Use of the transport specificity ratio and cysteine-scanning mutagenesis to detect multiple substrate specificity determinants in 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, and other members of the APC (amine/polyamine/choline) transporter superfamily share a CAR (consensus amphipathic region) that probably contributes to solute translocation. If true, then the CAR should contain structural features that act as determinants of substrate specificity (k_{cat}/K_m). In order to address this question, we have developed a novel, expression-independent TSR (transport specificity ratio) analysis, and applied it to a series of 69 cysteine-scanning (single-cysteine) variants. The results indicate that GabP has multiple specificity determinants (i.e. residues at which an amino acid substitution substantially perturbs the TSR). Specificity determinants were found: (i) on a hydrophobic surface of the CAR (from Leu-267 to Ala-285), (ii) on a hydrophilic surface of the CAR (from Ser-299 to Arg-318), and (iii) in a cytoplasmic loop (His-233) between transmembrane seg-

ments 6 and 7. Overall, these observations show that (i) structural features within the CAR have a role in substrate discrimination (as might be anticipated for a transport conduit) and, interestingly, (ii) the substrate discrimination task is shared among specificity determinants that appear too widely dispersed across the GabP molecule to be in simultaneous contact with the substrates. We conclude that GabP exhibits behaviour consistent with a broadly applicable specificity delocalization principle, which is demonstrated to follow naturally from the classical notion that translocation occurs synchronously with conformational transitions that change the chemical potential of the bound ligand [Tanford (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2882–2884].

Key words: γ -aminobutyric acid (GABA), carrier, catalysis, mutagenesis, permease, transport.

INTRODUCTION

The *gab* permease (TC 2.A.3.1.4) [1] known as GabP (encoded by *gabP*) is a 12-helix [2] plasma membrane protein that functions as the exclusive mediator of GABA (γ -aminobutyric acid) accumulation by *Escherichia coli* [3,4]. GabP, an archetypal member of the APC (amine/polyamine/choline) transporter superfamily [5,6], has been cloned [7], overexpressed under the control of the *lac* promoter [4], and subjected to extensive pharmacological characterization, both in whole cells [4,8,9] and in soluble preparations [10]. These studies have shown that the *E. coli* GABA transporter recognizes classical inhibitors [e.g. NA (nipecotic acid), guvacine, muscimol and *cis*-4-aminocyclohexylcarboxylic acid] of the mammalian GAT-1 GABA transporter, even though the two proteins belong to distinct superfamilies, and share limited primary sequence similarity in a region known as the CAR (consensus amphipathic region) [11].

Structural perturbations on the polar surface of the CAR are strongly correlated with a deleterious impact on the transport function of GabP, leading to the identification of an SPS (sensitive polar surface) [12], which plays a role in substrate translocation, possibly as part of the translocation pathway. The SPS contains a so-called 'signature cysteine' (Cys-300) that is common to the sequences of many different bacterial and mammalian GABA transporters. GABA transport is known to have a significant

quantitative dependence upon the structural integrity of Cys-300, which lies near the beginning of the SPS at the bilayer core. However, the qualitative behaviour of GabP variants modified at position 300 is normal. The Cys-less GabP variant exhibits the same affinities and rank order of preference as the wild type across a spectrum of structurally distinctive competitive ligands (kojic amine, 5-aminovaleric acid, GABA, NA, *cis*-4-aminocrotonic acid) [13]. These data suggest that the active-site architecture of the Cys-less GabP cannot differ markedly from that of the wild type. Thus, like its wild-type parent, the Cys-less variant of *E. coli* GabP recognizes classical inhibitors of mammalian GAT-1, and can serve as a model to study the underlying basis for this pharmacological similarity – a functional characteristic that is absent from *Bacillus subtilis* GabP, which rejects all of the classical heterocyclic neural-active GABA uptake inhibitors [14].

In order to understand the basis of the pharmacological similarity between GAT-1 and *E. coli* GabP, it will be important to locate the substrate binding domains in both of these proteins. The present study uses Cys-less GabP as the point of reference to investigate whether the CAR plays a role in determining substrate specificity. Amino acid residues that line a translocation pathway would be expected to (i) make contact with (i.e. bind to) transported substrates, and to thereby act as substrate specificity determinants [15,16], and (ii) be involved in the helix–helix realignments that are needed to change substrate binding affinity

Abbreviations used: APC superfamily, amine/polyamine/choline superfamily; CAR, consensus amphipathic region; GABA, γ -aminobutyric acid (4-aminobutyric acid); His₁₀, decahistidine tag; IPTG, isopropyl β -D-thiogalactopyranoside; LB, Luria broth; NA, nipecotic acid (3-piperidine carboxylic acid); PBS-TM, PBS supplemented with 0.05% (v/v) Tween 20 and 0.5% (w/v) non-fat dry milk; PBS-TTX, PBS supplemented with 0.05% (v/v) Tween 20 and 0.2% (v/v) Triton X-100; 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

Plasmids	Relevant genotype or comments	Source or reference
pSCK-380	<i>amp^r lac^P tacO⁺ P⁺</i>	[4]
pSCK-380-Hx10	pSCK-380 with a His ₁₀ -encoding cassette in the <i>Pst</i> I/ <i>Xho</i> I sites	This work
pSCK-GP11	pBluescript II KS(–) derivative in which <i>gabP</i> encodes a cysteine-less [13] transporter (Cys to Ala at codons 158, 251, 291, 300 and 443), and has its stop codon replaced by a <i>Pst</i> I restriction site to allow construction of GabP–His ₁₀ fusions	This work
pSCK-GP11-XYZ	Generic nomenclature to describe pSCK-GP11 derivatives in which codon number Y is altered in order to substitute amino acid Z for amino acid X; e.g. pSCK-GP11-S299C denotes the <i>gabP</i> mutant that encodes a cysteine at position 299	This work
pSCK-380-CL-Hx10	A pSCK-380-Hx10 derivative in which the chloramphenicol-resistance cassette is replaced by the <i>gabP</i> cassette taken from pSCK-GP11 using <i>Xba</i> I/ <i>Pst</i> I	This work
pSCK-380-XYZ-Hx10	Generic nomenclature to describe a pSCK-380-Hx10 derivatives in which the chloramphenicol-resistance cassette is replaced by the <i>gabP</i> cassette taken from pSCK-GP11-XYZ using <i>Xba</i> I/ <i>Pst</i> I	This work

Strain	Chromosome/plasmid	Source or reference
SK35	<i>lac</i> ⁺ Δ(<i>ZY</i>) <i>gab</i> Δ <i>P</i> (<i>kan</i> ^r –1)/–	[4]
SK45	SK35/pSCK-380	[4]
SK11	SK35/pSCK-380-CL-Hx10	This work

during transit and to provide bound substrates with ‘alternating access’ to the aqueous compartments on either side of the membrane [17,18].

