4-Aminobutyrate (GABA) transporters from the amine-polyamine-choline superfamily: substrate specificity and ligand recognition profile of the 4-aminobutyrate permease from *Bacillus subtilis*

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A previous study [Ferson, Wray and Fisher (1996) Mol. Microbiol. **22**, 693–701] has shown that transposon-mediated disruption of a protein 47% identical to the *Escherichia coli* GABA (4-aminobutyrate) transporter abolishes the ability of nitrogen-limited culture conditions to induce expression of a GABA transport activity in *Bacillus subtilis*. Here it is demonstrated directly that the *B. subtilis* GABA permease (*gabP*) gene can complement the transport defect in the *gabP*-negative *E. coli* strain. Unexpectedly, the ligand-recognition profile of the *B. subtilis* GabP was found to differ substantially from that of the highly homologous *E. coli* GabP. Unlike the *E. coli* GabP, the *B. subtilis* GabP: (i) exhibits approx. equal preference for the 3-carbon (β-alanine, $K_m = 9.6\ \mu M$) and the 4-carbon (GABA, $K_m = 37\ \mu M$) amino acids, and (ii) resists inhibition by bulky, conformationally constrained compounds (e.g. nipecotic acid, guvacine), which are active against GABA transporters from brain. The present study shows additionally that the *B. subtilis* GabP can translocate several open-chain GABA analogues (3-aminobutyrate, 3-aminopropanoate, *cis*-4-aminobutenoate) across the membrane via counterflow against [^{3}H]GABA. Thus, consistent with the idea that the ligand-recognition domain of the *B. subtilis* GabP is less spacious than that of the close homologue from *E. coli*, the former exhibits more stringent requirements than the latter for substrate recognition and translocation. These distinct functional characteristics of the *E. coli* and *B. subtilis* GABA transporters provide a basis by which to identify ligand-recognition domains within the amine-polyamine-choline transporter superfamily.

INTRODUCTION

High-affinity 4-aminobutyrate (γ-aminobutyric acid; GABA) transport in *Bacillus subtilis* is mediated by a *gab* permease (GabP) [1], which exhibits 47 percent sequence identity to the *Escherichia coli* GABA transporter, indicating that both proteins are evolutionarily related members of the APC (amine-polyamine-choline) superfamily [2], which includes transporters from bacteria, yeast and mammals. Although nitrogen-limited culture conditions are known to induce GABA transport in both *E. coli* and *B. subtilis* [3,4], it appears that the underlying metabolic machinery and regulatory events in GABA metabolism could be substantially different. In *E. coli* there is a co-ordinately regulated *gab* gene cluster [5,6], which includes the GabP as well as glutamate:succinic semialdehyde transaminase (GSST) and succinic semialdehyde dehydrogenase (SSDH), i.e. the same enzymes that metabolize GABA in the nervous systems of mammals [7]. In *B. subtilis*, however, genes specifying the transporter and the metabolic enzymes are not physically clustered [1], and there is no evidence that induction of *gab* permease is accompanied by induction of GSST and SSDH. The observed differences in gene structure and regulation suggest that the *B. subtilis* GabP (bsGabP) might transport a unique set of amino acids to and/or exhibit functional characteristics distinct from the *E. coli* GabP (ecGabP).

In order to compare explicitly the ecGabP with bsGabP, the latter has been expressed in a GabP-negative *E. coli* strain and subjected to pharmacological analysis using 18 GABA analogues that are known to be recognized by GABA receptors and transporters in brain [8] and in *E. coli* [9–11]. The results indicate that the pharmacological characteristics of the ecGabP are more closely reflected in the non-homologous GABA transporters and receptors of the mammalian brain than in the closely related bsGabP. The substantial pharmacological disparity between the homologous GABA transporters of *E. coli* and *B. subtilis* should be exploitable in structure–function studies aimed at locating and studying the protein domains that determine the substrate specificity of transporters from the APC superfamily.

EXPERIMENTAL

Reagents

GABA was from Sigma (St. Louis, MO, U.S.A.); Miller's Luria Broth medium was from Gibco-BRL (Grand Island, NY, U.S.A.); the plasmid pBluescript II KS (−) was from Stratagene (La Jolla, CA, U.S.A.); Sequenase version 2.0 DNA sequencing kit was from Amersham (Cleveland, OH, U.S.A.); restriction enzymes and T4 ligase were from New England Biolabs (Beverly, MA, U.S.A.); bicinchoninic acid protein determination reagents were from Pierce (Rockford, IL, U.S.A.); transport inhibitor compounds were either from Research Biochemicals Inc. (Natick, MA, U.S.A.) or Sigma; cellulose acetate filters (0.45 μm) were from Micron Separations, Inc. (Westboro, MA, U.S.A.); [^{3}H]GABA (31.6 Ci/mmol) and [^{3}H]muscimol (20.0 Ci/mmol) were from DuPont–New England Nuclear (Boston, MA. U.S.A.); [^{14}C]β-alanine (46.1 mCi/mmol) was from Sigma; Liquiscint™ scintillation cocktail was from National Diagnostics (Atlanta, GA, U.S.A.); and isopropyl β-D-thiogalactopyranoside (IPTG) was from Ambion Inc. (Austin, TX, U.S.A.). All other chemicals were from the usual sources and of molecular biological grade.

