Identification of the amine–polyamine–choline transporter superfamily ‘consensus amphipathic region’ as the target for inactivation of the *Escherichia coli* GABA transporter GabP by thiol modification reagents

Role of Cys-300 in restoring thiol sensitivity to GabP lacking Cys

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The *Escherichia coli* γ -aminobutyric acid transporter GabP (*gab* permease) contains a functionally significant cysteine residue (Cys-300) within its consensus amphipathic region (CAR), a putative channel-forming structure that extends out of transmembrane helix 8 and into the adjoining cytoplasmic loop 8–9 of transporters from the amine–polyamine–choline (APC) superfamily. Here we show that of the five cysteine residues (positions 158, 251, 291, 300 and 443) in the *E. coli* GabP, Cys-300 is the one that renders the transport activity sensitive to inhibition by thiol modification reagents: whereas substituting Ala for Cys-300 mimics the inhibitory effect of thiol modification, substituting Ala at position 158, 251, 291 or 443 preserves robust transport activity and confers no resistance to thiol inactivation; and whereas the robustly active Cys-300 single-Cys mutant is fully sensitive to thiol modification, other single-Cys mutants (Cys at 158, 251, 291 or 443) exhibit kinetically compromised transport activities that resist further chemical inactivation by thiol reagents. The present study reveals additionally that Cys-300 exhibits (1) sensitivity to hydrophobic thiol reagents, (2) general

resistance to bulky (fluorescein 5-maleimide) and/or charged {2-sulphonatoethyl methanethiosulphonate or [2-(trimethylammonium)ethyl] methanethiosulphonate} thiol reagents and (3) a peculiar sensitivity to *p*-chloromercuribenzenesulphonate (PCMBs). The accessibility of PCMBs to Cys-300 (located midway through the lipid bilayer) might be related to the structural similarity that it shares with guvacine (1,2,3,6-tetrahydro-3-pyridinecarboxylic acid), a transported GabP substrate. These structural requirements for thiol sensitivity provide the first chemical evidence consistent with channel-like access to the polar surface of the CAR, a physical configuration that might provide a basis for understanding how this region impacts the function of APC transporters generally [Closs, Lyons, Kelly and Cunningham (1993) *J. Biol. Chem.* **268**, 20796–20800] and the *gab* permease particularly [Hu and King (1998) *Biochem. J.* **300**, 771–776].

Key words: 4-aminobutyrate, carrier, membrane protein, permease.

INTRODUCTION

The *Escherichia coli* γ -aminobutyric acid (GABA) transporter GabP (*gab* permease) is a prototype amine–polyamine–choline transporter (APC) superfamily transporter, which has been sequenced [1], expressed under *lac* control [2] and extensively characterized with respect to its ligand recognition properties [2–5]. Gram-negative [2–4,6] and Gram-positive [5,7] organisms express homologous *gab* permeases that function as the exclusive mediators of GABA transport across the plasma membrane. Although the mechanism of GABA recognition and translocation remains unknown, a well-conserved consensus amphipathic region (CAR) has come under scrutiny as a putative transport conduit because of its channel-forming potential [8] and functional significance [9,10] in several proteins. For example, the CAR is known to specify *en bloc* the kinetic distinctions between different MCAT (mouse cationic amino acid transporter) protein subtypes [9,10]. The broad distribution of CAR-like structures in transporters throughout Nature suggests that they might have a fundamental role in the transport mechanism. The CAR is found in the helix 8 and loop 8–9 domain of a multitude of carriers from the APC superfamily [8]; CAR-like structures have been noted in

the topologically equivalent domain mammalian GABA transporters [8,11].

Although the channel-forming potential of an amphipathic segment such as the CAR prompts speculation that it could form part of a translocation pathway [8,12], there has been no physical or chemical evidence that functionally significant elements of the CAR might actually reside in a channel-like environment. However, recent work on GabP Cys-300 [12] has provided a basis for probing the CAR chemically for evidence that residues on the functionally significant polar face of transmembrane segment 8 (TM8) might be accessed through a channel. Here we show that Cys-300 (1) is the major target site at which thiol modification reagents inactivate the *E. coli* GabP and (2) resists modification by bulky and charged thiol reagents that do not resemble the transported substrates. The observation that reactivity towards Cys-300 depends on thiol reagent structure is consistent with the idea that access to this functionally significant residue requires solutes to pass through channel-like occluded spaces that impart structural selectivity. These results suggest the possibility that other portions of TM8 and/or the GabP ‘sensitive polar surface’ (SPS) [8] might similarly exist within a spatially restricted channel-like environment.

