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Structural Features of the Glutamate Transporter Family

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INTRODUCTION

Eukaryotic, bacterial, and archaeal cells contain a large number of integral membrane proteins and protein complexes involved in solute transport across the membrane (48). In *Escherichia coli* as many as 10.8% of all chromosomal genes code for membrane transport proteins (65). All known and putative transport proteins have recently been classified in different families based on their sequence similarities. This led to the identification of 159 transporter families, of which secondary transporters represent the largest functional category, comprising 59 families (73).

Secondary transporters use the free energy stored in ion and/or solute gradients over the membrane to drive transport. Since three-dimensional structures of secondary transporters have not yet been solved, models for their structures are based on biochemical data and on (computational) analysis of their amino acid sequences. A typical secondary transporter consists of a single polypeptide component that forms a bundle of membrane-spanning α-helices connected by loops of various sizes. Many of these proteins contain 12 membrane-spanning segments, but different numbers are not exceptional (73). The 59 families of secondary transporters do not represent as many different global structures. It has been shown by hydropathy profile analysis that many of the families belong to the same structural class (53, 54). One of the families that forms a separate structural class is the glutamate transporter family, also called dicarboxylate/cation symporters (DCS; transporter classification 2.23) (73).

The glutamate transporter family includes transporters that are found in neurons and glial cells in the mammalian central nervous system and catalyze the reuptake of the excitatory neurotransmitter glutamate from the synaptic cleft (26, 38, 67, 81). Excessive amounts of extracellular glutamate, associated with several diseases, cause neuronal destruction via activation of the *N*-methyl-D-aspartate receptors (57, 60). The neuronal and glial glutamate transporters are believed to prevent excitotoxicity of glutamate (52, 71, 83) and may help to end the excitatory signal together with diffusion (58, 63). Since these proteins have been implicated in numerous neurological disorders, they have attracted much attention and have been well characterized.

The number of known transporters in the glutamate transporter family has grown rapidly during the last years, and many members of the family have now been characterized, including retinal glutamate transporters from vertebrates (3, 22), mammalian neutral-amino-acid transporters (5, 51, 75), and bacterial nutrient uptake proteins (23, 85, 86). Because of the variety of transporters that are being studied in the glutamate transporter family and the numerous techniques used to study them, the structural features of the transporter family are now rapidly being unraveled. This review focuses on the diversity of transporters in the family and on their structural properties. Physiological and pathological aspects have recently been reviewed elsewhere (37, 94).

THE FAMILY

Substrate Specificity

The members of the glutamate transporter family that have been functionally characterized can be classified in three groups based on their substrate specificity (Table 1): C_4-dicarboxylate transporters (found in bacteria), glutamate/aspartate transporters (found in bacteria and eukaryotes) and neutral-amino-acid transporters (found in bacteria and eukaryotes). The bacterial C_4-dicarboxylate carriers transport the tricarboxylic acid cycle intermediates succinate, fumarate, and malate (28, 29). In addition, the transporter from *Rhizobium meliloti* uses aspartate (106) and the transporters from *Escherichia coli* and *Salmonella typhimurium* use orotate as substrate (6). It is not known which transmembrane ion or solute gradient(s) provides the free energy to drive transport.

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TABLE 1. Substrate specificity of the members of the glutamate transporter family

Subfamily	Kingdom[a]	Substrates[b]	Mode of energy coupling
C_4-dicarboxylate transporters	B	Succinate, fumarate, malate (orotate, aspartate)	Unknown
Glutamate/aspartate transporters	B, E	Glutamate, aspartate, glutamate analogues	H^+ symport; H^+/Na^+ symport; H^+/Na^+ symport-K^+ antiport
Neutral-amino-acid transporters	B, E	Alanine, serine, cysteine, threonine (asparagine, glutamine)	Exchange; Na^+ symport

[a] B, *Bacteria*; E, *Eucarya*.
[b] High-affinity substrates are shown; substrates in parentheses are not accepted by all members of the subfamilies.

All glutamate transporters use L-glutamate and L-aspartate as high-affinity substrates (K_m [Glu] $< 100\mu M$). In addition, several (nonphysiological) glutamate analogues are transported (38, 67, 81, 85, 88, 104). The bacterial proteins catalyze the electrogenic symport of glutamate with at least two cations (85, 87, 88). The GltP proteins from *E. coli* and *Bacillus subtilis* use protons to drive the uptake of glutamate, whereas the GltT proteins from *Bacillus stearothermophilus* and *Bacillus caldotenax* can replace one proton with a sodium ion (34). Na^+ is preferentially used at elevated temperatures, but the selectivity for sodium ions is lost when these transporters are expressed in *E. coli* (87). These observations indicate that minor conformational changes may alter the cation specificity.

The eukaryotic glutamate transporters catalyze the electrogenic symport of glutamate with two or three sodium ions and one proton, whereas one potassium ion is antiported (2, 7, 24, 43, 112). The exact number of transported sodium ions is still a matter of debate (37, 39, 50, 112), but a stoichiometry of three sodium ions per glutamate is generally favored (50, 112). Transport of glutamate by the eukaryotic proteins is strictly dependent on sodium ions, but not all Na^+ binding sites are equally selective. For the glutamate transporter EAAT2 of rats (also known as GLT-1), it was shown that only one Na^+ binding site strictly requires sodium ions whereas lithium ions can replace Na^+ in the others (33). Cesium, rubidium, and ammonium ions can replace K^+ to various extents (44).

The high-affinity substrates for the mammalian neutral-amino-acid transporters are alanine, serine, cysteine, and threonine ($K_m < 100\ \mu M$), but some members (ACST2 from mice, humans, and rabbits) show a broader substrate specificity and accept glutamine and asparagine with high affinity and several other amino acids, including glutamate, with lower affinity ($K_m > 300\ \mu M$) (5, 45, 46, 82, 93). The human neutral amino acid transporter ASCT1 is an obligate exchanger that does not mediate a net flux of amino acids (111). Exchange of the amino acids is electroneutral and accompanied by a symmetrical exchange of one Na^+. In contrast, mouse ASCT2 may exchange Na^+ for K^+ during turnover (93). Recently, a serine transporter from *E. coli* that is an Na^+ symporter (61) was characterized.

Both the eukaryotic glutamate transporters and the eukaryotic neutral-amino-acid carriers mediate thermodynamically uncoupled ion fluxes. Besides substrate-independent cation leaks, substrate-dependent Cl^- currents have been reported (3, 8, 22, 26, 39, 66, 79, 95, 101, 111). The extent of the substrate-dependent Cl^- flux differs for the various members of the family but is very high in the glutamate transporters EAAT4 and EAAT5 from the human brain and retina and EAAT5A from the salamander retina (3, 22, 26). Thus, these proteins have a dual function as glutamate transporters and glutamate-gated chloride channels.

Diversity and Phylogeny

Sixty protein sequences from the *Eucarya*, *Bacteria*, and *Archaea*, belonging to the glutamate transporter family, were found in the protein and translated nucleotide databases by using the BLAST facility (1) (Table 2). The sequences vary in length from 396 to 581 residues, with the bacterial ones being significantly shorter (396 to 491 residues) than the eukaryotic ones (479 to 581 residues). Several organisms contain multiple paralogues of the family. The bacteria *E. coli* and *B. subtilis* have four paralogues each, whereas in eukaryotes (*Homo sapiens*) up to seven have been found so far. A multiple-sequence alignment was made with the CLUSTALX program (84). A stretch of about 150 residues in the C-terminal part of the sequences is better conserved than the N-terminal part (Fig. 1). This C-terminal part of the alignment, which is unambiguous and contains very few gaps, was used to construct the phylogenetic tree shown in Fig. 2.

The tree includes a subset of 35 members of the family that do not contain pairs of sequences with more than 70% identical residues. Since more similar pairs cluster closely together in the tree, they are less informative. Several distinct subfamilies can be recognized in the phylogenetic tree of the glutamate transporter family. All eukaryotic members cluster in one branch of the tree. They have at least 31% identical residues over the full length. The neutral amino acid transporters and the glutamate transporters clearly form separate groups within the eukaryotic branch, supporting the notion that substrate specificity correlates with phylogenetic classification (64). However, the eukaryotic and bacterial glutamate transporters that have similar substrate specificity do not cluster in the tree but belong to separate subfamilies. Likewise, the eukaryotic neutral-amino-acid transporters and the bacterial serine transporters are found in different branches of the tree. The analysis suggests that phylogenetic clustering of members of the family is determined hierarchically, first by the evolutionary position of the host organism and second by the substrate specificity (72).

The bacterial glutamate transporters are found in the same major branch as the bacterial C_4-dicarboxylate transporters, but within this branch they form separate clusters. The bacterial glutamate transporters have more than 44% identical residues, whereas the C_4-dicarboxylate transporters have at least 40% identical residues. The percentage of identical residues between the members of the GltP and DctA subfamilies varies from 27 to 42%. In both groups, a protein from *B. subtilis* that has not been functionally characterized is present (YhfG and YdbH, respectively). It is likely that these proteins have the same substrate specificity as the other members of the subfamilies to which they belong. The two bacterial subfamilies are more closely related to each other than to the eukaryotic subfamily. The bacterial and eukaryotic members have 18 to 28% identical residues.

