Functional Significance of the “Signature Cysteine” in Helix 8 of the *Escherichia coli* 4-Aminobutyrate Transporter from the Amine-Polyamine-Choline Superfamily

RESTORATION OF CYS-300 TO THE CYS-LESS GabP*

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**Site-directed Mutagenesis—Mutagenesis was performed using the method of Kunkel (6) as implemented commercially by Bio-Rad. Single-stranded phagemid DNA (from pSCK-GPS or pSCK-GP9) containing uracil was produced from the *E. coli* strain CJ236 (obtained from Bio-Rad), infected with the kanamycin-resistant helper phage, VCSM13 (obtained from Stratagene). All mutations were verified by complete sequencing of gabP. The fully sequenced gabP inserts were subcloned into the expression vectors pSCK-380Z.

**Culture Conditions—LB medium (1% Bacto-tryptone, 0.5% Bacto-casein extract, 1% NaCl) was used to grow bacterial cells. Antibiotic supplements (ampicillin, 100 μg/ml; kanamycin, 40 μg/ml; chloramphenicol, 40 μg/ml) were added as appropriate for different constructs. For transport experiments, cells grown overnight (16 h) were diluted 100-fold into fresh medium containing 1 mM IPTG (to induce gabP expression from the plasmid) or diluted into medium supplemented with 0.5% glucose, 0.2% ammonium sulfate (to cause catabolite repression of chromosomal genes in CC118), and 1 mM IPTG. The cells were harvested in logarithmic phase after about 4 h of growth at 37 °C with vigorous shaking.

**Uptake—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 Δ*o* was 6–7. Transport reactions were initiated by adding 90 μl of washed cells with rapid vortex mixing to 10 μl of solution containing 100 μM [3H]GABA (31.6 Ci/mmol). Uptake was rapidly quenched by adding 1 ml of a "stop solu-

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*gab* permease (GabP) is the exclusive mediator of 4-aminobutyrate (GABA) transport across the *Escherichia coli* plasma membrane. Helix 8 and a portion of the adjoining cytoplasmic region (loop 8–9) constitute the GabP "consensus amphipathic region" (CAR), a potential channel-forming domain that is found to be evolutionarily conserved within the APC (amine-polyamine-choline) transporter superfamily. Upon the polar surface of the CAR, all known *gab* permeases display a “signature cysteine” not found in other members of the APC superfamily, suggesting that discrete features within the CAR might play a role in imparting specificity for the translocation reaction. Here we show that among the five cysteine residues in the *E. coli* GabP, only Cys-300, the signature cysteine, can restore wild type properties to the Cys-less GabP mutant. We conclude (i) from partial reaction studies (equilibrium exchange, counterflow) that rapid translocation of the GabP binding site from one side of the membrane to the other is greatly facilitated by Cys-300 and (ii) from pharmacological studies that loss of Cys-300 has little effect on the affinity that GabP exhibits for a structurally diverse array (kojic amine, 5-aminovaleric acid, GABA, nipecotic acid, and cis-4-aminoacrotonic acid) of competitive ligands. These results raise the possibility that other GABA transporters might rely analogously upon conserved cysteine residues positioned within the amphipathic helix 8 and loop 8–9 regions.

The *Escherichia coli* GabP (gab permease) is an archetypal APC superfamily transporter that catalyzes 4-aminobutyrate (GABA) translocation across the plasma membrane. The GabP has been cloned (1), expressed under lac control (2), and characterized extensively with respect to its ligand recognition properties (2–4). Recent evidence indicates that a “consensus amphipathic region” (or CAR) within transmembrane helix 8 and/or the adjoining cytoplasmic loop (loop 8–9) could play a significant role in the recognition and/or the translocation of these ligands. The association of functional identity with the CAR prompts consideration that this domain might play a role in the mechanism of many transporters from the APC superfamily.