Abbreviations used: APC superfamily, amine–polyamine–choline transporter superfamily; BM, benzophenone-4-maleimide; CAR, consensus amphipathic region; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; FM, fluorescein 5-maleimide; GABA, γ -aminobutyric acid; guvacine, 1,2,3,6-tetrahydro-3-pyridinecarboxylic acid; IAA, iodoacetamide; MMTS, methyl methanethiosulphonate; MTSEA, 2-aminoethyl methanethiosulphonate; MTSES, 2-sulphonatoethyl methanethiosulphonate; MTSET, [2-(trimethylammonium)ethyl] methanethiosulphonate; NEM, *N*-ethylmaleimide; PCMB, *p*-hydroxymercuribenzoate; PCMBs, *p*-chloromercuribenzenesulphonate; SPS, ‘sensitive polar surface’; TM, transmembrane segment.

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EXPERIMENTAL

Materials

[³H]GABA (31.6 Ci/mmol) was from DuPont–NEN (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.). Miller's Luria–Bertani medium was from Life Technologies (Gaithersburg, MD, U.S.A.). Sequenase® DNA sequencing kits were from Amersham Life Science (Arlington Heights, IL, U.S.A.). Cellulose acetate filters (0.45 µm pore size) were from Micron Separations (Westboro, MA, U.S.A.). Liquiscint® scintillation cocktail was from National Diagnostics (Atlanta, GA, U.S.A.). Antibiotics, GABA, isopropyl β-D-thiogalactoside, dithiothreitol (DTT) and the thiol reagents iodoacetamide (IAA), *p*-chloromercuribenzenesulphonate (PCMB), *p*-hydroxymercuribenzoate (PCMB, sodium salt) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were from Sigma (St. Louis, MO, U.S.A.). Benzophenone-4-maleimide (BM) and fluorescein 5-maleimide (FM) were from Molecular Probes (Eugene, OR, U.S.A.). Methyl methanethiosulphonate (MMTS), 2-aminoethyl methanethiosulphonate (MTSEA), 2-sulphonatoethyl methanethiosulphonate (MTSES) and [2-(trimethylammonium)ethyl] methanethiosulphonate (MTSET) were from Toronto Research Chemicals (North York, Ontario, Canada). Kits for plasmid preparation were from Qiagen (Chatsworth, CA, U.S.A.). Chemicals were from usual sources. Bacterial strains and plasmids are detailed in Table 1.

Site-directed mutagenesis

Mutagenesis was performed as described previously [12] by the method of Kunkel [13]. The fully sequenced *gabP* inserts were subcloned directionally into the *XbaI*/*PstI* sites of pSCK-380Z [8] to create expression constructs (Table 1) that were placed in *E. coli* SK35 for all transport experiments. *E. coli* SK45 served as the *GabP*-negative control in all experiments.

Standard preparation of cells for transport

The *E. coli* were grown overnight (16 h) in Luria–Bertani medium [1 % (w/v) Bacto tryptone/0.5 % Bacto yeast extract/1 % (w/v) NaCl] supplemented with ampicillin (100 µg/ml), then diluted 1:100 into fresh medium supplemented with 1 mM isopropyl β-D-thiogalactoside (to induce *gabP* expression from the plasmid). The cells were harvested in the exponential phase after approx. 4 h of growth at 37 °C with vigorous shaking. Freshly grown cells were centrifuged and washed twice with buffer A (100 mM potassium phosphate, pH 7.0). The resulting cell pellets were resuspended in the same buffer to provide a D_{600} of 6–7.

Preparation of EDTA-treated cells

Freshly grown cells were centrifuged and washed twice with buffer A, then finally resuspended in approx. 1/30 of the culture volume. The cells were then equilibrated to 37 °C in a water bath and an equal volume of buffer A containing 2 mM EDTA was added. After exactly 3 min, 50 vol. of buffer A containing 10 mM MgSO₄ was added. The EDTA-treated cells were harvested by centrifugation and then resuspended in the same solution to provide a D_{600} of 6–7.

Transport reactions

Transport reactions were initiated by adding, with rapid vortex-mixing, 90 µl of washed cells to 10 µl of solution containing 100 µM [³H]GABA (3 µCi/ml). Transport reactions were rapidly quenched, filtered and processed for scintillation counting as described previously [12].