Abbreviations used: APC, amine-polyamine-choline; GABA, 4-aminobutyrate or γ-aminobutyric acid; GabP, *gab* permease; 3-PCA (nipecotic acid); bsGabP, *Bacillus subtilis* GabP; ecGabP, *Escherichia coli* GabP; IPTG, isopropyl β-D-thiogalactopyranoside; LDR, log dose–response.

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Table 1 Origin of genotypes and strains

Strain or plasmid	Chromosome or strain	Plasmid	Reference
SK35	*lacI*$^+$ Δ(*ZY*) *gab* Δ*P*(*Kan*^{r}-*1*)		[9]
pSCK-380 (*gabP*-negative)		*Amp*^{r} *lacI*^{q} *tac O*$^+$*P*$^+$	[9]
SK45	*SK35*	pSCK-380	[9]
pSCK-472 (*E. coli gabP*)		*Amp*^{r} *lacI*^{q} *tac O*$^+$*P*$^+$ *gabP*$^+$	[9]
SK55	*SK35*	pSCK-472	[9]
pSCK-572 (*B. subtilus gabP*)		*Amp*^{r} *lacI*^{q} *tac O*$^+$*P*$^+$*gabP*$^+$	This work
SK57	*SK35*	pSCK-572	This work

Cloning and strain development

The *gabP* from *B. subtilis* chromosomal DNA (American Type Culture Collection strain BD170) was amplified by PCR using phosphorylated forward (5′-AGT TTA AAC GAG AGG ATT AAG ATG AAC AGT CTC AAT CAG G-3′) and reverse (5′-GAG CTT AAT TAA GGC AGG ATA TCG GGT TGC TGC-3′) primers. The PCR product was isolated and cloned into the *Eco*RV site of the phagemid, pBluescript II KS(−). The *gabP* insert was sequenced [12] to ensure conformity to the GenBank sequence (accession number U31756). A restriction fragment containing *gabP*, bounded by *Xho*I and *Xba*I restriction-enzyme sites, was subcloned into the IPTG-inducible expression plasmid pSCK-380 [9] to create the plasmid pSCK-572 (*Amp*^{r} *LacI*^{q} *tac O*$^+$*P*$^+$ *B. subtilis gabP*$^+$). The pSCK-572 was introduced by transformation into the *gabP*-negative *E. coli* SK35 [9], creating the strain SK57. Table 1 summarizes the relevant strains used.

Culture conditions

Cells grown overnight in Miller's Luria Broth medium plus ampicillin (100 μg/ml) were diluted 100-fold into fresh medium containing ampicillin and IPTG (0.5 mM), and grown for 3–4 doublings (log phase). Cells were harvested by centrifugation and washed twice in 100 mM potassium phosphate buffer (pH 7.0). These 'washed' cells were resuspended to an attenuance of Klett 1000 (#42 Blue filter), corresponding to a protein concentration of 1.5 mg/ml.

Transport

Transport reactions were initiated by adding 90 μl of washed SK57 to 10 μl of a solution containing radiolabelled substrate (typically, 100 μM [^{3}H]GABA, 3 μCi/ml; 100 μM [^{14}C]β-alanine, 1 μCi/ml; or 1 mM [^{3}H]muscimol, 4 μCi/ml). Transport kinetics (i.e. K_m measurements) were determined using an appropriate dilution of either 400 μM [^{3}H]GABA (6 μCi/ml) or [^{14}C]β-alanine (2 μCi/ml). A linear time course was obtained with five concentrations of either substrate, and the time course slope (calculated by linear regression) at each concentration was taken as the transport velocity. Reactions were quenched by adding 1 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 20 mM $HgCl_2$. Quenched reactions were vacuum-filtered and processed for scintillation counting as described previously [9]. Disintegrations per minute were assessed in a Beckman LS3801 scintillation spectrometer with automatic quench compensation (H number determination).

Substrate specificity ratio

A dual-label strategy was used to compare the active sites of ecGabP and bsGabP with regard to their specificity for either 3-carbon (β-alanine) or 4-carbon (GABA) substrates. The term 'specificity' is defined explicitly as the ratio, k_{cat}/K_m. The transport velocities measured simultaneously for two competing substrates (e.g. [^{3}H]GABA and [^{14}C]β-alanine) is proportional to their 'specificity ratio' (eqn. 1):

$$\frac{v_A[\mathrm{B}]}{v_B[\mathrm{A}]} = \frac{(k_{cat}/K_m)_A}{(k_{cat}/K_m)_B} \quad (1)$$

where [A] and [B] are the substrate concentrations, v_A and v_B are the initial velocities for transport of either substrate in the presence of the other, and $(k_{cat}/K_m)_A/(k_{cat}/K_m)_B$ is the 'specificity ratio' by which the active site discriminates between the two substrates. A large 'specificity ratio' indicates that the active site is better designed to translocate substrate A than substrate B. The relationship in equation 1 '… holds at all concentrations of substrates …' [13].

