Review

ASCT1 and ASCT2: Brother and Sister?

SLAS Discovery
1–16

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Mariafrancesca Scalise[1], Lara Console[1], Jessica Cosco[1], Lorena Pochini[1], Michele Galluccio[1], and Cesare Indiveri[1,2]

Abstract

The SLC1 family includes seven members divided into two groups, namely, EAATs and ASCTs, that share similar 3D architecture; the first one includes high-affinity glutamate transporters, and the second one includes SLC1A4 and SLC1A5, known as ASCT1 and ASCT2, respectively, responsible for the traffic of neutral amino acids across the cell plasma membrane. The physiological role of ASCT1 and ASCT2 has been investigated over the years, revealing different properties in terms of substrate specificities, affinities, and regulation by physiological effectors and posttranslational modifications. Furthermore, ASCT1 and ASCT2 are involved in pathological conditions, such as neurodegenerative disorders and cancer. This has driven research in the pharmaceutical field aimed to find drugs able to target the two proteins.

This review focuses on structural, functional, and regulatory aspects of ASCT1 and ASCT2, highlighting similarities and differences.

Keywords

ASCTs, EAATs, SLC1, membrane transport, cancer, neurological disorders

Introduction

The SLC1 family is a relatively small group of membrane transporters involved in amino acid traffic in cells. The family encompasses seven members, which are divided in two groups sharing substrate specificity and transport mode.[1–3] The first group includes SLC1A1, A2, A3, A6, and A7, collectively known as EAATs (excitatory amino acid transporters), exhibiting high affinity for the negatively charged amino acids glutamate and aspartate and localized mainly in the brain; the second group includes SLC1A4 and A5, known as ASCTs (alanine, serine, cysteine transporters), involved in the traffic of several neutral amino acids in a broad set of tissues.[1–3] The functional classification used in the pregenomic era has been later confirmed by the evolutionary analysis brought to the SLC classification. The seven members of the SLC1 family share a low percentage of identity, that is, 21% (**Suppl. Fig. S1A**); while, when analyzing the two groups separately, the percentage of identity increases up to 58% for ASCTs (**Suppl. Fig. S1B**) and up to 29% for EAATs (**Suppl. Fig. S1C**). The sequence comparison data reflect the differences between the two groups in terms of biochemical properties, specificity to substrates, sensitivity to inhibitors, and tissue distribution, and hence the different physiological roles. Despite these differences, the two groups share an almost overlapping three-dimensional (3D) architecture according to their belonging to the same family.[1–3] As mentioned above, EAATs are mainly expressed in glutamatergic synapses in the brain, where EAATs are fundamental for avoiding glutamate excitotoxicity and then for maintaining normal development and functioning of the brain.[4,5] Therefore, any alteration of glutamate handling in synapses may trigger pathological conditions and injuries, including neurological disorders and stroke.[4,5] Due to this relevance to the excitatory neurotransmission, extensive biochemical, physiological, and structural studies have been performed on the EAATs over the years.[2] In line with this, several eminent reviews are available on the mentioned aspects of the SLC1 family with a special focus on the EAAT group.[1–3,6,7] A systematic and up-to-date report of the ASCT group is, to our knowledge, lacking or incomplete; therefore, the scope of the present review is to compare the two members, namely, ASCT1 (SLC1A4) and ASCT2 (SLC1A5), to furnish a

[1]Department DiBEST (Biologia, Ecologia e Scienze della Terra), Unit of Biochemistry and Molecular Biotechnology, University of Calabria, Arcavacata di Rende, Italy
[2]CNR Institute of Biomembranes, Bioenergetics and Molecular Biotechnology (IBIOM), Bari, Italy

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Corresponding Author:
Cesare Indiveri, Department DiBEST (Biologia, Ecologia e Scienze della Terra), University of Calabria, Via P. Bucci cubo 4C, Arcavacata di Rende (CS), 87036, Italy.
Email: cesare.indiveri@unical.it

more refined picture of their structural, physiological, and possibly regulatory features. It has to be stressed that ASCT1 and ASCT2 deserved a different degree of attention from the scientific community. Indeed, besides the basic functional and kinetic characterization, ASCT2 became a hot topic in the last decades due to the discovery of its overexpression in virtually all human cancers; this triggered a boost of publication rate on ASCT2 not only on structural and functional aspects, but also on pathological and pharmaceutical/pharmacological ones. Indeed, the number of papers retrieved using the wildcard ASCT2 [Title] OR SLC1A5 [Title] is much higher (161 on PubMed) than that retrieved using the wildcard ASCT1 [Title] OR SLC1A4[Title] (37 on PubMed).

Gene Chromosome Location and Natural Variants

The human ASCT1 (SLC1A4) gene contains eight exons and maps on chromosome 2p14.[8] The ASCT1 gene is found in 341 organisms (https://www.ncbi.nlm.nih.gov/gene/6509), among which several are vertebrates. In the National Center for Biotechnology Information (NCBI) database, four different isoforms are listed. The first isoform is the longest one, encoding for a peptide of 532 amino acids (NM_003038.5), with a theoretical molecular mass of 55 KDa. The other three transcripts are shorter, being 312 (NM_001348407.2), 214 (NP_001180422.1), and 312 (NP_001335335.1) amino acids long. The isoforms differ in their 5′-UTR, coding sequences, and codon start. Several single-nucleotide polymorphisms (SNPs) have been found on the ASCT1 human gene, some of which have been associated with clinical conditions. In particular, rs201278558 and rs761533681 are responsible for the c766G-A and c.1369C-T transitions, respectively. These two mutations correspond to the substitution of the highly conserved residues E256 and R457 into Lys and Trp, respectively. Interestingly, these variants are associated with a disease called SPATCCM (OMIM 616657), that is, spastic tetraplegia, thin corpus callosum, and progressive microcephaly.[9]

The human ASCT2 (SLC1A5) gene contains eight exons and maps on the chromosome 19q13.32. The ASCT2 gene is found in 56 different organisms (https://www.ncbi.nlm.nih.gov/gene/6510) and is virtually present in all vertebrates. In the NCBI database, three different isoforms are listed. The first isoform is the longest one, encoding for a peptide of 541 amino acids (NM_005628), with a theoretical molecular mass of 57 kDa. The second one differs in the 5′-UTR from the first variant and encodes for a shorter peptide of 313 amino acids (NM_001145144). The third isoform has a different translation start, lacks the first exon (NM_001145145), and encodes for a peptide of 339 amino acids. A longer transcript XM_005259167 has been identified by computational analysis and is only reported in the NCBI database. Several SNPs have been reported for the *SLC1A5* gene, some of which have been characterized; as an example, the variants rs3027956 and rs11668878 are associated with breast cancer and chronic lymphocytic leukaemia, respectively.[10,11] On the contrary, the other two variants, rs3027985 and rs1644343, have been linked to longevity.[12]

Localization and Subcellular Localization in Physiological Conditions

The protein encoded by the *SLC1A4* gene has also different aliases: ASCT1, SATT, and SPATCCM, with a unique localization at the plasma membrane of cells. The ASCT1 protein is widely distributed in the human body; high levels of ASCT1 are found in skeletal muscle, lung, kidney, ovaries, and heart and across the digestive tract. In particular, it is found at the basolateral membrane of stratified squamous epithelia from the oral cavity to the nonglandular stomach and in the basolateral membrane of Paneth's cells.[13] Furthermore, ASCT1 is ubiquitously expressed in the brain, while its presence in the blood–brain barrier (BBB) has not been established yet. The physiological role of ASCT1 has been investigated since its first identification, even though no much information is available on this issue; the most acknowledged function of ASCT1 seems to be linked with brain homeostasis due to the transport of D-serine that is considered a gliotransmitter.[13–15] In good agreement, a significant decrease of the transport of L- and D-serine in astrocytes from ASCT1-KO mice has been observed.[14] ASCT1 is also relevant for the reuptake of cysteine in the brain; the levels of this amino acid, indeed, need to be kept under strict control due to toxicity.[13] The described function of ASCT1 is confirmed by some pathological conditions characterized by alterations of brain development and function,[14] such as schizophrenia,[16] visual dysfunction,[16] amyotrophic lateral sclerosis (ALS),[17] and microcephaly in children.[18] In line with this, an inherited disease, namely, SPATCCM (OMIM 616657), has been also linked with alterations of ASCT1 gene.[9] Furthermore, a role for ASCT1 has been proposed during hypoxia-ischemia injury in the brain.[19]