Counterflow reactions

Twice-washed cells were resuspended in buffer A, incubated at room temperature with or without a thiol modification reagent and then washed with ice-cold buffer A. Resuspended cells (1.5 ml) were brought to 30 mM NaN₃ and incubated for 30 min

Table 1 Plasmids and *E. coli* strains

Plasmid	Relevant genotype or comment	Source or reference
pSCK-380	amp ^r lacI ^q tacO ⁺ P ⁺	[2]
pSCK-380Z	pSCK-380 with lacZ insertion in <i>PstI</i> / <i>XhoI</i> sites	[8]
pSCK-GP7	pBluescript II KS(–) derivative carrying the <i>gabP</i>	[2]
pSCK-GP8	pSCK-GP7 derivative in which the <i>gabP</i> stop codon is replaced by a <i>PstI</i> restriction enzyme site	[8]
pSCK-GP8-X	Generic nomenclature for the series of pSCK-GP8 derivatives in which the <i>gabP</i> encodes amino acid substitution X, where X = C158A, C251A, C291A, C300A or C443A	[12]
pSCK-GP9	pSCK-GP8 derivative in which the <i>gabP</i> encodes a cysteine-less GABA transporter (CL) specified by Cys → Ala substitutions at codons 158, 251, 291, 300 and 443	[12]
pSCK-GP9CX	Generic nomenclature for the series of pSCK-GP9 derivatives in which the <i>gabP</i> encodes a single-Cys <i>GabP</i> (CX), where codon X = 158, 251, 291, 300 or 443	[12]
pSCK-CL380Z	pSCK-380Z derivative expressing <i>gabP</i> (Cys-less) from pSCK-GP9	[12]
pSCK-475	pSCK-380Z derivative, expressing the <i>gabP</i> (wild type) from pSCK-GP8	[8]
pSCK-475-X	Generic nomenclature for a series of pSCK-380Z derivatives expressing <i>gabP</i> taken from the pSCK-GP8-X series	[12]
pSCK-CXZ	Generic nomenclature for a series of pSCK-380Z derivatives expressing <i>gabP</i> taken from the pSCK-GP9CX series	[12]
Strain	Chromosome/plasmid	Source or reference
SK35	<i>lac</i> I ⁺ Δ(ZY) <i>gab</i> ΔP(kan ^r -1)/–	[2]
SK45	SK35/pSCK-380	[2]
SK475	SK35/pSCK-475	[8]
SK95	SK35/pSCK-CL380Z	[12]

at room temperature. The cells were then centrifuged at 4 °C and resuspended in 30 μ l of solution having the same composition as supernatant (control) or the same solution supplemented with 10 mM GABA to 'preload' the cells with substrate. Counterflow was initiated by diluting 5 μ l of preloaded cells into 1 ml of buffer A containing 10 μ M [3 H]GABA (0.25 μ Ci/ml). The reaction was quenched at timed intervals and filtered as described above. The results were expressed as an uptake ratio, being the [3 H]GABA uptake achieved by preloaded or non-preloaded *gabP*-positive strains divided by the equilibrium uptake achieved by the *gabP*-negative SK45 strain.

Thiol modification reagents

Thiol reagents were added to experiments from 100-fold concentrated stock solutions prepared in water [*N*-ethylmaleimide (NEM), IAA, PCMB, MTSES, MTSEA and MTSET], 0.2 M NaOH (PCMB), *N,N*-dimethylformamide (DMF, FM and DTNB) or DMSO (MTS). These solvents (1%, v/v) were without measurable effect on [3 H]GABA transport. Most notably, a 1% (v/v) addition of 0.2 M NaOH to buffer A caused an alkaline shift of only 0.04 pH units and was similarly without measurable effect on [3 H]GABA transport. Where indicated, the reaction with maleimide derivatives was quenched by adding DTT to a concentration of 5 mM. To avoid reversing [14] thiol modifications in GabP, reactions involving PCMB and PCMBs were not quenched with DTT.

