Induction of substrate specificity shifts by placement of alanine insertions within the consensus amphipathic region of the *Escherichia coli* GABA (γ -aminobutyric acid) transporter encoded by *gabP*

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The *Escherichia coli* GABA (γ -aminobutyric acid) permease GabP is a prototypical APC (amine/polyamine/choline) superfamily transporter that has a CAR (consensus amphipathic region) containing multiple specificity determinants, ostensibly organized on two helical surfaces, one hydrophobic [SHS (sensitive hydrophobic surface)] and the other hydrophilic [SPS (sensitive polar surface)]. To gauge the functional effects of placing alanine insertions at close intervals across the entire GabP CAR, 64 insertion variants were constructed. Insertions, particularly those in the SHS and the SPS, were highly detrimental to steady-state [³H]GABA accumulation. TSR (transport specificity ratio) analysis, employing [³H]nipecotic acid and [¹⁴C]GABA, showed that certain alanine insertions were associated with a specificity shift (i.e. a change in k_{cat}/K_m). An insertion (INS Ala-269) located N-terminal to the SHS increased specificity for [³H]nipecotic

acid relative to [¹⁴C]GABA, whereas an insertion (INS Ala-321) located C-terminal to the SPS had the opposite effect. Overall, the results are consistent with a working hypothesis that the GabP CAR contains extensive functional surfaces that may be manipulated by insertion mutagenesis to alter the specificity (k_{cat}/K_m) phenotype. The thermodynamic basis of TSR analysis provides generality, suggesting that amino acid insertions could affect specificity in many other transporters, particularly those such as the *E. coli* phenylalanine permease PheP [Pi, Chow and Pittard (2002) J. Bacteriol. 184, 5842–5847] that have a functionally significant CAR-like domain.

Key words: carrier, catalysis, mutagenesis, permease, specificity, transport.

INTRODUCTION

GABA (γ -aminobutyric acid) transport in *Escherichia coli* is mediated by the *gab* permease [1,2] GabP (TC 2.A.3.1.4) [3], a 12-helix integral membrane protein [4] which serves as the prototype for a large collection of carriers from the APC (amine/polyamine/choline) superfamily [5]. The ligand recognition properties of GabP have been studied extensively [2,6–8], and although many details of the GabP transport mechanism remain obscure, it is known that the CAR (consensus amphipathic region) plays a functionally significant role.

The CAR is a 70-residue segment that lies between TM7 (transmembrane segment 7) and TM9, and which exhibits amphipathic, channel-forming character when modelled as an α -helix. Rapid [³H]GABA translocation can be abolished by mutations that perturb polar residues positioned along the length of a discrete 20-residue amphipathic segment [9] known as the SPS (sensitive polar surface). Among the functionally significant residues displayed on the SPS is Cys-300 [10], a residue that lies at the bilayer core and serves as the exclusive target site for inactivation of GABA transport activity by thiol-modifying reagents [11]. Interestingly, sequence alignments between GabP and the mammalian GAT-1 GABA transporter show that the latter has a CAR-like domain [9,12], containing an analogous cysteine residue (Cys-399), which among 15 candidate Cys residues has also turned out to be the target site of transporter inactivation by thiol-

modifying reagents, prompting these authors to suggest that the cysteine residue might be close to the substrate binding site [13].

Consistent with the idea that Cys-300 may be closely associated with substrate translocation, TSR (transport specificity ratio) analysis (accompanying paper: [14]) of GabP cysteine-scanning variants has shown that Cys-300 is flanked on either side by multiple substrate specificity determinants, i.e. residues at which the cysteine substituent causes a substantial change in TSR, a parameter calculated from results of competitive uptake between [¹⁴C]GABA and [³H]NA ([³H]nipecotic acid) [14]. These TSR analyses revealed the presence of specificity determinants in two distinct parts of the GabP CAR: (i) the SPS, which is of functional significance in GabP [9] and the phenylalanine permease PheP [15], and (ii) the SHS (sensitive hydrophobic surface), which is ostensibly located on the hydrophobic surface of a functionally significant 20-residue domain at the N-terminal margin of the CAR [14].

If the SPS and the SHS display their specificity determinants in the context of a helical secondary structure, then a collection of GabP insertion-scanning variants with inserts arrayed across the CAR would be expected to include many transport-negative phenotypes, since lysozyme crystal structures show that on a helical surface the most probable effect of an alanine insertion would be to preserve the helix by residue translocation [16], thereby offsetting the specificity determinants by 100° on either side of the insertion. In order to investigate whether insertions

Abbreviations used: APC superfamily, amine/polyamine/choline superfamily; CAR, consensus amphipathic region; GABA, γ -aminobutyric acid (4-aminobutyrate); NA, nipecotic acid; β -ONPG, *o*-nitrophenyl β -D-galactopyranoside; SHS, sensitive hydrophobic surface; SPS, sensitive polar surface; TM7 (etc.), transmembrane segment 7 (etc.); TSR, transport specificity ratio.