The protein encoded by the *SLC1A5* gene has different aliases, besides ASCT2, that is, AAAT, ATBO, M7V1, M7VS1, R16, and RDRC, that are much less used. The protein has a predominant localization at the plasma membrane of cells. However, the shortest ASCT2 variant (NM_001145145) has been localized at the inner membrane of mitochondria.[20] This finding opened an important perspective in the field of bioenergetics, considering that the search for a mitochondrial glutamine transporter has been a

treasure hunt for decades.[21] However, the actual role of an ASCT2 variant in mitochondria has been questioned.[22] ASCT2 is widely distributed in the human body, with higher expression in lung, skeletal muscle, large intestine, kidney, testis, T cells, and adipose tissue.[1,23] The described broad expression underlies the physiological role of the ASCT2 in harmonizing the amino acid pools in cells, being in line with its transport mechanism and with the functional and kinetic asymmetry of the catalyzed reaction.[21] This role is also relevant in pathological conditions in which the request for specific amino acids increases.[24] Finally, ASCT2 is also expressed in different areas of the brain and in the BBB, where it seems to regulate the flux of glutamate and aspartate, besides that of neutral amino acids.[25,26] This alternative function, in line with the novel glutamate/proton transport recently demonstrated in vitro,[26] may underline a role for ASCT2 in neurological disorders and brain cancers.[16,27]

Structural and Functional Features

The main challenge in the study of structural properties of human membrane transporters is the scarcity of refined 3D structures, due to the intrinsic difficulties in handling such hydrophobic proteins. This paradigm also holds for ASCT1 and ASCT2 until the solution of the ASCT2 structure in 2018.[28] However, most of the available structure–function relationship information on the SLC1 family members, and hence for ASCTs, derives from comparisons with a bacterial glutamate transporter from *Pyrococcus horikoshii*, namely, GLT_{Ph}, whose structure was solved in 2004.[29] The overall architecture of GLT_{Ph} was clearly defined as a homotrimer with a concave aqueous basin and a triangular shape. Then, over almost two decades, other 3D structures of GLT_{Ph} in different conformations have been solved, together with those of other bacterial transporters for glutamate/aspartate, such as GLT_{tk}.[2,7,30] These studies confirmed the homotrimeric architecture and gave further insights into the molecular determinants of the transport mechanism and allowed studying the human members of the SLC1 family by homology modeling coupled to biochemical approaches.[31–33] Later, in 2017, the 3D structure of the human isoform of EAAT1 was solved, demonstrating that the eukaryotic proteins show the same homotrimer organization as the bacterial counterparts; since then, more refined homology models have been built for the other members of the SLC1 family, including ASCT2.[33,34] As stated in the introduction, the interest around ASCT2 increased a lot due to potential outcomes in human therapy, and its 3D structure was solved by cryo-EM in an inward occluded conformation in the presence of the favorite substrate, Gln (Protein Data Bank [PDB] ID: 6GCT).[28] Soon after, other structures were obtained in an outward open conformation, in the presence (6MPB) or absence (6MP6) of Gln, that helped in visualizing how the protein may move in the membrane during the transport cycle.[35] To solve the structure of ASCT2 in the inward open conformation, a specific mutation in the substrate binding site, namely, C467R, had to be introduced to obtain a suitable protein for the cryo-EM assay (6RVX and 6RVY).[36] Based on the information on the 3D ASCT2 and EAAT1 structures, some general features could be drawn for both ASCT1 and ASCT2: (1) the assembly of a homotrimer in the cell membrane, as for the prokaryotic proteins and EAATs (**Fig. 1A**, side view); and (2) the presence, in each monomer, of a scaffold domain and a transport domain (**Fig. 1B**, top view). In particular, the scaffold domain remains fixed and embedded in the membrane while, and the transport domain of each monomer slides over the scaffold, crossing the membrane, giving rise to a peculiar mechanism known as elevator mechanism,[37] described as such in 2011 for GLT_{Ph}.[38]

To better compare ASCT1 (SLC1A4) and ASCT2 (SLC1A5) in terms of structural properties, we built the homology model of ASCT1 based on the ASCT2 3D structure (**Fig. 2A–C**). The two proteins share a sequence identity of 58% (**Suppl. Figs. S1C and S2D**), and both ASCT1 and ASCT2 are formed by eight transmembrane (TM) helices organized in a scaffold and a transport domain as highlighted in the two-dimensional (2D) schematic representation (**Fig. 2E**). In both cases, the scaffold domain is formed by TMs 1, 2, 4, and 5, while the transport domain consists of TMs 3, 6, 7, and 8 and two helical hairpins (HPs), HP1 between TMs 6 and 7 and HP2 between TMs 7 and 8 (**Fig. 2D,E**). These HPs have been initially proposed as gate elements for amino acid exchange through the cell membrane;[28] however, based on a structural comparison with a specific mutant of GLT_{Ph} and with the bacterial folate transporter belonging to the ECF (energy coupling factor)-type transporters, it has recently been proposed that HP2 may act as both an intracellular and an extracellular gate, defining the SLC1 family members as one-gate elevator transporters.[36] This observation, supported by structural analyses, still requires experimental pieces of evidence to be definitively confirmed.[6,39] The one-gate elevator mechanism is shared by all the SLC1 family members;[2,7] during the cycle, the substrate is occluded in the transport domain that performs a large movement from the extracellular to the intracellular milieu and vice versa (**Fig. 3**). This is in contrast with the transport cycle of other membrane transporters, such as those characterized by a LeuT fold, in which the substrate is occluded between two domains that move in the membrane with a rocking movement.[40,41] The similar organization in cell membrane and the conservation across the evolution of all the SLC1 family members are somewhat in contrast with the differences in terms of substrate specificity and ion coupling described for the EAATs and the ASCTs. Therefore, it can be argued that these differences may reside in narrower areas of the proteins. As an example, one structural difference between EAATs and ASCTs is

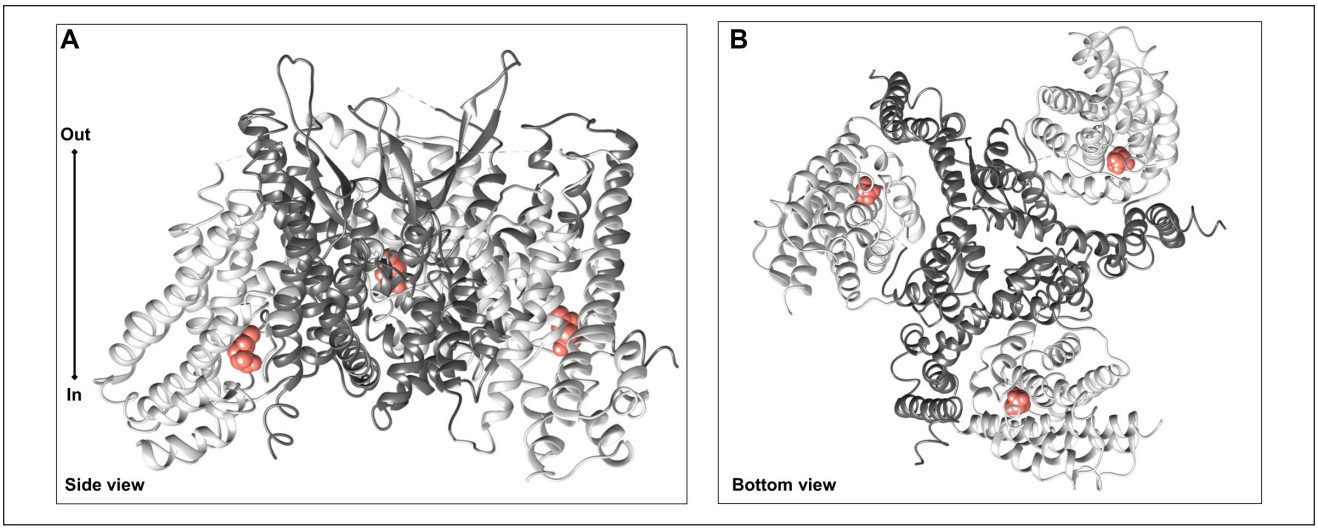

Figure 1. Cryo-EM structure of the human ASCT2. Homotrimers of human ASCT2 in the inward-facing conformation (PDB ID: 6GCT) are represented using Chimera v1.14 software (https://www.cgl.ucsf.edu/chimera). In dark gray is the scaffold domain, and in light gray, the transport domain. (**A**) Side view of the trimer with indicated membrane sidedness. (**B**) Bottom view. In pink is glutamine docked into the monomers.

that revealed by analyzing the inward open conformation of ASCT2; here, an additional cavity, formed by residues F393, V436, A433, and V46, is present (**Fig. 4**) that is absent in EAATs. The cavity is also present in the ASCT1 homology model (**Fig. 4**). This may have some relationships with the regulation of the transporters, even though further analyses are required to deepen this aspect.

