Functional sensitivity of polar surfaces on transmembrane helix 8 and cytoplasmic loop 8-9 of the *Escherichia coli* GABA (4-aminobutyrate) transporter encoded by *gabP*: mutagenic analysis of a consensus amphipathic region found in transporters from bacteria to mammals

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The *gab* permease (GabP) catalyses transport of GABA (4-aminobutyrate) into *Escherichia coli*. Although GabP can recognize and transport many GABA analogues that exhibit activity at GABAergic synapses in the nervous system, the protein domains responsible for these transport and ligand recognition properties have not been studied. Here we report that an amphipathic domain extending through putative transmembrane helix 8 and into the adjoining cytoplasmic region (loop 8-9) contains a critical 20 residue zone within which mutagenesis of polar amino acids has a deleterious effect on [³H]GABA transport activity. This functionally important amphipathic domain is

found to be highly conserved in the many APC family transporters that are homologous to GabP. And even though members of the GAT family of GABA transporters from the animal nervous system are not homologous to GabP, an analogous amphipathic structure is found in their loop 8-9 region. These results and observations suggest: (1) that the consensus amphipathic region (CAR) in the putative helix 8 and loop 8-9 region of GabP has functional significance, and (2) that nature has repeatedly used this CAR in transporters from bacteria to mammals.

INTRODUCTION

The *Escherichia coli* GABA (γ -aminobutyric acid) transporter (GabP) is encoded by *gabP*. Recently, the *gabP* has been cloned [1], sequenced [2] and overexpressed under *lac* control [3]. Hydropathy analysis of the predicted amino acid sequence suggests that GabP can be modelled as an intrinsic membrane protein with 12 transmembrane α -helical domains [4]. The GabP translocation pathway is known to accommodate a number of conformationally constrained GABA analogues [3,5,6] that are also known to be recognized by receptors and transporters in brain synapses that utilize GABA as a neurotransmitter [4]. The zwitterionic nature of GABA and its neurochemical analogues suggests that the *E. coli* GabP should contain a polar transmembrane transport conduit. However, protein domains that are capable of constituting a polar transport conduit have yet to be identified or studied in GabP or any of its homologues.

Here we focus attention on a long amphipathic domain in GabP which includes putative transmembrane helix 8 plus as portion of the adjoining hydrophilic region (loop 8-9) on the cytoplasmic side of the membrane. The amphipathic nature of transmembrane helix 8 suggests that it could be involved in pore formation, shielding the highly charged GabP cosubstrates from the hydrophobic lipid environment. Moreover, the length of the amphipathic domain suggests an additional thermodynamic feasibility of this region to undergo transport-related motions perpendicular to the plane of the lipid bilayer, a capability that might be anticipated for any structural element that was to participate directly in any of the gating [7–11] and/or solvation-substitution [12] reactions envisioned by some authors to be an integral part of carrier-mediated transport mechanisms.

Regardless of their actual role, polar residues are well-conserved within numerous homologous amino acid, polyamine,

and choline (APC family) transporters, suggesting that this amphipathic structural motif could be important in many different prokaryotic and eukaryotic transporters that share ancestry with the *E. coli* GabP. It is additionally intriguing that non-homologous GABA transporters from brain (GAT family) also exhibit within their helix 8 and loop 8-9 region a potentially amphipathic secondary structure that extends out of the transmembrane domain and well into the cytoplasm. Consistent with the notion that such a broadly distributed consensus amphipathic region (CAR) ought to be functionally significant, we show that the representation of CAR contained in the *E. coli* GabP includes a functionally sensitive twenty residue amphipathic segment that may, according to hydropathy analysis, extend out of the lipid bilayer and into the cytoplasm.