Here we describe a novel dual-isotope TSR (transport specificity ratio) method that detects perturbations in specificity (k_{cat}/K_m) that are attributable to changes in intrinsic substrate binding energy. The TSR analysis was applied to a bank of 69 GabP cysteine-scanning mutants constructed to span the region between TM7 (transmembrane segment 7) and TM9 (i.e. the entire CAR). Several substrate specificity determinants that are able to discriminate GABA from NA were found along the SPS and, surprisingly, also on a hydrophobic surface of the CAR that was not previously recognized as having any functional significance. The presence of these specificity determinants indicates that elements of the CAR are involved in discriminating between structurally distinct substrates, and suggests a close association with interfaces involved in transport-related conformational transitions.

EXPERIMENTAL

Materials

E. coli strains and plasmids are detailed in Table 1. GABA and PMSF were from Sigma (St. Louis, MO, U.S.A.); NA was from Research Biochemicals International (Natick, MA, U.S.A.); Miller’s LB (Luria broth) medium was from Gibco-BRL (Grand Island, NY, U.S.A.); agar and ampicillin were from Fisher Biotech (Fair Lawn, NJ, U.S.A.); the plasmid pBluescript II KS(–) was from Stratagene (La Jolla, CA, U.S.A.); restriction enzymes *Xba*I, *Pst*I and *Sna*BI, T4 DNA ligase and T7 DNA polymerase were from New England Biolabs (Beverly, MA, U.S.A.); oligonucleotides were obtained from Invitrogen (Carlsbad, CA, U.S.A.); bicinchoninic acid protein determination reagents were from Pierce (Rockford, IL, U.S.A.); cellulose acetate filters (0.45 µm; 25 mm) either were from Millipore (Bedford, MA, U.S.A.) or were MicronSep (cellulosic) from Osmonics Inc. (Minnetonka, MN, U.S.A.); [³H]NA (40 Ci/mmol) was a custom synthesis from Moravsek Biochemicals (Brea, CA, U.S.A.); [¹⁴C]GABA was from Dupont-New England Nuclear (Boston, MA, U.S.A.); Ultima GoldTM scintillation cocktail was from Packard BioScience (Meriden, CT, U.S.A.); anti-penta-His monoclonal antibody was from Qiagen (Valencia, CA, U.S.A.); goat anti-mouse alkaline phosphatase-conjugated antibody was

from Kirkegaard and Perry Laboratories (Gaithersburg, MD, U.S.A.); IPTG (isopropyl β-D-thiogalactopyranoside) was from Anatrace (Maumee, OH, U.S.A.); protease inhibitor cocktail tablets and PCR nucleotide mix were from Roche (Mannheim, Germany); Immobilon-PTM transfer membranes (0.45 µm) were from Millipore; the chemiluminescence reagent for alkaline phosphatase detection (Western Lightning) was from Perkin-Elmer Life Sciences, Inc. (Boston, MA, U.S.A.); TEMED (*N,N,N,N*’-tetramethylethylenediamine), ammonium persulphate and the acrylamide/Bis solution were from Bio-Rad Laboratories (Hercules, CA, U.S.A.); KathonTM was from Supelco (Bellefonte, PA, U.S.A.).

Construction of pSCK-380-Hx10

The plasmid pSCK-475 [12] expresses a GabP–LacZ hybrid protein (i.e. a LacZ-tagged GabP). In order to construct an analogous vector that would produce His-tagged GabP, the *lacZ* cassette from pSCK-475 was excised using *Pst*I/*Xho*I, and replaced with the synthetic His₁₀ cassette, CTGCAGaccaccac-caccaccaccatcatcatcaccatggcgcgccgtaaCTCGAG, where the *Pst*I and *Xho*I restriction enzyme sites are shown in upper case, and the sequence encoding the decahistidine sequence tag is shown in bold. The *gabP* cassette in the *Xba*I/*Pst*I sites of the resulting construct was replaced with a chloramphenicol-resistance cassette, which was derived by PCR from the phagemid pBC (Stratagene). The resulting plasmid, called pSCK-380-Hx10, was used to subclone *gabP* cassettes for expression of transporters bearing a C-terminal His₁₀ tag.

Site-directed mutagenesis

Mutagenesis was performed as described previously [12,13] by the method of Kunkel [19]. Briefly, single-stranded DNA from the phagemid pSCK-GP11 was produced from the *E. coli* strain CJ236 (from Bio-Rad), following infection with the kanamycin-resistant helper phage VCSM13 (obtained from Stratagene). Three internal primers were used to sequence *gabP* at the OHSU-MMI Research Core Facility using ‘Big-Dye’ terminators (Applied Biosystems Inc.) and a model 377 Applied Biosystems Inc. automated fluorescence sequencer. The *gabP* was subcloned into the *Xba*I/*Pst*I sites of the expression vector pSCK-380-Hx10. In order to evaluate the transport phenotype, the desired GabP–His₁₀ expression constructs were placed in *E. coli* SK35 (*gabP*-negative) by transformation. For mutants exhibiting a substantial

phenotypic change (e.g. see Figure 9), *gabP* was additionally sequenced from the expression vector to exclude any possibility of misidentification through mishandled subcloning, etc.

Standard preparation of cells for transport

E. coli strains were always streaked from frozen (-80°C) glycerol stocks to produce single colonies on LB agar supplemented with ampicillin ($100\text{ }\mu\text{g/ml}$). A single colony was picked to LB broth supplemented with ampicillin ($100\text{ }\mu\text{g/ml}$), and then shaken overnight (16 h) at 37°C . The overnight cultures were diluted 100-fold into fresh medium, shaken for 2 h at 37°C prior to adding IPTG (0.2 mM), and then shaken for 2 h more. Cells were then harvested by centrifugation (3000 g for 10 min in a Sorvall SS-34 rotor), washed twice with ice-cold 100 mM potassium phosphate, pH 7.0, and resuspended to 2 mg of protein/ml in the same buffer (20% of the original culture volume). Cultures treated in this manner are referred to hereafter as washed cells. Washed cells were stored on ice, and then equilibrated to 30°C in a heat block (25 min) prior to initiating transport reactions. Cultures treated in this manner are referred to hereafter as prewarmed cells.

Transport reactions

Transport reactions were initiated by adding $80\text{ }\mu\text{l}$ of prewarmed cells with rapid vortexing to $20\text{ }\mu\text{l}$ of a prewarmed substrate mixture containing $35\text{ }\mu\text{M}$ [^3H]NA ($2.1\text{ }\mu\text{Ci/ml}$) and $15\text{ }\mu\text{M}$ [^{14}C]GABA ($0.3\text{ }\mu\text{Ci/ml}$). A 60 or 120 Hz metronome was used to time the reactions, which were quenched rapidly with 1 ml of ice-cold stop solution (100 mM potassium phosphate, pH 7.0, containing 20 mM HgCl_2), and then vacuum-filtered ($0.45\text{ }\mu\text{m}$ pore). The reaction vessel was then rinsed with 1 ml of wash buffer (100 mM potassium phosphate, pH 7.0, containing 5 mM HgCl_2) and this was applied to the same filter. Finally, 4 ml of the wash buffer was applied to the filter. The filter was then dissolved in Ultima GoldTM scintillation cocktail, and ^3H and ^{14}C radioactivity (d.p.m.) was analysed with a Packard BioScience Tri-Carb 2900 TR liquid scintillation counter using stored Ultima GoldTM quench curves and automatic quench compensation. Where indicated, results are reported as TSRs:

$$\text{TSR} = (v_{\text{GABA}}/v_{\text{NA}}) \times ([\text{NA}]/[\text{GABA}]) \quad (1)$$

where v_{GABA} and v_{NA} are the initial rates of transport (10 s time points) for [^{14}C]GABA and [^3H]NA respectively. The easily measured TSR is an expression-independent parameter with the algebraic equivalencies (see Appendix) set forth in eqn (2):

$$\text{TSR} = \frac{(\text{specificity})_{\text{GABA}}}{(\text{specificity})_{\text{NA}}} = \frac{(k_{\text{cat}}/K_{\text{m}})_{\text{GABA}}}{(k_{\text{cat}}/K_{\text{m}})_{\text{NA}}} = e^{(-\Delta\Delta G_{\text{b}}/RT)} \quad (2)$$

where R is the gas constant, T is the absolute temperature, and $\Delta\Delta G_{\text{b}}$ is the change in intrinsic ligand binding energy due to the structural difference between GABA and NA.

Plasma membrane vesicle preparation

Washed cells (0.4 ml) were added to a microfuge tube containing 1 ml of Tris buffer (150 mM Tris, adjusted to pH 7.0 with HCl) supplemented with 0.3 mM PMSF (added from a 300 mM stock solution freshly prepared in ethanol). The microfuge tube was

centrifuged at 10000 g for 60 s at 4°C , the supernatant removed by aspiration, and the cell pellet resuspended in $450\text{ }\mu\text{l}$ of anti-protease cocktail (1 CompleteTM Mini anti-protease pill per 10 ml of Tris buffer). Cells were broken using a KontesTM micro ultrasonic cell disrupter for 15 s with the power setting at 5. Sonication was repeated two more times, allowing 5 min of cooling on ice between sonications. Unbroken cells were removed by centrifugation at 10000 g for 60 s in a microfuge at 4°C . Plasma membrane vesicles were harvested from the supernatant ($400\text{ }\mu\text{l}$) by ultracentrifugation in a Beckman A-95 Airfuge rotor operated at 94000 rev./min for 5 min at room temperature. The supernatant was removed, and the nearly transparent pellet was rinsed (not resuspended) with $500\text{ }\mu\text{l}$ of Tris buffer containing 0.3 mM PMSF. After aspirating the rinse buffer, the pellet was dissolved in $30\text{ }\mu\text{l}$ of 2% (w/v) SDS plus $30\text{ }\mu\text{l}$ of anti-protease cocktail. Solubilized membrane protein preparations ($1\text{--}2\text{ mg/ml}$) were stored at -80°C , and then thawed on ice to prepare samples for SDS/PAGE.

Immunoblots

SDS/PAGE was performed with a 5% (w/v) acrylamide stacking gel and a 10% (w/v) acrylamide resolving gel. Proteins were transferred to PVDF membranes using a Bio-Rad Trans-Blot SD semi-dry transfer cell with Towbin transfer buffer (15.6 mM Tris base and 120 mM glycine). Following transfer, the membranes were rinsed briefly in water, and then blocked in PBS-TM [PBS (136 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4 and 8.3 mM Na_2HPO_4 , pH 7.4) supplemented with 0.05% (v/v) Tween 20 and 0.5% (w/v) non-fat dry milk] for 60 min at room temperature. PBS-TM was prepared by adding 10 g of non-fat dry milk powder and 1 ml of Tween 20 to 2 litres of PBS. The mixture was heated to 70°C , and then stirred at 4°C overnight. The solution was filtered ($0.45\text{ }\mu\text{m}$; Millipore HVLP), and preserved by addition of either KathonTM (0.04%) or sodium azide (0.05%).

The blocked membranes were washed for 5 min in PBS-TTX [PBS supplemented with 0.05% (v/v) Tween 20 and 0.2% (v/v) Triton X-100] and then incubated overnight at room temperature with anti-penta-His monoclonal antibody (1:3000 dilution) in PBS-TM. The next day, the membranes were washed for $2 \times 10\text{ min}$ in PBS-TTX and $1 \times 10\text{ min}$ in PBS before being incubated for 60 min at room temperature with an alkaline phosphatase-conjugated anti-mouse antibody (1:20000 dilution) in PBS supplemented with 10% (w/v) non-fat dry milk. The membrane was then washed for 10 min in PBS supplemented with 10% non-fat dry milk, for 10 min in PBS supplemented with 5% non-fat dry milk, for $2 \times 10\text{ min}$ in TBS-TTX (TBS supplemented with 0.05% Tween and 0.2% Triton X-100), for 5 min in PBS, and finally for 5 min in 0.1 M Tris/HCl (pH 9.5).

Immunoblots were developed with a chemiluminescent alkaline phosphatase substrate (Western LightningTM), and visualized with a cooled CCD camera (Kodak Image Station 440 CF). Chemiluminescent intensities were quantified with Kodak 1D software.

RESULTS

In order to test the idea that the GabP CAR (Figure 1) has a role in determining substrate specificity ($k_{\text{cat}}/K_{\text{m}}$), a novel TSR analysis was used to detect substrate-selective perturbations in $k_{\text{cat}}/K_{\text{m}}$ in a series of 69 Cys-scanning mutants. The TSR method is a dual-label transport assay in which one measures the initial rate of uptake of two substrates – e.g. [^{14}C]GABA and [^3H]NA – competing for the same active site. Competition between $3\text{ }\mu\text{M}$

Figure 1 Working model of secondary structure across the GabP CAR

A generic 12-helix membrane protein is shown with several 'expanded views' depicting a hypothetical secondary structural arrangement in which the GabP amino acid sequence exhibits α -helical character both (i) within the periplasmic loop 7–8 region between TM7 and TM8, and (ii) within the TM8/loop 8–9 region that leads into the cytoplasm. Although a break in the helical structure is shown at Lys-286, this serves mainly to limit the vertical size of the drawing, and the precise distribution of secondary structure remains unknown (note that the black and white ovals emphasize opposite sides of the same periplasmic segment). The helical region(s) depicted in this drawing comprise an evolutionarily conserved segment known as the CAR, that is found in GabP and other members of the APC transporter superfamily [12]. The CAR exhibits an extensive polar surface (black ribbon) that could be part of a conduit involved in the translocation of polar solutes. Indeed, portions of the CAR may exist in a channel-like environment, since Cys-300 (at the centre of the membrane) reacts with *p*-chloromercuribenzenesulphonate (which resembles guvacine, a transported substrate [9]), but not with other hydrophilic thiol reagents [20]. Consistent with a role in substrate translocation, the GabP CAR includes a 20-residue segment (the SPS; grey box) within which functional integrity is correlated with structural integrity of the polar surface [12]. Residues from the SPS region are also important to the function of PheP [21], suggesting that the model has generality. The present study extends these observations, showing that specificity determinants, able to distinguish GABA from NA, are found both (i) on the SPS (Figure 9) and, unexpectedly, also (ii) on an ostensibly periplasmic hydrophobic surface (white ribbon) of the helical segment extending from residues 267 to 285 (see Figures 3 and 4) where transport defects are observed at intervals of three to four residues.