Substrate Binding Site

Identification of ASCT1 and ASCT2 substrate binding sites has been attempted over the years by employing combined approaches of homology modeling, molecular docking, and site-directed mutagenesis.[2,31,33] These studies highlighted the crucial role of a single amino acid residue that, very intriguingly, determines the differences existing between EAAT and ASCT groups,[2,21,31,33,34] namely, T459 in ASCT1 and C467 in ASCT2 (**Suppl. Fig. S1C and Fig. 5A,B**). At the same position, EAATs harbor an Arg residue in line with the interaction with the net negative charge of glutamate and aspartate, that is, the favorite substrates of EAATs.[2,34] These findings, derived from structure–function relationship studies, have been confirmed by the resolution of the human ASCT2 3D structure:[28,36] it has been shown that the substrate binding site of ASCT2 is formed, besides C467, by the residues S351, S353, D460, D464, T468, and N471 (**Fig. 5A,B**). The substrate binding site of ASCT1 is well conserved and is formed, besides the already mentioned T459, by the residues C343, S345, D452, D456, T460, and N463 (**Fig. 5A,B**). Different from EAATs, the two transporters share the amino acid antiport mechanism and a different range of recognized substrates. Differences in amino acid specificity are also reported between ASCT1 and ASCT2, which are not reflected by the acronyms of the two transporters: Ala, Ser, and Cys transporters 1 and 2. The functional features have been described over more than 30 years using radiolabeled amino acids in uptake and efflux experiments performed in intact cells and oocytes expressing both human and murine isoforms of ASCT1 and ASCT2.[13,14,42–47] Later, the up-to-date technology of reconstitution in proteoliposomes was set up for murine and human isoforms of ASCT2.[48,49] In this experimental model, the function and kinetics of ASCT2 have been characterized, confirming, on the one hand, the data on substrate specificity collected in cell systems and clarifying, on the other hand, some dark sides of ASCT2 function and kinetics. As an example, ASCT2 is functionally asymmetric with glutamine, serine, threonine, and asparagine being bidirectionally transported, while alanine is only inwardly transported.[49,50] The same asymmetry applies to kinetics; indeed, the external Km toward substrates is in the micromolar range, while the internal Km toward substrates is in the millimolar range.[49,50] Interestingly, cysteine that was first considered a substrate of ASCT2[43,44] revealed an allosteric modulator of transport reaction in cells and the proteoliposome system.[51] It has to be stressed that the preferred substrate of ASCT2 is glutamine, in good agreement with the physiological role of ASCT2 as the main transporter responsible for providing a great amount of glutamine when highly required.[24,52,53] This phenomenon is typical of both physiological and pathological conditions; indeed, cells undergoing high proliferation rates are characterized by an increased

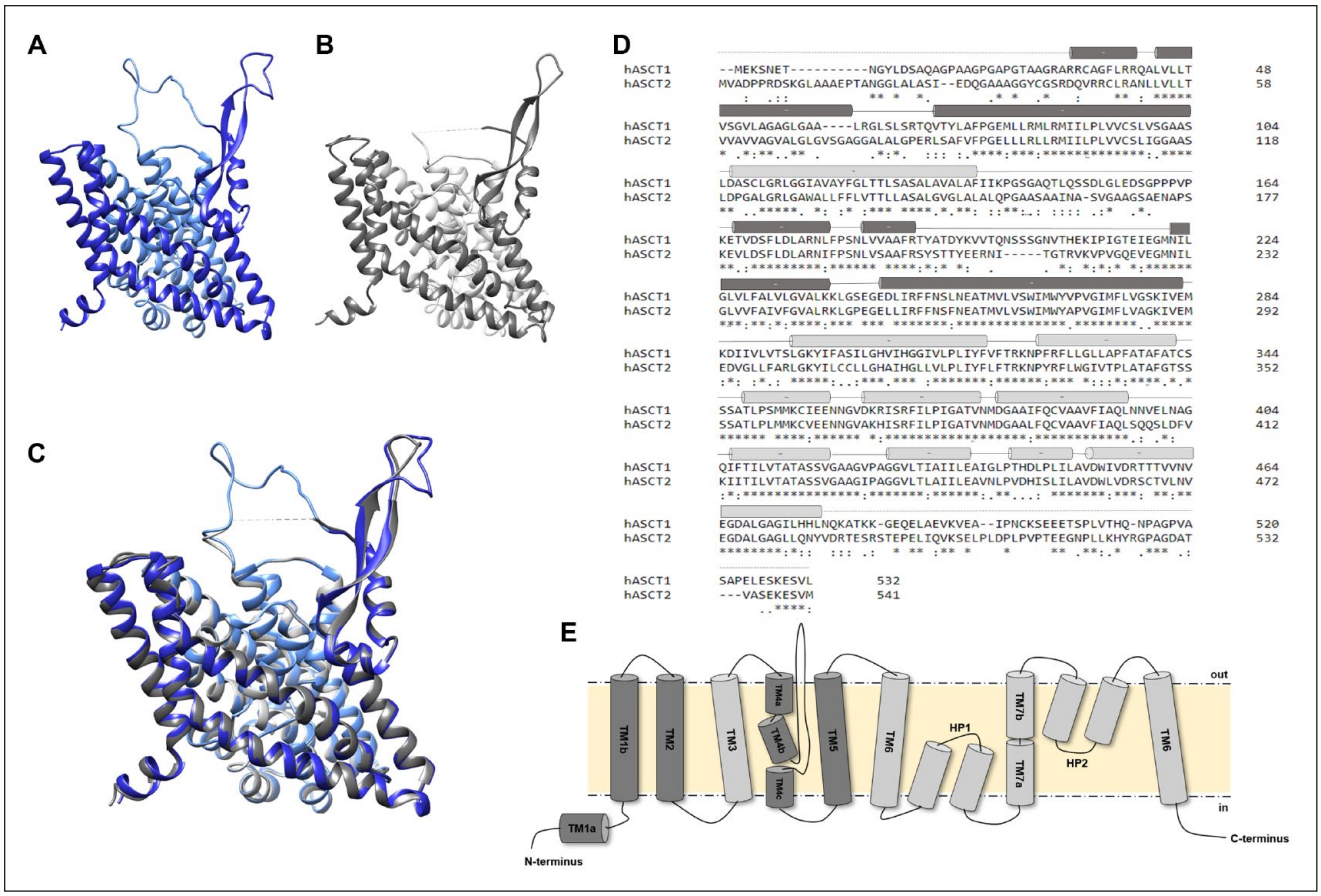

Figure 2. Human ASCT1 versus human ASCT2 structures. (**A**) The ASCT1 homology model has been built with Modeller v9, using the human ASCT2 structure (PDB ID: 6GCT). The structure is represented as a monomer with the scaffold domain in dark blue ribbon and the transport domain in light blue ribbon. (**B**) The cryo-EM structure of ASCT2 in an inward-facing conformation (PDB ID: 6GCT). The structure is represented as a monomer with the scaffold domain in dark gray and the transport domain in light gray. (**C**) Superimposition of ASCT1 and ASCT2 structure. Both structures and the superimposition are represented using Chimera v1.14 software (https://www.cgl.ucsf.edu/chimera). (**D**) Alignment of ASCT1 and ASCT2 performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo) with the indication of TM domains, extracellular loops (ECL), and HPs. In dark gray are shown TMs of the scaffold domain; in light gray, TMs and HPs of the transport domain; and in lines, ECLs. Dotted boxes indicate the portions of the protein interacting with cholesterol molecules conserved between ASCT1 and ASCT2. (**E**) 2D schematic representation of each monomer organization. In dark gray are shown TM domains of the scaffold domain, and in light gray, TMs and HPs of the transport domain. ECLs between TMs are indicated in lines. N- and C-termini of the protein face to the intracellular space are delimited by the membrane in yellow.