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Table 1 Plasmids and *E. coli* strains

Plasmid	Relevant genotype or comments	Source or reference
pSCK-380	<i>amp^r lac^R tacO⁺ P⁺</i>	[2]
pSCK-380Z	pSCK-380 with <i>lacZ</i> insertion in <i>Pst</i> I/ <i>Xho</i> I sites	[9]
pSCK-380-Hx10	pSCK-380 with a His ₁₀ -encoding cassette in the <i>Pst</i> I/ <i>Xho</i> I sites	[14]
pSCK-GP7	pBluescript II KS(−) derivative carrying <i>gabP</i>	[2]
pSCK-GP8	pSCK-GP7 derivative in which the <i>gabP</i> stop codon is replaced by a <i>Pst</i> I restriction enzyme site	[9]
pSCK-GP8-INS AlaX	Generic nomenclature for the series of pSCK-GP8 derivatives in which codon X specifies insertion of an alanine residue into <i>GabP</i>	This work
pSCK-475	pSCK-380Z derivative containing <i>gabP</i> (wild type) from pSCK-GP8	[9]
pSCK-88	pSCK-380-Hx10 derivative in which the chloramphenicol resistance cassette is replaced by the <i>gabP</i> cassette taken from pSCK-GP8 using <i>Xba</i> I/ <i>Pst</i> I	This work
pSCK-475-INS AlaX	Generic nomenclature for the series of pSCK-380Z derivatives in which the <i>gabP</i> cassette is derived from the pSCK-GP8-INS AlaX series	This work
pSCK-88-INS AlaX	Generic nomenclature for the series of pSCK-380-Hx10 derivatives in which the <i>gabP</i> cassette is derived from the pSCK-GP8-INS AlaX series	This work

Strain	Chromosome/plasmid	Source or reference
SK35	<i>lac I⁺ Δ(ZY) gabΔP(kan^r -1)/−</i>	[2]
SK45	SK35/pSCK-380	[2]
SK88	SK35/pSCK-88	This work
SK475	SK35/pSCK-475	[9]

produce these anticipated phenotypic effects, we have studied 64 *GabP* variants that have an alanine residue inserted at unique locations across the CAR. Insertions placed within either the SHS or the SPS were especially devastating to function. Interestingly, the present study has also revealed select locations at which the insertions were better tolerated, leaving a residual transport activity that could be subjected to TSR analysis with [¹⁴C]GABA and [³H]NA. Overall, the largest residual transport activities and the most obvious Δ(TSR) phenotypes were associated with alanine insertions placed at the margins of presumptive secondary structural elements in the CAR.

EXPERIMENTAL

Materials

The source and characteristics of bacterial strains and plasmids are detailed in Table 1. Other chemicals and reagents were from usual sources [14].

Site-directed mutagenesis

Mutagenesis was performed by the Kunkel method [17], as detailed in [14], using single-stranded phagemid uracil-DNA produced from pSCK-GP8 in *E. coli* strain CJ236. Sometimes the desired mutations were not isolated, creating several small gaps in the insertion series.

Cell growth and preparation

The *gab* permeases were expressed from either the pSCK-475-INS AlaX series or the pSCK-88-INS AlaX series (Table 1) to produce *GabP*-LacZ or *GabP*-His₁₀ proteins respectively in the *GabP*-negative *E. coli* SK35 host. Cells were prepared for transport as described in [14].

Steady-state transport assay

Transport reactions were initiated by adding 80 μl of prewarmed washed cells to 20 μl of 50 μM [³H]GABA (1.5 μCi/ml). The

reactions were terminated after 4 min at 30 °C (steady state with respect to the wild-type *GabP*) by adding ice-cold 100 mM potassium phosphate, pH 7.0 (1 ml), and harvesting the cells by vacuum filtration. Filters were washed with ice-cold 100 mM potassium phosphate, pH 7.0 (4 ml), and dissolved in scintillation cocktail (4 ml). Radioactivity (d.p.m.) was quantified on a Packard Tri-Carb 2900TR scintillation spectrometer using automatic quench compensation and stored quench curves. Results are reported as an uptake ratio, which is defined as the uptake exhibited by the test strain divided by the uptake exhibited by *E. coli* SK45 (*GabP*-negative).