[^{14}C]GABA (0.06 $\mu\text{Ci/ml}$) and 7 μM [^3H]NA (0.42 $\mu\text{Ci/ml}$) was found empirically to produce uptake that was linear with time to 20 s at 30 °C with almost identical signal levels from both isotopes (Figure 2). Establishment of signal equality is not required, but does facilitate the visual recognition of changes that either increase or decrease the TSR.

With respect to *E. coli* strain SK11, which expresses the Cys-less GabP, correction for background [^{14}C]GABA or [^3H]NA uptake could be accomplished equivalently either by performing parallel experiments with an excess of unlabelled GABA (Figure 2A) or by performing parallel experiments with the GabP-negative strain SK45 (Figure 2B). However, with respect to analysing uncharacterized mutants, the latter approach was adopted as the more reliable option, since GABA might sometimes fail

Figure 2 Time course for competitive uptake of GABA and NA catalysed by the Cys-less GabP

E. coli SK11 or SK45 (GabP-negative) was 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 7 μM [^3H]NA (0.42 $\mu\text{Ci/ml}$) and 3 μM [^{14}C]GABA (0.06 $\mu\text{Ci/ml}$). After the indicated intervals at 30 °C, the reactions were quenched, filtered and processed for dual-label scintillation counting as described in the Experimental section. Background was assessed in parallel experiments either by addition of excess GABA (2 mM) along with the labelled substrates (A) or by using the GabP-negative strain SK45 (B). In both panels, the GabP-dependent component of competitive uptake (SK11 signal minus background) is expressed as d.p.m. to emphasize that, for the Cys-less construct, the chosen conditions provide the same signal intensity from [^3H]NA (■) and [^{14}C]GABA (▲). A skewing of results in favour of one isotope or the other will thus reflect effects on k_{cat}/K_m that are substrate-selective, altering the ability of a GabP variant to discriminate between the structures shown in the inset of (A).

to adequately inhibit GabP-mediated [^3H]NA uptake, perhaps especially in the case of mutants that impact on substrate specificity.

Effects of IPTG on competitive uptake and TSR

Plasmid pSCK-380-Hx10 allows His-tagged GabP to be expressed under the control of the *lac* promoter, such that when cells are grown with exposure to a range of IPTG concentrations (0–1000 μM), both substrate uptake and GabP-His₁₀ protein levels (assessed by luminometry of immunoblots probed with an anti-penta-His monoclonal antibody) are increased 40-fold (results not shown). Despite a 40-fold change in transport activity and protein expression levels, the TSR held steady at approx. 8 across the entire range of IPTG concentrations. The TSR for a given substrate pair is theoretically (see the Appendix) an intrinsic property of the transport protein itself, independent of its expression level. Thus, if the TSR phenotype of a transporter variant is found to differ dramatically from its parent, then this reflects an underlying change in substrate preference (i.e. a change in the specificity parameter, k_{cat}/K_m), whether or not there has been any change in the transporter expression level.

Cys scanning in the GabP CAR

A collection of 69 GabP Cys-scanning mutants was constructed to investigate whether substrate specificity determinants might exist within the region from Gly-266 to Pro-334. Cultures expressing these mutants were divided into two parts so that both expression

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

The plasma membrane fraction was prepared from three independent cultures of strains expressing the indicated GabP mutants. The membrane proteins (2 μ g 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 $\pm$ S.E.M. ($n = 3$) relative to the intensity exhibited by the Cys-less parent ($= 1.0$).

GabP variant	Expression	GabP variant	Expression
Cys-less	1.0 $\pm$ 0.00	A300C	0.89 $\pm$ 0.46
G266C	0.78 $\pm$ 0.13	L301C	0.76 $\pm$ 0.19
L267C	0.97 $\pm$ 0.15	N302C	0.93 $\pm$ 0.35
K268C	0.73 $\pm$ 0.18	S303C	1.00 $\pm$ 0.26
A269C	1.1 $\pm$ 0.19	A304C	1.28 $\pm$ 0.25
V270C	0.69 $\pm$ 0.68	L305C	1.1 $\pm$ 0.39
G271C	0.91 $\pm$ 0.12	Y306C	0.45 $\pm$ 0.17
S272C	0.69 $\pm$ 0.11	T307C	1.0 $\pm$ 0.69
Y273C	0.85 $\pm$ 0.05	A308C	0.51 $\pm$ 0.03
R274C	1.2 $\pm$ 0.18	S309C	0.72 $\pm$ 0.11
S275C	1.1 $\pm$ 0.27	R310C	0.41 $\pm$ 0.03
V276C	0.80 $\pm$ 0.18	M311C	1.0 $\pm$ 0.25
L277C	0.62 $\pm$ 0.09	L312C	0.44 $\pm$ 0.09
E278C	0.63 $\pm$ 0.17	Y313C	0.89 $\pm$ 0.09
L279C	1.0 $\pm$ 0.23	S314C	0.56 $\pm$ 0.11
L280C	0.60 $\pm$ 0.18	L315C	0.71 $\pm$ 0.34
N281C	0.91 $\pm$ 0.29	S316C	0.84 $\pm$ 0.43
I282C	0.69 $\pm$ 0.26	R317C	0.54 $\pm$ 0.12
P283C	0.81 $\pm$ 0.31	R318C	0.99 $\pm$ 0.22
H284C	0.70 $\pm$ 0.31	G319C	0.77 $\pm$ 0.22
A285C	0.92 $\pm$ 0.21	D320C	0.91 $\pm$ 0.45
K286C	0.66 $\pm$ 0.39	A321C	1.1 $\pm$ 0.39
L287C	0.88 $\pm$ 0.19	P322C	0.51 $\pm$ 0.19
I288C	1.3 $\pm$ 0.37	A323C	0.75 $\pm$ 0.06
M289C	0.82 $\pm$ 0.35	V324C	0.56 $\pm$ 0.11
D290C	0.74 $\pm$ 0.15	M325C	0.76 $\pm$ 0.11
A291C	1.2 $\pm$ 0.34	G326C	0.26 $\pm$ 0.11
V292C	1.2 $\pm$ 0.20	K327C	0.56 $\pm$ 0.29
I293C	0.91 $\pm$ 0.09	I328C	0.68 $\pm$ 0.13
L294C	1.4 $\pm$ 0.32	N329C	0.31 $\pm$ 0.12
L295C	1.4 $\pm$ 0.12	R330C	0.85 $\pm$ 0.40
S296C	1.3 $\pm$ 0.18	S331C	0.42 $\pm$ 0.05
V297C	1.6 $\pm$ 0.27	K332C	0.43 $\pm$ 0.16
T298C	1.8 $\pm$ 0.33	T333C	0.51 $\pm$ 0.12
S299C	1.2 $\pm$ 0.38	P334C	0.48 $\pm$ 0.18

and competitive uptake of [14 C]GABA and [3 H]NA could be evaluated using the same preparation. Results from these studies are shown in a series of six figures (Figures 3–8) that have been prepared in a two-panel layout to facilitate comparisons between: (a) the absolute rates of competitive substrate transport, and (b) the TSR.

