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# Expressed human hippocampal ASCT1 amino acid transporter exhibits a pH-dependent change in substrate specificity

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**Abstract**

In mammalian cells, the basal $Na^+$-dependent uptake for many of the neutral amino acids is mediated by a transport activity designated System ASC. A cloned human brain cDNA sequence, ASCT1, encodes a $Na^+$-dependent neutral amino acid transport activity that exhibits a substrate specificity similar to that commonly associated with System ASC. However, the characteristics of ASC activity varies significantly between cell types and not all tissues contain detectable levels of ASCT1 mRNA. A unique property of System ASC activity is an altered substrate selectivity such that at pH values below 7.4 anionic amino acids function as inhibitors and substrates. The experiments in this report were designed to determine if the cloned ASCT1 transporter exhibited this pH-dependent anionic transport. Following transfection of HeLa cells with the ASCT1 cDNA, transport strongly favored neutral zwitterionic) amino acids when uptake was measured at a physiologic pH value of 7.5. However, lowering the assay pH to 5.5 significantly enhanced the interaction of the ASCT1 carrier with anionic amino acids such as cysteate, in a pH-dependent manner. The apparent p$K$ for the titratable group was in the range of 6.5–7.0. These results provide evidence that the human brain ASCT1 transporter exhibits the most distinguishing characteristic known for System ASC and provides a model system to investigate the molecular basis for this shift in substrate acceptance.

*Keywords:* Brain; Anionic amino acid; Vaccinia; Serine

## 1. Introduction

The concentrative uptake of amino acids across the plasma membrane is mediated by alkali ion-driven transporters which exhibit overlapping substrate specificities [1,2]. These activities have been extensively characterized in tissues, whole cells, and plasma membrane vesicles [3]. Until recently, little molecular characterization of these transporters has been possible due to the lack of cDNA probes or antibodies. Two cDNA sequences ASCT and SATT) that had homology to a family of $Na^+$-dependent glutamate transporters [4–6], were independently cloned from human hippocampal cDNA libraries [7,8]. Although they were originally considered to be two different amino acid sequences, most of the differences have been resolved and the term ASCT1 has been adopted by Shafqat et al. [9]. Recently, the ASCT1 gene has been localized to human chromosome 2p13-p15 [10].

It was proposed that the ASCT1 clone encoded the human brain ASC transport activity, largely on the basis of increased $Na^+$-dependent uptake of substrates typical of System ASC by ASCT1 [7,8]. System ASC activity is expressed in most, if not all, mammalian cells. It mediates the basal uptake of a wide range of neutral amino acids, but exhibits a preference for those with hydroxyl- or sulfhydryl-containing sidechains such as serine, cysteine, and threonine [11,12]. Although System ASC has been considered a single activity, significant tissue variation in the most basic properties such as the range of neutral amino acid substrates and the acceptance of $Li^+$ to substitute for $Na^+$ is also consistent with the hypothesis that ASC activity is mediated by different proteins in different tissues [11–15]. A number of tissues do not contain detectable ASCT1 mRNA by Northern analysis [7,8], despite having measurable ASC transport activity. These results could be due to: (1) an ASCT1 mRNA level below the sensitivity of Northern analysis; (2) related genes encoding

Abbreviations: cholKRP, choline-containing Krebs Ringer phosphate buffer ($Na^+$ free buffer); MeAIB, 2-(methylamine)isobutyric acid; NaKRP, sodium-containing Krebs Ringer phosphate buffer; PMSF, phenylmethylsulfonyl fluoride; TPCK, tosylphenylalanine chloromethyl ketone; TLCK, tosyllysine chloromethyl ketone; $C_{12}E_9$, polyoxyethylene-9-lauryl ether.

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similar activities, but sufficiently different in sequence to escape hybridization at high stringency (although attempts to screen cDNA libraries from these tissues at low stringency has not revealed such sequences); or (3) ASC-like activity resulting from expression of unrelated genes in a tissue-specific manner. A similar circumstance exists for the glutamate transporters in that there is cell specific expression of four different genes [4–6,16]. Therefore, Northern analysis of a given cell type might reveal the lack of a particular transporter mRNA, despite the presence of $Na^+$-dependent glutamate transport activity. By analogy, it is likely that ASCT1 represents one of a family of related genes that code for a similar transport activity and the other members have yet to be identified. If this is the case, the transport properties of the brain ASCT1-encoded protein may or may not correspond to those previously described for System ASC in other tissues.