Initial-rate competitive uptake and TSR analysis

Competitive uptake was performed as described in [14], except that transport reactions were initiated by adding 80 μl of prewarmed cells with rapid vortexing to 20 μl of a prewarmed substrate mixture containing 20 μM [³H]NA (1.2 μCi/ml) and 30 μM [¹⁴C]GABA (0.6 μCi/ml). Where indicated, results are reported as a TSR, which is readily calculated from the initial rates (60 s) of competitive uptake [14,18]. The TSR has the following algebraic equivalencies:

$$\text{TSR} = \frac{(k_{\text{cat}}/K_{\text{m}})_{\text{GABA}}}{(k_{\text{cat}}/K_{\text{m}})_{\text{NA}}} = e^{(-\Delta\Delta G_{\text{b}}/RT)} \quad (1)$$

where $k_{\text{cat}}/K_{\text{m}}$ is our formal definition of specificity, and $\Delta\Delta G_{\text{b}}$ is the change in intrinsic binding energy, ΔG_{b} , that is due to the structural difference between GABA and NA (R and T are the gas constant and absolute temperature respectively) [14]. The right-most part of eqn (1) indicates that an underlying substrate-specific effect on intrinsic binding energy may be held to account for any Δ(TSR) phenotype that might arise via mutagenesis. In turn, binding energy changes in a helix-rich carrier protein are thought [14,19] to consist of localized and delocalized components:

$$\Delta G_{\text{b}} = \Delta G_{\text{localized}} + \Delta G_{\text{delocalized}} \quad (2)$$

LacZ assay

Washed cells (15 μ l) were mixed with 135 μ l of 120 mM Tris (pH 7.0 with HCl), 150 μ l of permeabilization buffer [100 mM Tris/HCl, pH 7.8, 32 mM sodium phosphate, 8 mM dithiothreitol, 8 mM CDTA (*trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetra-acetic acid), 4% (w/v) Triton X-100 and 200 μ g/ml polymyxin B], and 500 μ l of Z buffer [20]. After warming to 37 °C, reactions were initiated by adding 200 μ l of β -ONPG (*o*-nitrophenyl β -D-galactopyranoside; 4 mg/ml). Typically, reactions were terminated 4 min later by adding 500 μ l of stop solution (1 M Na₂CO₃, 0.1 M Na₂EDTA, pH 10.6). Absorbance at 420 nm was recorded, using the LacZ-negative SK45 as a reaction blank. Results are reported as absorbance units/h per μ g of protein. Protein was quantified using bicinchoninic acid reagents and BSA as the standard.

Immunoblots

As described in [14], plasma membranes were isolated from sonicated cells, and the membrane proteins were transferred to PVDF following separation by SDS/PAGE (2 μ g of protein/lane). GabP-His₁₀ was detected with an anti-penta-His monoclonal antibody. The immunoblots were developed with a chemiluminescent alkaline phosphatase substrate, and the band intensities obtained from a cooled CCD imager were quantified with Kodak 1D software [14].

RESULTS AND DISCUSSION

The CAR is a putative channel-forming secondary structure, containing structural elements that play a functionally significant role (i) in GabP itself [9–11,14], (ii) in GabP homologues from the APC transporter superfamily (e.g. PheP [15]), and (iii) possibly in other GABA transporters that have a CAR-like domain [9] (e.g. GAT-1 [13]). When the GabP CAR is modelled as an amphipathic helical structure, its substrate specificity determinants are found to be displayed on two distinct surfaces (the SHS and SPS) positioned at either end of the CAR (Figure 1). The model implies that the transport function should be severely impacted by amino acid insertions placed at different positions within the CAR. In order to test this hypothesis, we constructed 64 alanine-insertion scanning mutants spanning the GabP CAR. Experiments were carried out to assess the impact of these insertions on [³H]GABA accumulation as well as the relative specificity (k_{cat}/K_m) for [¹⁴C]GABA and [³H]NA under competitive uptake conditions (i.e. TSR analysis). Overall, the results indicate that although insertions within the CAR have a generally deleterious effect on transport function, the precise location of the alanine insertion determines whether the defect exhibits a substrate-selective character, affecting either GABA or NA transport relatively more severely. The functional deficits exhibited by GabP insertion variants could not reasonably be attributed to anomalous expression levels in the vast majority of cases.

Whether gauged via LacZ reporter enzyme activity or immunoblot, expression levels of GabP insertion variants cannot account for most of the defective transport phenotypes

The *gabP* mutants were expressed either as GabP–LacZ hybrid proteins or as decahistidine-tagged (GabP–His₁₀) proteins. GabP–LacZ expression was assessed colorimetrically in permeabilized whole cells (Figures 2B and 3B), whereas GabP–His₁₀ expression was assessed luminometrically by immunoblotting plasma mem-

Figure 1 Putative distribution of secondary structural elements within the CAR of *Escherichia coli* GabP

The illustration shows a generic 12-helix membrane protein with two 'expanded views' that depict a predominantly α -helical arrangement of the GabP amino acid sequence within both (i) the loop 7–8 region between TM7 and TM8, and (ii) the TM8/loop 8–9 region (note that Lys-286 is drawn twice for continuity). Together, these two regions comprise an evolutionarily conserved segment known as the CAR that is found in GabP and other members of the APC transporter superfamily [9]. Since amphipathic helical domains have channel-forming potential, there has been speculation that the CAR could be part of a conduit involved in the translocation of polar solutes. Indeed, portions of the CAR appear to exist in a channel-like environment, since the reactivity of Cys-300 is highly dependent upon the structure (size and polarity) of a thiol-modification reagent [11]. The GabP CAR includes two 20-residue segments, the SHS (black box with white ribbon) and the SPS (grey box with black ribbon), which contain multiple substrate specificity determinants [14]. If these substrate specificity determinants are arrayed along helical surfaces, then alanine insertions placed within either the SHS or the SPS would be expected to cause devastating surface disruption. It should be noted that the actual secondary structure across the CAR remains unknown, and that the break at Lys-286 is introduced principally to limit the vertical height of the illustration.

brane proteins resolved by SDS/PAGE. The colorimetric and luminometric analyses were performed on materials derived from precisely the same cultures that were used to assess transport function (i.e. functional status and expression are directly comparable in Figures 2–4).