Cells used to determine specificity ratios were grown and processed as described above. However, in order to obtain initial rates of transport, expression from *E. coli* SK55 (ecGabP) and *E. coli* SK57 (bsGabP) was induced with either 1 mM or 0.5 mM IPTG, respectively. Transport was initiated by exposing: (i) the ecGabP to 10 μM [^{3}H]GABA (0.3 μCi/ml)/43 μM [^{14}C]β-alanine (2 μCi/ml), and (ii) the bsGabP to 10 μM [^{3}H]GABA (0.3 μCi/ml)/14 μM [^{14}C]β-alanine (0.1 μCi/ml). The transport reactions were quenched at intervals between 3 s and 15 s to provide linear uptake of both radiolabels. The samples were processed for scintillation counting, and disintegrations per min were calculated using a Beckman LS3801 scintillation spectrometer using a dual-label program, automatic quench compensation (H number) and stored quench curves [9].

Inhibition

For convenience, IPTG was omitted from the growth media to slow the initial rate of transport. Reactions were initiated by adding 80 μl of washed, non-induced *E. coli* SK57 (bsGabP) to 20 μl of a solution containing 10 μl of [^{3}H]GABA (100 μM, 3 μCi/ml) and 10 μl of the inhibitory compound. After 75 s (linear portion of uptake time course for non-induced cells), the reactions were quenched and processed for scintillation counting as described above. The results were corrected for non-specific uptake (assessed in the *gabP*-negative SK45) and expressed in the Figures as a percentage of the non-inhibited control (SK57).

GraphPad PRISM™ (version 2; Intuitive Software for Science, San Diego, CA, U.S.A.) was used to analyse statistically inhibition by pharmacological agents. Log dose–response (LDR) data were fitted by eqn. (2):

$$v_{obs} = v_{saturated} + \frac{(v_{control} - v_{saturated})}{1 + 10^{(log[I] - logIC_{50})}} \quad (2)$$

where $v_{control}$ is the uninhibited transport rate, v_{obs} is the transport rate observed in the presence of an inibitor at concentration [I],

$v_{saturated}$ is the rate observed with a saturating concentration of inhibitor and IC_{50} is the inhibitor concentration causing 50% inhibition (inflection point of the LDR curve). As described previously [9], eqn. (3) relates IC_{50} to the inhibitor K_I and to the K_m for substrate, [S]:

$$\frac{(IC_{50})K_m}{[S]+K_m} = K_I \quad (3)$$

Counterflow

Counterflow assays were performed as described previously [10,11]. Briefly, poisoned (30 mM NaN_3) cells were 'preloaded' by exposure to 10 mM unlabelled substrate (25 °C, 1 h). Counterflow was initiated by diluting 5 μl of preloaded cells 200-fold into 100 mM potassium phosphate buffer (pH 7.0) containing [^{3}H]GABA (10 μM, 0.2 μCi/ml) and 30 mM NaN_3. At various times the reactions were quenched by the addition of 1 ml of buffer, containing 100 mM $HgCl_2$. Cells were filtered, processed for scintillation counting, and expressed as an uptake ratio as described previously [11].

RESULTS AND DISCUSSION

The GABA transport activity induced in *B. subtilis* by growth on nitrogen-limited media has been attributed to a protein that exhibits 47% amino acid identity with the *E. coli* GABA permease (ecGabP). Ferson et al. [1] found that disrupting the *B. subtilis gabP* (bsGabP) gene by transposon insertion yields a *B. subtilis* strain that cannot transport GABA. The present study demonstrates directly that bsGabP, expressed under control of a *lac* promoter, confers upon a *gabP*-negative *E. coli* strain the ability to transport [^{3}H]GABA and [^{14}C]β-alanine, but not [^{3}H]muscimol (Figure 1). The bsGabP and ecGabP also exhibited highly significant ($P < 0.005$) differences in sensitivity to classical inhibitors of [^{3}H]GABA transport (Figure 2). Thus despite their sequence homology, the properties of bsGabP differ markedly from ecGabP, since the latter exhibits high affinity for muscimol (a transported substrate), and transports β-alanine only poorly (low affinity). In order to gauge the extent to which the ligand-binding sites differ in bsGabP and ecGabP, full dose–response data were acquired for a number of substrate analogues.