RESULTS AND DISCUSSION

A 20-residue segment from Ser-299 to Arg-318 of the *E. coli* GabP comprises the SPS, a putative channel-forming domain that is highly conserved within the APC superfamily. The SPS exhibits sensitivity to mutations that alter side chain polarity or charge [8]. Cys-300 lies midway through TM8 near the beginning of the SPS. To assess whether the membrane-embedded Cys-300

Figure 1 Time course for inactivation of wild-type GabP by thiol reagents

EDTA-treated cells expressing the wild-type GabP were incubated for the indicated durations with 0.2 mM PCMBs (○), 0.2 mM PCMB (□), 1 mM NEM (●), 2 mM IAA (■), 0.25 mM BM (▼), 0.25 mM FM (▲) or 1 mM DTNB (×), at which time the thiol modification reaction was terminated (and the transport reaction was simultaneously started) by rapidly mixing 90 μ l of the chemically modified cells with 10 μ l of 100 μ M [3 H]GABA (3 μ Ci/ml) with (●, ■, ▼, ▲) or without (○, □, ×) 50 mM DTT. After 1 min, the transport reaction was quenched and the samples were processed for scintillation counting. Results are expressed as a percentage of control (defined as the activity of EDTA-treated cells that were not exposed to a thiol modification reagent). The results shown are representative of several experiments.

Table 2 Effect of thiol reagents on the GABA transport activity exhibited by GabP mutants with alanine substituted for one of the five native cysteine residues

Washed cells were incubated for 10 min with 1 mM NEM, 0.2 mM PCMBs, 2 mM IAA or solvent (control) before the initiation of transport reactions, which were performed for 1 or 10 min (C300A) as described in the Experimental section. The results are expressed as percentages of the solvent control (means $\pm$ S.D.; $n = 4$), i.e. activity retained = 100 $\times$ (treated/untreated). The GabP mutant carrying the C300A substitution translocated [3 H]GABA at about 2% of the wild-type rate, although both its ligand affinity and its ability to transduce energy (i.e. generate a large accumulation ratio) were normal [12]. For the other mutants (Ala substituted for Cys at position 158, 251, 291 or 443), the residual activity remained greater than 50% of that of the wild type.

Amino acid substitution	Activity retained (% of control)		
	+ NEM	+ PCMBs	+ IAA
C158A	4.9 $\pm$ 4.2	8.8 $\pm$ 1.48	72.6 $\pm$ 8.53
C251A	32.3 $\pm$ 2.93	11.7 $\pm$ 1.82	66.3 $\pm$ 4.33
C291A	3.3 $\pm$ 0.2	7.0 $\pm$ 0.82	63.9 $\pm$ 5.33
C300A	193.5 $\pm$ 6.23	74.3 $\pm$ 4.32	150 $\pm$ 9.68
C443A	8.4 $\pm$ 1.02	8.5 $\pm$ 1.04	87.3 $\pm$ 3.9
None (wild type)	16.2 $\pm$ 1.77	8.4 $\pm$ 0.41	69.7 $\pm$ 8.51

is (1) sensitive to thiol modification, and/or (2) located in a channel-like environment, we probed several Cys $\rightarrow$ Ala, Cys-less and single-Cys GabP mutants (Table 1) with 11 structurally distinct thiol reagents.

GabP exhibits differential sensitivities to thiol reagents

The time courses for inactivation of [3 H]GABA transport activity (Figure 1) indicate that the *E. coli* GabP exhibits differential reactivity towards thiol reagents that differ markedly in terms of size and charge. Whereas some reagents (PCMB and PCMBs) reacted to completion within 1 min, others (FM and DTNB) were virtually without effect on transport activity after 2 h. Intermediate rates of inactivation were observed for several other thiol reagents (NEM, BM and IAA). To discern the site or sites responsible for inactivation of *gab* permease activity, we used site-directed mutagenesis to replace each of the five cysteine residues (at positions 158, 251, 291, 300 and 443) in GabP with Ala and compared their thiol sensitivity with that of the wild-type protein.

Sensitivity of GabP to thiol reagents is not conferred by cysteine residues 158, 251, 291 or 443

GabP mutants bearing the substitutions C158A, C251A, C291A and C443A remained robustly active and were as sensitive to thiol reagents as the wild-type GabP (Table 2). In contrast, the low basal activity of C300A revealed the presence of a reproducible 'stimulatory' effect that was detectable on short exposures to NEM and IAA (Table 2). Although the source of the 'stimulatory' effect is unknown, it did not arise from any effect that thiol reagents exerted at one or more of the GabP cysteines. It will be noted (1) that a 'stimulatory' phenomenon is observed when the Cys-less GabP is exposed to NEM for a short time, and (2) that this effect is abolished by longer exposures to NEM (see Figure 4). Therefore the stimulatory effect is 'extraneous' to GabP and appears in Table 2 as a 'unique property' of the slowly transporting C300A and Cys-less mutants only because the small stimulatory effect is 1/20 to 1/50 as prominent in wild-type GabP or in mutants that retain a robust transport activity.