The amphipathic nature of the CAR makes it structurally appropriate to form an interface between incompatible surfaces, such as between the lipid bilayer and a hydrophilic transport conduit. Consistent with such a function, the CAR is known to influence the catalytic properties of both the mouse cationic amino acid transport (MCAT) proteins and the GabP (1, 5). To investigate the possibility that discrete elements within the CAR have a significant role in determining transport specificity, we have focused on Cys-300 of the *E. coli* GabP, a “signature cysteine” found in the CAR of GABA transporters but not other members of the APC superfamily.

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EXPERIMENTAL PROCEDURES

Materials—[*H]GABA (31.6 Ci/mmol) were from NEN Life Science Products. Plasmid pSE380 was from Invitrogen (San Diego, CA), and pBluescript II KS(−) was from Stratagene (La Jolla, CA). Restriction enzymes were from New England Biolabs (Beverly, MA). Miller’s LB medium was from Life Technologies. DNA sequencing was performed with Sequenase™ from Amersham Pharmacia Biotec. Cellulose acetate filters (0.45 μm) were from Micron Separations, Inc. (Westboro, MA). Transporter inhibitors were obtained from Sigma or Research Biochemicals (Natwick, MA). Liquiscint™ scintillation mixture was from National Diagnostics (Atlanta, GA). Antibiotics, GABA, IPTG, o-nitrophenyl β-D-galactopyranoside were from Sigma. Plasmid DNA was prepared with a kit from Qiagen (Chatsworth, CA). Chemicals were from the usual sources. Bacterial strains and plasmids are detailed in Table I.

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**RESULTS**

Amino acid sequence alignments show that the CAR of all known gab permeases (i.e. from *E. coli*, *Bacillus subtilis*, and *Mycobacterium tuberculosis*) contains a signature cysteine that is not found at this position in the CAR of other APC superfamily members (Fig. 1). To probe the functional significance of these conserved cysteine residues, we have studied the role of all five cysteines in the *E. coli* GabP.

Cys-300 Is Functionally Significant in the *E. coli* GabP—GabP tolerates loss of cysteine residues at four (158, 251, 291, and 443) out of five positions, where 50% of wild type activity was observed following site-directed replacement with Ala and somewhat less with Ser (Fig. 2A). In contrast, a dramatic loss of transport activity was observed when Cys-300 was replaced by Ser (Fig. 2A), and similar results (i.e. indistinguishable from GabP-negative) were obtained when Cys-300 was replaced with Gly, Lys, Asp, Tyr, or Pro (not shown). Alanine was the best substituent for Cys-300, yielding a residual activity that was perhaps 5% percent of wild type (Fig. 2A).

Cys-300 Restores Activity to Cys-less GabP—Beginning with the C300A mutant, we found that without further loss of activity, a Cys-less GabP (i.e. pSCK-GP9) could be constructed by replacing the remaining four cysteine residues at positions 158, 251, 291, and 443 with Ala. Subsequent site-directed mutagenesis of the Cys-less GabP (Fig. 2B) showed that restoring the signature cysteine at position 300 also restored transport activity (>50% of wild type), whereas restoring a single cysteine at either position 158, 251, 291, or 443 did not (Fig. 2B). Does loss of Cys-300 impact energy coupling or the kinetics of GABA translocation?

Net Transport Is Slowed Substantially by Loss of Cys-300—Whereas [3H]GABA transport catalyzed by the wild type GabP is measured on the time scale of seconds (Fig. 3A), transport catalyzed by the Cys-less GabP mutant occurs over tens of minutes (Fig. 3B). A similar transport defect is fairly evident in the C300A point mutant, which is likewise slow, requiring many tens of minutes to approach steady state (not shown).

These results indicate that loss of Cys-300 slows the kinetics of one or more steps in the GabP cycle but does not abolish energy coupling *per se*. Transport kinetics are of paramount importance in real biological systems since “slow” acquisition of GABA (5% of the normal rate) under nitrogen-limited conditions would place the organism at a competitive disadvantage (accounts for evolutionary conservation of Cys-300). In nature, organisms losing Cys-300 would actually encounter far greater disadvantages because whereas the Ser substituent (complete-
ly defective) can be attained via a single missense mutation, the more functional Cys to Ala conversion requires two changes in codon 300.