LacZ activities measured in permeabilized whole cells indicated that expression levels of GabP–LacZ hybrid proteins were quite consistent across the entire collection of GabP insertion variants. The lowest LacZ activity (variant with insertion at position 293) was about one-third that of the highest LacZ activity (position 323), and the entire collection exhibited a S.D. that was approx. 24% of the mean (0.3 A_{420} unit/h per μ g of protein). Immunoblotting of plasma membrane proteins resolved by SDS/PAGE indicated that GabP insertion variants were expressed at levels ranging from approx. 27% to 220% of that of the wild type (Table 2). The entire collection of His-tagged GabP variants exhibited a S.D. that was approx. 40% of the mean expression levels.

Since GabP expression levels, whether gauged from whole cell analysis or from isolated plasma membranes, were never less than 25% of that of the wild-type control, factors other than the expression level must be held to account for the poor functional activity exhibited by GabP variants with an alanine insertion

Figure 2 Effects of alanine insertion mutagenesis of the *gabP* region encoding the putative TM7 and loop 7–8 domains

Cells expressing alanine insertion (INS) mutants of GabP as GabP–LacZ hybrids were grown and processed as described in the Experimental section. **(A)** Transport: transport reactions were carried out by exposing washed cells to 10 μM [³H]GABA (0.3 μCi/ml) for 4 min at 30 °C prior to stopping the reactions and processing the samples for scintillation counting. Error bars represent S.E.M. (*n* = 4). Transport by each mutant may be compared with that by the positive control, SK475, and the GabP-negative strain, SK45 (in which the uptake ratio is 1.0, by definition). **(B)** Expression level: cells from the same washed suspensions used for transport experiments in **(A)** were permeabilized with polymyxin B, then exposed to β-ONPG for 4 min at 37 °C prior to stopping the reactions and processing the samples to read absorbances (see the Experimental section). Error bars represent S.E.M. (*n* = 3).

Figure 3 Effects of alanine insertion mutagenesis of the *gabP* region encoding the putative TM8 and loop 8–9 domains

For details, see the legend to Figure 2.

Table 2 Levels of expression of *E. coli* GabP alanine-scanning mutants

The plasma membrane fraction was prepared from three independent cultures of strains expressing the indicated alanine insertion (INS) GabP mutants. Membrane proteins (2 μg of protein/lane) were separated by SDS/PAGE. Immunoblots were probed with an anti-penta-His monoclonal antibody and developed with a chemiluminescent alkaline phosphatase substrate. Chemiluminescent intensity (measured with a CCD imager) is reported as the mean ± S.E.M. (*n* = 3) of the intensity exhibited by the wild-type parent (= 1.0).

