

## Short External Loops as Potential Substrate Binding Site of $\gamma$ -Aminobutyric Acid Transporters\*

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While the  $\gamma$ -aminobutyric acid (GABA) transporter GAT1 exclusively transports GABA, GAT2, -3, and -4 also transport  $\beta$ -alanine. Cross-mutations in the external loops IV, V, and VI among the various GABA transporters were performed by site-directed mutagenesis. The affinity of GABA transport as well as inhibitor sensitivity of the modified transporters was analyzed. Kinetic analysis revealed that a cross-mutation in which loop IV of GAT1 was modified to resemble GAT4 resulted in increased affinity to GABA from  $K_m = 8.7$  to  $2.0 \mu\text{M}$  without changing the  $V_{\text{max}}$ . A cross-mutation in loop VI, which swapped the amino acid sequence of GAT2 for GAT1, decreased the affinity to GABA ( $K_m$ ,  $35 \mu\text{M}$ ). These results suggest that loops IV and VI contribute to the binding affinity of GABA transporters. A substitution of three amino acids in loop V of GAT1 by the corresponding sequence of GAT3 resulted in  $\beta$ -alanine sensitivity of its GABA uptake activity. These three amino acids in loop V seem to participate in the  $\beta$ -alanine binding domain of GAT3. It is suggested that those three external loops (IV, V, and VI) form a pocket in which the substrate binds to the GABA transporters.

$\gamma$ -Aminobutyric acid (GABA)<sup>1</sup> is a major inhibitory neurotransmitter in the mammalian brain and is widely distributed throughout the nervous system (1, 2). Termination of GABA neurotransmission is achieved by a rapid sodium-dependent uptake system (3). Molecular cloning studies have resulted in the isolation and characterization of cDNAs encoding four different GABA transporters, GAT1, GAT2, GAT3, and GAT4 (4–9). These cDNAs are part of a family of neurotransmitter transporters sharing similar structure and amino acid sequences but are different in their substrate and pharmacological specificities. Selective and differential localization of GABA transporters has been shown in GABAergic neurons and glial cells (6–12), and it is apparent that each of the four GABA transporters functions in different tissues in a variety of biological processes. The main feature separating GAT1 from the other three GABA transporters is the substrate specificity. While GAT1 exclusively transports GABA, the other transporters are also able to transport  $\beta$ -alanine and taurine (3–12). Consequently,  $\beta$ -alanine and taurine inhibit GABA transport by GAT2, GAT3, and GAT4 but have no effect on GABA transport by GAT1.

Information from sequence analysis and site-directed mu-

tagenesis may pinpoint regions and specific amino acids that are important for the various functions of the transporters. Site-directed mutagenesis revealed that three residues in the transmembrane helices are essential for the activity or the assembly of GAT1. It was suggested that Arg-69 functions in the binding of negatively charged chloride ions (13), Trp-222 functions in the binding of substrate in a manner analogous to the proposed function of a tryptophan residue in acetylcholine esterase (14), and substitution of Trp-230 prevented the proper sorting of the transporter into the plasma membrane (14). The precise substrate binding domain has not yet been defined. Recently, we and others (6–9) reported that four GABA transporters have different pharmacological properties and GABA binding affinities. It was suggested that these variabilities in drug sensitivity were caused by the different structures of the substrate binding domain.