EXPERIMENTAL

Materials

[³H]GABA (31.6 Ci/mmol) was from DuPont New England Nuclear (Boston, MA, U.S.A.). Plasmid pSE380 was from Invitrogen (San Diego, CA, U.S.A.) and pBluescript II KS(-) was from Stratagene (La Jolla, CA, U.S.A.). Restriction enzymes were from New England Biolabs (Beverly, MA, U.S.A.). Bacteriological media were Difco brand supplied by Fisher (Pittsburgh, PA, U.S.A.). DNA sequencing was performed with Sequenase[®] from Amersham Life Science (Arlington Heights, IL, U.S.A.). Cellulose acetate filters (0.45 μ m) were from Micron Separations (Westboro, MA, U.S.A.). Liquescent[®] scintillation cocktail was from National Diagnostics (Atlanta, GA, U.S.A.). *o*-Nitrophenyl- β -D-galactopyranoside (β -ONPG) was from Sigma (St. Louis, MO, U.S.A.). Plasmid DNAs were prepared

Abbreviations used: GABA, γ -aminobutyric acid or 4-aminobutyrate; IPTG, isopropyl- β -D-thiogalactopyranoside; β -ONPG, *o*-nitrophenyl- β -D-galactopyranoside; PCR, polymerase chain reaction; CAR, consensus amphipathic region; APC, amine, polyamine and choline transporters.

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Table 1 Plasmids and *E. coli* strains

Plasmids	Relevant genotype or comments	Source or reference
pSCK-380	<i>amp^r lac^I lacO⁺ P⁺</i>	[5]
pSCK-472A	pSCK-380 with <i>gabP</i> gene	[5]
pSCK-380Z	pSCK-380 with <i>lacZ</i> insertion in <i>PstI/XhoI</i> sites	This work
pSCK-GP7	pBluescript II KS(-) derivative carrying the <i>gabP</i>	[5]
pSCK-GP8	pSCK-GP7 derivative in which the <i>gabP</i> stop codon is replaced by a <i>PstI</i> restriction enzyme site	This work
pSCK-475	pSCK-380Z derivative (Figure 1), containing the <i>gabP</i> from pSCK-GP8	This work
Strains	Chromosome/plasmid	Source or reference
CC118	<i>araD139Δ(ara leu)7697 ΔlacX74 ΔphoA20 galEgaK thi rpsE rpoB recA1/-</i>	Dr. Colin Manoil [41]
SK35	<i>lac^r Δ(ZY) gabΔP(kan^r1)-</i>	[5]
SK45	SK35/pSCK-380	[5]
SK55	SK35/pSCK-472A	[5]
CK475	CC118/pSCK-475	This work
SK475	SK35/pSCK-475	This work
CK472	CC118/pSCK-472A	This work

Figure 1 Structure of pSCK-475

In-frame *gabP-lacZ* fusions can be easily produced by subcloning a *gabP* cassette (*XbaI-PstI* restriction fragment from phagemid pSCK-GP8) into the expression plasmid, pSCK-380Z. The resulting plasmid, pSCK-475 (shown), specifies an in-frame amino acid linker that separates the GabP and LacZ polypeptides. Production of the GabP-LacZ hybrid protein is IPTG-inducible (Figure 2).

with kit from Qiagen (Chatsworth, CA, U.S.A.). Chemicals were from usual sources. Bacterial strains and plasmids are detailed in Table 1.

Plasmid construction

A two-step strategy was used to construct the plasmid, pSCK-475. First, *LacZ* was amplified by PCR from the plasmid, p472-G9Z (L. Hu and S. C. King, unpublished work), which contains a *gabP-lacZ* gene fusion constructed using λ Tn/*lacZ*/In [13]. The forward primer was 5'-GTC GCA ATT AGC GGT TTT TAC CCTGCAGAC TCT TAT ACA CAA GTA-3' (*PstI* site underlined), and the reverse primer was 5'-TGA TCT ACG CTCGAG TTA TTT TTG ACA CCA GAC-3' (*XhoI* site underlined). The resulting PCR product, containing *lacZ*, was blunt-end ligated into the *EcoRV* site of the phagemid, pBluescript II KS(-); the

phagemid-borne *lacZ* was subcloned in the *PstI-XhoI* sites of the expression vector, pSCK-380, to create the plasmid, pSCK-380Z.