The translocation defect associated with loss of Cys-300 is also apparent when the membrane is de-energized. Efflux of [3H]GABA from azide-poisoned cells was rapidly catalyzed by either the wild type GabP or by the mutant with a single Cys at position 300 (C300). In contrast, efflux from cells expressing the Cys-less GabP was comparable with background leakage from the GabP-negative strain, SK45 (Fig. 4A). These results indicate that Cys-300 is the only cysteine that has a dramatic effect on the ability of GabP to catalyze rapid net translocation of substrate when poisoned cells are preloaded with [3H]GABA and diluted into GABA-free medium. Can the translocation defect be localized to a discrete portion of the transport cycle?

The Exchange Partial Reaction Is Inhibited by Loss of Cys-300—Equilibrium exchange of [3H]GABA across the membrane of azide-poisoned cells was rapidly catalyzed by either the wild type GabP or by the mutant with a single Cysteine at position 300 (C300). In contrast, exchange from cells expressing the Cys-less GabP was comparable with background leakage from the GabP-negative strain, SK45 (Fig. 4A). These results indicate that Cys-300 is the only cysteine that has a dramatic effect on the ability of GabP to catalyze rapid net translocation of substrate when poisoned cells are preloaded with [3H]GABA and diluted into GABA-free medium. Can the translocation defect be localized to a discrete portion of the transport cycle?

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position 300 (Fig. 4B). These experiments indicate that Cys-300 is the only cysteine that has a dramatic effect on the ability of GabP to catalyze rapid equilibrium exchange (no net flux) of cytoplasmic [3H]GABA for unlabeled GABA in the extracellular medium.

Defects in GabP-mediated efflux (Fig. 4A) and exchange (Fig. 4B) associated with loss of Cys-300 are also reflected in counterflow experiments (Fig. 5). In counterflow (8, 9), the rising phase reflects the exchange rate (rapid for wild type GabP), whereas the falling phase reflects the rate of net efflux (likewise rapid for wild type GabP). The rising phase of counterflow catalyzed by the C300A point mutant was found: (a) to be markedly slower than wild type (indicating a severe exchange defect) and (b) to require preloading with unlabeled GABA. Like GabP C300A mutant, the Cys-less GabP exhibits a slow but significant counterflow activity that depends upon preloading (not shown). Counterflow cannot occur if net efflux is faster than exchange, and therefore these counterflow experiments involving Cys-less GabP or the C300A mutant (Fig. 5) indicate that both efflux and exchange are slow, confirming that loss of Cys-300 compromises net translocation via effects on the exchange partial reaction (Fig. 4). Is a defect in the substrate binding domain likely to account for poor exchange activity in GabP mutants lacking Cys-300?

The Cys-less GabP Retains High-affinity Ligand Binding—Although the rates of [3H]GABA uptake, efflux, and equilibrium exchange are all greatly diminished in mutants lacking the signature cysteine, there is strong evidence to indicate that loss of Cys-300 is not accompanied by denaturation of the
The GabP Signature Cysteine

Fig. 5. Comparison of the counterflow in SK475 and C300A. E. coli SK475 (wild type GabP), SK95 (GabP-negative), C300A (●, ○), or SK45 (GabP-negative; ▲) were poisoned with 30 mM sodium azide and then incubated with (solid symbols) or without (open symbols) 10 mM unlabeled GABA. The counterflow reactions were initiated by diluting the poisoned cells 200-fold into potassium phosphate buffer (pH 7.0) containing 10 μM [3H]GABA (0.25 μCi/ml) and 30 mM sodium azide. The reactions were quenched at the indicated times by adding 1 ml of buffer containing 20 mM HgCl₂. The cells were processed for scintillation counting as described under "Experimental Procedures."