TABLE 2. Members of the glutamate transporter family[a]

Name	Organism	Kingdom[b]	Description	No. of residues	Accession no.
ASCT2 Ocu	*Oryctolagus cuniculus*	E	Neutral-amino-acid transporter	541	O19105
ASCT2 Hs	*Homo sapiens*	E	Neutral-amino-acid transporter	541	O15758
ASCT2 Mmu	*Mus musculus*	E	Neutral-amino-acid transporter	553	P51912
ASCT1 Hs	*Homo sapiens*	E	Neutral-amino-acid transporter	532	P43007
ASCT1 Mmu	*Mus musculus*	E	Neutral-amino-acid transporter	532	O35874
EAAT1 Tni	*Trichoplusia ni*	E	Glu/Asp transporter	479	AF003006
EAAT3 Mmu	*Mus musculus*	E	Glu/Asp transporter	523	P51906
EAAT3 Rno	*Rattus norvegicus*	E	Glu/Asp transporter	523	P51907
EAAT3 Bta	*Bos taurus*	E	Glu/Asp transporter	524	O95135
EAAT3 Ocu[c]	*Oryctolagus cuniculus*	E	Glu/Asp transporter	524	P31597
EAAT3 Hs	*Homo sapiens*	E	Glu/Asp transporter	524	P43005
EAAT1 Hs	*Homo sapiens*	E	Glu/Asp transporter	542	P43003
EAAT1 Bta	*Bos taurus*	E	Glu/Asp transporter	542	P46411
EAAT1 Rno[c]	*Rattus norvegicus*	E	Glu/Asp transporter	543	P24942
EAAT1 Mmu	*Mus musculus*	E	Glu/Asp transporter	543	737473
EAAT1 Ati	*Ambystoma tigrinum*	E	Glu/Asp transporter	543	AF018256
EAAT4 Mmu	*Mus musculus*	E	Glu/Asp transporter	561	O35544
EAAT4 Rno	*Rattus norvegicus*	E	Glu/Asp transporter	561	O35921
EAAT4 Hs	*Homo sapiens*	E	Glu/Asp transporter	564	P48664
EAAT5 Hs	*Homo sapiens*	E	Glu/Asp transporter	560	O00341
GLT5A Ati	*Ambystoma tigrinum*	E	Glu/Asp transporter	564	AF018259
GLT5B Ati	*Ambystoma tigrinum*	E	Glu/Asp transporter	544	AF018260
EAAT2 Hs	*Homo sapiens*	E	Glu/Asp transporter	572	P43004
EAAT2 Mmu	*Mus musculus*	E	Glu/Asp transporter	572	P43006
EAAT2 Rno[c]	*Rattus norvegicus*	E	Glu/Asp transporter	573	P31596
GLT2A Ati	*Ambystoma tigrinum*	E	Glu/Asp transporter	579	AF018257
GLT2B Ati	*Ambystoma tigrinum*	E	Glu/Asp transporter	581	AF018258
EAAT Cel	*Caenorhabditis elegans*	E	Putative Glu/Asp transporter	503	O10901
EAAT Ovo	*Onchocerca volvulus*	E	Putative Glu/Asp transporter	492	O25605
EAT2 Cel	*Caenorhabditis elegans*	E	Putative Glu/Asp transporter	532	O21353
EAT3 Cel	*Caenorhabditis elegans*	E	Putative Glu/Asp transporter	575	O21751
GLT Cel	*Caenorhabditis elegans*	E	Putative Glu/Asp transporter	491	Z99277
EAT4 Cel	*Caenorhabditis elegans*	E	Putative Glu/Asp transporter	502	O22682
GltT Bca	*Bacillus caldotenax*	B	Glu/Asp transporter	421	P24944
GltT Bst	*Bacillus stearothermophilus*	B	Glu/Asp transporter	421	P24943
YhfG Bsu	*Bacillus subtilis*	B	Putative Glu/Asp transporter	429	Y14083
GltP Ec	*Escherichia coli*	B	Glu/Asp transporter	437	P21345
GltP Bsu	*Bacillus subtilis*	B	Glu/Asp transporter	414	P39817
DctA Ec	*Escherichia coli*	B	C_4-dicarboxylate transporter	428	P37312
DctA Sty	*Salmonella typhimurium*	B	C_4-dicarboxylate transporter	428	P50334
DctA RleN	*Rhizobium leguminosarum* NGR234	B	C_4-dicarboxylate transporter	449	S38912
DctA Sme	*Sinorhizobium meliloti*	B	C_4-dicarboxylate transporter	441	P20672
DctA Rle	*Rhizobium leguminosarum*	B	C_4-dicarboxylate transporter	444	Q01857
YdbH Bsu	*Bacillus subtilis*	B	Putative C_4-dicarboxylate transporter	421	AB001488
DctA Mtu	*Mycobacterium tuberculosis*	B	Putative C_4-dicarboxylate transporter	491	Z81451
b1729 Ec	*Escherichia coli*	B	Unknown	463	AE000268
YB54 Hin	*Haemophilus influenzae*	B	Unknown	440	P45079
YhcL Bsu	*Bacillus subtilis*	B	Unknown	463	P54596
GltP Bbu	*Borrelia burgdorferi*	B	Unknown	463	AE001172
YgjU Ec	*Escherichia coli*	B	Serine transporter	414	P42602
YgjU Hin	*Haemophilus influenzae*	B	Putative serine transporter	414	P45246
GltP Aae	*Aquifex aeolicus*	B	Unknown	398	AE000735
GltP Ser	*Saccharopolyspora erythraea*	B	Unknown	449	S71005
GltP Cps	*Chlamydia psittaci*	B	Unknown	406	AF017105
GltP2 Ctr	*Chlamydia trachomatis*	B	Unknown	415	AE001296
GltP Tpa	*Treponema pallidum*	B	Unknown	396	AE001231
GltP2 Tpa	*Treponema pallidum*	B	Unknown	407	AE001262
GltP2 Bbu	*Borrelia burgdorferi*	B	Unknown	400	AE001145
GltP Ctr	*Chlamydia trachomatis*	B	Unknown	412	AE001313
GltP Pho	*Pyrococcus horikoshii*	A	Unknown	425	AB009510

[a] The BLAST search was performed at the BLAST Website (8a) using the Advance BLAST 2.0 mode. Only sequences from the databases that do not have obvious sequencing errors are shown; (splicing) variants of the same protein are not shown.

[b] A, *Archaea*; B, *Bacteria*; E, *Eucarya*.

[c] Rat EAAT1 and EAAT2 and rabbit EAAT3 are also named GLAST, GLT-1, and EAAC1, respectively. *E. coli* YgjU is also named SstT.

The serine transporter YgjU of *E. coli* is only distantly related to the other characterized members of the family (with at most 20% identical residues). It is likely that the related protein YgjU of *Haemophilus influenzae* (56% identity) is also a serine transporter. The bacterial serine transporters are found in a cluster of proteins that branches off from a point near the center of the tree, indicating that they have diverged from the other sequences early in evolution (64). The cluster contains a