Secondly, the *gabP* was amplified by PCR from a pSCK-GP7 template using the reverse primer, 5'-GAA T CTGCAG CA CGC GTA TTA TGA ACG GGT-3' (*PstI* site underlined), and the forward primer, 5'-GCTCTAGAG AGA GGA TTC AGA TGG GGC AAT CAT CGC AA-3' (*XbaI* site underlined). The resulting PCR product was blunt-end ligated in the *EcoRV* site of the phagemid, pBluescript II KS(-), to create pSCK-GP8. The *gabP* insert of pSCK-GP8 lacks a stop codon. The stop codon was replaced by a *PstI* site to enable formation of a *gabP-lacZ* fusion upon subcloning the *gabP* insert from pSCK-GP8 into the *XbaI-PstI* sites of pSCK-380Z. Thus, the resulting construct (called pSCK-475, Figure 1) expresses a GabP-LacZ hybrid protein under *lac* control.

Site-directed mutagenesis

Mutagenesis was performed using the method of Kunkel [14] as implemented commercially by BioRad. Single-stranded phagemid DNA (from pSCK-GP7 or pSCK-GP8) containing uracil was produced from the *E. coli* strain, CJ236 (obtained from BioRad), infected with the kanamycin-resistant helper phage, VCSM13 (obtained from Stratagene). All mutations (Table 3) were verified by complete sequencing of *gabP*. The fully sequenced *gabP* inserts were subcloned into the expression vectors, pSCK-380 or pSCK-380Z, as indicated in the Figures.

Culture conditions

LB medium (1 % Bacto tryptone/0.5 % Bacto yeast extract/1 % NaCl) was used to grow bacterial cells. Antibiotic supplements (ampicillin 100 μ g/ml; kanamycin 40 μ g/ml; chloramphenicol 40 μ g/ml) were added as appropriate for different constructs (Table 1). For transport experiments, cells grown overnight (16 h) were diluted 100-fold into fresh medium supplemented with 0.5 % glucose, 0.2 % ammonium sulphate (to cause catabolite repression of chromosomal *gabP*), and 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) (to induce *gabP* expression from the plasmid). The cells were harvested in logarithmic phase after about 4 h growth at 37 °C with vigorous shaking.

Transport

Cells were harvested in logarithmic growth and washed twice with 100 mM potassium phosphate (pH 7.0). The cell pellet was resuspended in this buffer such that the A_{600} was 6–7. Transport reactions were initiated by adding 90 μ l of washed cells with rapid vortex mixing to 10 μ l of solution containing 100 μ M [3 H]GABA (3 μ Ci/ml). Uptake was rapidly quenched by adding 1 ml of a 'stop solution' (buffer containing 20 mM HgCl_2) to the rapidly mixed reaction solution. The 'quenched' reaction was vacuum filtered. The reaction vessel was then rinsed with 1 ml of 'wash buffer' [100 mM potassium phosphate (pH 7.0) containing 5 mM HgCl_2]. Finally, the filter was rinsed with 4 ml of 'wash buffer'. The filter was dissolved in scintillation cocktail and the radioactivity (d.p.m.) was calculated using a Beckman LS3801 scintillation spectrophotometer using stored quench curves and automatic quench compensation (H number determination).

β -Galactosidase assay

Cells were grown to early log phase and placed on ice for 20 min. The β -galactosidase (LacZ) activity (28 °C) was measured using the assay described by Miller [15]. Cells were permeabilized by adding 2 drops of chloroform and 1 drop of 0.1 % SDS per ml of reaction mixture.