The most unique feature reported for System ASC is a pH-dependent alteration of substrate selectivity [17–19]. When assayed at neutral pH, System ASC primarily mediates the uptake of neutral amino acids, whereas at an acidic pH ($< 7.0$) both neutral and anionic amino acids are effective inhibitors and substrates. Given that previous reports relied almost solely on neutral amino acid uptake at pH 7.5 to assign ASC activity to the ASCT1 clone, the present experiments were designed to determine if the human hippocampal ASCT1 transporter exhibited a pH-dependent change in reactivity with anionic amino acids. The anionic amino acid analog cysteate was chosen as a test inhibitor because its sidechain p$K$ of less than 1.5 eliminates titration of the inhibitor to a zwitterion as a possible interpretation. The data demonstrate that brain ASCT1-mediated uptake is not functional in the presence of $Li^+$ instead of $Na^+$ and is inhibited by cysteate in a pH-dependent manner. The observed acceptance of anionic amino acids in the presence of a decreased extracellular pH may help to explain the apparent evolutionary relationship between ASCT1 and the four previously identified numbers of the so-called 'glutamate transporter family' [4–6,16].

## 2. Methods

### 2.1. Cell transfection

HeLa cells were maintained in tissue culture and transporter cDNA expression was performed as described previously [8]. Approx. 100 000–150 000 cells/well in 24-well cell culture plates were infected with a recombinant vaccinia virus strain $VTF_{7\text{-}4}$ 15 plaque forming units/cell) that expresses T7 polymerase. Using 3 $\mu$l/well of lipofectin (BRL, Gaithersburg, MD), 30 min after virus infection the cells were transfected with 1 $\mu$g/well of the plasmid vector pcDNA3 alone (Invitrogen, San Diego, CA) or plasmid containing the full-length ASCT1 insert cloned downstream of a T7 promoter sequence [8]. The plasmid without insert, or the ASCT1 sequence in the anti-sense orientation, was used in control transfections to demonstrate that the infection/transfection process itself did not induce an endogenous transport activity.

### 2.2. Transport assays

8 h post-transfection the cells were washed twice in 2 ml/well of $Na^+$-free Krebs Ringer phosphate buffer (cholKRP) at 37°C, each of the $Na^+$ salts was replaced with the corresponding choline salts [20]. The uptake of 5 $\mu$M [$^3$H]serine (Amersham Corp., Arlington Heights, IL) was initiated by the addition of 250 $\mu$l of uptake buffer/well at 37°C, as described previously [20]. The uptake buffer, either NaKRP ($Na^+$-containing buffer) or cholKRP, always contained 2 mM MeAIB, a System A-specific analog to eliminate contribution of that activity to the uptake of the test amino acid [21]. The $Na^+$-dependent transport was calculated as the difference in uptake in $Na^+$-containing (NaKRP) and $Na^+$-free cholKRP) medium and is reported as the averages $\pm$ S.D of at least four determinations. All variables and the appropriate controls were contained within a given experiment and each experiment was repeated with multiple cell preparations to demonstrate qualitative reproducibility.

### 2.3. Northern analysis

The method of Chomczynski and Sacchi was used to prepare total cellular RNA [22] and poly(A) mRNA was isolated by the PolyATract system according to the manufacturer's protocol (Promega, Madison, WI). ASCT1 mRNA content was detected by Northern analysis as described by Church and Gilbert [23]. Following UV cross-linking to a nylon membrane, the RNA was hybridized at 65°C with a cDNA probe for either ASCT1 or $\beta$-actin, radioactively labelled prior to hybridization with [$^{32}$P]dCTP using a random primer DNA labelling kit (BRL, Gaithersburg, MD). Blots were subjected to autoradiography.