need for glutamine that is used for energy production and cell signaling, besides protein production.[24,54] This is a typical feature of cancer,[55,56] stem, and activated immune cells[57] that give rise to an alternative utilization of the glutamine carbon skeleton in a truncated form of the tricarboxylic acid (TCA) cycle for oxygen-independent production of ATP.[56,58,59] In these situations, glutamine becomes a conditionally essential amino acid and endogenous synthesis is not sufficient anymore, triggering an overexpression of ASCT2 among other transporters.[21,52,60]

Regarding studies on ASCT1 characterization, kinetics has been evaluated only for the external side, due to technical limitations of the intact cell model; Km values are in the micromolar range.[13,61] Different from ASCT2, ASCT1 also recognizes D-amino acids, in particular D-serine that is a physiological co-agonist of the *N*-methyl D-aspartate (NMDA) type of glutamate receptor in the brain.[62] The roles of C467 in ASCT2 and of T459 in ASCT1 have been demonstrated by site-directed mutagenesis approaches in different studies; the mutation of T459R in ASCT1 abolished the serine transport and enabled the transport of the negatively charged amino acids glutamate, aspartate, and cysteate at physiological pH.[31] Conversely, the mutation of R447 in EAAT1 to cysteine, glutamate, glycine, or serine desensitized the transporter toward glutamate while conferring some specificity toward neutral amino acids; however, no

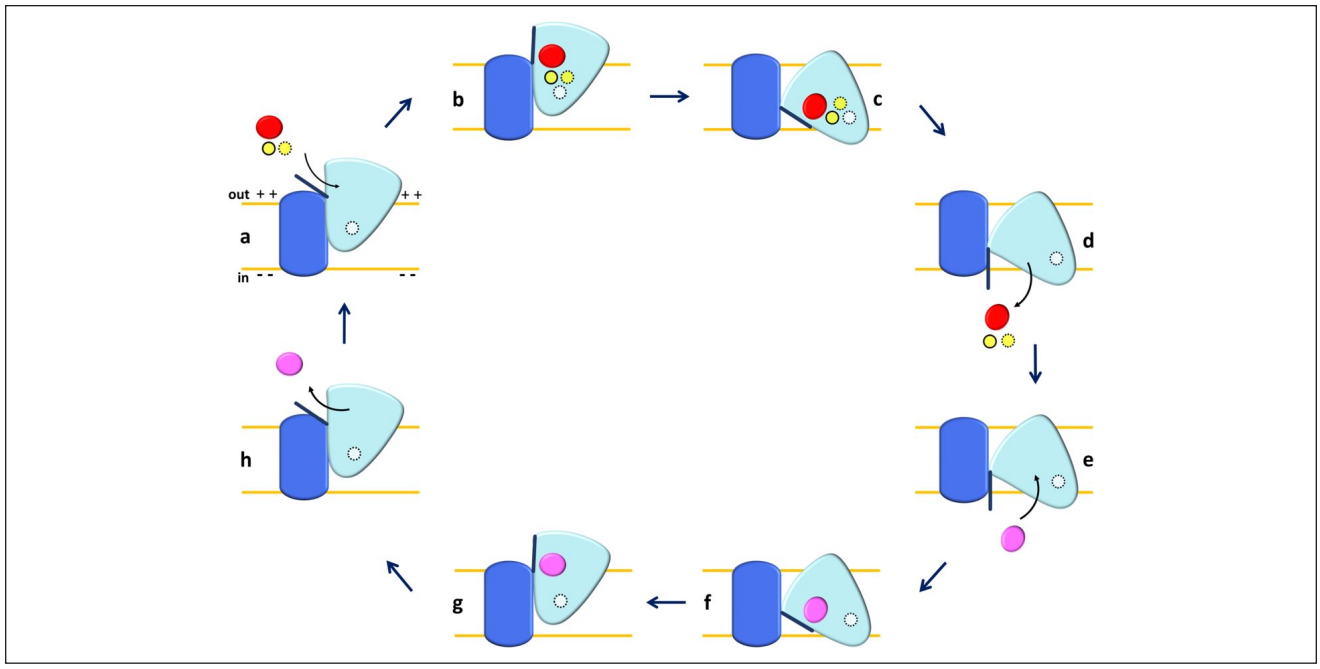

Figure 3. Schematic representation of the one-gate elevator mechanism in ASCT2. The scaffold domain is in dark blue, and the transport domain is in light blue. External substrate is depicted in red; internal substrate is depicted in pink; and Na^+ ions are shown in a yellow solid line (Na3), yellow dotted line (Na1), and white dotted line (Na2). (**a**) The transport cycle begins with the transporter in an outward open state (PDB ID: 6MPB/6MP6)[35] to allow for access of external substrates. (**b**) The transport domain switches to the outward occluded state. (**c**) Then, the transport domain shuttles toward the cytoplasm forming the inward occluded state (PDB ID: 6GCT).[28] (**d**) The substrate is released into the cytoplasm and the transporter is in the inward open state (PDB ID: 6RVX/6RVY).[36] Upon the substrate release, (**e**) the cycle continues backward with the internal substrate binding (pink) to the inward open state, (**f**) the switch toward the inward occluded state, (**g**) the movement to the outward occluded state, and (**h**) the release of the substrate in the outward open state. Not all the states have been structurally determined. For the sake of clarity, only in panel **a** are indications shown of the external (out) and internal (in) sides of the membrane and membrane potential (+, –) across the depicted membrane.

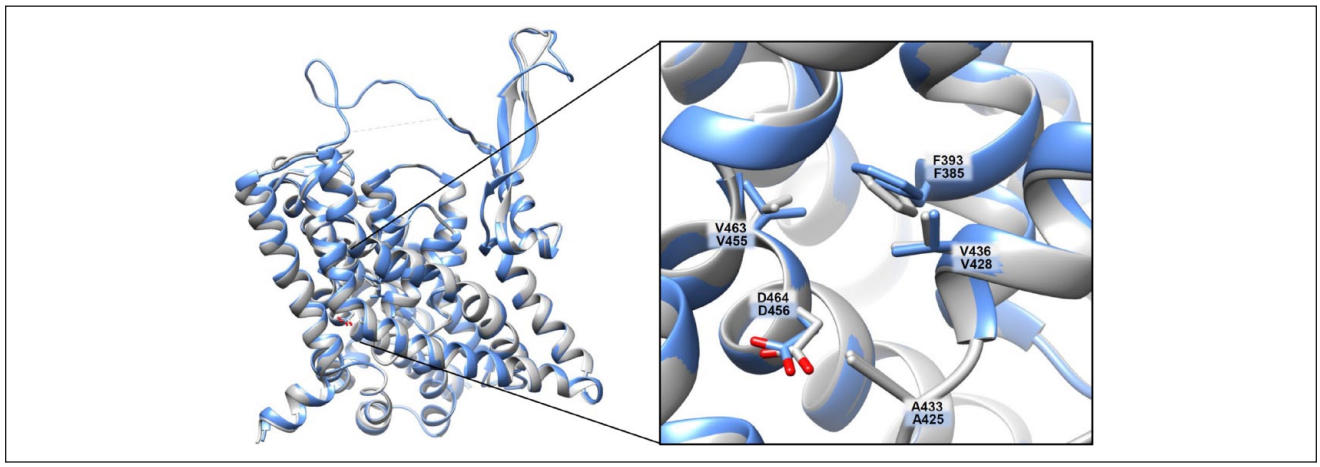

Figure 4. Human ASCT1 versus human ASCT2 structures: additional cavity. The homology model of ASCT1 and the 3D structure of ASCT2 have been superimposed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo). In the zoomed box, residues belonging to the additional cavity were compared between ASCT1 and ASCT2 and indicated as labeled stick. In blue are those of ASCT1, and in gray are those of ASCT2.