### EXPERIMENTAL PROCEDURES

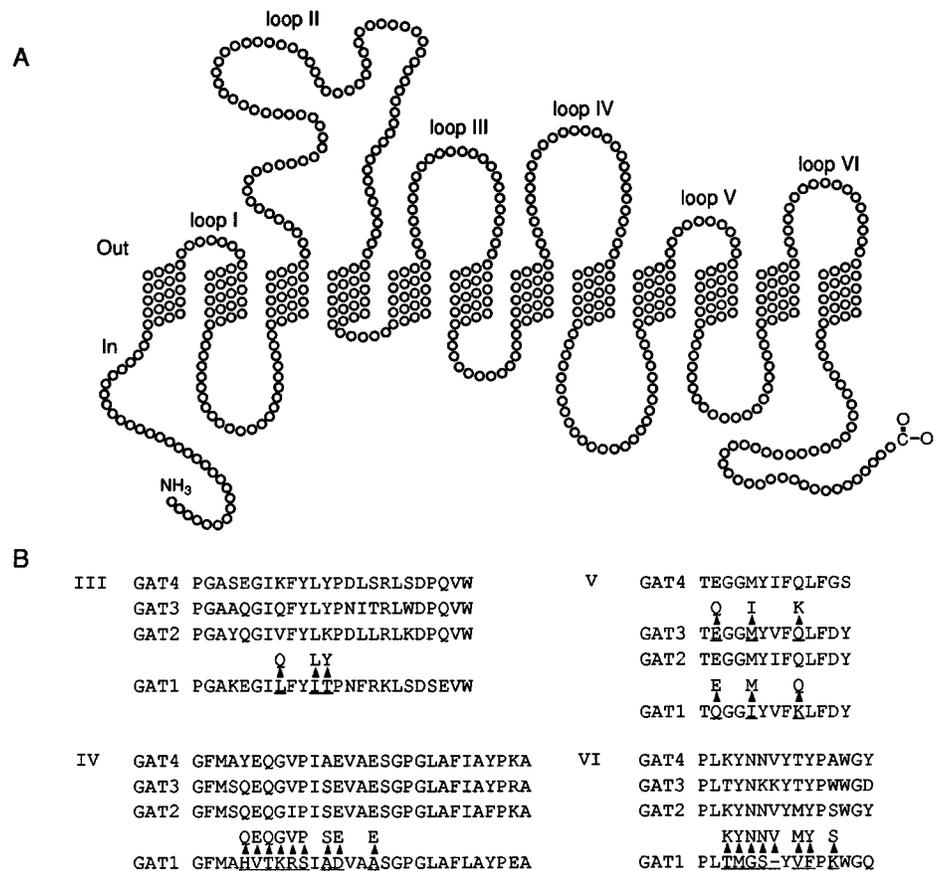
**Generation of Mutants**—Oligonucleotide-directed site-specific mutagenesis was performed by overlap extension using the polymerase chain reaction (PCR) (15–17). Bluescript (0.1 ng) harboring GAT1 cDNA was used as a template for PCR mutagenesis. Two sets of reactions were carried out as follows: the first using primers of N1351 (5'-CTACTTCTCAGCCACGTAC-3'), corresponding to bases 858–876 of GAT1 cDNA, and antisense mutagenic oligonucleotide (200 pmol of each); the second using N1271 (5'-GGTGTGGGTACAGCCAGTC-3'), corresponding to bases 1981–1994 of GAT1 cDNA, and sense mutagenic oligonucleotide (200 pmol of each). The mutagenic oligonucleotides used were loop III, 5'-CCAAGGAGGGGATCCTCTTCTACCTATCCCAA-CTTCCGAAAGCTGT-3'; loop IV, 5'-CAGGAACAAGGGGTGCCCAT-ATCTGAGGTGGCAGAGTCAGGCCCGGGGCTGGCATTCT-3'; 5'-TAGGGCACCCCTTGTTCCTGAGCCATGAAGCCCACGATG-3'; loop V, 5'-ACCGAGGGTGGCATGTATGTCTTCCAACCTGTTTGATTACT-3'; 5'-GACATACATGCCACCCTCGGTGATGTTAGACAG-3'; loop VI, 5'-AAATATAATAATTATATGTACCCCTCTGGGGCCAGGGCGTGG-GCTG-3'; 5'-GGTACATATAATTATTATTTGAGTGGTGTTCATCTG-CACAGCA-3'. Base changes are underlined. The two amplified products were combined (0.2 ng of each) and used as a template for a second PCR, using N1351 and N1271 as primers. Second PCR produced a 1136-base pair amplified fragment containing the mutation and harboring *Bam*HI and *Stu*I sites. Following digestion by *Bam*HI and *Stu*I, the fragment was directionally cloned into the *Bam*HI and *Stu*I sites of GAT1 cDNA. The following two sets of oligonucleotides were used for mutagenizing GAT3 cDNA. First, N1469 (5'-TTCCAGGAGTGACACTGC-3') corresponding to bases 749–766 of GAT3 cDNA and antisense-mutated oligonucleotide, and the second using sense-mutated oligonucleotide together with T3 primer. The mutagenic oligonucleotides used were loop V of GAT3, 5'-CAGGGCCGATTTACGTGT-TCAAGCTCTTTGACTACTAT-3'; 5'-AACACGTAATGCGGCCCTGT-GTGAGCATGATGAGCCCGAT-3'. Base changes are underlined. The resulting two DNA fragments were subjected to second PCR using N1469 and T3 primer. The PCR product of 1382 base pairs was digested by *Bg*III and *Hind*III and directionally cloned into the *Bg*III and *Hind*III sites of GAT3 cDNA. All the mutations were confirmed by sequence analysis (18).