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                                                                                   7
                                                                        ______________________
              399                                                                                           474
GltT Bst   253  IIKI-LKDELILAYSTASSETVLPKIMEKMEN-FGCPKAITSFVIPTGYSFNLDGST-LYQALAAIFIAQLYGI-DM 325
GltP Ec    263  LIRI-LKDELILAYSTASSESVLPRIIEKMEA-YGAPVSITSFVVPTGYSFNLDGST-LYQSIAAIFIAQLYGI-DL 335
GltP Bsu   247  VLKM-IWDLFLIAFSTTSTETILPQLMDRMEK-YGCPKRVVSFVVPSGLSLNCDGSS-LYLSVSCIFLAQAFQV-DM 319
DctA Ec    252  FIRY-IREELLIVLGTSSSESALPRMLDKMEK-LGCRKSVVGLVIPTGYSFNLDGTS-IYLTMAAVFIAQATNS-QM 324
DctA Rle   265  LLRY-IKEELLLVLGTSSSEAALPGLMNKMEK-AGCKRSVVGLVIPTGYSFNLDGTN-IYMTLAALFIAQATGI-HL 337
EAAT2 Rno  346  FFAG-IFQAWITALGTASSAGTLPVTFRCLEDNLGIDKRVTRFVLPVGATINMDGTA-LYEAVAAIFIAQMNGV-IL 419
EAAT Cel   310  FMRG-LFQAWITALGTASSSATLPITFNCLEENLGVDRRVTRFVLPVGATINMDGTA-LYEAVAAIFIAQINGV-HL 383
GLT Cel    297  FLKG-LGQAIMTALGTSSSAASLPVTFRCLNK-LGIDPRVTKFVLPVGAMVNMDGTA-LYEATASIFIAQMNGL-EL 369
EAAT1 Rno  348  FIGG-LLQALITALGTSSSSATLPITFKCLEENNGVDKRITRFVLPVGATINMDGTA-LYEALAAIFIAQVNNF-DL 421
EAAT4 Hs   373  FIGG-MLQALITAMGTSSSSATLPITFRCLEEGLGVDRRITRFVLPVGATVNMDGTA-LYEALAAIFIAQVNNY-EL 446
EAAT3 Ocu  316  FAMG-MTQALLTALMISSSSATLPVTFRCAEEKNRVDKRITRFVLPVGATINMDGTA-LYEAVAAVFIAQLNDM-DL 389
EAAT5 hs   327  FIRG-ILQALLIALATSSSSATLPITFKCLLENNHIDRRIARFVLPVGATINMDGTA-LYEAVAAIFIAQVNNY-EL 400
ASCT2 hs   336  FLWG-IVTPLATAFGTSSSSATLPLMMKCVEENNGVAKHISRFILPIGATVNMDGAA-LFQCVAAVFIAQLSQQ-SL 409
ASCT1 hs   328  FLLG-LLAPFATAFATCSSSATLPSMMKCIEENNGVDKRISRFILPIGATVNMDGAA-IFQCVAAVFIAQLNNV-EL 401
EAAT1 Trn  301  YIAK-MGQVMATAFGTASSSATMPITIGCCDD-MGLDPRITRFVIPIGATINMDGTA-LYEAVAAIFIAQLRKV-EM 373
YgjU Ec    249  LVLLCLRESGVYAFFTRSSAANIPVNMALCEK-LNLDRDTYSVSIPLGATINMAGAA-ITITVLTLAAVNTLGI-PV 322
YgjU Hin   254  LVWTCVRESGVTAFFTRSSAANIPVNIELAKR-LNLDEETYSVSIPLGANINMAGAA-ITITILTLAAVHTLGL-EV 327
B1729 Ec   292  YFRK-VWPVLTFAFTSRSSAASIPLNVEAQTRRLGVPESIASFAASFGATIGQNGCAGLYPAMLAVMVAPTVGINPL 366
YB54 Hin   286  YYKK-VLPTLSFAFTSRSSAATIPLNIETQTAKLGNNNVIANFAATFGATIGQNGCGGIYPAMLAVMVAPMVGIDPF 360
GltP Aae   246  YFIQ-VREALLLAFSTASSAATLPVSLELAIERGKVKKEVAGFVLPLGATINMDGTA-LYESVAAVYIANLYGI-DL 319
GltP Pho   261  FIKK-AKDAMLTAFVTRSSSGTLPVTMRVAKE-MGISEGIYSFTLPLGATINMDGTA-LYQGVCTFFIANALGS-HL 333
GltP Ctr   244  FLSS-MMDAISCAVSTASSSATLPVTMRCVSKNLGVSSEVSGFVLPLGATVNMNGTA-IFQGMAAVFIAQAYNC-PL 317
GltP Ser   268  FFAK-TWTVLQFAFVSRSSSATLPLSRQAAEN-LGVDRSYANFAVPLATTTKMDGCAAIYPAIGSMFIANLFGI-HL 341
GltP2 Ctr  251  TFKL-MSRPLATAFFSKSSAATLPLTMEVAEENLHIRPTISRFVFPLCSVINMNACA-AFILTTVLFVGVSNGI-VF 324
GltP Chp   248  IAKR-MSPALITAFFSKSSASTLPLTMELAEEELKIKPSLSRLAFPLCSVINMNGCA-AFILITVLFLGISNGI-SF 321
GltP Tpa   231  VLRH-YGAAYATALGTMSSAATLPVSLQCAHKSRALPAEIVDFAIPLGATTHLCGSV-LTETFFCLTIAQMLYGSMP 305
                               _____                              __________________    _________
                              Motif A                                  Motif B            Motif C

                   8          9                                               10
               __________  __________                                 ____________________
              475                                                                                             554
GltT Bst   326  PISQQISLLLVLMVTSKGIAGVPGVSFVVLLATLGTVG------IPIEGLAFIAGIDRILDMARTAVNVIGNSLAAIIMS 399
GltP Ec    336  SIWQEIILVLTLMVTSKGIAGVPGVSFVVLLATLGSVG------IPLEGLAFIAGVDRILDMARTALNVVGNALAVLVIA 409
GltP Bsu   320  TLSQQLLMMLVLVMTSKGIAAVPSGSLVVLLATANAVG------LPAEGVAIIAGVDRVMDMARTGVNVPGHAIACIVVS 393
DctA Ec    325  DIVHQITLLIVLLLSSKGAAGVTGSGFIVLAATLSAVGH-----LPVAGLALILGIDRFMSEARALTNLVGNGVATIVVA 399
DctA Rle   338  SWGDQILLLLVAMLSSKGAAGITGAGFITLAATLSVVPS-----VPVAGMALILGIDRFMSECRALTNLVGNAVATIVVA 412
EAAT2 Rno  420  DGGQIVTVSLTATLASIGAASIPSAGLVTMLLILTAVG------LPTEDISLLVAVDWLLDRMRTSVNVVGDSFGAGIVY 493
EAAT Cel   384  SFGQVVTVSLTATLASIGAASVPSAGLVTMLLVLTAVG------LPVKDVSLIVAVDWLLDRIRTSINVLGDAMGAGIVY 457
GLT Cel    370  SIGQIVTVSITATLASIGAASIPSAGLVTMLIVLTALG------LPANDISLILAVDWFLDRLRTSVNVIGDALGCGFVH 443
EAAT1 Rno  422  NFGQIITISITATAASIGAAGIPQAGLVTMVIVLTSVG------LPTDDITLIIAVDWFLDRLRTTTNVLGDSLGAGIVE 495
EAAT4 Hs   447  NLGQITTISITATAASVGAAGIPQAGLVTMVIVLTSVG------LPTEDITLIIAVDWFLDRLRTMTNVLGDSIGAAVIE 520
EAAT3 Ocu  390  SIGQIITISVTATAASIGAAGVPQAGLVTMVIVLSAVG------LPAEDVTLIIAVDWLLDRFRTVVNVLGDAFGTGIVE 463
EAAT5 Hs   401  DFGQIITISITGTAASIGAAGIPQAGLVTMVIVLTSVG------LPTDDITLIIGVDWALDRFRTMINVLGDALAAGIMA 474
ASCT2 Hs   410  DFVKIITILVTATASSVGAAGIPAGGVLTLAIILEAVN------LPVDHISLILAVDWLVDRSCTVLNVEGDALGAGLLQ 483
ASCT1 Hs   402  NAGQIFTILVTATASSVGAAGVPAGGVLTIAIILEAIG------LPTHDLPLILAVDWIVDRTTTVVNVEGDALGAGILH 475
EAAT1 Trn  374  SFGKIIAVSVTATAASIGAAGIPQAGLVTMVMVLDTVN------LPAEDVSLILAVDWLLDRFRTTINVVCDALGAIIVT 447
YgjU Ec    323  DLPTALLLSVVASLCACGASGVAGGSLLLIPLACNMFGI--SNDIAMQVVAVGFIIGVLQDSCETALNSSTDVLFTAAAC 400
YgjU Hin   328  SFVSALLLSIVAALCACGASGVAGGSLLLIPLACSLFGI--SDDVAAQMIGVGFIIGILQDSTETALNSSTDVLFTAAVC 405
B1729 Ec   367  DPMWIATLVGIVTVSSAGVAGVGGGATFAALIVLPAMG------LPVTLVALLISVEPLIDMGRTALNVSGSMTAGTLTS 441
YB54 Hin   361  SFSYILTLIFVVAISSFGIAGVGGGATFAAIVVLSTLG------LPLELIGLLISIEPIIDMGRTALNVNGAMVAGTITD 435
GltP Aae   320  SISQMVTIFLTATLASIGAAAIPGAGLVLLTLVLSSVG------IPLEGIGLIIAVDRFLDMLRTAVNVWGDLNGAKILN 393
GltP Pho   334  TVGQQLTIVLTAVLASIGTAGVPGAGAIMLAMVLESVGLPLTDPNVAAAYAMILGIDAILDMGRTMVNVTGDLTGTAIVA 413
GltP Ctr   318  SLTSLLLLVVTATFSAVGSAGVPGGGMITLGSVLTSVG------LPIQGIAVLAGIDRLRDIIGTPMNILGDAVVALYIA 391
GltP Ser   342  GPAQYLTIIAVAVFGAIATAGVT-GWFTMLTLTVGALGFP--PEVVATGIAIAYAVDPILDMMRTATNVAGQIVVPTVVA 418
GltP2 Ctr  325  SPLSLISWVFIATLAAVGNAGVPMGCYFLTSSLLASMN------VPLCLLGLILPAYALLDMLETLINVWSDCCVVSVIN 398
GltP Chp   322  SLFSMIGWVFIATLCAVGNAGVPMGCFFLASSLITSMH------IPLHLLGLILPFYTILDMIETSLNVWSDCCVVSLTD 395
GltP Tpa   306  SLADMVLFSCLFGIFAVGAPGVPGGTVLASLGLVLDVL-----HFDTTGTGLLIAIFALQDSFGTACNITGDGALALMLR 380
                                                                                ____________________
                                                                                      Motif D
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FIG. 1. Multiple-sequence alignment of a stretch of approximately 150 residues near the C terminus of the transporters. The alignment was made with the program CLUSTALX (84). A representative set of 26 members of the glutamate transporter family is shown. Bold numbers refer to the positions in the multiple-sequence alignment and correspond to the numbers in Fig. 4. Other numbers refer to the residue numbers of the individual sequences. Bars below and above the sequences indicate the positions of the conserved motifs (motifs A to D, highlighted) and the positions of the transmembrane segments (as published by Grunewald et al. [32]), respectively. Abbreviated transporter names are taken from Table 2.