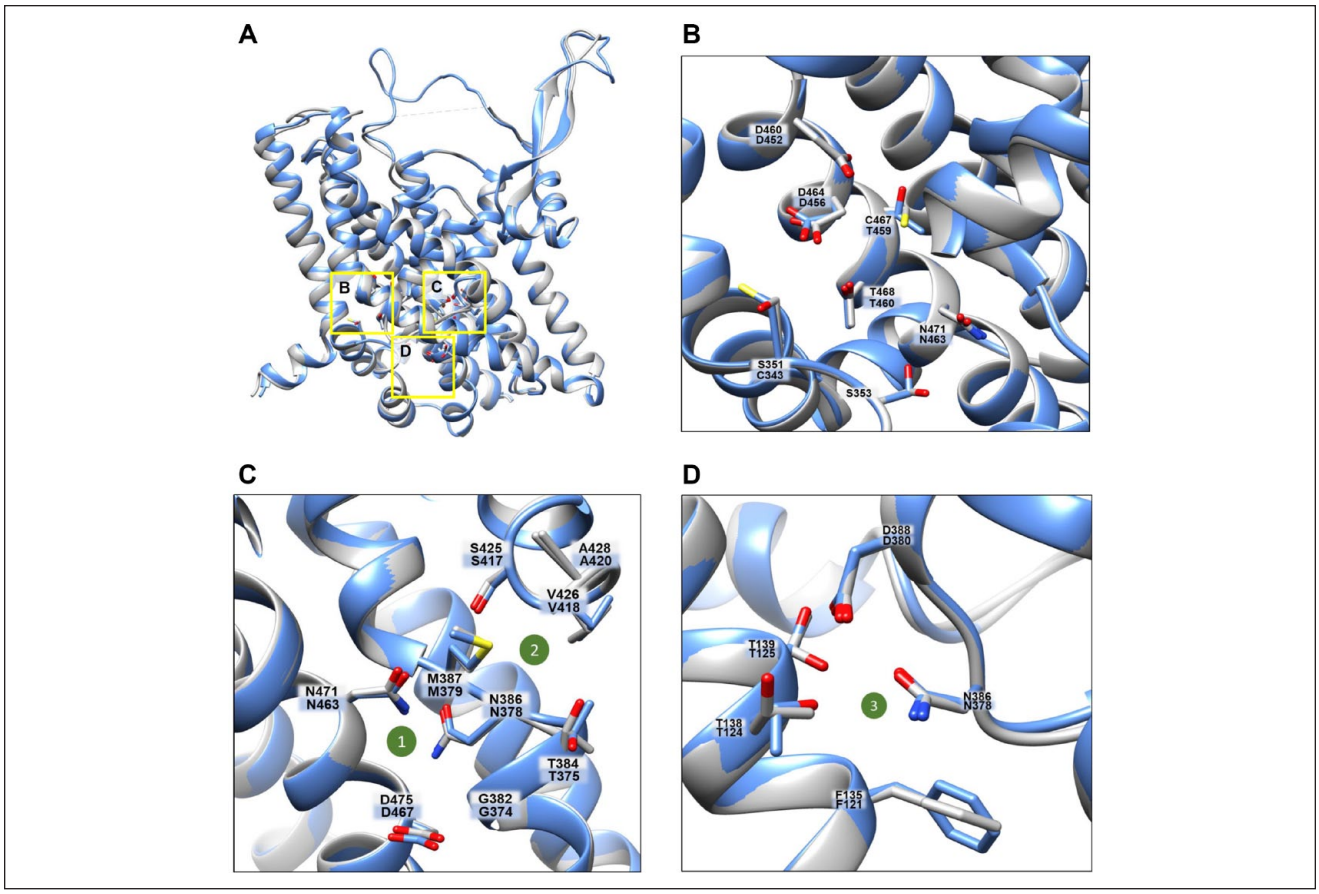

Figure 5. Human ASCT1 versus human ASCT2 structures: substrate binding site and sodium binding sites. (**A**) The homology model of ASCT1 and the 3D structure of ASCT2 have been superimposed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo). Residues were compared between ASCT1 and ASCT2 and are indicated as the labeled sticks: in blue for those of ASCT1 and in gray for those of ASCT2. (**B**) In the zoomed box, residues belonging to the substrate binding site. (**C**) In the zoomed box, residues belonging to the sodium binding sites Na1 and Na2. (**D**) In the zoomed box, residues belonging to the sodium binding site Na3.

data on possible changes in the transport mode were reported.[63] Recently, in line with the role of Thr and Cys residues in substrate recognition, the mutation of Thr into Cys in ASCT1 and vice versa in ASCT2 switched the substrate specificities of the two proteins. A particularly relevant change in the substrate specificity is that ASCT1 mutagenized to resemble ASCT2 becomes able to transport glutamine, while ASCT2 mutagenized to resemble ASCT1 loses the ability to transport glutamine.[32] Finally, the C467A substitution in ASCT2 strongly increased the Km toward glutamine;[21] furthermore, the C467R mutation switches the specificity of ASCT2 toward acidic amino acids.[36] However, a more detailed study performed by fluorometric assay revealed that the wild-type ASCT2 recognizes and transports glutamate at a rate comparable to that of other neutral amino acids. This novel ability resides in exploiting a different transport mode in which a proton is also involved in the overall transport cycle.[26] Therefore, even though C467 is a crucial residue in ASCT2, it may have roles other than the sole binding of glutamine. Besides the residues T459 of ASCT1 and C467 in ASCT2, another intriguing difference exists between the two proteins; indeed, both ASCT1 and ASCT2 binding sites have a Cys residue, even though in an opposite position (**Suppl. Fig. S1A, Figs. 2D and 5A,B**): the residue C343 of ASCT1 corresponds to S351 in ASCT2 (**Suppl. Fig. S1A, Figs. 2D and 5A,B**), and the same Ser residue is also conserved in EAATs (**Suppl. Fig. S1A**). This structural feature may suggest a functional consequence in regulating substrate recognition and/or mode of transport. Importantly, besides the range of amino acids transported by both the proteins, glutamine is the preferred substrate of ASCT2 but is not transported by ASCT1;[13] cysteine is a substrate of ASCT1 but is not transported by ASCT2.[51] Furthermore, as stated above, ASCT1 recognizes D-amino acids, while ASCT2 does not. Another residue that was found to be crucial in substrate binding site layout is located in TM7: in EAATs this helix harbors the motif NMDGT responsible for allosteric coupling of the substrate and

sodium binding. Interestingly, the last amino acid of the motif, that is, Thr, is not conserved in ASCTs that harbor an Ala residue[7,31] (**Fig. 5A,B**). A definitive explanation of the described differences is not available yet, even though a previous study conducted using site-directed mutagenesis of ASCT1 proposed that the hydroxyl group of Thr enables the transporter to discriminate between L- and D-aspartate.[50]

Sodium Binding Site

The classification of SLC1 family members in EAATs and ASCTs, besides the substrate specificity, is related to the different specificities for inorganic ions. The transport cycle of EAATs is based on the co-transport of a negatively charged amino acid with Na^+ and H^+ from the extracellular side and K^+ from the intracellular side,[1–3,64] while ASCTs were shown to obey an amino acid antiport cycle in which the exchange of neutral amino acids between the extracellular and intracellular sides is coupled to the co-transport of Na^+ ions from the extracellular side with no involvement of K^+ and H^+.[1,2] In line with this, site-directed mutagenesis on EAATs identified a Glu residue, not present in ASCTs, responsible for H^+ coordination during the transport cycle and hence for pH dependence.[65] However, as above outlined, a proton can participate in the ASCT2-catalyzed reaction in the case of glutamate transport, probably coordinating other residues of the substrate binding site.[26] Over the years, the molecular basis of ions' dependence of SLC1 family members has been investigated with site-directed mutagenesis coupled to functional analyses.[1–3,31,64] When 3D structures of the bacterial ortholog Glt_{Ph} became available,[29,37] structural biology studies added further details on the interaction of EAATs and ASCTs with ions. In particular, a 3D structure of Glt_{Ph} obtained in 2004[29] and then the EAAT structure in 2017[34] allowed for identifying two sodium binding sites in EAATs that are conserved in ASCTs (**Fig. 5A,C**). A third sodium binding site, named Na3, was not observed in the crystal structure of Glt_{Ph} but was predicted by molecular dynamics (MD) simulations.[29,37] The three sodium binding sites have been more recently described for another bacterial transporter, Glt_{tk}, by x-ray, molecular dynamics simulations, and substrate binding assays,[30,66] with the order of affinities being Na3 > Na1 > Na2. The third sodium binding site is also conserved in either EAATs and ASCTs (**Fig. 5A,D**). With particular reference to ASCTs, the studies on sodium binding sites are at different stages of investigation: in the case of ASCT1, the main residues of Na1-Na2-Na3 sites have been predicted by bioinformatics and identified by site-directed mutagenesis and functional assays,[61] while in the case of ASCT2, the binding/transport of sodium has been studied by functional assays in murine and human isoforms.[49,50,67–69] In the case of ASCT1, one of the key residues of the Na1 site, Asp467, has been mutated to Asn, Thr, and Ala, demonstrating that the D467 mutants still bind sodium even if transport activity is impaired.[61] The coordination of Na^+ in this site occurs through the β-carboxylate of the Asp residue and the backbone carbonyls of G374, N378, and N463 in TMs 7 and 8 (**Fig. 5A,C**), as shown for Glt_{tk}.[30] Regarding the Na2 binding site, the ion is coordinated by backbone interactions, as highlighted in Glt_{Ph}, of T375, M379, S417, V418, and A420 (**Fig. 5A,C**). Accordingly, mutation of a vicinal residue (G422) caused perturbation of the Na2 site, impairing the binding of sodium but not the amino acid translocation. An interesting role may be ascribed to the methionine residue (M387), as suggested for Glt_{tk}.[30] Indeed, the sulfur–Na interaction is not usual and MD simulation suggested that Met in the Na2 site could be necessary for the rearrangement of the site during the transport cycle. Altogether, the experimental and computational data suggest that the Na2 site might be the low-affinity binding site last to be loaded by Na^+ and first to be left.[30,61] The third sodium binding site is located between the unwound central region of helix 7 containing the conserved NMDGT motif and the TM3 (**Fig. 5A,D**). It has been previously shown that in ASCT1, the residues mainly responsible for the ion coordination at the Na3 site are T124, T125, and D380. Analogously to Glt_{tk}, it is plausible to assume that Na^+ is coordinated at Na3 by (1) the hydroxyl group of T124 and T125, (2) the carboxamide of N378, (3) the side-chain carboxyl of D380, and (4) the lateral chain of F121 (**Fig. 5A,D**).[30] It has to be highlighted that the Asn residue is located between the Na1 and Na3 sites (**Fig. 5A,C,D**). The described residues for the three sodium binding sites are fully conserved in ASCT2, as depicted in **Supplemental Figure S1B, Figure 2D,** and **Figure 5A,C,D**, even though no mutagenesis studies have been conducted so far. The dependence of ASCT2 transport activity on external sodium has been investigated in terms of kinetics in murine isoforms of ASCT2,[67,69] suggesting that at least one sodium ion may be involved in the amino acid antiport. More refined information has been obtained from the studies conducted using proteoliposomes reconstituted with human ASCT2; it has been shown that the Km for sodium is independent of the concentration of external glutamine.[50] Despite much data on sodium binding and translocation, the actual stoichiometry of sodium/amino acid co-transport (i.e., how many sodium ions are transported) and the direction of sodium flux are still up for debate (see also **Fig. 3**).