Figure 2 Effect of NEM on the GABA uptake mediated by the wild-type GabP or the C300A mutant

Washed cells (1 ml) were incubated with (●, ■) or without (○, □) 1 mM NEM for 1 h before the addition of 5 μ l of 1 M DTT to quench the thiol modification reaction. The NEM-treated cells (or untreated controls) were exposed to 10 μ M [3 H]GABA for the indicated durations and then processed for scintillation counting as described in the Experimental section. The transport activities for wild-type GabP (●, ○) and the C300A mutant (■, □) were normalized to the uptake exhibited by the *gabP*-negative strain SK45 (△). Whereas the main figure emphasizes that the NEM-treated GabP remains measurably active on the 1 h time scale, the inset emphasizes the effect that 1 mM NEM (1 h of exposure) has on rapid [3 H]GABA translocation. Similar results were obtained in additional experiments (results not shown).

Taken as a whole, the results from Table 2 imply that the sensitive thiol in GabP could not be Cys-158, Cys-251, Cys-291 or Cys-443. By inference, Cys-300 ought to be the cysteine that exhibits sensitivity to structural modification. Indeed, this is consistent with the observation that the C300A substitution causes a transport defect that mimics thiol modification.

NEM-modified GabP mimics the slow kinetics of the C300A substitution

Even though certain mutants (such as C300A, Cys-less and several single-Cys mutants) lacking a cysteine residue at position 300 were kinetically compromised, they retained the ability to transduce energy. Over tens of minutes, cells expressing these mutants accumulated far more [3 H]GABA than the GabP-negative background strain (Figure 2). As a result it was possible to observe the effect that added reagents exert on these defective mutants. For example, inhibitory effects were measured quantitatively when mutants lacking Cys-300 were exposed to competitive ligands [12]. In the present study, however, placing the C300A substitution into GabP mimicked all NEM-mediated effects; exhaustive treatment of the four remaining cysteines (positions 158, 251, 291 and 443) therefore had no further effect on activity. Whereas the wild-type GabP was sensitive to NEM (Figure 2, inset), the easily measured residual activity of the C300A mutant resisted chemical inactivation (Figure 2), suggesting that [3 H]GABA accumulation is highly resistant to thiol group modification at positions 158, 251, 291 and 443. Over tens of minutes both the C300A mutant and the NEM-treated wild-type GabP accumulated [3 H]GABA in excess of the GabP-

Figure 3 Effect of NEM on counterflow catalysed by the wild-type GabP or the C300A mutant

Washed cells were preincubated for 10 min with 1 mM NEM (▲) or left untreated (■, □, ○). The cells were then poisoned with azide and 'preloaded' with 10 mM GABA (▲, ■) or not (□, ○) as detailed in the Experimental section. Wild-type (■, □) and GabP-negative (○) controls were azide-poisoned, but were neither preloaded with GABA nor treated with NEM. These control experiments (□) ensure that active transport had been suppressed by azide-treatment because activity in excess of the GabP-negative (○) required preloading with 10 mM GABA. (A) Comparison of counterflow catalysed by wild-type GabP before (■) or after (▲) NEM exposure. (B) Comparison of counterflow catalysed by the C300A mutant before (■) or after (▲) NEM exposure. C300A (□) and GabP-negative (○) controls were azide-poisoned, but were neither preloaded with GABA nor treated with NEM. Similar results were obtained in other experiments (results not shown).

negative background (Figure 2), indicating that NEM affects the kinetics of substrate translocation more than energy coupling in itself. To separate energy coupling from translocation kinetics, we studied the effect of thiol agents on the GabP-mediated counterflow reaction in metabolically poisoned cells.

Thiol modification reagents affect the kinetics of transmembrane substrate exchange

Counterflow is a characteristically biphasic phenomenon that can be used to monitor the rate of carrier-mediated [3 H]GABA translocation across the de-energized plasma membrane of azide-poisoned cells [3–5, 12]. The rising phase of counterflow reflects the rapid exchange of extracellular [3 H]GABA with preloaded intracellular GABA, whereas the falling phase reflects the slower

Table 3 Effect of thiol reagents on the [³H]GABA transport activity of single-Cys GabP mutants

Washed cells were incubated for 90 min with 1 mM NEM, 0.2 mM PCMB, 2 mM IAA or solvent (control) before the initiation of transport reactions, which were performed for either 1 min (C300) or 10 min (all others) as described in the Experimental section. The results are expressed as percentages of the solvent control (means $\pm$ S.D.; $n = 4$), i.e. activity retained = $100 \times (\text{treated}/\text{untreated})$. The single-Cys and Cys-less GabP mutants exhibited residual [³H]GABA transport rates that were in the range of 5–10% of that of the wild type except for C300, which exhibited more than 60% of wild-type activity [12]. As described in the legend to Table 2, mutants lacking a cysteine at position 300 nevertheless recognized inhibitors and retained the ability to transduce energy.