**Expression in *Xenopus* Oocytes**—Following linearization of the plasmid by *Stu*I for GAT1 or *Hind*III for GAT3, RNA was synthesized using SP6 or T7 RNA polymerases with RNA synthesis and capping kit from Ambion Inc. The synthetic RNA was injected into *Xenopus* oocytes, and

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<sup>1</sup> The abbreviations used are: GABA,  $\gamma$ -aminobutyric acid; PCR, polymerase chain reaction.



**FIG. 1. Mutations introduced into the external loops (III–VI) of GAT1.** Putative external loops of GABA transporters are depicted in a transmembrane model (A). Each loop is marked in Roman numerals I to VI. B, amino acid sequences of the external loops III, IV, V, and VI of the various GABA transporters. Mutations were introduced into the *underlined residues* of GAT1 and GAT3. Swapped sequences are indicated by the *arrows*.

the GABA uptake was assayed as follows. Oocytes were surgically removed from frog and defolliculated by collagenase treatment for 2 h. After recovery at 16 °C for 24 h, the oocytes were injected with 50 nl containing 2–10 ng of synthetic RNA. After 3 days they were assayed for GABA transport. Prior to the uptake assay, the oocytes were washed three times with 1 ml of a solution containing 102 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM HEPES (pH 7.5) and preincubated in 0.8 ml of this solution for 15 min. After removal of the preincubation medium, the transport reaction was started by the addition of 0.8 ml of solution containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.5), about 0.1 μCi of [<sup>3</sup>H]GABA, and 1 μM or the specified amounts of cold GABA. After incubation for 20 min with shaking at room temperature, the oocytes were washed three times with 1 ml of the incubation solution in which the substrate was omitted. Individual oocytes were solubilized in 500 μl of 1% SDS, and the radioactivity was determined by scintillation counting. 5–10 oocytes were used for each experimental point, and the data were expressed as the average uptake/h.

#### RESULTS AND DISCUSSION

It has been proposed that the family of Na<sup>+</sup>/Cl<sup>-</sup> neurotransmitter transporters are composed of 12 transmembrane helices, and their N and C termini are situated at the cytoplasmic side of the membrane (19). This structure contains six external loops of various sizes that are denoted in Fig. 1A (I–VI). We looked for amino acid sequences in these putative external loops that were nearly identical among GAT2, GAT3, and GAT4 and were significantly different in GAT1. Fig. 1B compares the amino acid sequences in the external loops of the four GABA transporters (see Fig. 1A for their position). It is apparent that in these regions the amino acid sequences of GAT1 are quite unique. Site-directed mutagenesis was used to generate GAT1 transporters in which the amino acid sequences of these loops were identical to those of GAT3 (7, 9) (Fig. 1B). Each mutant was analyzed by following its GABA uptake expression in *Xenopus* oocytes (8, 9). The cRNA-injected oocytes accumulated up to 70 times as much GABA as uninjected oocytes,

oocytes injected with water, or oocytes injected with the glycine transporter cRNA. The expression levels for each mutant of GAT1 were evaluated by assay of [<sup>3</sup>H]GABA transport activities. Fig. 2 shows the kinetics of GABA uptake by the expressed mutants of GAT1. We introduced the loop structure of GAT3 into loops III and V of GAT1 (Fig. 1B). An Eadie-Hofstee plot revealed an apparent  $V_{\max} = 310$  and 190 pmol/oocyte/h for GABA uptake of loop III and V mutants (Fig. 2, A and C). These substitutions increased the  $V_{\max}$  value 3-fold over that of the wild-type GAT1. However, substitutions of GAT1 into the corresponding loops III and V structure of GAT3 did not significantly affect the  $K_m$  value. These mutants mimicked the  $V_{\max}$  value of GAT3 without changes in GABA binding affinity (Table I).