A corollary to the actual role of sodium ions involved in the transport cycle from the extracellular and/or intracellular side is the electrogenicity of the entire process, that is, the net accumulation of positive charges linked to sodium entry. This issue has been overlooked for a long time for both EAATs and ASCTs, also due to controversial results collected with the bacterial Glt_{Ph} that was shown to have a low electrogenicity.[70] Concerning ASCTs, the dependence on membrane potential has been observed only on ASCT2 with different and opposite results; indeed, using rat and murine

ASCT2, an electroneutral exchange of amino acids has been postulated simultaneous to an electroneutral exchange of external Na^+ with internal Na^+.[43,68] Then, a voltage-dependent electroneutral exchange was proposed for rat ASCT2, implying an electrogenic binding of Na^+/amino acid on both sides of the membrane, resulting in a net electroneutral bidirectional transport;[69] since that study, however, no further analyses have been performed for defining this mechanism in entire cell systems due to the lack of a methodology allowing for direct measurement of the Na^+ flux. Later on, using the proteoliposome tool, it has been shown that the transport cycle (Na^+_{ex}-Gln_{ex}/Gln_{in}) catalyzed by the human ASCT2 is stimulated by membrane potential generated as a K^+ diffusion potential in the presence of valinomycin. This correlates with an inwardly directed net flux of Na^+.[50] Interestingly, Na^+ is also needed in the intraliposomal compartment (intracellular side), but at a concentration much lower than the external one, corresponding to the physiological intracellular Na^+ concentration. This effect is based on the allosteric regulation of ASCT2, not on Na^+ transport from the intracellular side.[50] These data may also fit with a model in which three sodium ions bind to the protein, but not all of them are transported (**Fig. 3**).

Anion Channel Conductance

One of the most intriguing aspects still lacking a definitive molecular explanation is the presence of an anion conductance in ASCT1 and ASCT2, similar to that observed in the other members of the SLC1 family.[2,6,7] According to this property, the SLC1 family members reveal a unique double behavior of transporter and channel. The presence of these activities is acknowledged as a hallmark of the SLC1 family so that the permeation to anions has been used for both ASCT1 and ASCT2 as an indirect measure of the transport activity using patch-clamp methodology.[1,3,47,68] The anion current, associated with the Na^+-dependent uptake of neutral or negatively charged amino acids, showed the following preference: $SCN^- >> NO_3^- > I^- > Cl^- > F^- >>$ gluconate.[7,71] The anion conductance is evolutionary old, as proven by its presence in bacterial orthologs, such as in Glt_{Ph}.[6] For the EAATs, this phenomenon seems to be linked to regulation of synaptic transmission and excitability with relevance to human pathologies such as ataxia and epilepsy,[72] opening important perspectives in therapeutic strategies for neurological disorders, while for the two ASCTs, the actual role of anion conductance is not clarified yet, considering that the preferred ion, SCN^-, is unphysiological.[68] Based on investigations performed with Glt_{Ph}, it has been assessed that the anion flux occurs at the interface between the scaffold and transport domains in a flexible area formed when the Glt_{Ph} substrates (Na^+ and aspartate) are bound to the transporter.[6,73] Very recently, the chloride channel cavity has been solved by cryo-EM in an open-channel conformation, giving information on the other SLC1 family members.[74] It is worth noting that in ASCT1, different from the EAATs, the anion conductance is retained in all the mutants of the three sodium binding sites, although the amplitude of some substrate-activated currents is reduced compared to the wild type.[61]

Regulatory Aspects

One of the still undeveloped aspects concerning transporter studies is the regulation of their function/stability through posttranslational modifications and interactions with other proteins and regulatory factors. The great importance of this area of investigation is testified to by the increasing number of in silico predictions and by some ex vivo experiments, even if the complete collections of post translational modifications (PTMs) and interactions for ASCT1 and ASCT2 are not deciphered yet. The state of the art on some of these aspects is given below.

PTMs: N-Glycosylation, Phosphorylation, Acetylation, and Methylation

N-glycosylation is acknowledged as the canonical pathway responsible for driving proteins to their definitive location in cell membranes.[75–77] Based on sequence analysis, *N*-glycosylation sites are present, corresponding to N201 and N206 in ASCT1 and N163 and N212 in ASCT2 (**Fig. 6**). The role of *N*-glycosylation in ASCT2 has been deeply investigated in the last decade; indeed, the two glycosylated Asn residues, identified in the 3D structure of human ASCT2, have been definitively confirmed by site-directed mutagenesis. Indeed, the substitution N163Q and N212Q of human ASCT2 prevents its localization to the plasma membrane and decreases the protein stability,[78] while the N163Q and N212Q mutants retain full intrinsic transport activity, as shown by parallel assays in intact cells and proteoliposomes demonstrating that glycosylation/deglycosylation status does not affect the capacity and specificity of ASCT2 to catalyze the physiological Na^+-dependent amino acid antiport. Another study on *N*-glycosylation of human ASCT2 has been conducted using tunicamycin, an antibiotic that blocks the *N*-glycoprotein synthesis. Upon tunicamycin treatment, a link between the extent of ASCT2 *N*-glycosylation and glucose metabolism has been proposed, supporting the hypothesis that coordinated regulation of glucose and glutamine metabolism exists.[79] Much less is known about the role of the conserved *N*-glycosylation sites of human ASCT1. The only available data linked *N*-glycosylated ASCT1,[80] as well as ASCT2, to viral recognition at the cell membranes of a group of retroviruses named HERVs (human endogenous retrovirus).[81]

Besides *N*-glycosylation, the phosphorylation of serine, threonine, and tyrosine residues is one of the most common PTMs linked to the regulation of protein functions being