Single-Cys position	Activity retained (% of control)		
	+ NEM	+ PCMB	+ IAA
C158	108 $\pm$ 8.51	81 $\pm$ 2.34	145 $\pm$ 12.01
C251	44.5 $\pm$ 0.77	87.1 $\pm$ 3.51	145.7 $\pm$ 11.69
C291	103.5 $\pm$ 12.24	73.4 $\pm$ 1.86	115.0 $\pm$ 14.85
C300	8.1 $\pm$ 0.97	1.2 $\pm$ 0.35	1.2 $\pm$ 0.69
C443	54.4 $\pm$ 2.9	86.6 $\pm$ 4.75	143.4 $\pm$ 2.37
None (Cys-less)	78.6 $\pm$ 2.82	102.7 $\pm$ 6.08	96.2 $\pm$ 8.7

net efflux of preloaded GABA. When wild-type GabP was treated with NEM the rate of transmembrane [³H]GABA exchange was greatly inhibited, although not abolished (Figure 3A). The C300A mutant also catalysed a slow counterflow reaction (similar to that of the NEM-treated wild type) that resisted further inactivation by NEM (Figure 3B), suggesting that Cys residues at positions 158, 251, 291 and 443 are insensitive to thiol modification reagents. Thus both the uptake assay with the energized membrane (Figure 2) and the counterflow assays with the de-energized membrane (Figure 3) indicate that Cys-300 is necessary for NEM to produce substantial inhibitory effects.

It is noteworthy that loss of Cys-300 (either by mutagenesis or exposure to NEM) was associated with a counterflow uptake ratio that did not return to equilibrium over the time courses studied in Figure 2. Although this behaviour resembles the exchange-only phenotype observed in certain *lac* permease mutants [15,16], *gab* permeases lacking a thiol at position 300 retained the ability to catalyse [³H]GABA uptake (Figure 2) or net efflux from azide-poisoned cells [12] at rates in excess of GabP-negative controls.

Cys-300 is the only cysteine required for full sensitivity to thiol modification reagents

To discern whether Cys-300 is also a sufficient target to produce all the inhibitory effects of NEM, we studied the effect of thiol reagents on Cys-less and single-Cys GabP mutants. Exhaustive (90 min) treatment with NEM, PCMB or IAA failed to inhibit the residual activity of the Cys-less GabP (Table 3), a set of observations consistent with the total absence of a target cysteine. We next studied the effect of thiol reagents on five single-Cys GabP mutants. As with the Cys-less mutant, four of the single-Cys mutants (C158, C251, C291 and C443) exhibited a residual [³H]GABA transport activity that resisted further inactivation by thiol modification reagents (Table 3), suggesting that these proteins lack a sensitive target thiol. In contrast, full sensitivity to NEM, PCMB and IAA was observed in a robustly active GabP mutant having only a single cysteine residue in place at position 300 (C300).

Therefore the present results (Tables 2 and 3) show collectively that the sensitivity of GabP to thiol reagents (1) requires Cys-300

Figure 4 Thiol sensitivity accompanies the restoration of Cys-300 to the Cys-less GabP

Cells expressing the C300 single-Cys mutant ($\square, \triangle, \circ$) or the Cys-less mutant ($\blacksquare, \blacktriangle, \bullet$) were treated with EDTA and then exposed to 0.2 mM PCMB ($\blacksquare, \square$), 0.2 mM PCMBs ($\blacktriangle, \triangle$) or 1 mM NEM ($\bullet, \circ$) for the durations indicated on the abscissa. The reactions with NEM were quenched by the addition of DTT to a concentration of 5 mM. Transport activity was assessed by exposing the cells to $10 \mu\text{M}$ [³H]GABA for either 1 min (for C300 mutant) or 10 min (for the Cys-less mutant). The transport activities are expressed as percentages of control (defined as the activity exhibited by cells that were not exposed to thiol reagents). Some symbols have been offset slightly for clarity. Similar results were obtained in several independent experiments with these reagents.

and (2) does not require the other cysteine residues at positions 158, 251, 291 and 443. As a result, it is observed that high reactivity towards PCMB and PCMBs is a characteristic of both the wild-type GabP (Figure 1) and the C300 single-Cys mutant (Figure 4), but not any of the mutants that lack a cysteine residue at position 300.