Oocytes injected with cRNA containing loop IV mutation of GAT1 took up GABA by a high affinity mechanism (Fig. 2B). This mutant had a GAT4-type structure in loop IV (Fig. 1). Kinetic analysis revealed a  $K_m$  of 2.0 μM, which was similar to the  $K_m$  value reported for the high affinity GAT4 GABA uptake (6, 9). This  $K_m$  value was about 4-fold lower than that reported for GABA uptake by GAT1 ( $K_m = 8.7$  μM). The mutation in loop IV did not significantly alter the  $V_{\max}$  value from that of GAT1 (77 pmol/oocyte/h). This mutant mimicked the high affinity of GABA binding of GAT4 without change in  $V_{\max}$  value (Table I).

In addition, we exchanged the amino acid sequence of loop VI of GAT1 with that of GAT2 (Fig. 2D). Kinetic analysis revealed a  $K_m$  of 35 μM, which is 9-fold higher than the value of GAT1. The mutant showed low affinity GABA uptake, which was similar to the uptake by GAT2 ( $K_m = 79$  μM) (8). The mutation in loop VI increased the  $V_{\max}$  value more than 2-fold in comparison with that of GAT1. This mutant mimicked the low binding affinity and the  $V_{\max}$  value of GABA uptake by GAT2 (Table I).

β-Alanine inhibits the GABA uptake by the expressed GAT3

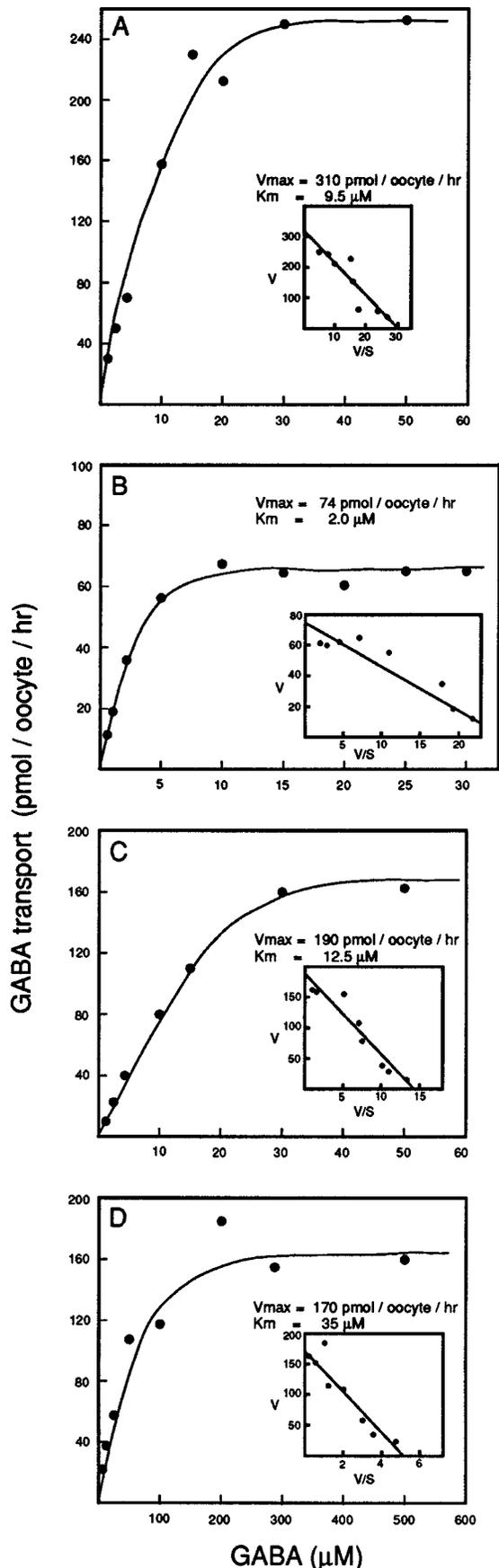


FIG. 2. Effect of GABA concentration on GABA uptake by *Xenopus* oocytes injected with synthetic mRNA of GAT1 and mutations in the extracellular loops. [ $^3\text{H}$ ]GABA uptake into mRNA-injected oocytes was assayed at the indicated GABA concentra-

TABLE I  
 $K_m$  and  $V_{max}$  of the GAT1 cross-mutants and the four wild-type GABA transporters

The experimental conditions were as described in Fig. 2.