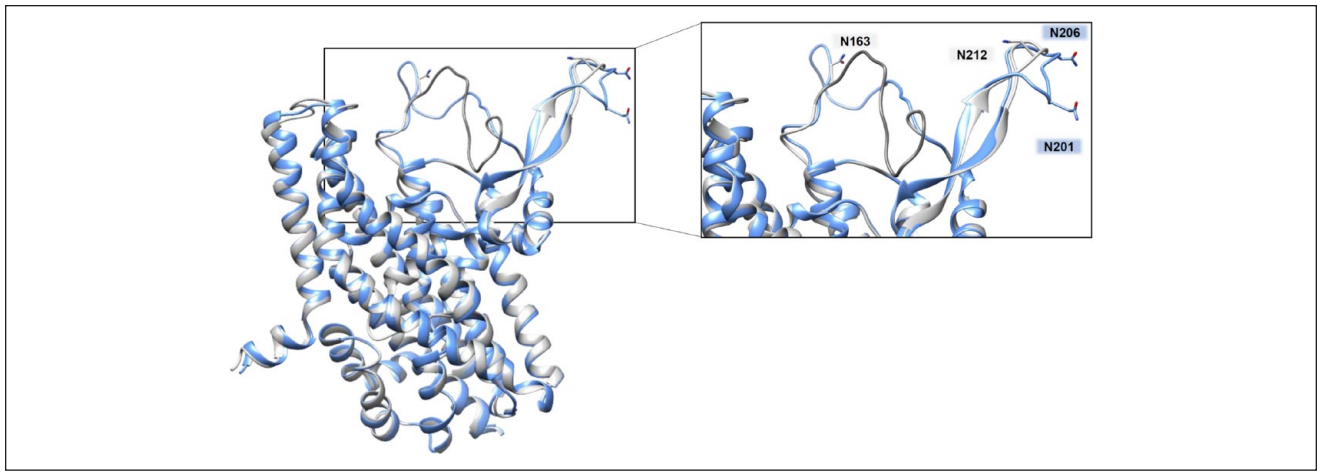

Figure 6. Human ASCT1 versus human ASCT2 structures: *N*-glycosylation sites. The homology model of ASCT1 and the 3D structure of ASCT2 have been superimposed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo). In the zoomed box, residues responsible for *N*-glycosylation were compared between ASCT1 and ASCT2 and are indicated as labeled sticks: in blue for those of ASCT1 and in gray for those of ASCT2.

Table 1. Post Translational Modifications of hASCT1 and hASCT2.

PTM Type	Residue of PTM Human ASCT1	Residue of PTM Human ASCT2
Serine phosphorylation	242, 502,507,521, 527, 530	9, 27, 183, 194, 198, 493, 503, 535, 539
Threonine phosphorylation	7, 506	494, 532
Tyrosine phosphorylation	10	38, 524
Lysine ubiquitination	196, 483, 484, 493, 501, 528	10, 178, 247, 372, 502, 522, 537
Lysine acetylation		537
Lysine sumoylation		522
Arginine methylation		525

either an activation or inhibition message.[82] Proteomic analyses performed using different databases such as Phosphositeplus and iPTMnet showed that both ASCT1 and ASCT2 are subjected to phosphorylation at multiple sites (**Table 1**). Concerning ASCT2, an old paper showed that phosphorylation of ASCT2 via SGK1, SGK3, and PKB kinase, activates ASCT2 in *Xenopus laevis* oocytes.[83] Moreover, it has been recently described that the extent of ASCT2 phosphorylation at position S503 decreased in a colon adenocarcinoma cell line after treatment with growth inhibitors.[84] Regarding ASCT1, a correlation between the increased levels of phosphorylated threonine and the decreased affinity of ASCT1 has been suggested as a result of leukotriene D4 (LTD4), an inflammatory mediator present in chronically inflamed intestine.[85] Moreover, the bioinformatics resource iPTMnet reports that ASCT1 is subjected to lysine ubiquitination and that ASCT2 is subjected to lysine ubiquitination, acetylation, sumoylation, and arginine methylation (**Table 1**). Interestingly, iPTMnet includes the list of some ASCT1 and ASCT2 residues, subjected to PTMs, that are mutated in some human cancers.[86] For instance, the S502 of ASCT1 has been found mutated to Cys in breast cancer and the Ser535 of ASCT2 has been found mutated to Phe in melanoma.[86] Moreover, the methylation site R525 and the acetylation site K537 of ASCT2 are mutated in uterine cancer.[86]

Protein–Protein Interactions and Cellular Effectors

Little detailed information is available regarding the regulation of ASCT1 and ASCT2 transport activity by interactions with other cellular proteins. Ubiquitin reconstruction proximity label–MS, affinity capture–MS, and the two-hybrid assay described some interactions of ASCT1 with cell proteins (https://thebiogrid.org/112400; https://www.ebi.ac.uk/intact/interactors/id:P43007*; https://mint.bio.uniroma2.it/index.php/results-interactions/?id=P43007; https://string-db.org/network/9606.ENSP00000234256). A few examples are the interactions of ASCT1 with (1) G-protein-coupled receptors, one of the most important protein classes involved in cell signaling in physiological and pathological

conditions,[87] and (2) Nek 4, one of the largest members of the serine–threonine kinase Nek family, related to the primary cilia formation and in DNA damage response.[88] Moreover, in the mentioned databases, interactors of ASCT1 have been suggested, among other amino acid transporters related to brain homeostasis, such as SLC7A5, SLC7A11, and SLC38A2 (https://string-db.org/network/9606.ENSP00000234256). Regarding ASCT2, in silico predictions as well as immunoprecipitation or double-hybrid technologies described several interactions of ASCT2 with cell proteins (https://thebiogrid.org/112401; https://string-db.org/network/9606.ENSP00000444408; https://string-db.org/network/9606.ENSP00000444408; https://mint.bio.uniroma2.it/index.php/results-interactions/?id=slc1a5; https://www.ebi.ac.uk/intact/interactors/id:Q15758*). As an example, ASCT2 has been shown to interact with CD147/MCT1 and CD98/LAT1, forming a super-complex able to respond to metabolic changes.[89] Furthermore, ASCT2 physically interacts with PDZK1 and SNX27, two proteins containing a PDZ domain, one of the most common domains in the human genome.[50,90–92] Recently, the serotonin transporter (SERT) has been shown to physically interact with ASCT2, stimulating the uptake of serotonin in cells and thus contributing to the homeostasis of neurons.[93] Furthermore, physical interaction of ASCT2 with EGFR in cancer has been described as a consequence of EGF-mediated regulation of ASCT2.[94]

Regulation by Physiological Molecules

The regulation of ASCT1 and ASCT2 by endogenous effectors is still at the initial stage of the investigation. Human ASCT2 harbors in its primary structure eight Cys residues that have been substituted by alanine to evaluate their role in the sensitivity of ASCT2 to reducing and oxidizing reagents. Interestingly, the C467, crucial for substrate recognition, is also responsible for an ON/OFF regulation of ASCT2 by physiological cysteine-targeting reagents triggering redox responsiveness. In particular, it has been proposed that the C467 residue could be involved in the formation of a disulfide bridge with the vicinal C308 or C309 triggering the switch between an active (SH state) to an inactive (S–S state) protein.[33] The regulation of ASCT1 is much less studied, and an inhibitory effect by the potent oxidant peroxynitrite has been reported in chronic inflammation even though the molecular mechanism is still not clarified.[95] It is important to highlight that even if ASCT1 harbors seven Cys residues, only three are homologous with ASCT2 (**Suppl. Figs. S1 and S2D**) that do not correspond to C308, C309, and C467, indicating a different involvement of the Cys residues in the regulation and redox responsiveness of the two proteins. A recent finding in ASCT2 biology is the regulation of cholesterol by physical interaction with the protein.[96] This finding has been achieved, in parallel, with different approaches: (1) identification of cholesterol-like densities in the 3D ASCT2 structure by cryo-EM[28,36] and (2) stimulation of transport activity by biochemical assays in proteoliposomes prepared with different cholesterol contents.[96] Computational analysis suggested six different poses for cholesterol interaction, including the acknowledged cholesterol binding motif CARC/CRAC. The chemical targeting strategy, employed in proteoliposomes, suggested that cholesterol binding occurs at the level of tryptophan and cysteine residues.[96] Cholesterol triggers the increase of ASCT2 transport rate with no effect on the affinity for substrates. So far, no effect of cholesterol has been described for ASCT1, even though most of the residues putatively involved in the cholesterol–-ASCT2 interaction are conserved (**Fig. 2D**, dotted box), suggesting that ASCT1 might also be regulated by cholesterol.