### 2.4. Immunoblotting of ASCT1 transport protein

To detect the ASCT1 protein by immunoblotting, the DNA sequence corresponding to the FLAG epitope sequence DYKDDDDK was subcloned between amino acid residues 477 and 478 to generate an ASCT1/FLAG construct. This epitope is recognized by a commercially-available (Kodak Co., New Haven, CT) monoclonal antibody [24]. In parallel transfections for both transport and protein measurements, cells were extracted in 0.2 N NaOH/0.2% SDS and then 10 $\mu$g protein per lane was subjected to one-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis [25,26], transferred to nitrocellulose [26], and the transporter protein detected by enhanced chemiluminescence (Amersham Corp., UK) immunoblotting according to the manufacturer's directions. The anti-FLAG

antibody was used at 10 μg/ml and rabbit anti-mouse linked to horseradish peroxidase (1:20000) was the secondary antibody.

## 3. Results

HeLa cells or *Xenopus* oocytes that express the ASCT1 transporter exhibit a significant increase in the $Na^+$-dependent uptake of serine, threonine, and cysteine that is not blocked by the System A specific analog MeAIB [7–9]. We have documented the expression of the corresponding ASCT1 mRNA by Northern analysis following transfection of HeLa cells with pcDNA3 plasmid alone or plasmid containing the ASCT1 insert (Fig. 1A). Endogenous ASCT1 mRNA, if expressed at all in HeLa cells, was not detectable, whereas an intense signal was obtained from ASCT1 transfected cells.

To demonstrate the expression of the ASCT1 transporter protein itself HeLa cells were transfected with a cDNA construct in which the FLAG epitope sequence was inserted to permit detection with the FLAG monoclonal antibody [24]. This epitope-tagged protein (ASCT1/FLAG) yielded approximately the same level of 5 μM [$^3$H]serine transport as the native sequence (ASCT1-Mediated = 452 ± 48, ASCT1/FLAG-Mediated = 551 ± 51 pmol serine/mg protein per min). In two separate experiments, immunoblotting of a total cell extract from cells transfected with the ASCT1/FLAG construct revealed a significant amount of protein expression (Fig. 1B). At the present time, the molecular basis for the multiple bands is unknown, but similar patterns for both endogenous and expressed transporters have been shown to be due to heterogeneity in glycosylation. Cells transfected with the native ASCT1 cDNA served as the negative control and no immunoreactive protein was observed. Parallel experiments in which protein expression was measured instead by immunoprecipitation of $^{35}$S-labelled cells using anti-FLAG antibody showed a similarly broad band of immunoreactive protein in the same size range (60–70 kDa) as that obtained by immunoblotting data not shown).

Shafqat et al. [8] previously demonstrated that ASCT1-mediated serine uptake was unaffected by the replacement of chloride in the uptake buffer with anions such as thiocyanate, gluconate, or acetate. With regard to the co-transported cation, the ability of System ASC to accept $Li^+$-for-$Na^+$ substitution varies widely between cell types. For example, in Ehrlich ascites cells [11,13] and certain hepatoma cell lines [12] $Li^+$ is not an effective co-substrate for System ASC but in rat hepatocytes a significant amount of $Li^+$-dependent ASC activity is measurable [14,15]. As shown in Table 1, the human brain-derived ASCT1 transporter was completely inactive when $Na^+$ was replaced with $Li^+$.

The System ASC activity in Ehrlich ascites tumor cells and rat hepatocytes was shown to exhibit a unique pH-dependent alteration of substrate specificity that converted the transporter from one that accepts only neutral amino acids to one that also exhibits affinity for anionic substrates [17–19]. Inhibition or transport of anionic amino acids that have sidechain p$K$ values of less than 1.5, such as cysteate and cysteinesulfinate, has demonstrated that this pH effect on System ASC was associated with titration of the transporter rather than the substrate [18,19]. To determine if the human hippocampal ASCT1 transporter