RESULTS AND DISCUSSION

Characterization of the GabP-LacZ hybrid system

An expression plasmid, pSCK-475, has been developed (Figure 1) to facilitate construction of GabP-LacZ hybrid proteins that can be used to study topology as well as structure–function relationships in GabP. With respect to [3 H]GABA transport activity there appears to be no distinction between otherwise equivalent *E. coli* strains expressing either GabP from pSCK-380 or GabP-LacZ hybrids from pSCK-380Z (Table 2). Furthermore, β -ONPG hydrolysis rates were found to be directly correlated with [3 H]GABA transport rates in strains expressing the GabP-LacZ hybrid (Figure 2), making it possible to eliminate low steady-state protein production as an explanation for the transport-negative phenotype exhibited by many mutants. All of the GabP mutants described below were produced as GabP-LacZ

Table 2 Comparison of GabP mutants with the same mutants expressed as GabP-LacZ hybrids

The measured transport activities were normalized to the activity of either CK472 (the wild-type for constructs expressing GabP) or CK475 (the wild-type for constructs expressing the GabP-LacZ hybrid). There was no difference between the wild-type GabP transport activities expressed from CK472 and CK475 (not shown). The values are means $\pm$ S.E. of eight measurements in at least two independent experiments.

Mutations	Transport activity (% of control)	
	GabP	GabP-LacZ
S299A	-0.19 ± 0.23	1.02 ± 0.16
C300A	3.3 ± 0.33	4.84 ± 0.29
S303A	6.34 ± 0.69	6.42 ± 0.57
Y306F	22.06 ± 5.01	33.15 ± 4.58
T307I	11.83 ± 2.23	11.83 ± 2.23
S309A	11.43 ± 0.68	12.14 ± 2.06
Y313F	52.91 ± 4.12	57.86 ± 8.92
R317N	22.05 ± 3.76	22.75 ± 3.57
R318N	4.88 ± 1.27	1.25 ± 0.05

Figure 2 Induction of GABA transport activity and β -galactosidase activity in host cells harbouring pSCK-475

E. coli CK475 were grown overnight and diluted 100-fold into the fresh medium containing the indicated concentrations of IPTG. The cells were harvested after approx. 4 h growth with shaking at 37 °C, and both the GABA transport activity (●) and the β -galactosidase activity (○) were measured. The results were normalized to the control activities obtained from cells grown without IPTG.

Table 3 Site-directed GabP mutants and their expression as GabP-LacZ hybrids

The indicated β -galactosidase activities are normalized to the activity of *E. coli* CK475 (wild-type GabP in the GabP-LacZ hybrid) which is 1.0.

Amino acid change	Codon change	β -Galactosidase activity
H284N	CAT $\rightarrow$ AAT	0.73
K288N	AAA $\rightarrow$ AAC	0.82
D290A	GAC $\rightarrow$ GCC	0.88
C291A	TGC $\rightarrow$ GCC	0.99
S296A	TCC $\rightarrow$ GCC	1.34
T298I	ACC $\rightarrow$ ATC	0.83
S299A	AGT $\rightarrow$ GCT	1.00
C300A	TGC $\rightarrow$ GCC	1.34
N302H	AAC $\rightarrow$ CAT	1.06
S303A	TCG $\rightarrow$ GCG	1.70
Y306F	TAT $\rightarrow$ TTT	1.23
T307I	ACC $\rightarrow$ ATC	1.00
S309A	TCA $\rightarrow$ GCA	1.44
R310N	AGG $\rightarrow$ AAC	0.97
Y313F	TAC $\rightarrow$ TTC	0.98
S314A	TCC $\rightarrow$ GCC	0.95
S316A	AGT $\rightarrow$ GCT	0.93
R317N	CGT $\rightarrow$ AAT	0.98
R318N	CGC $\rightarrow$ AAC	1.26
D320A	GAT $\rightarrow$ GCT	0.84

hybrids at steady-state levels comparable to the wild-type control (Table 3).