Cys-scanning mutagenesis across the 11-residue region from Gly-266 to Val-276 had a modest overall effect on transporter expression levels, which by immunoblot luminometry ranged from 68% to 114% of that of the Cys-less parent (Table 2). This generally high level of expression is quantitatively insufficient to account for the severe competitive uptake defects exhibited by mutants L267C, G271C, S272C and Y273C (Figure 3, upper panel). Mutant G271C was totally defective, exhibiting [14 C]GABA and [3 H]NA uptake levels that could not be distinguished from background. In addition to diminished absolute levels of [14 C]GABA and [3 H]NA uptake, mutants L267C and Y273C also exhibited a low TSR (Figure 3, lower panel), suggesting the presence of an intrinsic catalytic defect that affects GABA uptake more severely than NA uptake.

Figure 3 Effects of Cys-scanning mutagenesis within the polypeptide segment containing amino acid residues 266–276 of *E. coli* GabP

The indicated single-Cys variants were compared with the Cys-less parent (*E. coli* SK11) in terms of initial GABA and NA transport rates and TSR. Upper panel: competitive transport reactions were initiated by exposing *E. coli* strains expressing the indicated GabP variants to 3 μ M [14 C]GABA (black bars) and 7 μ M [3 H]NA (grey bars) for 10 s prior to quenching and processing of samples for dual-label scintillation counting (see the Experimental section). Error bars represent S.E.M. ($n = 3$). Lower panel: the characteristic TSR, which is given by $(k_{cat}/K_m)_{GABA}/(k_{cat}/K_m)_{NA}$, is shown for each of the indicated GabP variants. Error bars represent S.E.M. ($n = 3$). The asterisks (*) denote cases in which a TSR was not calculated due to occurrence of 'zero' in the denominator (i.e. NA transport was not significantly greater than that exhibited by the GabP-negative strain, SK45).

Cys-scanning mutagenesis across the 11-residue region from Leu-277 to Leu-287 had generally modest effects on transporter expression levels, which by immunoblot luminometry ranged from a low of 60% (L280C) to 100% (L279C) of that of the Cys-less parent (Table 2). The highly expressed A285C mutant exhibited quite a severe defect in competitive uptake, which affected NA (indistinguishable from background) more severely than GABA (Figure 4). Although it is safe to conclude that the A285C variant has a substantial TSR, an asterisk is shown in Figure 4 (lower panel), since NA uptake (the denominator in the TSR) is zero. Mutant I282C exhibited a low TSR, indicating discrimination against GABA. Mutant L277C was highly defective, but as with A285C a TSR value cannot be reported. Other single-Cys substitutions between positions 277 to 287 were without marked effect on the TSR, indicating that observed defects in competitive uptake affected GABA and NA to a similar extent.

Cys-scanning mutagenesis across the 11-residue region from Ile-288 to Thr-298 had a modest overall effect on transporter expression levels, which by immunoblot luminometry ranged from 73% to 174% of that of the Cys-less parent (Table 2). Mutants D290C and T298C were expressed at 74% and 174% respectively of the Cys-less parent, accounting for significant portions of their differences from the parent (Figure 5, upper

Figure 4 Effects of Cys-scanning mutagenesis within the polypeptide segment containing amino acid residues 277–287 of *E. coli* GabP

The indicated single-Cys variants were compared with the Cys-less parent (*E. coli* SK11) in terms of initial GABA and NA transport rates, and TSR. Details are provided in the legend to Figure 3.

panel). Mutant S296C was well expressed, and although highly defective in competitive uptake, its defect did not lead to much discrimination between GABA and NA, since the TSR was relatively normal (perhaps 50 % of that of the Cys-less parent). In general, Cys-scanning in the first half of TM8 had minimal impact on the TSR, suggesting that these side chains have little role in distinguishing GABA from NA. It should be noted also that wild-type *gabP* encodes cysteine at codon 291, and the present results confirm previous studies which showed that the transporter is relatively indifferent to the loss of a thiol function at this position [13].

The 11-residue region from Ser-299 to Ser-309 includes the first half of the SPS (Figure 1), wherein previous studies have disclosed a number of functionally significant amino acid residues in both GabP and the phenylalanine permease PheP [12]. Cys-scanning mutagenesis across the first half of the SPS produced variable effects on transporter expression levels, which by immunoblot luminometry were generally modest, ranging from 45 % to 128 % of that of the Cys-less parent (Table 2). Several mutants (L301C, N302C, S303C, A304C, T307C, A308C) exhibited modest competitive uptake defects (Figure 6, upper panel), and TSR values similar to that of Cys-less GabP (Figure 6, lower panel). Overall, these results suggest that the affected amino acid side chains have no substantial role either in catalysis or in discriminating GABA from NA.

Mutant A300C is a special case, since wild-type *gabP* encodes Cys at codon 300. Cys-300 (or its equivalent in amino acid se-

Figure 5 Effects of Cys-scanning mutagenesis within the polypeptide segment containing amino acid residues 288–298 of *E. coli* GabP

The indicated single-Cys variants were compared with the Cys-less parent (*E. coli* SK11) in terms of initial GABA and NA transport rates, and TSR. Details are provided in the legend to Figure 3.

quence alignments) is known (i) to play a significant role in transport catalysis [13], (ii) to be uniquely associated with GABA transporters (bacterial and animal) [13], and (iii) to be the major target site for inactivation of GABA transporters (bacterial and animal) [20]. Because the A300C substitution restores the side chain to 'wild type,' the single-Cys mutant is highly active, and had to be grown without induction in order to capture initial rates of transport (i.e. when induced, the A300C mutant is expressed to about 90 % the level of Cys-less parent; Table 2). The high activity of mutant A300C confirms a prior study [13] which indicated that changing Cys-300 to Ala decreases the transport rate by approx. 50-fold (wild-type *gabP* encodes Cys at codon 300) – coincidentally equivalent to the extent of induction in this present expression system. Table 2 indicates that A300C can be induced to levels comparable with other mutants. It is worth noting that, although the rate defect associated with the C300A substitution is not accompanied by a change in the TSR (compare C-less and A300C; Figure 6, lower panel), substitutions involving other polar residues in this vicinity (i.e. S299C and Y306C) impact not only on the absolute rates of competitive uptake (Figure 6, upper panel), but also on the TSR (Figure 6, lower panel), indicating involvement both in catalytic throughput and in discriminating between the structures of GABA and NA.