GabP variant	Expression	GabP variant	Expression
Wild type	1.0	INS Ala-294	0.41 ± 0.06
INS Ala-6	0.77 ± 0.46	INS Ala-295	0.75 ± 0.18
INS Ala-236	0.98 ± 0.06	INS Ala-297	0.43 ± 0.08
INS Ala-258	0.63 ± 0.15	INS Ala-298	0.68 ± 0.26
INS Ala-260	1.3 ± 0.27	INS Ala-299	0.69 ± 0.08
INS Ala-261	1.0 ± 0.06	INS Ala-300	0.55 ± 0.05
INS Ala-262	1.1 ± 0.16	INS Ala-301	0.94 ± 0.41
INS Ala-263	1.3 ± 0.29	INS Ala-303	1.0 ± 0.18
INS Ala-264	1.1 ± 0.03	INS Ala-304	1.0 ± 0.18
INS Ala-265	0.97 ± 0.21	INS Ala-305	0.61 ± 0.26
INS Ala-266	0.87 ± 0.43	INS Ala-306	0.96 ± 0.30
INS Ala-267	1.3 ± 0.15	INS Ala-307	0.96 ± 0.29
INS Ala-268	0.87 ± 0.11	INS Ala-309	0.62 ± 0.11
INS Ala-269	1.3 ± 0.29	INS Ala-310	0.88 ± 0.30
INS Ala-270	0.97 ± 0.28	INS Ala-311	0.38 ± 0.06
INS Ala-271	2.2 ± 0.37	INS Ala-312	0.71 ± 0.12
INS Ala-272	1.7 ± 0.59	INS Ala-313	0.80 ± 0.18
INS Ala-273	1.1 ± 0.20	INS Ala-314	0.55 ± 0.10
INS Ala-274	1.8 ± 0.31	INS Ala-316	0.61 ± 0.27
INS Ala-277	1.3 ± 0.39	INS Ala-317	1.3 ± 0.80
INS Ala-278	1.2 ± 0.43	INS Ala-318	1.2 ± 0.40
INS Ala-279	1.4 ± 0.33	INS Ala-319	0.45 ± 0.27
INS Ala-280	1.2 ± 0.46	INS Ala-320	0.65 ± 0.09
INS Ala-281	0.95 ± 0.18	INS Ala-321	0.63 ± 0.25
INS Ala-282	0.70 ± 0.25	INS Ala-322	0.41 ± 0.08
INS Ala-283	0.47 ± 0.16	INS Ala-323	0.66 ± 0.23
INS Ala-284	0.92 ± 0.37	INS Ala-325	0.46 ± 0.06
INS Ala-285	0.89 ± 0.325	INS Ala-326	0.27 ± 0.07
INS Ala-286	0.73 ± 0.08	INS Ala-327	0.74 ± 0.10
INS Ala-290	0.42 ± 0.01	INS Ala-330	0.70 ± 0.20
INS Ala-291	0.69 ± 0.10	INS Ala-333	0.63 ± 0.08
INS Ala-292	0.81 ± 0.08	INS Ala-358	1.2 ± 0.34
INS Ala-293	0.65 ± 0.10		

located in the SHS/TM8/SPS domains (e.g. Figure 4). On the other hand, GabP variants exhibiting moderate transport activities (e.g. insertions in the loop 8–9 segment) probably owe an appreciable fraction of their observed functional deficit to moderately reduced levels of expression.

Insertions placed in selected hydrophilic loops have moderate effects on [³H]GABA accumulation

GabP probably contains multiple polypeptide sequences that serve to connect domains that are directly involved in the transport process itself. It would be anticipated that the insertion of an alanine residue into such a connecting segment would have a minimal effect on function. Such robustly active insertion mutants can serve as benchmarks against which to judge the functional consequence of insertions placed in more vulnerable regions that play a functional role or contain elements of secondary structure (e.g. α-helices) that interact in critical ways with other parts of the protein. Several insertion-tolerant positions that presumably lack these functional roles and/or structural interactions have been identified in the present study.

Figure 4 Effects of alanine-insertion scanning mutagenesis on GabP expression levels and competitive uptake characteristics for NA and GABA

E. coli strains expressing the indicated GabP variants were grown and processed as indicated in the Experimental section. Washed cells (in 100 mM potassium phosphate, pH 7.0) were divided into two portions, one of which was used for the competitive uptake analysis and the other for immunoblot analysis. Competitive uptake: prewarmed cells were exposed for 1 min at 30 °C (initial rate) to a mixture of 4 μ M [3 H]NA (0.24 μ Ci/ml) and 6 μ M [14 C]GABA (0.12 μ Ci/ml) prior to processing for dual-label scintillation counting (see Experimental). Results are expressed as mean $\pm$ S.E.M. ($n=3$) uptake of either NA (grey bars) or GABA (black bars). Immunoblots: plasma membrane vesicle proteins (2 μ g per lane) were resolved by SDS/PAGE, transferred to a PVDF support, probed with an anti-penta-His monoclonal antibody and developed with a chemiluminescent alkaline phosphatase substrate (see Experimental). A summary of luminometric data from immunoblot analysis of several independent membrane preparations from each GabP variant is found in Table 3.

The GabP variant with an alanine insertion at position 6 in the cytoplasmic N-terminal domain exhibited steady-state [3 H]GABA accumulation (Figure 2) that was approx. 40% of that of the wild-type control (*E. coli* SK475), and initial rates of transport that were also approx. 40% of normal (Figure 4). The insertion made at position 236 (in the cytoplasmic loop connecting TM6 and TM7) resulted in a protein that exhibited approx. 30% of normal steady-state [3 H]GABA accumulation (Figure 2), and initial transport rates that were $>50\%$ of normal (Figure 4). Additionally, variants containing insertions made

C-terminal to the SPS (i.e. beyond position 320) in the cytoplasmic loop between TM8 and TM9 retained significant transport activity, as judged from either steady-state (Figure 3) or initial-rate (Figure 4) analyses. An entirely different outcome may be expected for insertions that perturb crucial interfaces (e.g. protein-substrate, or transport-related helix-helix interactions). The GabP CAR contains two functionally significant 20-residue segments, the SHS and SPS [14], wherein we might anticipate that an amino acid insertion would produce a 'loss of function' phenotype.