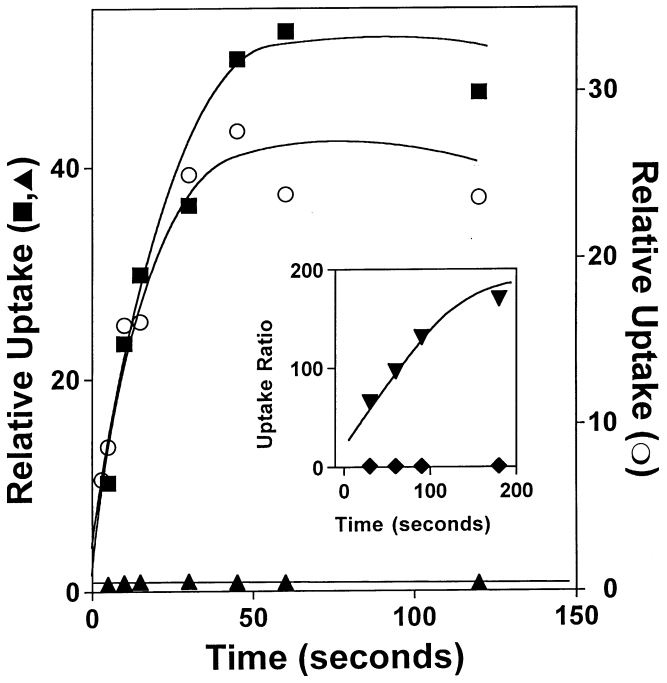

Figure 1 Time course of bsGabP-dependent uptake of GABA and β-alanine

SK57 cells (bsGabP) were grown in the presence of 1 mM IPTG and prepared for transport as indicated in the Experimental section. Washed cells were incubated in the presence of either 10 μM [^{3}H]GABA (■), 10 μM [^{14}C]β-alanine (○) or 100 μM [^{3}H]muscimol (▲) for the indicated times. The inset shows that in the presence of a genuine substrate such as 10 μM [^{3}H]GABA the background correction from SK45 (◆) is negligible compared with uptake observed in the GabP-positive strain, even if the latter is grown without IPTG (▼). There was, of course, no appreciable difference (▲, main figure) between SK57 and SK45 when the cells were exposed to 100 μM [^{3}H]muscimol, which is not a bsGabP substrate. Relative uptake is defined as (SK57 uptake − SK45 uptake)/(SK45 uptake); uptake ratio is defined as (observed uptake)/(SK45 uptake). The results shown are representative of multiple experiments that yielded similar results.

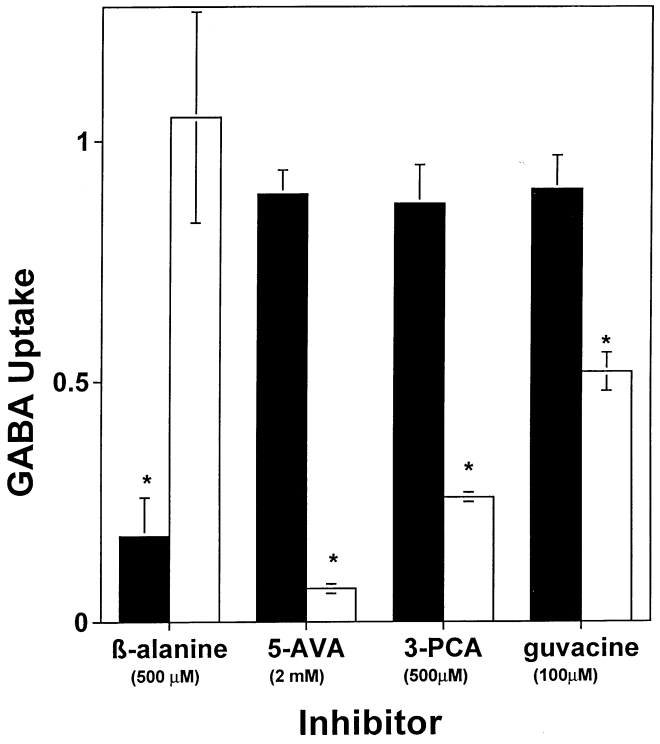

Figure 2 Ligand recognition differences between bsGabP and ecGabP

Cells expressing either the ecGabP (white bars) or the bsGabP (black bars) were exposed to 10 μM [^{3}H]GABA and one of either 500 μM β-alanine, 2 mM 5-aminovaleric acid (5-AVA), 500 μM nipecotic acid (3-PCA) or 100 μM guvacine. The transport reactions were quenched after either 45 s (IPTG-induced ecGabP) or 10 s (IPTG-induced bsGabP) to ensure initial rate conditions. Data are expressed as the mean ($n = 3$) fraction of the control [^{3}H]GABA uptake exhibited by cells that were not exposed to a test compound. Error bars represent the S.D.; * denotes highly significant differences ($P < 0.005$; unpaired *t*-test) between bsGabP and ecGabP with respect to inhibition by each compound at the indicated concentration.