The 12-residue region from Arg-310 to Ala-321 includes the second half of the SPS (Figure 1). Cys-scanning mutagenesis across the second half of the SPS produced variable effects on transporter expression levels, which by immunoblot luminometry

Figure 6 Effects of Cys-scanning mutagenesis within the polypeptide segment containing amino acid residues 299–309 of *E. coli* GabP

The indicated single-Cys variants were compared with the Cys-less parent (*E. coli* SK11) in terms of initial GABA and NA transport rates, and TSR. Details are provided in the legend to Figure 3. The asterisk (*) denotes that the highly active A300C mutant was grown without IPTG induction (in order to capture transport initial rates). A300C expression levels following induction may be found in Table 2.

were generally modest, ranging from 44 % to 108 % of that of the Cys-less parent (Table 2). Several mutants (e.g. L312C, Y313C, S314C, L315C, R317C) exhibited defects in competitive uptake (Figure 7, upper panel), along with a decrease in transporter expression levels. These mutants exhibited TSR values (Figure 7, lower panel) that are within 2-fold of that of the Cys-less parent, indicating no especially important role in discriminating GABA from NA. In contrast, mutant R310C exhibited defects in competitive uptake that were accompanied by quite a substantial decrease in TSR, suggesting that the side chain at position 310 impacts on the ability of GabP to discriminate between GABA and NA.

The 13-residue region from Pro-322 to Pro-334 includes the C-terminal end of the CAR (Figure 1). Cys-scanning mutagenesis across the end of the CAR produced variable effects on transporter expression levels, which by immunoblot luminometry ranged from 26 % to 85 % of that of the Cys-less parent (Table 2). Mutants P322C, G326C and N329C exhibited decreased expression levels that could explain in part the observed defects in competitive uptake, although the TSR values suggest that the side chains at positions 322 and 326 have a small influence on specificity (Figure 8). Mutant K327C was expressed approx. 56 % as well as its Cys-less parent, explaining much of its overall deficit in competitive uptake.

Figure 7 Effects of Cys-scanning mutagenesis within the polypeptide segment containing amino acid residues 310–321 of *E. coli* GabP

The indicated single-Cys variants were compared with the Cys-less parent (*E. coli* SK11) in terms of initial GABA and NA transport rates, and TSR. Details are provided in the legend to Figure 3.

Time courses of competitive uptake for selected single-Cys mutants

Competitive uptake conditions were set empirically to allow the control substrate specificity of the parental Cys-less GabP to be recognized graphically as a pair of overlapping time courses for the transport of the test substrates [14 C]GABA and [3 H]NA (Figure 2). Dramatic examples deviating from this 'overlapping' substrate specificity pattern occurred within the region from Ser-299 to Lys-327, wherein several single-Cys substitutions in the SPS were observed to impact on GABA transport in a substrate-selective manner (Figure 9). Mutants S299C, R310C, S314C and R318C exhibited competitive uptake time courses in which [14 C]GABA uptake remained near the baseline, whereas [3 H]NA uptake increased with time. Mutant Y306C behaved similarly (results not shown). Although the A300C substitution is known to have a large impact on transport rate, uptake of GABA and NA is affected uniformly, as indicated by the 'overlapping' competitive time courses for [14 C]GABA and [3 H]NA uptake (Figure 9, panel A300C).

The single-Cys substitution H233C, made within the cytoplasmic loop interconnecting TM6 and TM7, resulted in a TSR equal to 31 ± 2.8 , indicating that its specificity for GABA is increased relative to that for NA. Accordingly, the competitive uptake time course for mutant H233C features a [14 C]GABA signal that exceeds the [3 H]NA signal. These data provide evidence to indicate (i) that substrate specificity determinants do not lie exclusively within the CAR, (ii) that although structural

Figure 8 Effects of Cys-scanning mutagenesis within the polypeptide segment containing amino acid residues 322–334 of *E. coli* GabP

The indicated single-Cys variants were compared with the Cys-less parent (*E. coli* SK11) in terms of initial GABA and NA transport rates, and TSR. Details are provided in the legend to Figure 3.

perturbations within the CAR generally tend to impact on GABA specificity, this bias cannot be ascribed to the TSR methodology itself, which clearly is capable of detecting TSR increases caused by structural perturbations elsewhere in the protein (Figure 9, panel H233C), and (iii) that the CAR may interact with other parts of the transporter to determine overall substrate specificity characteristics, an observation consistent with recent work on the related transporter PheP [21].

DISCUSSION

Transport proteins from the APC superfamily contain a well conserved polypeptide segment known as the CAR [12,20] that, when modelled as an α -helix, exhibits amphipathic character across some 60 residues – easily more than double (and perhaps triple) the length required to traverse a lipid bilayer in α -helical conformation (Figure 1). Structural perturbations within a 20-residue CAR subdomain known as the SPS are known to disrupt transport function in GabP [12,13]. The related PheP protein also displays several sensitive polar residues in this region, lending generality to the notion that the CAR has important functional role family-wide [21]. The reactivity profile of Cys-300 of GabP towards a battery of structurally distinct thiol-modification reagents has suggested that the SPS may be associated with a solute-sieving, channel-like domain [20]. If the SPS is involved in substrate translocation, then the CAR should be found to contain substrate specificity determinants, i.e. loci at which structural

perturbations are observed to have a greater impact on some substrates than others.

The GabP CAR has two specificity-determining surfaces, the SPS and the SHS (sensitive hydrophobic surface)

Cys-scanning mutagenesis across the GabP CAR has revealed a number of loci (e.g. Leu-267, Leu-277, Ser-299, Tyr-306, Ser-309, Arg-310, Ser-314 and Arg-318) that substantially decrease the TSR. Except for Leu-267 and Leu-277, all of these residues lie within or near the SPS (Figure 1) and are potentially capable of interacting with the amino and/or carboxyl functions on GABA and NA via hydrogen bonds. Leu-267, on the hypothetical periplasmic α -helix shown in Figure 1, is associated with Gly-271, which appears to be functionally significant, since their replacement by Cys abolishes transport of both [14 C]GABA and [3 H]NA (Figure 3). As modelled (Figure 1), Leu-267, Gly-271, Tyr-273, Leu-277, Ile-282 and Ala-285 would seem to functionally define a SHS in the N-terminal region of the GabP CAR (Figure 1, white ribbon).

Cys-300 exerts isosteric effects on GABA and NA

Since the presence of a Δ (TSR) phenotype is taken to imply that mutagenesis has impacted on substrate binding (eqn 2), it is tempting to conclude from the absence of a change in TSR that substrate binding is not impacted. This is, however, an unwarranted conclusion, since $\Delta\Delta G_b$ can remain constant if a particular structural perturbation isosterically affects both members of the substrate pair used in the TSR analysis. Such may be the case at position 300 (located near the beginning of the SPS), where replacement of Cys-300 of GabP by either Ser or Ala is known to have a substantial (50-fold) impact on the rate of transport of [3 H]GABA [13]. However, the present study shows that the A300C and C300A variants have virtually the same TSR (Figure 6), so that overlapping [14 C]GABA and [3 H]NA competitive uptake time courses are observed for both mutants (compare Figures 2 and 9). It is additionally noteworthy that other specificity determinants in the SPS (e.g. Ser-299, Arg-310, Ser-314 and Arg-318) appear to operate independently of Cys-300, since specificity shifts similar to those reported here (Figure 9) were observed in the double-Cys GabP variants containing the wild-type cysteine residue at position 300 plus the respective cysteine replacements (results not shown).