Alanine insertions placed within the SHS exert a highly deleterious effect on transport function

The GabP region containing residues 266–285 is modelled as an amphipathic helical segment, displaying both polar and non-polar surfaces. This region contains a functionally defined SHS, within which cysteine-scanning mutagenesis has led to identification of GabP variants that exhibit loss of function and $\Delta(\text{TSR})$ phenotypes at positions 267, 271, 272, 273, 277, 282 and 285 [14], indicating that the SHS contains specificity determinants that influence intrinsic substrate binding energy, ΔG_b (eqn 1). Thus inserting an extra alanine residue at points throughout SHS would be anticipated to perturb ΔG_b via effects on its delocalized component (eqn 2).

Indeed, the present study shows that, of the 18 GabP variants with alanine insertions located within the SHS, all exhibited a seriously compromised transport function. Steady-state [^3H]GABA accumulation levels (after 4 min) were only slightly in excess of those in the GabP-negative strain SK45 for all insertion variants involving the SHS region (Figure 2). This contrasts with results obtained with (i) SK475, the GabP-positive control strain, which accumulated [^3H]GABA to levels that were about 50-fold above those by SK45 (Figure 2), and (ii) other GabP variants with alanine insertions located in presumptive hydrophilic loops (e.g. positions 6, 236, 329 and 330), for which measured [^3H]GABA accumulation ranged from 20- to 60-fold above that of the GabP-negative control (Figures 2 and 3).

Initial transport rates were also severely affected in GabP variants having alanine insertions located within the SHS. Initial-rate competitive uptake assays performed with [^3H]NA and [^{14}C]GABA (Figure 4) showed that transport activity was reduced essentially to background levels in cases where alanine insertions were placed within the C-terminal two-thirds (residues 271–285) of the SHS. Somewhat greater residual transport activities (approx. 5–20% of normal) were observed for insertions affecting the N-terminal one-third (residues 264–270) of the SHS (i.e. near the periplasmic end of TM7). These greater residual transport activities are hypothesized to result from the limited surface element offsets that might be expected at locations where helical secondary structures meld into random coil.

Alanine insertions placed within the SPS exert a highly deleterious effect on transport function

The GabP region from positions 299 to 318 has been modelled as amphipathic helical segment containing a functionally defined [9] SPS. A cysteine-scanning study revealed that the SPS contains multiple specificity determinants [14], i.e. loci at which a change in the amino acid side-chain structure is associated with a $\Delta(\text{TSR})$ phenotype. Since a change in the TSR reflects an underlying change in the intrinsic substrate binding energy, ΔG_b (eqn 1), it may reasonably be anticipated that the insertion of an extra alanine residue at points throughout the SPS would likewise perturb ΔG_b via effects on its delocalized component (eqn 2).

In keeping with this expectation, steady-state [^3H]GABA accumulation exhibited by the 19 GabP variants having alanine insertions within the SPS were 15–50-fold below that of the GabP-positive control strain, *E. coli* SK475 (Figure 3), leaving residual activities that in many cases were well above that of the GabP-negative strain, SK45. Alanine insertions across the SPS were also observed to have a severe impact on initial transport rates measured in competitive uptake assays with [^{14}C]GABA and [^3H]NA (Figure 4), and this pattern persisted for insertions made several residues to the C-terminal side of the SPS (up to position 320). For insertions beyond position 320, larger competitive

Table 3 TSR values of GabP alanine insertion variants

Prewarmed cells were exposed for 1 min at 30°C (initial rate) to a mixture of 4 μM [^3H]NA (0.24 $\mu\text{Ci/ml}$) and 6 μM [^{14}C]GABA (0.12 $\mu\text{Ci/ml}$) prior to processing the sample for dual-label scintillation counting as described in the Experimental section. Results are given as the mean $\pm$ S.E.M. ($n = 3$). The TSR is an expression-independent, intrinsic property of the carrier–substrate interaction [14]. Strains having a TSR that differs from that of the GabP–His₁₀ control are said to exhibit a $\Delta(\text{TSR})$ phenotype, and this reflects a substrate-specific change in the intrinsic binding energy (see eqn 2). Many strains have been omitted from the Table, since insertion mutagenesis frequently abolished transport activity. Representative time courses are shown in Figure 5 for several exemplar strains that exhibit a substantial $\Delta(\text{TSR})$ phenotype.