Detection of a 'sensitive polar surface' within an extended amphipathic domain

Site-directed mutagenesis has been used to remove certain functional groups from select positions in GabP while minimizing steric alterations (e.g. side chain volume, branching, etc.) that

Figure 3 Relationship between the putative helix 8 structure and the measured activity of GabP mutants expressed as GabP-LacZ hybrid proteins

The transport experiments (left) were performed using cells grown in LB medium supplemented with 0.5% glucose, 0.2% ammonium sulphate, and 1 mM IPTG. After washing, the cells were resuspended in 100 mM potassium phosphate (pH 7.0) and exposed for 1 min to 10 μ M [3 H]GABA (0.3 μ Ci/ml) prior to quenching the transport reactions with a stop solution and harvesting the cells by vacuum filtration as described in the Experimental section. The transport activities were normalized to the activity of β -galactosidase (Table 3), and are displayed as a percent of the control activity measured from *E. coli* CK475 (wild-type GabP expressed as the GabP-LacZ hybrid). The error bars represent standard error of the mean ($n = 8$). The presumed structure of the GabP helix 8 region (right) is presented to emphasize its relationship to the measured [3 H]GABA transport activity of various GabP mutants (left). Residues on the hydrophilic face of the helix 8 region are shown contained within the irregular black ribbon. Note that the functionally important residues cluster on the 'sensitive polar surface' of a 20 residue helical region (Ser-299 to Arg-317) contained within the grey box. Hydropathy analysis suggests that the 'sensitive polar surface' is only partially imbedded in the lipid bilayer, here represented by the area between two bold lines.

might disrupt secondary structure *per se*. The pattern of mutagenesis follows from a working hypothesis that the putative helix 8 and loop 8-9 regions of GabP probably exist as a single, long (30 residue), amphipathic, α -helical domain. Systematic mutagenesis (Figure 3) of residues along the hydrophilic face of this putative α -helical domain has highlighted a 'sensitive polar surface' on a helical segment containing 20 residues (Ser-299 to Arg-317); exactly the number needed to traverse the lipid bilayer.

The GabP topography inferred from hydropathy analysis suggests that the 'sensitive polar surface' is only partially imbedded in the lipid bilayer (Figure 3). Taken at face value, this interpretation of the topology would indicate that cytoplasmic elements in the helix 8 region may play an important role in the transport reaction. This is reminiscent of the cystic fibrosis transmembrane conductance regulator in which residues extending beyond a channel-forming helical domain (and originally believed to be a cytoplasmic loop) are now suspected of forming the selectivity filter in an extended transmembrane channel [16]. Alternatively, the putative 30 residue amphipathic helical region may traverse the bilayer at an angle off the perpendicular so that the entire segment might be membrane-imbedded. Regardless, the functional studies indicate that the 'sensitive polar surface' spans only 20 of the 30 residues within the amphipathic domain studied here.

Potential roles in co-transport for a transmembrane amphipathic domain

GabP should contain within its structure a membrane-spanning hydrophilic core capable of shielding zwitterionic GABA or protons from the lipid environment during translocation across

the membrane [3]. An amphipathic α -helical structure is chemically well suited to forming a compatible interface between the lipid membrane and the charged substrate. In addition, the nature of carrier-mediated transport suggests that protein conformational changes ought to somehow provide 'alternating access' (the process that exposes a substrate binding site first to one side of the membrane and then to the other). Although the precise nature of such conformational changes remains obscure, some ideas in the literature suggest the process should involve motions perpendicular to the lipid bilayer. For example, the classical 'mobile barrier' concept discussed by Mitchell [12] indicates that helical elements themselves move across a relatively stationary bound solute to achieve 'solvation substitution' and translocation. Additionally, there is evidence that ion-coupled co-transporters [11,17–20], including GABA transporters [9–11], display ion-channel gating activity. In bonafide ion channels, it is felt that gating involves protein rearrangements perpendicular to the bilayer [21–24]. To the extent that 'mobile barrier' and 'gating' motions are actually relevant to co-transport, it is worth pointing out that a 30 residue amphipathic domain would be thermodynamically suited to move in and out of the membrane. Regardless, the amphipathic nature of this domain also makes it an attractive candidate to participate in formation of a static pore – gated by entirely different mechanisms that may not involve large conformational changes [25].