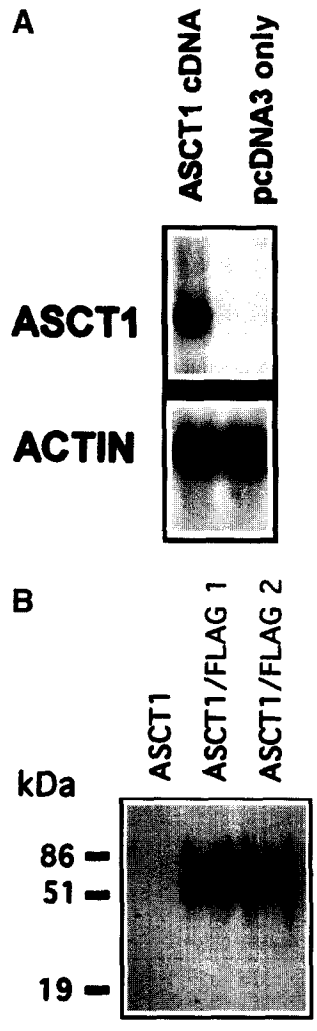


Fig. 1. ASCT1 mRNA and protein content in transfected HeLa cells (A) Poly(A) mRNA was isolated from HeLa cells 8 h after they were transfected with pcDNA3 containing the ASCT1 cDNA or pcDNA3 plasmid only. The mRNA (2 μg/lane) was subjected to Northern analysis with $^{32}$P-labelled ASCT1 cDNA. Exposure time for the blot shown was approx. 30 min. Ethidium bromide staining of the rRNA bands and the β-actin mRNA content served as internal references for comparison of the RNA loaded in each lane. (B) HeLa cells were solubilized in 0.2 N NaOH/0.2% SDS 8 h after they were transfected with ASCT1 cDNA or the ASCT1/FLAG cDNA containing the 8 amino acid FLAG insertion after residue 477. The ASCT1/FLAG transporter was detected by immunoblotting following one-dimensional SDS-PAGE using the FLAG monoclonal antibody as described in Section 2. Expression of protein in two independent experiments is shown (ASCT1/FLAG1 and ASCT1/FLAG2). Where not shown, the standard deviation bars are contained within the symbol.

Table 1
Effect of cation substitution on ASCT1-mediated serine uptake

| Cation | Serine uptake (pmol/mg protein per min) | |
|---|---|---|
| | control | transfected |
| NaKRP | 380±56 | 782±70 ($P < 0.005$) |
| LiKRP | 38±12 | 34±14 (N.S.) |
| CholKRP | 22± 4 | 38±12 ($P < 0.05$) |

8 h after transfection, the MeAIB-insensitive uptake of 5 μM [$^3$H]serine was measured for 1 min at 37°C in NaKRP, cholKRP, or LiKRP. The control cells were transfected with pCDNA3 vector alone. The data are reported as the averages±S.D. of at least four determinations. The indicated $P$ values were determined by the Student's $t$-test. For each medium, the transport rates in the ASCT1-transfected cells were compared to those cells obtained with cells transfected with plasmid only. Several replicate experiments with different cell preparations yielded similar results. (N.S. = not statistically different).

exhibited a pH-dependent acceptance of anionic amino acids, ASCT1-mediated serine uptake was assayed at pH 7.5 or 5.5 in the presence of increasing amounts of unlabelled cysteate or serine itself (Fig. 2). At pH 7.5, cysteate produced only modest inhibition at concentrations up to 1 mM, whereas unlabelled serine completely saturated its own transport at 1 mM (Fig. 2A). However, at pH 5.5, the inhibition of serine uptake by cysteate was half-maximal at approx. 100 μM and was nearly complete at a concentration of 1 mM (Fig. 2B). Inhibition by the anionic amino acids, aspartate, glutamate, and cysteinesulfinate gave similar results (data not shown).