	$K_m$	$V_{max}$	Reference
	$\mu\text{M}$	pmol/h/oocyte	
GAT4	0.7	2	9
GAT3	18	217	9
GAT2	79	197	8
GAT1	8.7	77	This work
GAT1 cross-mutants			
Loop III	9.5	310	This work
Loop IV	2.0	74	This work
Loop V	12.5	190	This work
Loop VI	35.0	170	This work

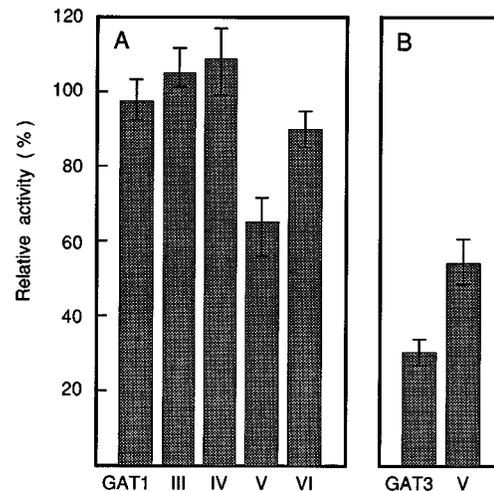


FIG. 3. The effect of  $\beta$ -alanine on GABA transport by GAT1, GAT3, and cross-mutants in their external loops. GABA transport was assayed in the absence or presence of  $200 \mu\text{M}$  cold  $\beta$ -alanine during the transport reaction. 5–10 oocytes were used for each experimental point, and the data are expressed as the average uptake as pmol/h/oocyte. Vertical bars give the standard deviation. The results are given as percentage of the control activity (100%) without  $\beta$ -alanine by GAT1 and cross-mutants (A) and by GAT3 and cross-mutants (B). Mutations introduced into each external loop are shown in Fig. 1B.

and GAT4, and to a much lesser extent by GAT2 (9). However, GAT1 is not sensitive to  $\beta$ -alanine. These differences in  $\beta$ -alanine sensitivities prompted us to search for the location of the  $\beta$ -alanine binding site. Fig. 3 shows the different  $\beta$ -alanine sensitivities to GABA uptake by GAT1, GAT3, and cross-mutants in their loops. As reported previously,  $\beta$ -alanine did not compete with GABA uptake by GAT1; however, GABA uptake by GAT3 was sensitive to  $\beta$ -alanine. When amino acids in loop V of GAT1 were substituted to give identical amino acid sequence as in the corresponding loop of GAT3,  $\beta$ -alanine inhibited the GABA uptake activity of the mutated transporter. When similar substitutions were done in loops III, IV, and VI of GAT1,  $\beta$ -alanine did not compete with GABA uptake into the oocytes. Strikingly, only three amino acid substitutions in loop V resulted in  $\beta$ -alanine sensitivity of GABA uptake. These three amino acid substitutions rendered loop V of GAT1 identical to that of GAT3 (Fig. 1, V).

To determine whether the three amino acids in loop V of GAT3 were important for  $\beta$ -alanine binding, we substituted the three residues of GAT3 to the corresponding amino acids in GAT1. This reverse mutation of GAT3 exhibited less sensitivity

tions (8, 9). Expression levels for each construct were evaluated by [ $^3\text{H}$ ]GABA transport activity. Eadie-Hofstee analysis is depicted in the inset of each panel. Panels: A, loop III; B, loop IV; C, loop V; D, loop VI.

TABLE II  
Inhibitor sensitivity of the various native and mutated GABA transporters

See Fig. 1 for location and sequences of the mutated transporters. The specified chemicals were present during the preincubation and assay periods at the following concentrations: 200  $\mu\text{M}$   $\beta$ -alanine, 500  $\mu\text{M}$  betaine, 100  $\mu\text{M}$  nipecotic acid, 100  $\mu\text{M}$   $\beta$ -guanidinopropionic acid ( $\beta$ -GPA), and 100  $\mu\text{M}$  L-2,3-diaminopropionic acid (DAPA). 6–10 oocytes were used for each experimental point, and the data are expressed as the average uptake calculated as pmol/h/oocyte.