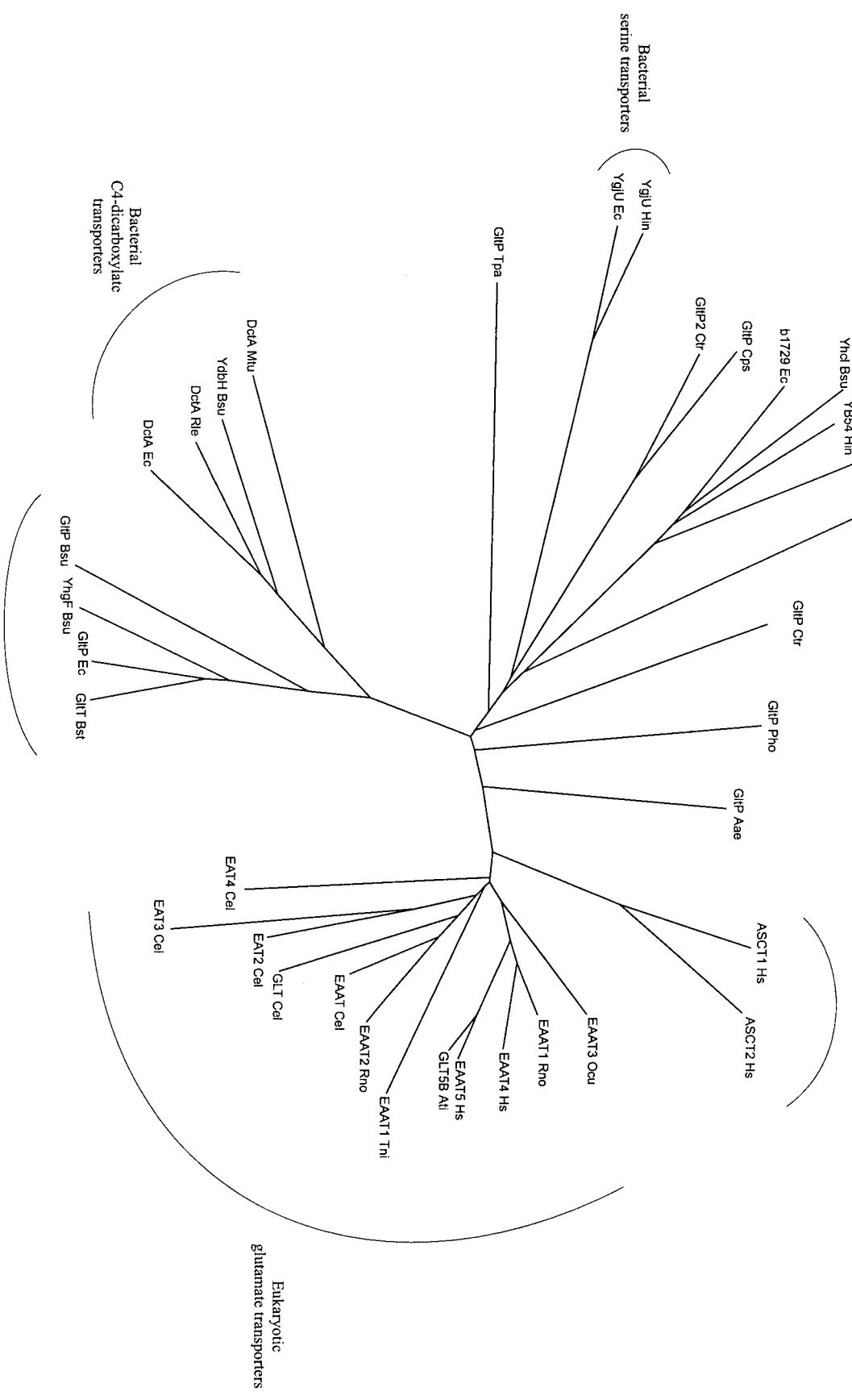

FIG. 2. Phylogenetic tree of 35 members of the glutamate transporter family. The set of 35 members does not contain pairs of sequences with more than 70% identical residues. The tree is based on the part of the multiple-sequence alignment shown in Fig. 1. It was drawn with the DRAWTREE program from the PHYLIP package (27). Abbreviated transporter names are taken from Table 2.

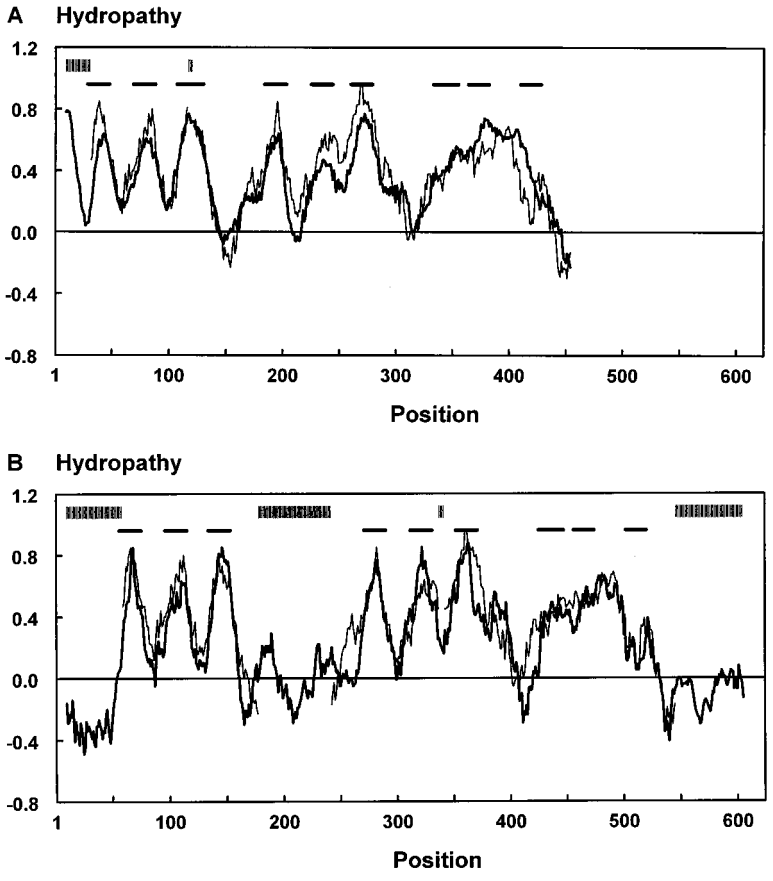

FIG. 3. Alignment of the average hydropathy profiles of the subfamily of bacterial glutamate transporters (thin line) and the subfamily consisting of b1729 of *E. coli*, YB54 of *H. influenzae*, GltP of *B. burgdorferi*, and YhcL of *B. subtilis* (thick line) (A) and the subfamilies of bacterial and eukaryotic glutamate transporters (thin and thick lines, respectively) (B). The profiles were aligned as specified by Lolkema and Slotboom (53) with a window of 19 amino acids. Vertical and horizontal bars above the profiles indicate the positions of gaps in the sequences and the positions of the transmembrane segments (32), respectively. The profiles are almost superimposable even though the sequences have considerably diverged. The bacterial glutamate transporters and the subfamily containing YB54 of *H. influenzae* have 18 to 24% identical residues (A), whereas the subfamilies of bacterial and eukaryotic glutamate transporters have 22 to 29% identical residues (B). The subfamily containing YB54 of *H. influenzae* has an extra hydrophobic segment at the N terminus.

large number of uncharacterized proteins that are only distantly related to the characterized members of the glutamate transporter family. The uncharacterized proteins are found not only in organisms like the chlamydiae (107), which are not closely related to the organisms in the clusters with characterized proteins, but also in organisms like *E. coli* and *B. subtilis*, which have well-characterized members. Therefore, it is likely that at least some of the uncharacterized members of the family are proteins with different substrate specificity from the transporters that have been characterized so far. Among the uncharacterized members, several subfamilies can be distinguished that are likely to consist of proteins with similar, currently unknown substrate specificity. Hence, b1729 of *E. coli*, YB54 of *H. influenzae*, GltP of *Borrelia burgdorferi*, and YhcL of *B. subtilis* probably are orthologues with the same function. The same applies to GltP2 of *Chlamydia trachomatis* and GltP of *Chlamydia psittaci*. Two other proteins, GltP2 of *Treponema pallidum* and GltP2 of *B. burgdorferi*, are not included in the phylogenetic tree (Fig. 2) because they have diverged so far that it is difficult to unambiguously align them (they have at most 19 and 17% of residues identical to those of other members of the family, respectively). Nevertheless, they are likely to belong to the family because of the (partial) presence of conserved motifs and characteristic features of the hydropathy profiles (see below).

Finally, two sequences occupy separate positions in the tree. The glutamate transporter homologue GltP Aae from the hyperthermophilic bacterium *Aquifex aeolicus* is more closely related to the eukaryotic transporters (32 to 39% identical residues) than to the bacterial members (22 to 32% identical residues). The separate position of GltP of *A. aeolicus* in the tree reflects the unique evolutionary position of this organism (16). The only archaeal sequence in the tree (GltP Pho) is equally closely related to the bacterial and eukaryotic sequences (approximately 30% of identical residues) and branches from a point near the center of the tree, reflecting the distinct phylogenetic position of the archaea (108).