High reactivity of PCMBs towards Cys-300 is idiosyncratic

The ability of PCMBs (charged) to inactivate the C300 single-Cys GabP rapidly (Figure 4) runs contrary to the notion that Cys-300 lies membrane-embedded at a position midway through the lipid bilayer [8,17]. To test the alternative hypothesis that Cys-300 lies in the periplasmic space, we challenged the wild-type GabP and the C300 single-Cys mutant with MTSES and MTSET, a homologous pair of membrane-impermeable [18] thiol reagents bearing fixed negative and positive charges respectively. Whereas exhaustive treatment with these charged reagents failed to abolish GABA transport activity, their membrane-permeable [18] congeners, MMTS and MTSEA, were highly inhibitory (Figure 5). These experiments thus fail to support the idea that Cys-300 has prominent exposure to the periplasm and suggest instead that PCMBs has idiosyncratic structural features that allow it to access Cys-300 by pathways that are not available to other charged reagents such as MTSES and MTSET.

Cys-300 could lie within a channel-like environment

The peculiar reactivity of PCMBs towards certain membrane-embedded thiols is thought to derive from its ability to mimic weakly other cyclic molecules that enter the translocation pore [14,19]. The *E. coli* GabP translocates several cyclic substrates [2–4], including 1,2,3,6-tetrahydro-3-pyridinecarboxylic acid (guvacine), which bears a superficial structural similarity to

Figure 5 Reactivity of Cys-300 towards membrane-permeable reagents from the methanethiosulphonate family

Cells expressing either the wild-type GabP (filled bars) or the C300 single-Cys mutant (hatched bars) were exposed to the indicated reagents (1 mM) for 90 min at room temperature. Transport activity was then assessed by exposing the cells to 10 μ M [3 H]GABA (0.3 μ Ci/ml) for 1 min. Results are expressed as percentages of the control activity (exhibited by cells that were not exposed to a thiol reagent). Error bars indicate the S.D. ($n = 4$).

Figure 6 Reactivity of Cys-300 towards bulky thiol modification reagents

Cells expressing the wild-type GabP (WT), the C300 single-Cys mutant (C300) or the Cys-less mutant (CL) were treated with EDTA and then incubated with 0.25 mM BM (filled bars), 0.25 mM FM (hatched bars), 1 mM DTNB (stippled bars) or solvent (control) for 2 h after which the thiol modification reactions were quenched with 5 mM DTT. The cells were then exposed to 10 μ M [3 H]GABA for either 1 min (wild-type and single Cys-300) or 10 min (Cys-less). Results are expressed as percentages of the control activity (exhibited by cells that were not exposed to a thiol reagent). Error bars indicate the S.D. ($n = 4$).

PCMB and PCMBS. Apart from its idiosyncratic reactivity towards PCMBS, we looked for evidence that Cys-300 is associated simultaneously with both the lipid bilayer and a dimensionally constrained hydrophilic space by comparing bulky thiol reagents that were either highly hydrophobic or highly hydrophilic.

BM is a bulky, hydrophobic thiol reagent that partitions strongly into the lipid bilayer so that it reacts exclusively with buried thiol groups [20]. Whereas the residual activity of the Cys-less mutant resisted further inactivation by BM, wild-type GabP and the C300 single-Cys were highly sensitive to this reagent (Figure 6), suggesting the presence of a hydrophobic pathway to

Figure 7 Hypothetical basis for differential reactivity of thiol reagents towards Cys-300

On the basis of hydropathy analysis and data from topological reporter enzymes [17], GabP Cys-300 (black highlight) is thought to lie within TM8. All polar residues in TM8, including Cys-300, are located on the same side of the helix, a potential channel-forming configuration that is well conserved throughout the APC transporter superfamily [8,12]. This study suggests a division of thiol reagents into three categories on the basis of their reactivity towards Cys-300: (1) membrane-permeable reagents, capable of adopting a neutral form, were reactive at Cys-300; (2) membrane-impermeable reagents, bulky and/or bearing ionized or ionizable groups, were unreactive towards the membrane-embedded Cys-300 (poor reactivity denoted by the cross); and (3) guvacine-like reagents, superficially resembling the transported GabP substrate [3] guvacine (circled), exhibited idiosyncratic reactivity that was not observed with other charged reagents. Taken together, the results suggest the presence of both a hydrophobic route and a hydrophilic route to Cys-300. The hydrophilic pathway seems to include features that impart structural selectivity to the reaction of thiol reagents with Cys-300.