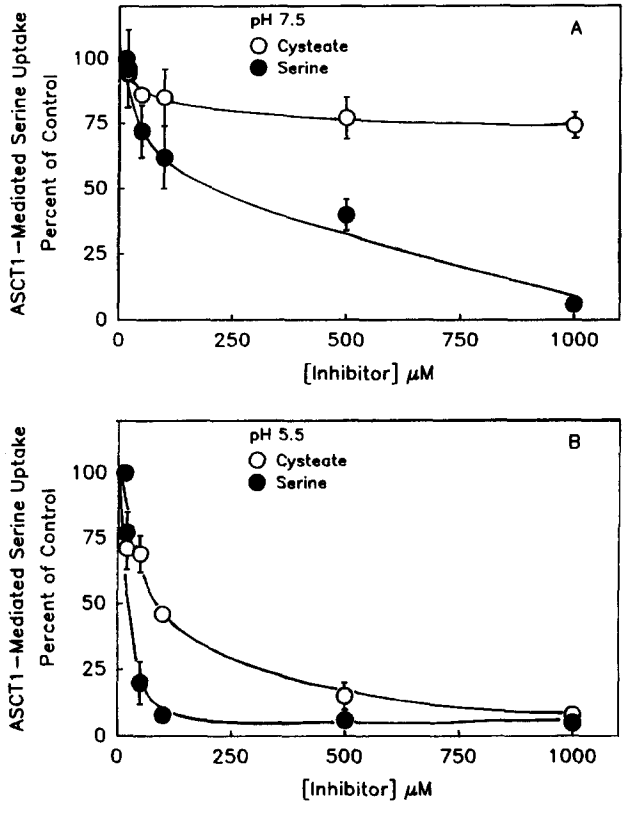


Fig. 2. Concentration-dependence of cysteate inhibition of ASCT1-mediated serine uptake Sodium-dependent uptake of 5 μM [$^3$H]serine, in the presence of 2 mM MeAIB, was measured for 1 min at 37°C at pH 7.5 (A) or pH 5.5 (B). ASCT1-mediated uptake was obtained by subtracting the $Na^+$-dependent uptake in cells transfected with vector only from that in cells transfected with ASCT1 cDNA at each concentration. The concentration of unlabelled serine or cysteate as inhibitor was varied from 0 to 2 mM as shown. The data are the averages of assays in triplicate, and repetitive experiments with different cell preparations yielded qualitatively similar results. Where not shown, the standard deviation bars are contained within the symbol. The control velocities corresponding to 100% were 600±10 and 300±17 pmol/mg protein per min at pH 7.5 and 5.5, respectively.

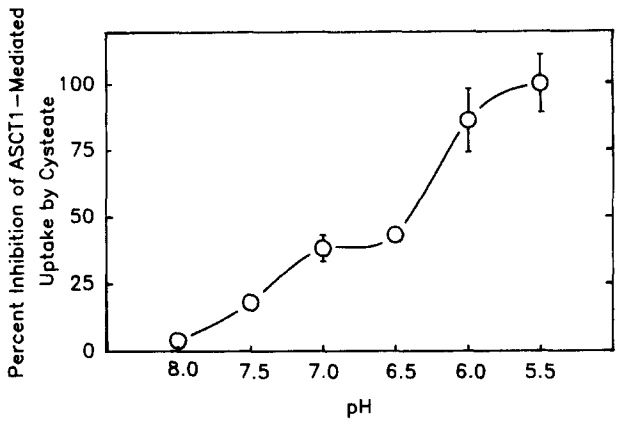


Fig. 3. The pH-dependent inhibition of ASCT1-mediated serine uptake by cysteate Inhibition of the MeAIB-insensitive ASCT1-mediated uptake of 5 μM [$^3$H]serine was measured in the presence and absence of 2 mM cysteate for 1 min at 37°C. The $Na^+$-dependent transport rates in cells transfected with plasmid only were subtracted from those transfected with plasmid containing ASCT1 to obtain the values shown. The pH of the transport assay medium was varied between 5.5 and 8.0. The control velocity, ASCT1-mediated serine uptake at pH 7.5 in the absence of cysteate, was 600±10 pmol/mg protein per min. Where not shown, the standard deviation bars are contained within the symbol.

To establish the pH-dependent profile for the substrate conversion of the ASCT1 transporter, transfected cells were tested for inhibition of serine uptake by 2 mM cysteate at several assay pH values between 8.0 and 5.5. At pH 7.5, ASCT1-mediated serine uptake was inhibited by less than 20% in the presence of cysteate (Fig. 3). However, as the extracellular pH was decreased, the cysteate inhibition of ASCT1-mediated serine uptake increased until the serine transport was completely abolished at pH 5.5. The data obtained suggest titration of a group with a p$K$ value of approx. 6.5–7.0. The most likely residue to account for this response is a histidine for which the imidazole group has a p$K$ value of about 6.0 when determined as a free amino acid in solution.