Inhibitory potencies

The bsGabP appears to differ markedly from the previously characterized ecGabP with respect to both (i) its chain-length requirement for recognition of open-chain amino acids and (ii) the ability of its active site to accommodate bulky cyclic amino acid analogues that are classical ligands of GABA transporters and receptors in brain. For example, whereas several cyclic GABA analogues (kojic amine, *cis*-3-aminocyclohexylcarboxlic acid, nipecotic acid and guvacine) were found to act 40–300-fold

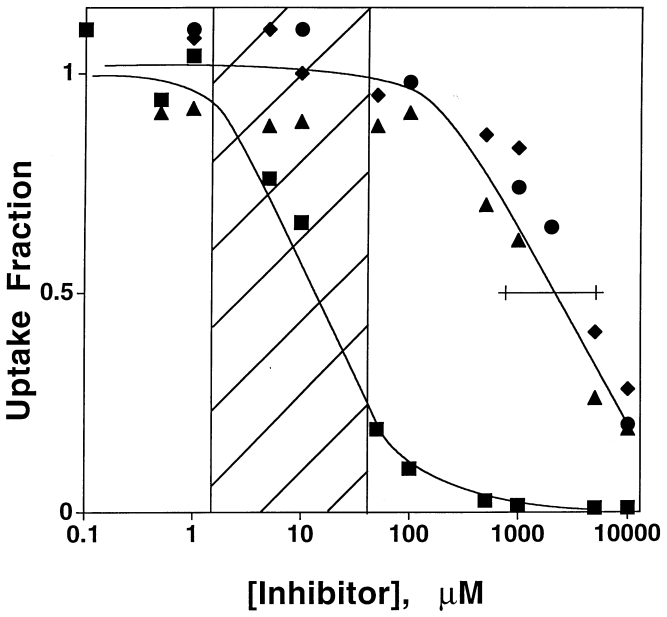

Figure 3 Inhibition of bsGabP-dependent [^{3}H]GABA uptake by linear and cyclic GABA analogues

Washed SK57 cells (bsGabP) were incubated for 75 s in the presence of [^{3}H]GABA (10 μM, 0.3 μCi/ml) and either the 3-carbon open-chain compound, β-alanine (■), or the heterocyclic GABA analogues, guvacine (◆), nipecotic acid (●) and kojic amine (▲) at concentrations indicated in the Figure. These data are presented as a fraction of the uptake exhibited by the uninhibited control. The hatched region represents the concentration range which spans all three 95% confidence intervals for previously published data [11] describing the IC_{50} for inhibition of the ecGabP by guvacine, nipecotic acid and kojic amine. The smooth curves are provided to emphasize the qualitative disparity between β-alanine and the cyclic analogues. The horizontal bar, on the other hand, is quantitatively significant, including a concentration range that spans all three 95% confidence intervals for the IC_{50} values obtained by fitting Equation 2 to the LDR data for guvacine, nipecotic acid and kojic amine.

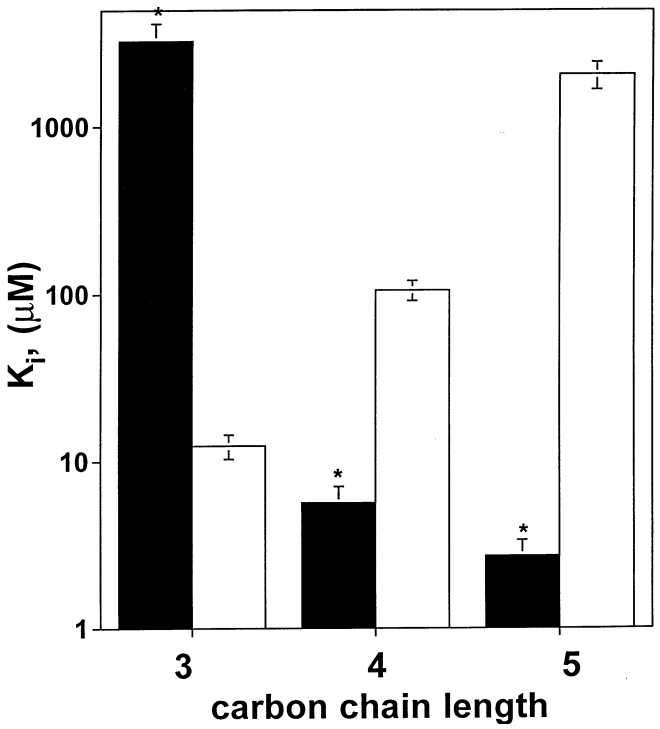

Figure 4 The distinct ligand chain-length preferences of the GabP proteins from *E. coli* and *B. subtilis*

Dose–response relationships (e.g. Figure 3) were generated to assess the potency (IC_{50}) of 3-carbon (β-alanine), 4-carbon (GABA) and 5-carbon (5-aminovaleric acid) compounds as inhibitors of [^{3}H]GABA (10 μM; 0.3 μCi/ml) uptake by either the ecGabP (white bars) or the bsGabP (black bars). Error bars represent the S.D.; * denotes significant differences ($P < 0.05$; unpaired *t*-test) between bsGabP and ecGabP with respect to inhibitory potency. The K_i values (shown) are offset from IC_{50} by a small factor (1.3-fold for bsGabP and 1.8-fold for ecGabP) obtained by applying Equation 3 together with the K_m values from Table 2. Note: whereas there is overall agreement that β-alanine is poorly recognized by ecGabP, the current study indicates that the affinity for β-alanine is even lower (50-fold) than previously reported [9].

more potently as [^{3}H]GABA transport inhibitors in ecGabP than in bsGabP (Figure 3), the 3-carbon open-chain amino acid, β-alanine, was found to act 500-fold more potently on the bsGabP than on the ecGabP. A series of similar studies (summarized in Figure 4) establish that bsGabP favours open-chain amino acids with 3 or 4 carbons, whereas the ecGabP favours amino acids with 4 or 5 carbons.