STRUCTURAL ANALYSIS

Hydropathy Profile Analysis

Although the amino acid sequences of the members of the glutamate transporter family have diverged considerably, their hydropathy profiles are remarkably similar (40, 53, 77) (Fig. 3). It is a general property of families of integral membrane proteins that the hydropathy profiles of the members are better

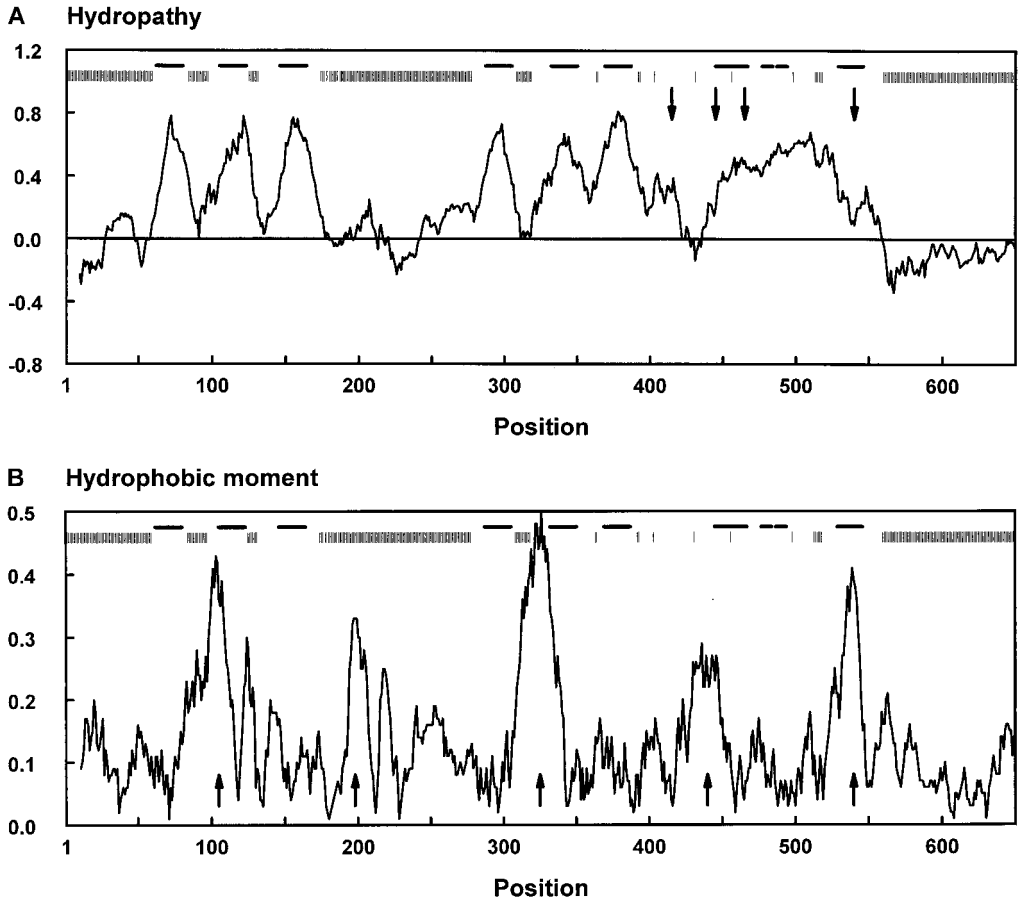

FIG. 4. Average profiles of hydropathy (A) and hydrophobic moment (B) of the glutamate transporter family. The set of 35 members (Fig. 2) which does not contain pairs of sequences with more than 70% identical residues was used. Vertical and horizontal bars above the profiles indicate the positions of gaps in the sequences and the positions of the transmembrane segments (32), respectively. Position numbers refer to positions in the multiple-sequence alignment and correspond to the bold numbers in Fig. 1. In panel A, a window of 19 residues was used and the arrows point to the positions of the conserved motifs A to D. In panel B, a window of 21 residues and a period of 3.6 residues, appropriate for α-helical structures, was used and the arrows point to the five conserved putative amphipatic helices (AH1 to AH5 from left to right).

conserved than the primary structures. The conservation of hydropathy profiles is a reflection of the conservation of the global structure of the members of a family (53, 54). Quantitative comparison of the hydropathy profiles of different families of secondary transporters has recently been used to group the transporters into four major structural classes. It was shown that the hydropathy profile of the glutamate transporter family is likely to reflect a global structure that is unique among secondary transporters (53).

The average hydropathy profile derived from the multiple sequence alignment of a subset of 35 members of the family (Fig. 4A) emphasizes the conserved characteristics of the profiles, which include six alternating hydrophobic and hydrophilic regions in the N-terminal half and a large hydrophobic region in the C-terminal half. The subset of 35 members does not contain any pairs of sequences with more than 70% identical residues, so that the average profile is not biased toward the profile of a particular subfamily. The eukaryotic and prokaryotic members differ predominantly in the length of their hydrophilic regions. Three hydrophilic stretches are considerably longer in the eukaryotic proteins: the N-terminal and C-terminal extensions and the region between the third and fourth hydrophobic peaks, which is glycosylated in the eukaryotic members (13). As is commonly observed for families of integral membrane proteins, the gaps in the multiple-sequence alignment are found almost exclusively in the hydrophilic stretches (Fig. 4A).

Membrane Topology

The six hydrophobic segments in the N-terminal part of the glutamate transporters were predicted to be transmembrane helices in several reports (38, 67, 81, 88). Experimentally, the membrane topology of rat EAAT1 (GLAST) was determined by monitoring the glycosylation state of C-terminally truncated transporters fused to a reporter peptide with glycosylation sites (103) and the topology of rat EAAT2 (GLT-1) was assessed by labeling of single-cysteine mutants (32). Both studies confirmed the presence of six membrane-spanning segments in the N-terminal half of the transporters. Also, the results of trypsin digestion experiments on rat EAAT2 in membrane vesicles are consistent with the presence of six N-terminal membrane helices. These studies showed that the N terminus of the protein is located at the cytoplasmic side of the membrane whereas the large hydrophilic region between the third and fourth hydrophobic segments is extracellular (33), which is in agreement with the observed glycosylation of this loop in the eukaryotic transporters (13).

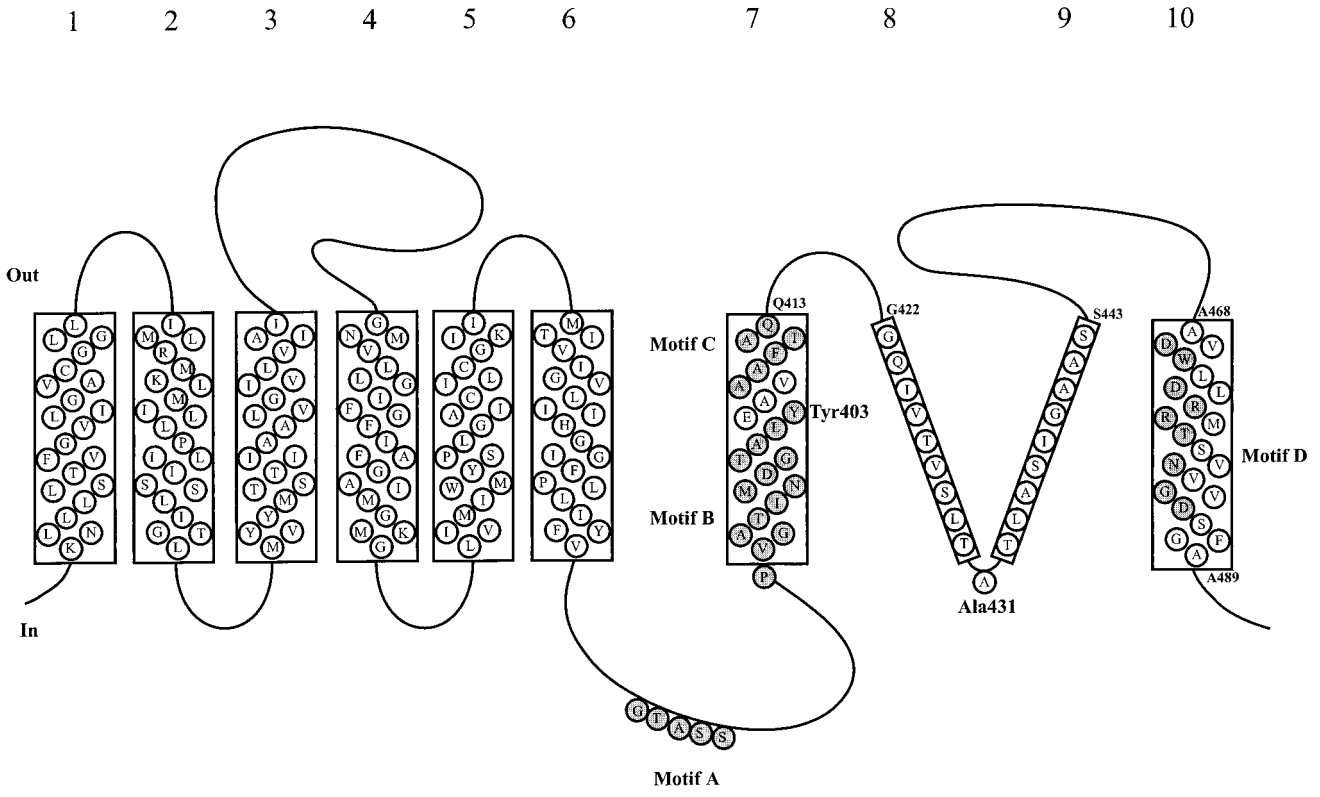

FIG. 5. Model for the membrane topology of the C-terminal part of the glutamate transporters comprising membrane-spanning segments 7 to 10 (based on the model of Grunewald et al. [32]). The sequence of rat EAAT2 (GLT-1) is shown. Membrane segments 7 and 10 are likely to be helices and were also proposed in the model of Slotboom et al. (77). Membrane segments 8 and 9 form a reentrant loop or loop-pore structure according to Grunewald et al. (32). Conserved motifs B and C and the conserved hydrophilic face of helix 10 (motif D) are shaded. Tyrosine 403, which is involved in potassium binding and is accessible from either side of the membrane depending on the presence of substrates, is located in the middle of helix 7.