Specificity determinant delocalization is a predicted consequence of the 'alternating access' transport mechanism

It is appealing to imagine on structural grounds that a putative substrate translocation conduit should be lined with multiple substrate specificity determinants that in essence trace out the path of the substrate through the core of the protein. There is, however, a potentially very significant alternative view that many specificity determinants along the length of the CAR are not directly in contact with the substrate. Instead, it can be argued that many (perhaps most) specificity determinants will be found at conformationally active interfaces distributed throughout the protein fold. How can substrate specificity determinants be delocalized from the protein–substrate interface?

We can understand the role of the protein fold in determining substrate specificity by beginning with the formal definition of specificity, k_{cat}/K_m , and following through with quantitative arguments (see Appendix) which show that the TSR depends strictly upon binding energies realized in the transition state

Figure 9 Time courses for competitive uptake of GABA and NA by selected GabP mutants that have a single cysteine residue on the putative polar surface of the CAR

E. coli SK45 (GabP-negative) and strains expressing GabP mutants with the indicated single-Cys substitutions S299C, R310C, S314C, R318C or H233C were grown to early exponential phase and washed with 100 mM potassium phosphate buffer (pH 7.0), as described in the Experimental section. In order to capture initial rates, expression of the highly active GabP mutant with the A300C substitution (panel A300C) was not induced, since 0.2 mM IPTG increases activity by approx. 40-fold (not shown). Dual-label competitive transport reactions were initiated by exposing the cells to 7 μ M [3 H]NA (■; 0.42 μ Ci/ml) and 3 μ M [14 C]GABA (▲; 0.06 μ Ci/ml). In all panels, the GabP-dependent component of competitive uptake (signal from the GabP mutant minus the SK45 signal) is expressed as d.p.m. for facile comparison with the Cys-less GabP (Figure 2), which exhibits an equal signal in both isotope channels.

(eqn 2) [15,22]. Binding energy is recognized [18] as consisting of two parts, localized and delocalized (eqn 3):

$$\Delta G_b = \Delta G_{\text{localized}} + \Delta G_{\text{delocalized}} \quad (3)$$

The localized component accounts for the contribution from direct molecular contacts at the substrate–protein interface. The delocalized component accounts for the contribution from molecular contacts at all other interfaces (e.g. helix–helix) that reconfigure in synchrony with formation of the localized contacts (Figure 10). Eqn (3) tells us that if the helical bundles of a transporter reconfigure to ‘surround’ the substrate molecule, then the binding energy, ΔG_b , is obligated to consist of a delocalized component equal to the Gibbs energy change of the conformation transition itself (substrate absent). Stated differently, in a helix-rich carrier operating by an alternating access mechanism, the TSR phenotype (eqn 2) can easily come to depend upon a network of coupled interactions between helical domains, exhibiting rigid-body behaviour. The idea that active-site catalytic properties (i.e. the specificity parameters) can be meaningfully shaped by delocalized interactions in the protein fold is a topic of great current interest in enzymic catalysis [23–28], and the present

study, along with many previous studies (see below), suggests applicability to transport catalysis as well.

Retrospective support for specificity delocalization in transporters

In the ‘paradoxical selection’ of co-transporter mutants, a selective pressure applied with a particular organic substrate (e.g. competitive inhibition of nutrient uptake) has been found to result ‘paradoxically’ in the natural selection of mutants with altered specificity for the nutrient-coupled cation, and vice versa [16,29]. Paradoxical selection is explained by eqns (2) and (3) applied in the context of a synchronous transport mechanism (Figure 10), wherein $\Delta G_{\text{delocalized}}$ would include any synchronous influence that helical twists and tilts may have on the architecture of the remote binding site(s) for coupled co-substrates.

It may be worth noting that eqns (2) and (3) do embrace particular cases (e.g. [29]) in which the organic substrate is hypothesized to provide groups that ‘directly ligand’ the cationic co-substrate. This scenario is a ‘limiting case’ of specificity delocalization in which the localized and delocalized sites have become sufficiently close together as to cause a semantic problem, but not a mathematical problem. In direct liganding, all of the interfaces in question may be identified as chemically distinct

entities, which remain subject to the mathematical constraints of eqns (2) and (3), wherein the helix–substrate interface may be considered localized, while other interfaces (cation–substrate and cation–helix) may be considered mathematically as making contributions to $\Delta G_{\text{delocalized}}$ (eqn 3), even though all of these interfaces are hypothesized to be quite proximal to one another in the direct liganding model [29].

Summary

Here we have provided experimental evidence that, in association with the GabP CAR, there is a transmembrane trail of amino acid residues that define surfaces upon which side-chain structural perturbations cause a $\Delta(\text{TSR})$ phenotype, discernable via competitive uptake of [^{14}C]GABA and [^3H]NA. These $\Delta(\text{TSR})$ phenotypes imply that the CAR has a role in determining intrinsic substrate binding energy, and from eqn (3) we understand further that it is the protein–substrate interaction ($\Delta G_{\text{localized}}$) together with protein–solvent and/or protein–protein interactions ($\Delta G_{\text{delocalized}}$) that provide a rational basis to understand the wide dispersion of specificity determinants, independent from (but not exclusive of) any consideration as to whether or not these determinants lie within a genuine transport conduit. We conclude: (i) that the CAR is part of an interface (possibly the substrate conduit) that controls ΔG_b in GabP specifically [12,13,20], (ii) that the counterintuitive principle of specificity delocalization turns out to be in striking accordance with elements from classical and contemporary thinking on the role of substrate binding energy in transport [15–18,30] and catalysis [23,24,31,32], and therefore, (iii) that, on thermodynamic grounds, observations on GabP should extrapolate to other transporters, including GABA transporters and/or APC superfamily transporters such as PheP, which is known to display functionally significant residues on its CAR [21]. Finally, and perhaps most importantly, the TSR analysis provides a generally applicable experimental rationale to embrace not only localized protein–substrate interactions (which are well accepted), but also delocalized interactions, which are gaining recognition as fundamental catalytic elements that make a crucial contribution to the catalytic specificity of enzymes [23,24,26,27] and transporters [33].

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APPENDIX

This appendix provides a quantitative rationale for the propositions: (i) that the TSR is independent of the level of expression of the transporter in the membrane; (ii) that substrate specificity (k_{cat}/K_m) depends only upon the intrinsic substrate binding energy realized in the transition state; and (iii) that the TSR depends strictly upon changes in the transition-state substrate binding energy that are due to structural differences between the tested substrates.

