 mM Tris pH 7.4, 300 mM NaCl, 10% glycerol, 6 mM β-mercaptoethanol, 0.03% $C_{12}E_8$, and 50 mM imidazole) and incubated by gentle agitation for 3 h at 4 °C. The Ni-NTA resin was subsequently packed into a plastic 1 mL column. The resin was washed with 30 mL of the equilibration buffer. Then, 4 mL of the