Translocation specificities

The possibility that bsGabP might translocate some of the compounds that block [^{3}H]GABA transport was investigated by characterizing 18 compounds with respect to (i) their potency as [^{3}H]GABA transport inhibitors, and (ii) their ability to drive [^{3}H]GABA counterflow. The counterflow assay provided a means to infer whether an unlabelled intracellular compound could fully traverse the bsGabP transport channel via exchange with extracellular [^{3}H]GABA. For example, bonafide substrates such as β-alanine and GABA (Figure 1) support [^{3}H]GABA counterflow (Figure 5), whereas non-transported compounds such as muscimol (Figure 1) do not (Figure 5). A similar series of counterflow experiments (summarized in Figure 6) failed to provide evidence that cyclic GABA analogues (i.e. compounds 5–10, 12, 13, 15 and 16) could be transported by bsGabP. Evidence of translocation (i.e. counterflow) was obtained only for compounds 1–4, the open-chain amino acids (β-aminobutyric acid, β-alanine, 4-amino-*cis*-2-butenoic acid and GABA, respectively), which inhibited bsGabP with high-affinity ($K_i \leqslant 100$ μM).

Table 2 Specificity and apparent affinity for GABA and β-alanine

Values for the 'specificity ratio' (Equation 1) and transport K_m were determined as described in the Experimental section. For ecGabP, the K_m for GABA is the published value [4]. As indicated by the high 'specificity ratio', β-alanine is a poor substrate for ecGabP and a K_m value has therefore not been determined.

	GabP homologue	
	bsGabP	ecGabP
Specificity ratio (GABA/β-alanine)	0.73 ($\pm$0.094)	50.1 ($\pm$16.1)
K_m GABA	37 μM ($\pm$13.2)	12 μM
K_m β-alanine	9.6 μM ($\pm$5.3)	Not available

Summary

The present study is consistent with a model in which the substrate-recognition domain of bsGabP is smaller than that of ecGabP (Figure 7). Whereas the bsGabP can accommodate amino acids with up to 4 carbons, the ecGab binding domain

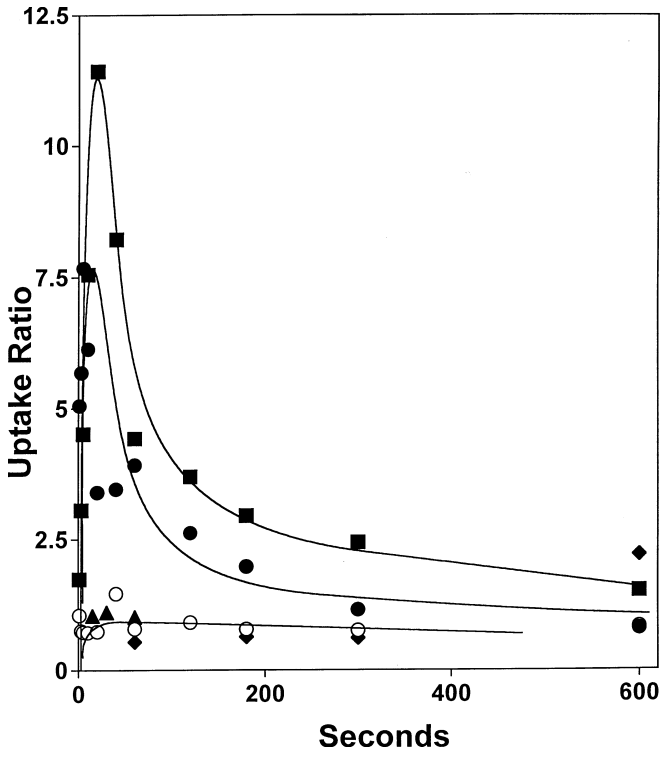

Figure 5 The bsGabP-dependent counterflow of [^{3}H]GABA driven by non-radiolabelled substrates

E. coli SK57 (bsGabP) incubated with 30 mM sodium azide in the presence (solid symbols), or absence (○) of 10 mM unlabelled GABA (●), β-alanine (■) or muscimol (▲) were diluted 200-fold into [^{3}H]GABA (10 μM, 0.2 μCi/ml) to initiate counterflow. A control (◆) to demonstrate the GabP-dependence of counterflow was performed by preloading SK45 (*gabP*-negative) with each of the above-mentioned substrates. Note that the final extracellular chemical concentration of [^{3}H]GABA was 60 μM when cells were preloaded with 10 mM GABA.