The C-terminal half of the proteins does not contain clear alternating regions of high and low hydrophobicity. Therefore, it is difficult to predict the position of transmembrane segments from the hydropathy profile. This has led to the proposal of several different models for the membrane topology of the proteins (35, 38, 67, 81, 88). The topologies of the C-terminal halves of rat EAAT1 and EAAT2, *B. stearothermophilus* GltT, and a small part of human EAAT1 were experimentally assessed (32, 74, 77, 103). The four studies gave conflicting results. In the bacterial transporter, four putative membrane-spanning helices were found (helices 7 to 10) when alkaline phosphatase was used as the reporter protein. Accessibility scanning of single-cysteine mutants of rat EAAT2 revealed two membrane-spanning helices at the same positions as helices 7 and 10 in the bacterial system. In addition, two short membrane-spanning segments of approximately 10 residues each were found in the region where bacterial helices 8 and 9 were proposed (Fig. 5). The two short membrane-spanning segments may form a reentrant loop or a loop-pore structure reminiscent of those found in ion channels (19, 55, 56). In the third study, on rat EAAT1, four membrane-spanning β-strands were proposed at significantly different positions from the membrane-spanning segments in the other studies. This model is not consistent with results of trypsin digestion studies on membrane vesicles containing rat EAAT2 (GLT-1). These experiments showed that the C terminus of the protein and the hydrophilic region following transmembrane segment 6 are located at the cytoplasmic side of the membrane (32, 33). The other two models are both consistent with these results and display several similar features, most importantly the presence of two membrane-spanning segments (numbered 7 and 10) that may form an important part of the substrate recognition site and translocation pore (see below). The only difference between the models is the position and length of membrane-spanning segments 8 and 9. At present, we tentatively favor the model with two helices and the loop-pore structure for the C-terminal part of the proteins, since it is based on the study of full-length and active carriers. Truncated transporters fused to reporter proteins may fold in a nonnative conformation (89). This may be especially the case when the protein does not consist of a conventional bundle of membrane-spanning α-helices.

A small part of the topology of human EAAT1 was also determined by accessibility scanning of single-cysteine mutants (74). The examined region includes the putative membrane-spanning segment 7 and its flanking sequences. It was suggested that rather than being a membrane-spanning helix, this region forms another reentrant loop with both ends positioned at the extracellular side of the membrane. This implies that an extra membrane-spanning segment must be present between the cytoplasmic end of helix 6 and the extracellular beginning of this putative reentrant loop. At present it is not possible to explain the discrepancies between the models for the membrane topology of rat EAAT2 and human EAAT1, since both studies are based on a similar experimental approach, the analysis of active single-cysteine versions of the transporters. Again we tentatively favor the model proposed by Grunewald et al. (32). Only their model is consistent with the trypsin

TABLE 3. Sequence motifs in the glutamate transporter family

Motif	Start position[a]	Sequence[b]	End position[a]
A	414	(ST)(STARK)S(ST)	417
B	443	PxGx(TS)xN(ML)DGxx(LI)(FY)	457
C	461	Ax(IVL)F(LI)AQ	467
D (eukaryotic glutamate transporters)	531	DWxLDRxRTxxNVxGD	546
D (eukaryotic neutral-amino-acid transporters)	531	DWxVDRxxTxxNVEGD	546
D (bacterial glutamate transporters)	531	DRxxDMARTxxNxxG(NH)	546
D (bacterial C_4-dicarboxylate transporters)	531	DRFMSExRxxxNxxGN	546
D (bacterial serine transporters)	531	GxLQDSxETALNSSTD	546

[a] Numbers refer to positions in the multiple-sequence alignment of Fig. 1.
[b] When two or more residues are indicated in parentheses, either of the them is found; x refers to nonconserved residues.

digestion studies, discussed above, that demonstrated the intracellular location of the region between putative helices 6 and 7. Clearly, the topology of the C-terminal part of the transporters has to be further examined, not only because of the conflicts between the different models but also since loop-pore structures which are found in several ion channels (19, 55, 56) have not been found in secondary transporters before. A model for the membrane topology of the C-terminal half of the transporters is shown in Fig. 5. The positions of the membrane-spanning segments are indicated in Fig. 1 and 4a.

Periodicity Properties

The members of the glutamate transporter family were examined for patterns of residue hydrophobicity and residue conservation (or substitution) in their amino acid sequences (21, 77). Figure 4B shows the average periodicity profile of amino acid hydrophobicity of the family (amphipathy profile) with a period of 3.6 residues, appropriate for α-helical structures. The glutamate transporter family contains five regions with conserved hydrophobic moments that could form amphipathic α-helices (AH1 to AH5, Fig. 4B). AH1 to AH4 are found in loop regions connecting the putative transmembrane helices and may therefore form membrane surface helices with the axis parallel to the plane of the membrane and the hydrophilic residues exposed to the aqueous phase. AH2 and AH4 are found exclusively in the eukaryotic transporters. The hydrophobic moment of AH5 is conserved in all members of the family and has a particularly large value of 0.45 to 0.6/residue in the glutamate and C_4-dicarboxylate transporters, which is larger than that of the peptide melittin, a considerably amphipatic peptide with a known α-helical structure (20). The hydrophobic moment is somewhat smaller in the mammalian neutral-amino-acid transporters and the bacterial serine transporters (0.3 to 0.4/residue). The putative amphipathic α-helix AH5 coincides with transmembrane segment 10 and thus provides a hydrophilic path through the membrane. It was suggested that this amphipathic membrane-spanning helix could provide part of the translocation pore (77). This suggestion recently gained experimental support in studies on chimeric transporters (see below).

Within transmembrane helices, the pattern of residue conservation (or substitution) can be used to discriminate between buried and lipid-exposed residues (18). The amphipathic membrane-spanning helix 10 has an exceptionally large substitution moment with α-helical periodicity (0.09/residue) (77). The hydrophobic face of the helix is less well conserved than the hydrophilic face, and it is likely that the membrane-spanning helix has a lipid-exposed hydrophobic face and a protein-buried, well-conserved hydrophilic face. The other putative membrane-spanning segments have considerably smaller substitution moments, but the moments of helices 1 and 3 (0.06 and 0.05/residue, respectively) may be significant.

Sequence Motifs

The glutamate transporter family does not contain residues that are conserved in all members. This is due to the inclusion of several distantly related sequences in the family that have emerged from recent genome-sequencing projects. For the same reason, most of the previously described "signature sequences" for the glutamate transporter family are not found in all the members listed in Table 2 (70). Only one sequence motif is conserved in all members of the family. This is a serine- and threonine-rich stretch in the hydrophilic region of the proteins following membrane-spanning helix 6 (motif A, Table 3 and Fig. 1 and 4A). It is located intracellularly in the topology model proposed by Grunewald et al. (32). Serine clusters were found in the ligand binding sites of the metabotropic glutamate receptors and in G-protein-coupled acetylcholine and biogenic amine receptors (62, 105). It was suggested that the stretch in the glutamate transporters may have a similar function (40), though there is as yet no conclusive experimental indication of such a function (59).

A second motif that was suggested to be involved in substrate binding (67) is AX(I/V/L)F(L/I)AQ (motif C, Table 3 and Fig. 1), which is located in the putative membrane-spanning helix 7 (32, 77, 109). The motif is conserved in the glutamate, neutral-amino-acid, and C_4-dicarboxylate carriers but not in most of the uncharacterized bacterial proteins. It may therefore be involved in binding of a carboxylate group of the substrate, which is the only functional group common to all substrates of the carriers that contain the motif. The motif is part of a stretch of 76 residues that is involved in the binding of dihydrokainate, a glutamate analogue that competitively inhibits glutamate transport (95) (see below). This observation is consistent with the hypothesis that the motif is involved in substrate binding.

The motif PXGX(T/S)XN(M/L)DGXX(L/I)(F/Y), located at the cytoplasmic interface of membrane helix 7, is present in all of the functionally characterized and most of the putative transporters (motif B, Table 3 and Fig. 1). This motif has been subjected to extensive mutagenesis studies and is involved in cation binding (see below). The stretch that forms the amphipathic membrane helix 10 (motif D) is conserved in most members of the family, but its exact amino acid composition varies along with the substrate specificity of the transporters (Table 3). The substrate-specific differences in this stretch are consistent with the hypothesis that the amphipathic membrane-spanning helix 10 may be part of the translocation pore (59, 77). Finally, the human glutamate transporter EAAT5 contains a C-terminal consensus motif for interaction with

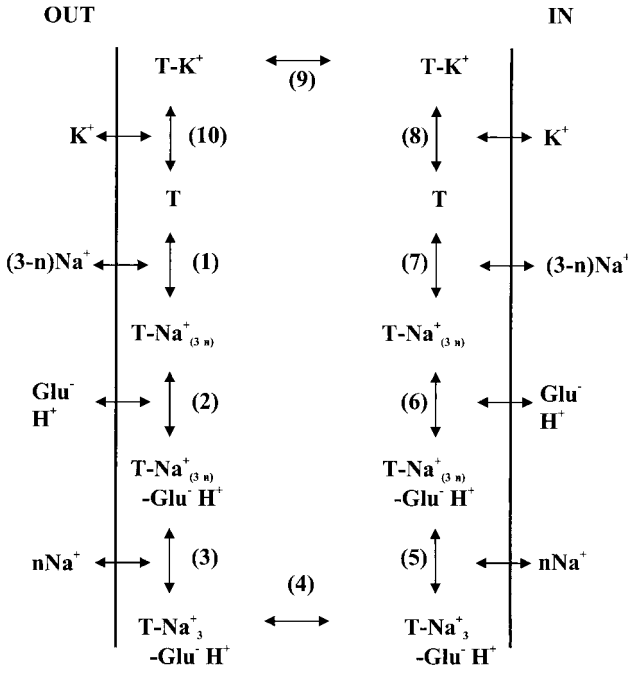

FIG. 6. Schematic representation of the transport cycle of the eukaryotic glutamate transporters (44). T, transporter; glu^-, glutamate; n, the number of sodium ions that bind after glutamate binding. A description is given in the text.