DISCUSSION

Cys-300 Lies at the Beginning of a Functionally Sensitive Amphipathic Domain—Cys-300, the signature cysteine residue in the E. coli GabP, lies physically about midway through transmembrane helix 8 where the GabP SPS begins (1). The GabP SPS is a functionally defined, 20-residue domain that extends out of helix 8 and into the adjoining cytoplasmic loop 8–9. The GabP SPS is in fact part of a larger amphipathic loop 8–9. The GabP SPS is a functionally defined domain that connects the eighth and ninth transmembrane segments. The GabP SPS begins (1) the signature cysteine Cys-300 affects the elements of catalytic specificity, defined by the ratio, kₗ/Kₘ (12). Our results show that neither the rank order of potency for competitive inhibitors nor the absolute affinity for the individual ligands is substantially affected by loss of Cys-300 (Fig. 6), reflecting that the signature cysteine has little role in mediating carrier-cosubstrate interactions in the ground state. In contrast, the rate of [3H]GABA translocation is greatly diminished when Cys-300 is removed (Fig. 4), suggesting that a rate-limiting step (i.e., a transition state) in the translocation reaction does depend upon Cys-300. To what extent might the present results and concepts be extended to other gab permeases, as well as to other transporters from the APC superfamily and perhaps beyond?

Interrelationships within the Functional Family of Cotransporters—The hazard in extrapolating from the properties of E. coli GabP to other systems clearly increases as one moves away from discussing close GabP homologs and toward the discussion of more distant homologs or non-homologs. Nevertheless, a gathering together of information from homologous and analogous systems may provide a useful framework for future progress in this area.

Although the signature cysteines described here are restricted to GABA transporters, the kinetic significance of the CAR as a whole can be traced to distant APC homologs like the MCAT proteins, which lack these cysteines. Closs et al. (5) found that different MCAT subtypes exhibit kinetics that are determined exclusively by “... a divergent stretch of 41 amino acids...” that connect the eighth and ninth membrane-spanning domains. Likewise, GABA transporters from the nervous system also contain an amphipathic loop 8–9 segment.

These CAR-like domains in mammalian GABA transporters are structurally analogous to the GabP SPS (1) in that (i) they are positioned directly opposite the extracellular loops that have been implicated as determinants of substrate selectivity (13), (ii) they exhibit amphipathic character (1), (iii) they con-
tain a highly conserved Cys residue that aligns\(^2\) with the GabP signature cysteine (14) and which may serve as the sensitive target for the subset of inhibitory sulfhydryl reagents that are able to gain access to the cytoplasm (15). These characteristics are consistent with the notion that GABA transporters have a translocation pathway guarded symmetrically on either side of the membrane by functionally significant loops. Other transporters may also have cytoplasmic loop domains that affect translocation (16–20).

The foregoing inter-relationships are consistent with the notion that nature may have created analogous \(^{\text{structures and mechanisms via evolutionary convergence from distinct ancestral genes}}\) (21) in order to meet common exigencies associated with controlling transmembrane solute distribution. Wright \textit{et al.} (22) rephrase this concept with their 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 ...” In any event, the overall thrust of the present study is to underscore that kinetically significant, transporter-specific features (\textit{e.g.} the signature cysteines of GabP) may be superimposed on the general amphipathic character of a CAR that appears to exist in a multitude of transporters throughout nature.

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\(^2\) Alignments between the CAR and CAR-like regions of bacterial and mammalian GABA transporters can be criticized on grounds of “poor homology.” But this is to misunderstand both the definition (21, 23) of “homology” and our stated hypothesis which is that these amphipathic structures are analogous (not homologous). Analogous structures \textit{arise} by evolutionary convergence from distinct primordial structures, whereas homologous structures \textit{diverge} from one and only one primordial structure. As a result, \textit{homologous} protein structures are obliged to exhibit sequence similarity. In contrast, \textit{analogous} protein structures need not exhibit any sequence similarity. Clearly, two independent protein segments can converge toward amphipathicity with only incidental primary sequence similarity. Moreover, gaps of 3 or 4 residues (instead of 1 or 2 residues) in these “incidental” or fortuitous sequence similarities can serve to enhance amphipathicity in the secondary structure, even though gaps “penalize” the sequence similarity score. In the “convergence scenario,” a high similarity score is not relevant \textit{per se} since primary sequence similarity provides only one of many potential paths toward a functionally significant (evolutionarily selectable) amphipathic \textit{analogy} in the secondary structure.