Interaction with Drugs

The involvement of the two proteins in human diseases together with the availability of the 3D structure of ASCT2 and the homology model of ASCT1 opened important perspectives in the field of drug design. Notwithstanding, the International Transporter Consortium has so far not included ASCT1 and ASCT2 as relevant for drug–transporter interactions.[97] It has to be stressed that almost no studies are available for ASCT1-targeting drugs, while a lot of effort has been made in the last 15 years to design novel molecules as drugs targeting ASCT2 transport activity. In particular, these studies have been conducted on both murine and human isoforms (**Fig. 7**). The first attempt, realized in a pre-3D era, was performed using substrate-mimicking molecules on the rat isoform identifying two molecules, benzylcysteine (**Fig. 7A**) and benzylserine (**Fig. 7B**), as competitive inhibitors able to block ASCT2 transport activity, measured as glutamine-induced anion currents, even if at high concentrations.[98] This study was conceived as a "proof of principle" to demonstrate that the competitive inhibitors are able to selectively block amino acid transport. Then, glutamine (**Fig. 7C**)[99] and serine (**Fig. 7D**)[100] structures were used as a scaffold for other substrate-like compounds, either substrates or inhibitors. In these cases, the efficacy of inhibitors has been tested as a block of anion currents as well. The serine analogs were revealed to be threefold stronger than glutamine analogs. More recently, other molecules based on the proline scaffold have been designed, able to activate or inhibit rat ASCT2 transport activity in intact cells.[32,101] These findings are very intriguing because proline, per se, is not a substrate of ASCT2. Notwithstanding such a premise, the cis-3-hydroxyproline (**Fig. 7E**) revealed a small activator of ASCT2, while the proline fluorobenzyl substituent (g-FBP) was found to be a potent inhibitor with cytotoxic effects (**Fig. 7F**). Very interestingly, despite the differences existing

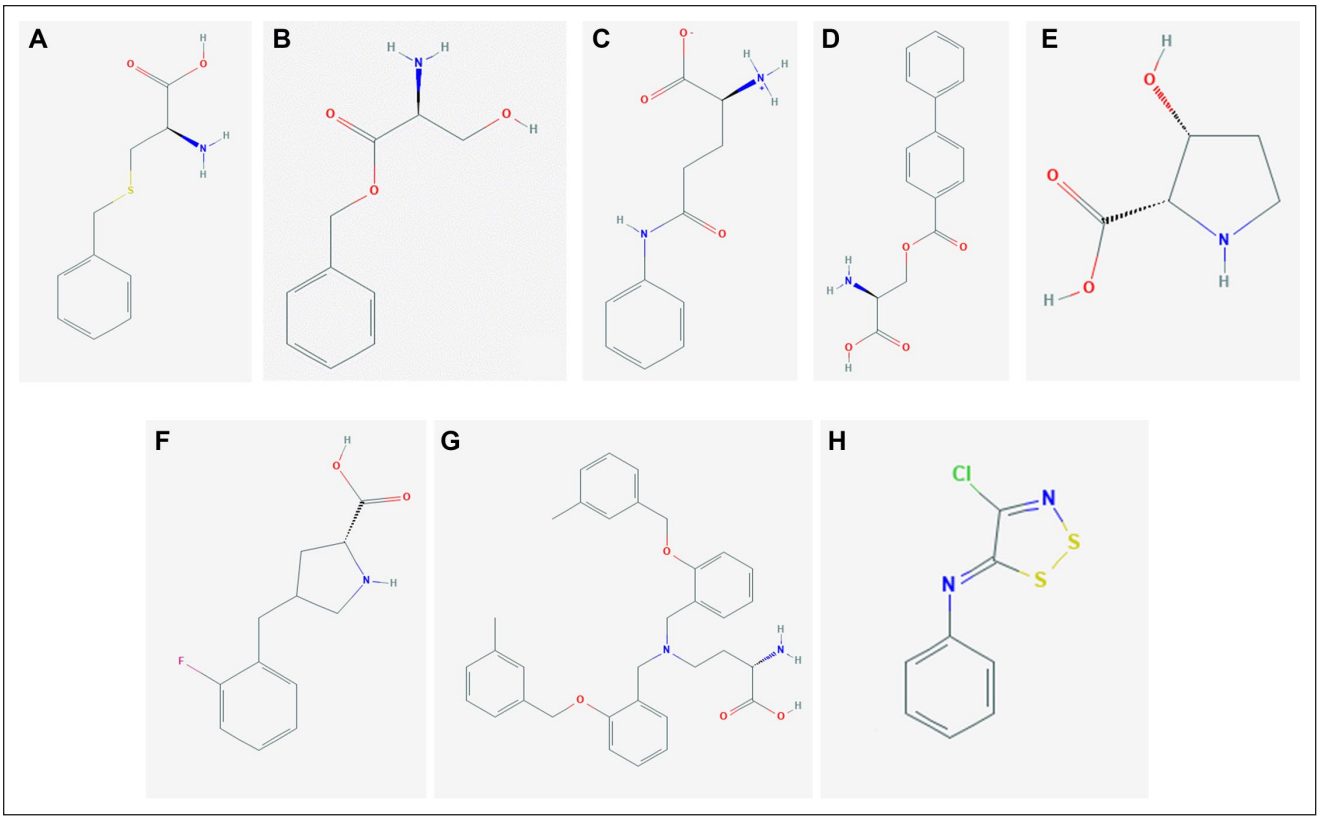

Figure 7. Molecules interacting with ASCT2. (**A**) Benzylcysteine and (**B**) benzylserine.[98] (**C**) *g*-Glutamylanilide.[99] (**D**) Serine–biphenylcarboxylate.[101] (**E**) Hydroxyproline and (**F**) FBP.[102] (**G**) V-9302.[103] (**H**) Dithiazole-based scaffold molecule.[104] (**A–G**) Competitive inhibitors. (**H**) Covalent inhibitor.

between rat and human isoforms, these molecules were also active on the human melanoma cell line.[101] These observations triggered the development of drugs based on the scaffold of proline derivatives. Recently, a competitive inhibitor of human ASCT2 has been designed, namely, V-9302 (**Fig. 7F**), moving from a screening conducted on 2-amino-4-bis (aryloxybenzyl)aminobutanoic acid analogs.[102] The V-9302 reached a preclinical phase of investigation due to its ability in inhibiting ASCT2 transport in vitro and in mice harboring a patient-derived xenograft (PDX) tumor.[103] Besides the competitive inhibitors, an alternative strategy has been employed, using the rat isoform, for designing and testing covalent inhibitors able to irreversibly block ASCT2 transport activity, identifying six compounds with dithiazole (**Fig. 7H**) moieties with IC50 in the micromolar range.[104] Based on computational analysis, it was predicted that dithiazole-based molecules can interact with Cys residues of ASCT2, probably at the CXXC motif present in the rat isoform. The covalent drug approach is an old-fashioned strategy that, in the case of molecules targeting amino acid transporters, would have the great advantage of dramatically increasing the specificity and potency of a drug.[104] The covalent binding, indeed, should avoid the pitfalls linked to substrate analogs that can be displaced from the binding site by endogenous amino acids whose concentrations may increase under some specific conditions.[105,106] Finally, another path has been pursued for ASCT2 inhibition, that is, anti-ASCT2 monoclonal antibodies, first identified as responsible for the inhibition of colorectal cancer cells in vitro[107] and a PDX model of gastric cancer.[108] Finally, a monoclonal antibody recently reached the phase I clinical trials in hematological cancers.[2]

Conclusions

The proteins forming the ASCT group are intriguing players of cell biology due to their ability to mediate a Na^+-dependent flux of amino acids in cells, despite their antiport mechanism (**Fig. 3**). This peculiar transport mode results in a more efficient accumulation of the inwardly transported amino acids in cells. Indeed, the maintenance of amino acid homeostasis is critical for cell survival given the plethora of metabolisms in which amino acids are involved, besides protein production.[109] Even though ASCT1 and ASCT2 share several structural features, they differ in tissue localization and the specificity of crucial amino acids such as glutamine and cysteine. These apparently small divergences have great implications for physiology and hence for

pathological conditions characterized by altered expression/function of ASCT1 and ASCT2. Indeed, as above described, while ASCT1 has been mainly linked to neurological disorders, ASCT2 overexpression is nowadays considered a hallmark of virtually all human cancers. This difference is not as sharp as it is commonly described in the literature; indeed, ASCT1 overexpression has been reported in some human cancer, and a role for ASCT2 in neurological disorders has been proposed as well.

Altogether, the available data on ASCT1 and ASCT2 may help to answer the question raised in the title: notwithstanding the structural similarities existing between ASCT1 and ASCT2, the actual differences in terms of substrate specificities and physiological roles suggest that the two proteins may be considered cousins rather than brother and sister.

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Supplemental Material

Supplemental material is available online with this article.

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