## 4. Discussion

The ASCT1 transporter cloned from human hippocampus mediates the uptake of neutral amino acids such as alanine, serine, threonine, and cysteine [7,8]. Based on this substrate specificity and a few other features, the ASCT1 clone was proposed to code for a transport activity origi-

nally described 3 decades ago and called System ASC [11]. System ASC is a generic term for similar activities in a wide variety of mammalian cells, but significant differences between cell types have been noted [12]. In a few cell lines, a unique characteristic of System ASC is a novel pH-dependent alteration of its substrate specificity [18,19]. The results reported here show that the human brain ASCT1 transporter exhibits this property. The present investigation, as well as the original reports of this phenomenon, have used amino acids with sidechain p$K$ values of less than 1.5 to document that titration of the transporter rather than the substrate is responsible for the acceptance of the anionic amino acid. Interestingly, serine itself was a more effective inhibitor at pH 5.5, an observation consistent with the results of Vadgama and Christensen for threonine inhibition of System ASC in Ehrlich ascites tumor cells [17].

Protonation of a histidine residue would be consistent with the pH-dependent profile for this change of substrate specificity. For GLT1, one of the glutamate transporters related to ASCT1 [7,8], site-directed mutagenesis of histidine 326 abolished transport [27]. Other transporters for which a histidine residue may play a critical role include the lactose transporter [28] and the $Na^+/H^+$ exchanger [29] of *E. coli*, the neuronal monoamine vesicle transporter [30] and mammalian $Ca^{2+}$-ATPase [31]. Conversion of a neutral residue such as histidine to a site of positive charge within the ASCT1 protein might provide a recognition site for the anionic sidechain. It is interesting to speculate that the ability of ASCT1 to undergo this interconvertibility is also reflected in the sequence similarity between ASCT1 and the numerous glutamate/aspartate transporters that have been cloned [4–6,16]. Recently [16,32], four human glutamate transporter clones (EAAT1-4) were reported, three of which are homologs to the rat GLAST1 [4], rat GLT1 [5], and rabbit [6] or rat [15] EAAC1 clones. The primary amino acid sequence of EAAT3 cloned by Arriza et al. [32] is identical to that of a human kidney EAAC1 clone obtained by Kanai et al. [33]. Human ASCT1 shows 60.7%, 57.0%, and 58.8% homology to the human EAAT1-3 transporters, respectively.

Beyond the obvious importance of this pH-dependent substrate interconversion as an experimental tool to probe structure-function relationships within the ASCT1 transporter protein, this alteration in amino acid recognition also may have physiologic significance. ASCT1 mRNA is expressed at high levels in intestinal tissue [8], and it has been demonstrated both in vitro and in vivo that there are pH microclimates in specific regions of the intestinal mucosa. Lucas [34], using exteriorized rat intestinal loops, measured intestinal microvillus surface pH. When incubated in a medium at pH 7.2, the surface pH in the proximal jejunum was 6.1, whereas the surface pH in the mid gut was 6.5, and in the distal ileum it was 7.3. Causing anoxia of the tissue by blocking blood supply caused the surface pH in the jejunum to increase to that of the incubation medium. Daniel et al. [35] used microelectrode measurements of rat jejunum in vitro to demonstrate that the surface pH was most acidic in a 10–100 $\mu$m region just below the tip of the villus, but the surface pH in the villus crypt was actually slightly alkaline. In a separate study [36], it was shown that this pH gradient, from the villus tip to the crypt, occurred in the jejunum, but not in the ileum. Those authors also showed that treatment of the tissue in situ with theophylline caused an increase in the jejunum pH microclimate, suggesting regulation of this pH microclimate in response to the metabolic status of the animal. If the ASCT1 transporter is expressed in the jejunal brush border domain and the acidic microclimate at the membrane surface permits acceptance of anionic amino acids by this transporter, regulation of this microclimate, either acutely or chronically, would permit modulation of the substrates transported and thus, alter tissue availability.

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