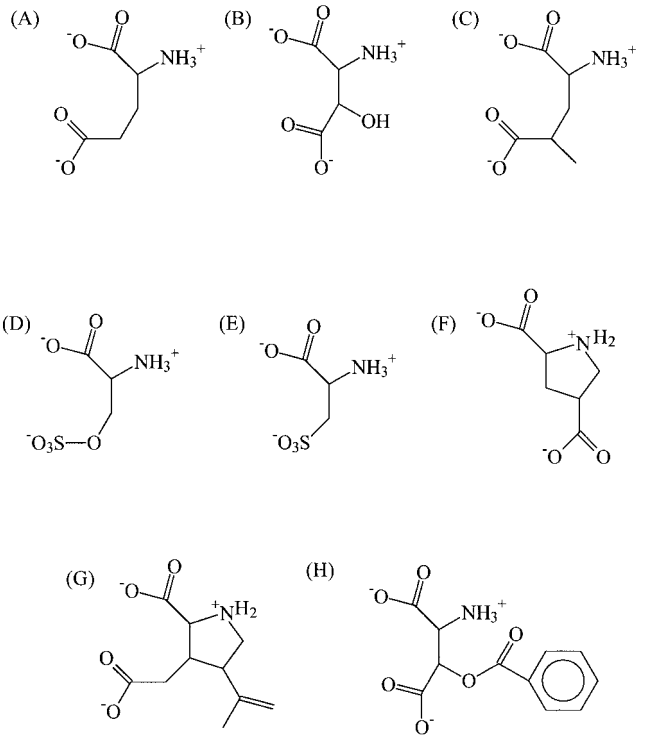

FIG. 7. Structures of some transported substrates (A, B, D, E, and F) and competitive inhibitors (G and H) of the glutamate transporters. 4-Methylglutamate (C) is a substrate of some transporters but a competitive inhibitor of others. (A) Glutamate; (B) β-hydroxyaspartate; (C) 4-methylglutamate; (D) serine-*O*-sulfate; (E) cysteate; (F) pyrrodiline-2,4-dicarboxylate; (G) kainate; (H) β-benzyloxyaspartate.

synaptic proteins that promote ion channel clustering (3). EAAT5 is one of the glutamate transporters that shows large substrate-dependent chloride currents (see above), and clustering with components of the signal transduction pathway may point to the function of the chloride currents.

FUNCTIONAL ANALYSIS

Catalytic Cycle

Extensive characterization of the eukaryotic glutamate carriers EAAT1 to EAAT3 has resulted in the model for the transport cycle that is shown in Fig. 6 (44). The transporters catalyze the uptake, efflux, and exchange of glutamate. In uptake and efflux, the complete cycle is followed in the forward and reverse directions, respectively, whereas during exchange, steps 1 to 7 (Fig. 6) are followed alternately in the forward and reverse directions. K^+ is required only to reorient the empty carrier (steps 8 to 10). Exchange is not dependent on potassium ions, since reorientation of the empty carrier is omitted. The bacterial glutamate transporters also catalyze uptake, efflux and exchange, but none of the transport modes is believed to require potassium ions (87); the empty carrier reorients spontaneously.

The binding order of Na^+, H^+, and glutamate (steps 1 to 3) is a matter of debate. Kanner and coworkers (42, 68) showed that exchange catalyzed by rat EAAT2 does not require external Na^+ at saturating glutamate concentrations whereas it does at nonsaturating glutamate concentrations. They concluded that all cotransported sodium ions bind to the transporter prior to glutamate binding. In contrast, Kanai et al. (39) found that binding of sodium ions is modulated by glutamate, and in their model one Na^+ ion binds after the binding of glutamate (see also references 17 and 47). The observations indicate that the glutamate and Na^+ binding sites intimately interact, which is in agreement with the results of recent mutagenesis studies (69, 114) (see below). The binding order may depend on the conditions used. The catalytic cycle of the neutral-amino-acid transporters may be similar to that of the exchange mode of the glutamate transporters. The human neutral-amino-acid transporter ASCT1 catalyzes electroneutral exchange of amino acids that is dependent on Na^+ but not on K^+, just like exchange catalyzed by the glutamate transporters (111). One difference is the Na^+ stoichiometry, since only one sodium ion was found to interact with the neutral-amino-acid transporters (93, 111).

Substrate Binding Site

Several inhibitors of the glutamate transporters have been found, some of which have different specificities for different members of the family (4, 49, 96, 98). Noncompetitive inhibitors include oxidizing agents like $ONOO^-$, which covalently interact with glutamate transporters and specifically inhibit the V_{max} of transport (90, 91, 99, 100), and Zn^{2+}, which is a partial inhibitor of the human and salamander glutamate transporters EAAT1 (80, 97). Arachidonic acid inhibits some glutamate transporter subtypes but stimulates others (92, 110).

Most of the competitive inhibitors of the glutamate transporters were found to be substrates of the proteins that are transported with a high affinity ($K_m < 100$ μM), e.g., D-aspartate, cysteate, and *threo*-β-hydroxyaspartate (Fig. 7) (38, 67, 81). Only a few competitive inhibitors that are not transported have been identified, such as *threo*-β-benzyloxyaspartate and kainate, a specific inhibitor of the EAAT2 subtypes (Fig. 7) (9, 49, 76, 96). The conformations of glutamate and aspartate in the substrate binding site have been modeled by comparing the

structures of transported glutamate analogues and competitive blockers of the transporters (9, 10, 14, 49, 96). It was originally proposed that glutamate binds to the transporters in a folded form and aspartate binds in the extended form. In these conformations, the functional groups of the transported substrates (one amino group and two carboxylate groups) can be superimposed. However, more recent data from the analysis of conformationally constrained glutamate analogues showed that compounds that mimic the folded conformation as well as compounds that mimic a stretched conformation of glutamate bind to glutamate transporters but that only the "stretched" compounds can be transported (25).

The nature and diastereomeric properties of the substituents at the β-carbon (C-3 position) of aspartate determine whether aspartate analogues are transported substrates or competitive blockers (49, 96). Thus, derivatives of aspartate with small groups at the β-carbon, such as β-hydroxyaspartate (Fig. 7B) and the related compounds β-acetoxyaspartate and β-propionyloxyaspartate, are transported by the bovine glutamate transporters EAAT1 and EAAT2 with high affinity (K_m 10 to 100 μM). Derivatives with bulkier groups at the β-carbon, such as β-benzyloxyaspartate (Fig. 7H), are competitive blockers (K_i < 20 μM) (49, 76).

The transporters are less tolerant in accepting derivatives of glutamate as substrates. (2*S*,4*S*)-4-Methylglutamate (Fig. 7C) is not recognized by human EAAT1 and EAAT2, whereas its stereoisomer (2*S*,4*R*)-4-methylglutamate is a substrate for EAAT1 (K_m = 54 μM) but a competitive blocker of EAAT2 (K_i = 3.4 μM). Both stereoisomers of 4-hydroxyglutamate are transported by the human glutamate transporters EAAT1 and EAAT2, but the affinity for *erythro*-4-hydroxyglutamate is very low (K_m > 1 mM) (96). Substitutions at the C-3 position are even less well tolerated. None of the stereoisomers of 3-methylglutamate is recognized by EAAT1, while only the stereoisomer *threo*-3-methylglutamate is bound, but not transported, by EAAT2 (K_i = 2.3 μM).