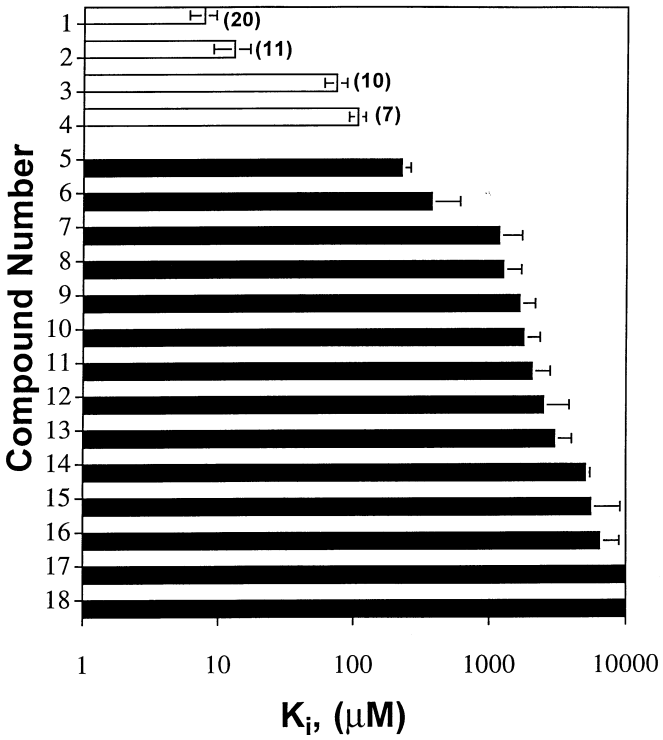

Figure 6 The inhibitory potency and ability of bsGabP ligands to support counterflow

Dose–response curves (e.g. Figure 3) were generated to assess the inhibitory potency of the 17 compounds shown. The procedure was as described in the Experimental section except that to assess the inhibitory potency of GABA, the cells were exposed to 10 μM [^{14}C]β-alanine (0.1 μCi/ml). White bars represent compounds that were able to support counterflow (e.g. Figure 5), whereas compounds that did not support counterflow are represented by black bars. The peak counterflow uptake ratio is shown parenthetically. Error bars represent the 95% confidence interval for the IC_{50} obtained by fitting Equation 2 to the dose–response data. From application of Equation 3 and the K_m values in Table 2, it is understood that the IC_{50} for compound 4 (GABA) is right-shifted 2.0-fold relative to K_i, and that for all the other compounds the offset is 1.3-fold. The chemical structure of compounds 1–18 can be found in Figure 7. The compounds (c) studied were numbered as follows: c1, β-aminobutyric acid; c2, 3-aminopropanoic acid (β-alanine); c3, 4-amino-*cis*-2-butenoic acid; c4, GABA; c5, 3-hydroxy-5-aminomethylisoxazole (muscimol); c6, 4,5,6,7-tetrahydroisoxazolo[5,4c]pyridin-3-ol; c7, 2-(aminomethyl)-5-hydroxy-4H-pyran-one (kojic amine); c8, 5-(aminomethyl)-3–2H-isothiazalone (thiomuscimol); c9, *cis*-3-aminocyclohexylcarboxlic acid; c10, 4-piperidine carboxylic acid (isonipecotic acid); c11, 5-aminovaleric acid; c12, 3-piperidine carboxylic acid (nipecotic acid); c13, 1,2,3,6-tetrahydro-3-pyridinecarboxylic acid (guvacine); c14, 6-aminohexanoic acid; c15, 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol; c16, 1,2,3,6-tetrahydro-4-pyridinecarboxylic acid (isoguvacine); c17, 3-aminopropylphosphonic acid; c18, 4-amino-*trans*-2-butenoic acid.

behaves as though it were larger, accommodating amino acids of up to 5 carbons in length (Figure 4), as well as several bulky cyclic GABA analogues that function as classical ligands of GABA transporters and receptors in brain (Figure 3, hatched region). Since the pharmacological profile of bsGabP is quite dissimilar from that of ecGabP, there is now a conceptual basis in the comparison of bsGabP and ecGabP to localize structural elements responsible for recognition (or rejection) of certain classical GABAergic ligands (i.e. Figure 7C).

The ligand-recognition relationships summarized in Figure 7 are consistent with the idea that the binding of simple amino acids (compounds 2, 4 and 11) ought to be dominated by interactions designed to accommodate the amino and carboxyl functions at either end of the substrate. To gauge the distances involved, we point out that β-alanine (compound 2), the shortest open-chain molecule transported by either bsGabP or ecGabP, has its zwitterionic groups separated by 4.6 Å in the energy-minimized configuration (calculated with the Alchemy III computer program, Tripos Assoc.). Also although GABA (compound 4) and 5-aminovalerate (compound 11) are larger (with potentially greater separation distances between the amino and carboxy groups), these compounds could presumably be recognized in a site complementary to β-alanine (3 carbons) provided there was sufficient room to accommodate folding of their longer methylene chains. Consistent with the notion that the bsGabP binding domain lacks this additional space, a bulky molecule such as nipecotic acid (compound **12**) is recognized poorly since it 'incorporates' both the 3-carbon (emphasized bottom right, Figure 7) and the 5-carbon (emphasized bottom left, Figure 7) amino acid backbone into the cyclic structure. In contrast, bulky cyclic ligands are accepted by ecGabP [10,11], suggesting that its binding domain may be larger than that of the bsGabP.