The TSR is expression-independent

When it is rewritten in terms of the concentration of the free carrier, [C], the Michaelis–Menten equation takes the form of a second-order rate equation:

$$v = (k_{\text{cat}}/K_m)[C][S] \quad (\text{A1})$$

Figure 10 Transport model with conformational changes that synchronously remodel protein–protein, protein–solvent and protein–co-substrate interfaces

Rigid-body transmembrane helices [34–37] behave as rods, so that all points on the helical surface are constrained to move in synchrony. As a consequence, helix-rich carrier proteins catalysing transport by ‘alternating access’ may be expected to undergo conformational transitions associated with multiple synchronous events that affect: (1) ligand accessibility from either side of the membrane, (2) molecular contacts between the ligand and its binding site, (3) protein–protein interfaces, (4) protein–solvent interfaces (at either aqueous or lipid interfaces), and (5) miscellaneous interfaces of any description (including remote co-substrate binding sites). Synchronous remodelling of multiple interfaces makes it impossible “... to separate free energy changes attributable to direct bonding [of ligands] to the proteins from free energy changes attributable to rearrangement of the protein structure that may accompany the binding process.” [17]. If this key thought, summarized by eqn (3), left anything unstated, then it was that a bound co-substrate at some remote location (item 5, above) may be considered in the same manner as any other synchronously remodelled interface. As a result, eqns (2) and (3) ensure that substrate specificity will always be determined jointly by item 2 (above) along with Gibbs energy contributions from the synchronous remodelling of remote interfaces (3–5, above). More concretely, one can envisage how a delocalized steric perturbation (mutation affecting the saw-tooth interface) may be transmitted down the helical axis to the active site, thereby synchronously altering active-site architecture, ΔG_b , and therefore substrate specificity (eqn 2). In a helix-rich transporter, it is entirely likely that active-site remodelling (necessary for alternating access) would couple synchronously to rearrangement of multiple helix–helix interfaces, thereby causing *bona fide* specificity determinants to be delocalized throughout the protein fold. It is to be emphasized that delocalized specificity determinants are evolutionarily selectable ‘design features.’ Thus networks of coupled motions promoting catalysis are of great current interest in enzymic catalysis [23–28,38]. TSR analysis provides a means to identify these synchronously remodelled interfaces – which may include (but are not limited to) direct protein–substrate interfaces (represented by the interaction between white circles and the black oval).

characterized by the apparent second-order rate constant, k_{cat}/K_m (units of $\text{M}^{-1} \cdot \text{s}^{-1}$). The reader will appreciate that as [S] goes to infinity, [C] goes to zero, creating the familiar asymptotic substrate–velocity relationship. Eqn (1) in the Experimental section is easily derived by taking the ratio of two instances of eqn (A1), one for GABA and one for NA. Algebraic elimination of [C] shows that the TSR [$(k_{\text{cat}}/K_m)_{\text{GABA}}/(k_{\text{cat}}/K_m)_{\text{NA}}$] is independent of carrier expression levels in the membrane. This prediction, borne out by the IPTG induction experiments (see the Results section), indicates that the present analysis, while simple, does not produce an oversimplified model that fails to predict experimental outcomes with GabP.

Specificity depends only upon the intrinsic transition-state binding energy

Catalysts generally [22,32], and transport catalysts particularly [15], improve reaction kinetics by decreasing the activation

Figure A1 The analysis of specificity is inherently simple

Despite the kinetic complexity of carrier-mediated transport, the analysis of specificity (k_{cat}/K_m) is inherently simple, because the pre-transition-state CS complex(es) have no role in determining $\Delta G_T^\ddagger$, which is (i) the activation energy associated with the apparent second-order rate constant k_{cat}/K_m , and as the Figure shows is also (ii) the thermodynamic distance between the free reactants (C + S) and the transition-state complex (CS ‡). The Figure contrasts two cases in which the Gibbs energy of the CS complex is either more positive (non-saturated carrier) or more negative (saturated carrier) than that of the free reactants. The magnitude of $\Delta G_T^\ddagger$ (and hence the value of k_{cat}/K_m) does not depend on saturation levels. Moreover, we could insert an arbitrary number of distinct CS complexes at different energy levels (complicated mechanism), and still the transport specificity constant, k_{cat}/K_m , is determined quite simply by $\Delta G_T^\ddagger$, the thermodynamic distance between the free reactants and the transition-state complex. This is in striking contrast with the value of the apparent first-order rate constant, k_{cat} , which varies with the thermodynamic distance between CS and CS ‡ .

energy. Kinetic specificity (k_{cat}/K_m) thus reflects the magnitude of the activation energy, as indicated by the following logarithmic form of Eyring's equation:

$$RT \ln (k_{\text{cat}}/K_m) = RT \ln (kT/h) - \Delta G_T^\ddagger \quad (\text{A2})$$

where k is the Boltzman constant, h is the Planck constant, R is the gas constant, T is the absolute temperature and $\Delta G_T^\ddagger$ is the activation energy associated with the second-order rate constant, k_{cat}/K_m . The magnitude of k_{cat}/K_m depends upon the thermodynamic distance ($\Delta G_T^\ddagger$) between the free reactants (C + S) and the transition-state complex (CS ‡) (Figure A1). The free reactants are relevant because, under second-order conditions (i.e. $[S] \ll K_m$), the carrier–substrate complex(es) are present in negligible amounts, so that a very simple reaction scheme without CS complexes is valid, despite any inherent complexity in the reaction mechanism: $C + S_1 \rightarrow C + S_2$ (subscripts indicate sides of the membrane). This ‘simple’ scheme does not ignore the obvious microscopic complexity of k_{cat} and K_m , nor the presence of CS complex(es) inherent in the mechanism of a carrier. The ‘simplicity’ arises because the CS complex(es) make no energetic contribution to the value of $\Delta G_T^\ddagger$, the activation energy of k_{cat}/K_m . Inspection of Figure A1 reveals that this is true whether the CS complex(es) are formed at energies above (second-order conditions) or below (first-order conditions) the energy of the reactants (C + S) in solution, a situation reflected in eqn (1) of the Experimental section, which indicates that the TSR may be determined at arbitrarily chosen substrate concentrations (i.e. the level of carrier saturation by either substrate is irrelevant).

Implicit in the foregoing is that transport specificity (k_{cat}/K_m) arises exclusively from binding interactions in the transition-state

complex [15] – none of the other complex(es) matter. Fersht cast this notion in thermodynamic terms [22]:

$$\Delta G_T^\ddagger = \Delta G_o^\ddagger + \Delta G_b \quad (\text{A3})$$

where $\Delta G_o^\ddagger$ is the uncatalysed chemical component of the free energy of activation, and ΔG_b is the intrinsic substrate binding energy.

TSR depends only on intrinsic binding energy changes

The explicit relationship between transport specificity (k_{cat}/K_m) and intrinsic substrate binding energy (ΔG_b) may be obtained by substituting eqn (A3) into eqn (A2):

$$RT \ln (k_{\text{cat}}/K_m) = RT \ln (kT/h) - (\Delta G_o^\ddagger + \Delta G_b) \quad (\text{A4})$$

Eqn (A4) relates specificity (k_{cat}/K_m) to ΔG_b . If, for two competing substrates (here GABA and NA), we take the difference between two instances of eqn (A4), we obtain eqn (2) in the Experimental section, which describes the relationship between the binding energies and specificity ratios (TSR) for a pair of substrates. The parameter $\Delta \Delta G_b$ is a change in binding energy, and thus eqn (2) reflects how the structural difference between the two substrates influences the binding energy that is made available to lower the activation energy in catalysis. A change in the TSR phenotype, $\Delta(\text{TSR})$, represents the impact of amino acid side chain structure on $\Delta \Delta G_b$. In other words, if a transporter variant exhibits a $\Delta(\text{TSR})$, then this reflects an underlying interplay between substrate structure and amino acid side chain structure involving the variant locus.

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