In the human glutamate transporter EAAT3, the stoichiometry of proton-substrate symport was found to depend on the charge of the transported substrate (112). Thus, transport of compounds which are anionic at physiological pH, like glutamate (pK_a = 4.5) and cysteate (pK_a = 1.5) (Fig. 7E), is associated with the symport of one proton. However, transport of cysteine, a low-affinity substrate for EAAT3 that is neutral at physiological pH (pK_a = 8.3), is transported without a proton. Based on these observations, it was suggested that the anionic substrates are transported in the protonated state, i.e., as glutamic acid or cysteic acid (112). This mechanism of proton transport is feasible if the pK_a of the anionic substrates is raised sufficiently in the substrate binding site of the transporter. This can be achieved by destabilizing the negative charge on the substrate in an apolar or negatively charged surrounding. However, this mechanism may be improbable since some substrates, like serine-*O*-sulfate (pK_a < 0) (Fig. 7D), are so acidic that protonation is not likely to occur (98). Alternatively, the observed substrate-dependent proton stoichiometry could be explained if the formation of a hydrogen bridge between the substrate and a residue in the substrate binding site were essential (Fig. 8). Then, transport of anionic substrates requires a proton to bind in the substrate binding site to form the hydrogen bridge whereas uncharged substrates, like cysteine and glutamic acid, provide the proton for the hydrogen bridge themselves. This mechanism implies that the substrate binding site can exist in a protonated state and a unprotonated state, which can be accounted for by, for instance, the presence of a histidine residue, although other residues can also be envisaged (Fig. 8). The symported proton

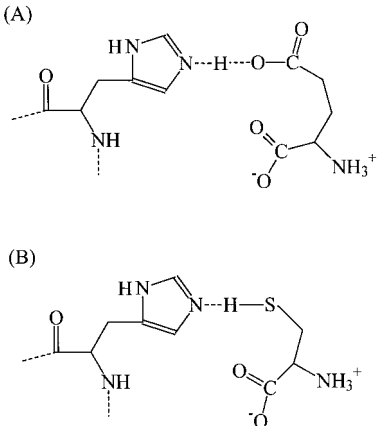

FIG. 8. Model for hydrogen bridge formation between a residue in the substrate binding site of the glutamate transporters (histidine is used as an example) and the substrates glutamate (A) and cysteine (B). Hydrogen bridge formation can explain the observed substrate-dependent proton stoichiometry (112) (see the text).

is shared by the substrate and the proton-accepting residue in the substrate binding site.

Only at low pH are glutamate and cysteate also transported by the neutral-amino-acid transporters ASCT2 from mice, rabbits, and humans (46, 82, 93). The pH dependence of transport of the anionic compounds is caused by protonation of a residue on the transporter, possibly a histidine (82). Glutamate may therefore be transported by ASCT2 in a similar way to that by the glutamate transporters (93). However, unlike the glutamate transporters that may have a protonated substrate binding site at physiological pH, the neutral-amino-acid transporters are optimized for transport of neutral compounds and are normally not protonated. Nevertheless, since glutamate is a substrate for both the glutamate transporters and the neutral-amino-acid transporters, the substrate binding pockets of the transporters may have a similar spatial structure (93). A further indication for this suggestion is the observation that cysteine, a common substrate for the neutral-amino-acid transporters, is transported with low affinity by the human glutamate transporter EAAT3 (K_m = 190 μM) (113).

Differences in inhibitor specificity as well as differences in Cl^- permeability between the human glutamate transporters EAAT1 and EAAT2 have been used to identify domains that may be part of the substrate binding site and translocation pore. Chimeras were constructed, and it was shown that a stretch of 76 residues comprising the conserved motifs B and C and membrane-spanning segment 7 (Fig. 5) is involved in the binding of dihydrokainate, a glutamate analogue and nontransported competitive inhibitor of EAAT2 (95). The region comprising membrane-spanning segment 10, including motif D, is also involved in inhibitor recognition and accounts for differences in the chloride permeability as well (59). Hence, membrane-spanning segments 7 and 10 may form an important part of the substrate recognition site and translocation pore. Kanai et al. constructed chimeras between mouse EAAT2 and EAAT3 and showed that a region of 38 residues comprising conserved motif A (Fig. 5) is also involved in the recognition of dihydrokainate (41). However, dihydrokainate binds to an external site of the transporters (102), and in the topology model proposed by Grunewald et al. (32) this region is located intracellularly (Fig. 5). Therefore, it is unlikely that the region is directly involved in dihydrokainate binding, unless the topology model of the region is not correct (59).

TABLE 4. Glutamate transporter mutants[a]

Mutated residue(s)[b]	Protein	Characteristic	Reference
H326(RKTN)	EAAT2 rno	Not functional	115
N396C	EAAT2 rno	Not functional	109
M397C	EAAT2 rno	Not functional	109
D398(CEGN)	EAAT2 rno	Not functional	69
G399C	EAAT2 rno	Not functional	109
T400(CSNA)	EAAT2 rno	Not functional	114
Y403(FWC)	EAAT2 rno	Cation binding affected	114
E404(CDGN)	EAAT2 rno	Cation binding affected	44
D470(EGN)	EAAT2 rno	Not functional	69
H146	EAAT1 hs	Zn^{2+} binding	97
H156	EAAT1 hs	Zn^{2+} binding	97
G394C	EAAT1 hs	Not functional	74
A395C	EAAT1 hs	Glutamate binding	74
N398C	EAAT1 hs	Not functional	74
M399C	EAAT1 hs	Not functional	74
D400C	EAAT1 hs	Not functional	74
G401C	EAAT1 hs	Targeting failure	74
T402C	EAAT1 hs	Not functional	74
F412C	EAAT1 hs	Targeting failure	74
I413C	EAAT1 hs	Targeting failure	74
Q415C	EAAT1 hs	Targeting failure	74
Y127F	EAAT1 rno	Not functional	11
N206T	EAAT1 rno	Glycosylation mutant	13
N216T	EAAT1 rno	Glycosylation mutant	13
L325H	EAAT1 rno	Targeting failure	11
E389F L390V[c]	EAAT1 rno	Not functional	103
Y405F	EAAT1 rno	Like Y403C of EAAT2 rno	109
R479T	EAAT1 rno	Not functional	12

[a] Only mutants that are significantly different from the wild-type proteins are listed.
[b] If a residue was replaced with multiple other amino acids, they are all indicated in parentheses.
[c] Only the activity of the double mutant was tested.

Cation Binding Sites

Differences in the amino acid sequences of members of the glutamate transporter family have been used as a guide to find residues of the substrate binding site or translocation pore (12, 97, 115). A list of single and multiple amino acid substitutions that have been made in members of the glutamate transporters family is shown in Table 4. Mutations that completely abolish transport may be useful to delineate functionally or structurally important regions in the proteins. Thus, it was shown that two of the conserved hydrophilic residues in helical membrane-spanning segment 10 (Asp-470 in rat EAAT2 [69] and Arg-479 in rat EAAT1 [12]) are indispensable for transport activity. This is consistent with the suggestion that the hydrophilic face of segment 10 is part of the translocation pore (77). The exact function of residues that are indispensable for transport function is, however, difficult to assess.

The most informative mutants are those that alter a specific property of the transporters. Extensive mutagenesis studies of conserved motif B at the cytoplasmic end of membrane-spanning helix 7 (Fig. 5) in rat EAAT2 have shown that two residues (Y403 and E404) are involved in the binding of potassium ions (44, 114). Transporters in which these residues are conservatively mutated are unable to complete the transport cycle shown in Fig. 6 because they cannot reorient the empty carrier, which is due to a defect in K^+ binding and/or translocation. These mutant transporters are, however, able to catalyze exchange that is K^+ independent. Cysteine modification and protection studies on the Y403C mutant showed that this residue is alternately accessible from either side of the membrane depending on the presence of substrates or transport blockers (109). This is in agreement with the alternating accessibility of the K^+ binding site from both sides of the membrane in the transport cycle (Fig. 6). Interestingly, E404 in rat EAAT2 is conserved in all eukaryotic glutamate transporters but not in the bacterial carriers and the neutral-amino-acid transporters (Fig. 1). This correlates with the observations that the neutral-amino-acid transporters are K^+-independent obligate exchangers and that the bacterial transporters are glutamate cation symporters. Y403 of rat EAAT2 is not conserved in the neutral-amino-acid transporters either, but it is conserved in the bacterial carriers. Mutations of Y403 and E404 in rat EAAT2 also affect the Na^+ and glutamate selectivity of the transporters, respectively, indicating that the three binding sites intimately interact (69, 114). The E404D mutant strongly prefers aspartate over glutamate, whereas the Y403W and Y403F mutants have an increased affinity for sodium ions but have lost the strict dependency on Na^+ and also accept lithium and cesium ions. The proximate residues 396 to 400 in rat EAAT2 that are indispensable for transport were suggested to be part of the Na^+ binding site(s) (109). These residues are also conserved in the bacterial glutamate transporters that do not use sodium ions. Furthermore, D304 of *B. stearothermophilus* GltT (the counterpart of D398 of rat EAAT2) is indispensable for function (78). Therefore these residues may be, more generally, involved in cation binding.

PROSPECTS

As long as a high-resolution structure of (one of) the members of the glutamate transporter family is not available, structural features must be obtained from biochemical and biophysical experiments. Mutagenesis studies and studies on chimeric transporters have so far proved to be very informative in providing information on the function of individual residues and domains, respectively. Mutagenesis is likely to remain useful as long as mutants with interesting properties can be found. One way to find interesting residues is to look for second-site revertants of mutant transporters that are inactive. For this, the bacterial members of the family can be used.

Cysteine-scanning mutagenesis is likely to become a major source of structural information. This technique has been extensively used in the study of the lactose transporter LacY from *E. coli* (30, 36), and the first results from this technique are now available for members of the glutamate transporter family. To fully benefit from the possibilities offered by cysteine-scanning mutagenesis, it will be necessary to use purified and reconstituted transporters. Such a pure system allows the use of a number of spectroscopic techniques to study both structural and dynamic properties of the transporters. The rat EAAT2 and *B. stearothermophilus* GltT glutamate transporters have been purified to homogeneity and reconstituted into proteoliposomes (15, 31). The bacterial system is suitable for the expression and large-scale purification of both wild-type and mutant transporters.

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