Cys-300. In contrast, there also seemed to be a selective hydrophilic pathway to Cys-300 that was available to PCMBS but not to bulky and/or charged hydrophilic reagents such as FM (Figure 6), which reacts exclusively with surface-exposed thiols [20,21].

The present study has provided the first indication that residues on the polar face of the APC superfamily CAR might be associated with a channel-like environment (summarized in Figure 7). Although Cys-300 was shown to be functionally significant, an unresolved question is whether its ionization state directly regulates the rate of GABA translocation. Preliminary studies indicate that GABA efflux is controlled by a neutral ionization rather than by a cysteine residue (pK_a 8.3). However, the pK_a of the membrane-embedded Cys-300 is not known. The present study has served to enable the site-directed labelling of

Cys-300 (Figure 6), a capability that promises to yield information on the ionization state of this cysteine residue particularly, and on the CAR environment generally. The hypothesis that Cys-300 exists within a channel-like structure affords a physical basis for understanding how structural perturbations within the CAR are able to affect the transport properties of both bacterial [8,12] and mammalian [9] representatives of the APC superfamily, as well as other transport proteins that exhibit amphipathic character in the helix 8 and loop 8–9 region [8,12].

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REFERENCES

- 1 Niegemann, E., Schulz, A. and Bartsch, K. (1993) *Arch. Microbiol.* **160**, 454–460
- 2 King, S. C., Fleming, S. R. and Brechtel, C. E. (1995) *J. Biol. Chem.* **270**, 19893–19897
- 3 King, S. C., Fleming, S. R. and Brechtel, C. E. (1995) *J. Bacteriol.* **177**, 5381–5382
- 4 Brechtel, C. E., Hu, L. and King, S. C. (1996) *J. Biol. Chem.* **271**, 783–788
- 5 Brechtel, C. and King, S. C. (1998) *Biochem. J.* **333**, 565–571
- 6 Kahane, S., Levitz, R. and Halpern, Y. S. (1978) *J. Bacteriol.* **135**, 295–9
- 7 Ferson, A. E., Wray, Jr., L. V. and Fisher, S. H. (1996) *Mol. Microbiol.* **22**, 693–701
- 8 Hu, L. A. and King, S. C. (1998) *Biochem. J.* **330**, 771–776
- 9 Closs, E. I., Lyons, C. R., Kelly, C. and Cunningham, J. M. (1993) *J. Biol. Chem.* **268**, 20796–20800
- 10 Sophianopoulou, V. and Dhalluin, G. (1995) *FEMS Microbiol. Rev.* **16**, 53–75
- 11 King, S. C. (1996) in *Handbook of Psychiatric Genetics* (Blum, K. and Noble, K. P., eds.), pp. 163–198, CRC Press, Boca Raton
- 12 Hu, L. A. and King, S. C. (1998) *J. Biol. Chem.* **273**, 20162–20167
- 13 Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488–492
- 14 Yan, R.-T. and Maloney, P. C. (1993) *Cell* **75**, 37–44
- 15 Franco, P. J. and Brooker, R. J. (1994) *J. Biol. Chem.* **269**, 7379–7386
- 16 Carrasco, N., Antes, L. M., Poonian, M. S. and Kaback, H. R. (1986) *Biochemistry* **25**, 4486–4488
- 17 Hu, L. A. and King, S. C. (1998) *Biochem. J.* **336**, 69–76
- 18 Holmgren, M., Liu, Y. and Yellen, G. (1996) *Neuropharmacology* **35**, 797–804
- 19 Lebendiker, M. and Schuldiner, S. (1996) *J. Biol. Chem.* **271**, 21193–21199
- 20 Jones, P. C., Sivaprasadarao, A., Wray, D. and Findlay, J. B. C. (1996) *Mol. Membrane Biol.* **13**, 53–60
- 21 Jones, P. C., Harrison, M. A., Kim, Y. I., Finbow, M. E. and Findlay, J. B. (1995) *Biochem. J.* **312**, 739–747

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