GabP variant	TSR	GabP variant	TSR
GabP–His ₁₀	3.8 $\pm$ 0.37	INS Ala-286	0.04 $\pm$ 0.01
INS Ala-6	5.6 $\pm$ 0.35	INS Ala-290	0.08 $\pm$ 0.02
INS Ala-236	2.4 $\pm$ 0.02	INS Ala-297	0.03 $\pm$ 0.01
INS Ala-260	0.07 $\pm$ 0.01	INS Ala-303	0.20 $\pm$ 0.09
INS Ala-262	0.50 $\pm$ 0.02	INS Ala-307	0.04 $\pm$ 0.01
INS Ala-263	4.0 $\pm$ 0.18	INS Ala-309	0.40 $\pm$ 0.36
INS Ala-264	7.5 $\pm$ 0.99	INS Ala-313	0.04 $\pm$ 0.01
INS Ala-265	1.6 $\pm$ 0.04	INS Ala-316	5.0 $\pm$ 0.51
INS Ala-266	0.43 $\pm$ 0.04	INS Ala-321	19.0 $\pm$ 0.55
INS Ala-267	0.52 $\pm$ 0.02	INS Ala-322	7.0 $\pm$ 0.24
INS Ala-268	3.0 $\pm$ 0.64	INS Ala-323	8.7 $\pm$ 3.07
INS Ala-269	0.55 $\pm$ 0.15	INS Ala-325	22.0 $\pm$ 1.01
INS Ala-270	1.4 $\pm$ 0.36	INS Ala-326	4.6 $\pm$ 1.81
INS Ala-278	0.14 $\pm$ 0.04	INS Ala-327	6.4 $\pm$ 0.69
INS Ala-279	2.8 $\pm$ 8.34	INS Ala-330	5.2 $\pm$ 0.32
INS Ala-280	7.2 $\pm$ 0.89	INS Ala-333	3.5 $\pm$ 0.09
INS Ala-281	0.63 $\pm$ 0.49	INS Ala-358	1.9 $\pm$ 0.51
INS Ala-285	0.13 $\pm$ 0.04		

uptake activities were observed, presumably owing to the smaller surface element offsets that are likely to occur in regions where helical secondary structures undergo transitions into nondescript loops.

Alanine insertions placed within TM7 or TM8 exert a deleterious effect on transport function

Based on topological studies with GabP–LacZ and GabP–PhoA constructs [4], TM7 and TM8 have been assigned to the approximate regions comprising residues 246–265 and 291–309 respectively. Thus the functionally defined SHS [14] is sandwiched tightly between TM7 and TM8, with little in the way of connecting loops between these three putative domains. If the entire TM7–SHS–TM8 segment were to behave as though composed primarily of α -helix, then surface group offsets up to 100° would be anticipated [16] on either side of alanine insertions throughout the region. Large offsets such as these would be expected to (i) interfere with helix–helix packing in regions of structural significance such as transmembrane segments, and to (ii) cause large disturbances in the delocalized component of ΔG_b (eqn 2) in regions of functional significance. As a result, many loss of function and/or $\Delta(\text{TSR})$ phenotypes would be anticipated among the collection of GabP variants with alanine insertions located within the TM7–SHS–TM8 segment.

Indeed, the pattern of deficits (Figure 2) in steady-state [^3H]GABA accumulation observed for GabP variants with insertions in TM7 (residues 258–265) continues through the SHS, through TM8, and all the way through the SPS (Figure 3), the latter domain commencing midway through the bilayer and extending into the cytoplasm (Figure 1). A pattern of greater residual transport activity is not observed until we encounter the GabP variants with insertions that are placed in the region comprising residues 320–330, well into the loop connecting the SPS to TM9.

Figure 5 Time courses for competitive uptake of GABA and NA by selected GabP mutants with an alanine insertion in the CAR

E. coli SK45 (GabP-negative) and strains expressing either GabP-His₁₀ (wild type) or its variants with the indicated alanine insertions were grown to early exponential phase and washed with 100 mM potassium phosphate buffer (pH 7.0) as described in the Experimental section. Dual-label competitive transport reactions were initiated by exposing the cells to 4 μ M [³H]NA (■; 0.24 μ Ci/ml) and 6 μ M [¹⁴C]GABA (▲; 0.12 μ Ci/ml). After the indicated times, the reactions were quenched, filtered and processed for dual-label scintillation counting as described in the Experimental section. In all panels, the GabP-dependent component of competitive uptake (signal from the GabP variant minus the SK45 signal) is expressed as d.p.m. for facile comparison with the GabP-His₁₀ control (top left panel). The indicated substrate mixture was adjusted empirically to exhibit an equal d.p.m. signal in both isotope channels under initial-rate conditions. Separation of the curves from one another (e.g. panel INS Ala285) thus reflects a change in TSR. It is noted that these empirically established substrate concentrations differ slightly from those employed with the previously studied Cys-less GabP mutant, since the latter exhibits a TSR value approx. 2.3-fold greater [14] than the wild-type control (which has five cysteine residues) studied here (see Table 3).

Overall, these results are consistent with the presence of functionally significant secondary structure across the entire CAR.

Residual transport activities and demonstrable Δ (TSR) phenotypes are associated with insertions placed either at the margins of functionally significant surfaces and/or in presumptive secondary structures

The displacement of surface residues on either side of an alanine insertion should depend upon whether the insertion is placed in an unstructured connecting loop or a helical segment. In the lower limit (zero displacement of surface residues from their natural positions), there is no basis to expect any intrinsic binding energy perturbations that would in turn change the measured TSR (eqn 1). However, engineering the insertion into a region with more helical character is likely to result [16] in greater surface group displacement – up to the 100° α -helical limit for which a profound change in specificity (i.e. loss of function) might be anticipated.