Generality of the amphipathic motif

If helix 8 is involved in pore formation, then one might anticipate (a) that additional amphipathic helical elements would be needed to complete the pore, and (b) that it might be possible to attack

Figure 4 Evolutionary distribution of a CAR that is functionally important in the *E. coli* GabP

GabP is the first transporter from the APC family in which polar residues in the amphipathic helix 8 and loop 8-9 region have been probed for functional significance. **(A)** Amino acid sequence alignments illustrate that the helix 8 region of GabP is homologous to many APC family transporters from both prokaryotic and eukaryotic membranes. These alignments were excerpted from the Protein Domain Database (Prodom release 34.1) by searching proteins with homology to the *E. coli* GabP (SwissProt accession #: P25527). There are in fact over forty proteins that contain this CAR, and many were omitted from these alignments to conserve space. The darkened regions emphasize that many of the polar residues subjected to mutagenesis in this study are: (a) highly conserved within the APC family, and (b) occur in a pattern that maintains amphipathic character family-wide. Positions marked by an asterisk correspond to functionally important residues lying within the 'sensitive polar surface' of GabP defined in Figure 3. The abbreviated gene products are: ALP1: basic amino acid permease; AROP: aromatic amino acid permease; ANSP: L-asparagine permease; BAP2: Leu/Val/Ile permease; CAN1: arginine permease; CYCA: D-serine/D-alanine/glycine transporter; DIP5: dicarboxylic amino acid permease; GABP: GABA permease; GAP1: general amino acid permease; GNP1: glutamine permease; HIP1: histidine permease; HUTM: putative histidine permease; INA1: amino acid permease; ISP5: putative amino acid permease; LYP1: lysine permease; LYSP: lysine-specific permease; PAP1: hypothetical amino acid permease; PHEP: phenylalanine-specific permease; PROY: proline-specific permease; PUT4: proline permease; ROCE and ROCC: putative amino acid permeases; TAT2: tryptophan permease; VAL1: valine and tyrosine permease; YCC5, YFF5, YBY2 and YIFK: hypothetical amino acid permeases. **(B)** Helical wheel projections of APC family sequence exhibit considerable structural similarity in terms of amphipathic secondary structure. The topmost wheel represents the CAR from *E. coli* GabP. The black arc emphasizes the hydrophobic helical face; black circles emphasize residues that exhibit identity with consensus sequence in the sequence alignments **(A)**; asterisks (*) have the same meaning as in **(A)**. The middle wheel represents the CAR from CTR-1 (cationic amino acid transporter-1). CTR-1 is one of several mammalian transporters from the APC family that exhibit an amphipathic domain analogous to that of GabP. These transporters include CTR-1 from human, mouse and rat; and CTR-2 from human and mouse [26]. **(C)** Helical wheel projections of the mouse GAT3 sequence. The black circles emphasize residues that exhibit identity with GabP [4]. The helical wheel projection provides an example in support of the idea that the broad family of co-transporters may share common structures and mechanisms despite primary sequence dissimilarity [27].

the putative pore with protein modification reagents. Indeed, helix 4 of GabP also exhibits amphipathic character, and most of its polar residues appear to be functionally important (C. Brechtel and S.C. King, unpublished work). Furthermore, ongoing studies indicate (a) that sulphhydryl reagents block transport exclusively via Cys-300 of helix 8, and (b) that Cys-300 is the only important sulphhydryl in the *E. coli* GabP. These observations strengthen the notion that helix 8 contains a polar face that constitutes a functionally significant domain.