Although the structural domains that account for the unique pharmacology of *B. subtilis* GabP remain unknown, it is attractive to speculate that the specificity of GabP for translocation of its ligands involves a region that begins in transmembrane helix 8 and extends into the adjoining cytoplasmic loop. A recent study [14] points out that this 'consensus amphipathic region': (i) is preserved in many members of the APC superfamily; (ii) is

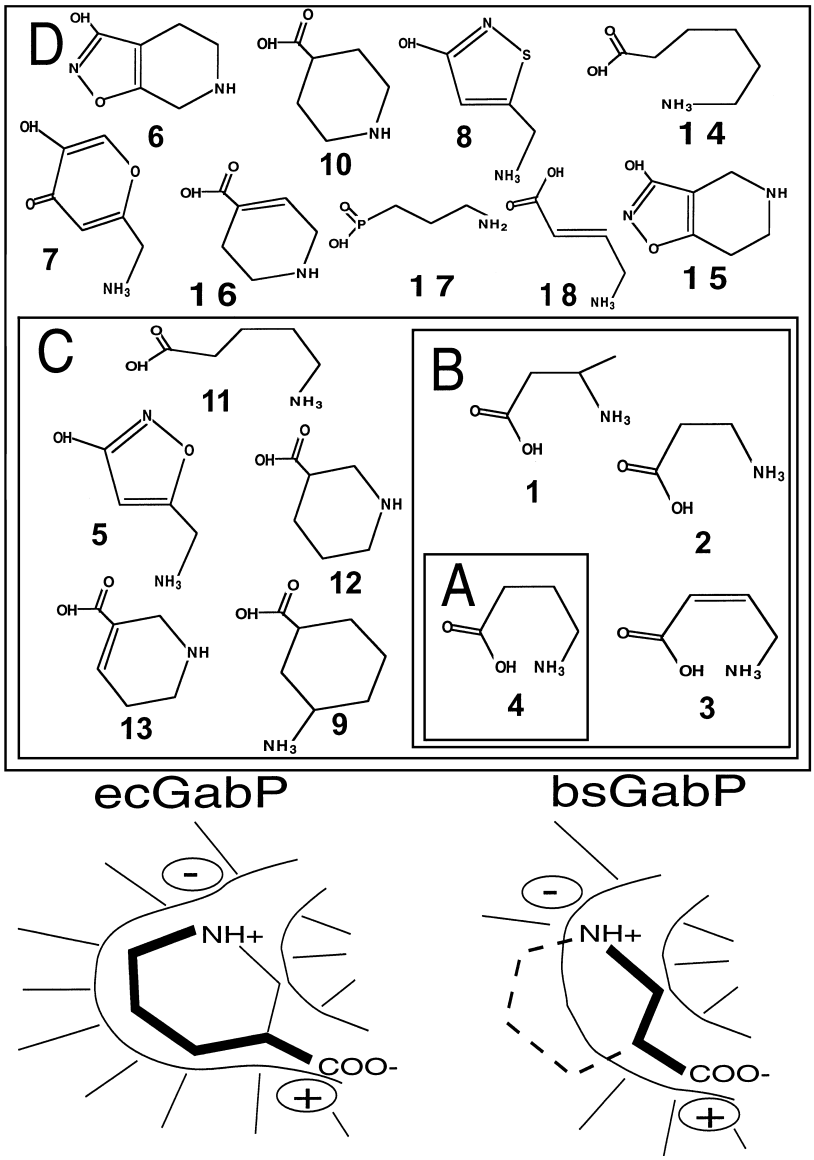

Figure 7 Summary and minimal structural interpretation

(**A**) Emphasizes the structure of GABA, the native substrate of ecGabP and bsGabP. (**B**) Encloses only the subset of compounds found to be transported by bsGabP. (**C**) Encloses the subset of compounds that are transported by the ecGabP [11], including several bulky cyclic compounds not transported by bsGabP (Figure 6). It should be noted that (**C**) does include all of the open-chain compounds enclosed by (**A**) and (**B**). (**D**) Encloses the entire set of compounds tested, irrespective of whether they were found to interact strongly with the bsGabP. The chemical names and relevant abbreviations are detailed in the legend to Figure 6.

the site in GABA transporters of a functionally important 'signature cysteine' [14a]; (iii) is functionally important in GabP and other APC-family proteins [15–17]; and (iv) is present outside the APC family (probably by evolutionary convergence) in the structure of GABA transporters from the nervous system. That such common secondary structural elements may exist in non-homologous transporters is consistent with the prediction that 'the functional family of co-transporters in bacteria, plants and animal membranes will turn out to have common tertiary structures and transport mechanisms, despite the lack of primary sequence homology' [18]. It will be for future structure–function studies to provide details on how such common secondary structural elements conspire to influence ligand specificity and to ensure co-substrate coupling in co-transport.

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