Thus with regard to the present study, it probably is not entirely coincidental that the more modest (i.e. functional with known substrates) specificity shifts were associated with GabP variants

having alanine insertions located near the end of a putative element of secondary structure – whether defined topologically (TM segments) [4] or functionally (SHS and SPS) [9,14]. Inspection of Figure 4 reveals a general pattern in which somewhat greater residual transport activities were associated with mutants that have an insertion near (i) the TM7–SHS margin, (ii) the SHS–TM8 margin, (iii) the SPS–(loop 8–9) margin, and (iv) the TM9–(loop 9–10) margin (position 358). Furthermore, insertions located near these boundaries often exhibited differential effects (Figure 4, compare relative heights of dark and light bars for different GabP variants) on the initial rates of [¹⁴C]GABA and [³H]NA transport, leading to a Δ (TSR) phenotype (Table 3). The TSR is an interesting parameter, because (i) it is independent of the amount of carrier protein expressed, and (ii) it changes as a simple function of the intrinsic binding energy (ΔG_b) realized in the rate-limiting transition state [14], which is the conformational state most crucial to the expression of specificity (k_{cat}/K_m) by carrier proteins [14,21] and enzymes [22].

Time courses (Figure 5) offer a convenient means by which to visualize the practical consequences of a Δ (TSR) phenotype with respect to a particular substrate pair, here [¹⁴C]GABA and [³H]NA. It is possible to empirically adjust substrate concentrations and specific radioactivities such that, for the wild-type control, a

pair of overlapping curves (Figure 5, Wild Type) is obtained in the initial-rate segment of the time course. Any separation between these curves is easily observed, reflects the presence of a Δ (TSR) phenotype and lends itself to a highly intuitive functional interpretation as to which member of the substrate pair is preferred by a given transporter variant. In the present study, it is clear that insertions within the CAR most commonly lead to a specificity shift in the direction favouring [3 H]NA uptake (Figure 5). Possibly, this relative 'down-shifting' of GABA specificity is due to optimization of the transporter for the natural substrate. If GabP is truly optimized for GABA transport, then structural changes would almost always be unfavourable. In any event, it is the case that specificity shifts that favour [14 C]GABA over [3 H]NA uptake are less common among the insertion mutants studied here (Figure 5), and this generalization applies also to missense mutations within the CAR [14]. Other examples of insertion-induced specificity shifts in either direction can be found in Table 3. It is worth noting that Δ (TSR) phenotypes induced by amino acid insertions (and probably deletions?) should be inherently selectable, a fact that could have quite broad implications with respect to the evolution of size differences among transporters [23].

Mid-helix insertion 'loop-out' is improbable

It is worth noting that inserted amino acid residues have been known to 'loop-out' of the helix body in certain proteins of known crystal structure – particularly when helix-breaking glycine residues are inserted [24,25]. But this is emphatically rare even for these soluble proteins, where helix-forming forces are generally weaker than in the lipid bilayer. The α -helix is favoured so strongly in the lipid bilayer that the canonical helix breakers [26], Pro and Gly, are in the membrane environment found to be good helix-formers [27,28], with significant roles in protein folding [29] and tertiary interactions [30]. In other words, helix-breaking loop-out is likely to be even more unusual in membrane proteins than in soluble proteins, where loop-out is atypical [16].

Regardless, how might insertion loop-out affect interpretation of the results of the present study? Loop-out acts to preserve secondary structure, and structural preservation does not offer a reasonable explanation for the present results – except possibly for proteins with insertions around positions 265, 285, 290 and 321, for which substrate specificity shifts (Figure 5) and larger than average residual activities (Figure 4) were noted. Consistent with the idea that these positions are associated with breaks in the helical structure of the CAR, loop-out is known to occur more frequently near helix termini than in helix bodies [16]. If we entertain the less likely [16] possibility that loop-outs have occurred in the body of a helix, then implicitly we are endorsing the idea that the CAR contains uninterrupted helical tracts that are far longer than the minimum required to span a lipid bilayer. Either way, the data are consistent with the presence of considerable secondary structure across the 70-residue GabP CAR.

Summary

A large collection of GabP variants with alanine insertions placed at unique locations across the CAR has been studied. Overall, the insertions produced deleterious functional effects across the entire TM7–SHS–TM8–SPS region, although somewhat larger residual transport activities were associated with proteins with alanine insertions positioned at the margins of each domain. Many of these residual activities were characterized as having a Δ (TSR) phenotype when assessed with [14 C]GABA and [3 H]NA. The work is consistent with the following: (i) the GabP CAR is

unlikely to contain large loops interconnecting its functionally significant elements of secondary structure, (ii) insertions can be used to manipulate substrate specificity (k_{cat}/K_m), and (iii) insertion-induced specificity shifts arise, at least in part, from classical concepts that underpin the specificity delocalization principle [14].

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