And sequence similarities (Figure 4) suggest that a region analogous to the helix 8 'sensitive polar surface' (Figure 3) will be found broadly distributed in Nature (as might be expected for a structure critically involved in transport function). Among all proteins that contain an amphipathic motif in their helix 8 and loop 8-9 regions, many are overtly homologous to GabP (Figure 4A). These homologous proteins are typically transporters, but members of the family appear to also function in a dual role as viral receptors (e.g. CTR; Figure 4B) [26]. Unlike APC proteins, the GAT family of GABA transporters from the nervous system display little overall sequence similarity to GabP. Nevertheless, secondary structural models of GAT family transporters contain

a potentially analogous amphipathic domain that extends out of helix 8 and into cytoplasmic loop 8-9 (Figure 4C). The presence of analogously positioned secondary structural elements in proteins having little or no sequence similarity supports the prediction that 'the functional family of cotransporters in bacteria, plants and animal membranes will turn out to have common tertiary structures and transport mechanisms, despite the lack of primary sequence homology.' [27].

Relationship to GAT gene structure

Convergent evolutionary mechanisms have been offered as an explanation for how common structural interrelationships might have arisen [28]. In this regard, it is precisely at the junction between coding exons 9 and 10 [4,29] that residues from GAT family of transporters exhibit secondary structural analogy to APC family CAR (Figure 4C). If not altogether coincidental, this observation may contribute something to the suggestion [29] that neurotransmitter transporter genes could have evolved via intron-mediated recombination of exons [30–33] that encode structural or functional modules. With each of their 12 membrane-spanning

domains encoded by a separate exon [4,29,34], transporters of the GAT family may stand as exemplars supporting the notion that complex transport systems could have arisen as evolutionary mosaics in which '...protein constituents or domains which are specifically responsible for energy coupling may have had distinct origins' [28].

Potential roles of helix 8 and loop 8-9 in GABA transport

Regardless of whether the observed (Figure 4C) secondary structural similarity between the GabP and GAT proteins arose by convergent or divergent evolutionary processes [35,36], it is most crucial to discern whether this similarity will predict functionality. Here we have demonstrated that the amphipathic helix 8 and loop 8-9 domain is functionally significant in the *E. coli* GabP (Figure 3). Although there has been no explicit test for functionality in other systems, the present results will probably extrapolate directly to the close homologues of GabP (Figure 4A). Extrapolation to the non-homologous GAT family is intriguing, but emphatically less certain. Nevertheless, the possible evolutionary and mechanistic significance of any existing relationship may warrant some consideration.

To the extent that their topology is actually known [37], we point out that in GAT proteins the CAR appears to lie in a putative cytoplasmic loop domain, directly opposite the extracellular loops IV and V. These extracellular loops impart selectivity to the extracellular opening of the GABA permeation pathway [38]. It is tempting to speculate, based on symmetry, that the periplasmic and cytoplasmic loops flanking helix 8 may likewise be involved in regulating the ligand specificity of GabP. It is emerging as a recurrent theme in nature that loops often provide for the precise placement of chemical groups outside the main structural framework of a protein. Loops that decorate a ligand recognition domain can impart specificity not only in enzymatic catalysis, but also in ion transport through channels; see MacKinnon [39] for a review. Modulation of ligand specificity may be a key feature of active transport since substrate affinity probably changes during translocation across the membrane [40]. It thus seems reasonable to suggest as a point of departure for further analysis that in either the GAT or GabP transporters, peptide segments on either side of helix 8 might co-operate to form part of the permeation pathway. Consistent with such roles, and with the observations of Tamura and co-workers [38], we find that the elevated [³H]GABA transport activity of the D320A mutant (Figure 3) is associated with a significant change in the affinity (L. Hu and S. C. King, unpublished work) for certain previously characterized [3,5,6] GabP substrates.

In summary, the helix 8 and loop 8-9 regions of GabP appear to exist as an extended α -helical domain, having a membrane-imbedded amphipathic segment plus an amphipathic cytoplasmic segment (both with potential to participate in pore formation). The present study has provided the first evidence that this amphipathic region contains a functionally 'sensitive polar surface' that may be of broad significance. Similar or potentially analogous amphipathic secondary structures appear in homologous transporters from the APC family (Figure 4B) as well as in the non-homologous GABA transporters (Figure 4C) from the mammalian nervous system.

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