	GABA transport activity							
	GABA transporters				Cross-mutants of GAT1			
	GAT4	GAT3	GAT2	GAT1	III	IV	V	VI
	% of control							
None	100 $\pm$ 6	100 $\pm$ 1	100 $\pm$ 15	100 $\pm$ 7	100 $\pm$ 15	100 $\pm$ 12	100 $\pm$ 7	100 $\pm$ 15
$\beta$ -Ala	23 $\pm$ 2	31 $\pm$ 8	57 $\pm$ 3	93 $\pm$ 10	110 $\pm$ 6	113 $\pm$ 12	65 $\pm$ 6	95 $\pm$ 10
Betaine	94 $\pm$ 10	123 $\pm$ 15	30 $\pm$ 5	94 $\pm$ 9	90 $\pm$ 9	111 $\pm$ 13	102 $\pm$ 12	79 $\pm$ 6
Nipecotic acid	39 $\pm$ 6	106 $\pm$ 11	100 $\pm$ 15	13 $\pm$ 2	25 $\pm$ 4	6 $\pm$ 2	18 $\pm$ 4	74 $\pm$ 12
$\beta$ -GPA	10 $\pm$ 2	12 $\pm$ 5	71 $\pm$ 10	81 $\pm$ 15	116 $\pm$ 6	44 $\pm$ 9	105 $\pm$ 7	83 $\pm$ 15
DAPA	9 $\pm$ 5	24 $\pm$ 4	43 $\pm$ 10	100 $\pm$ 10	124 $\pm$ 20	75 $\pm$ 10	119 $\pm$ 15	116 $\pm$ 15

of its GABA uptake to  $\beta$ -alanine. This result suggested that three amino acids in loop V participated in the  $\beta$ -alanine binding domain of GAT3.

The pharmacology of the mutated GABA transporters was assayed by following the effect of selective inhibitors of GABA uptake by the various transporters. We tested five drugs for their ability to block the GABA accumulation by the expressed mutant transporters. As shown in Table II, betaine inhibited the GABA transport by the expressed loop VI mutant of GAT1. GABA transport by the loop IV mutant of GAT1 was more sensitive to  $\beta$ -GPA and dansylarginine-*N*-(3-ethyl-1,5-pentanediy)amine than that of loops III, V, or VI mutants. However, loop III, IV, and V mutants retained the original sensitivity to nipecotic acid. The mutant in loop VI was less sensitive to the drug. It is apparent that substrate and inhibitor specificities of the GABA transporters were altered by introducing mutations into their external loops.

An important unanswered question for the mechanism of transport across the biological membrane is the location of the transporter's substrate binding site. An extensive study of bacterial and mammalian sugar transporters failed to situate the precise location of the substrate binding site in these transporters (20–22). Several reactive amino acids such as cysteines were proposed to participate in this activity, but further studies using site-directed mutagenesis revealed that these residues are not directly involved in transport or substrate binding (20, 21). Extended external loops and functional groups within the transmembrane helices were formally analyzed as potential substrate binding sites (22). Although it may be that both of these structures are involved in substrate binding and transport, our work points to the possibility that the short external loops are involved in primary substrate binding. The fact that a reciprocal effect was observed in two different GABA transporters endorses the possibility that indeed loop V in the GABA transporters is involved in the substrate binding. When three amino acids were substituted in this region of GAT1, which exclusively transports GABA, inhibition by  $\beta$ -alanine was observed. Conversely, when the same three amino acid residues found in GAT1 were substituted in GAT3, the inhibition of GABA uptake by  $\beta$ -alanine was decreased. This reciprocal effect suggests that indeed this short loop takes part in the substrate binding site of  $\beta$ -alanine. Although it is almost certain that other parts of the transporters are involved in this binding, we think that we positively identified at least one of the moieties that is involved in this binding. Substitutions in the two adjacent loops (IV and VI) affected the  $K_m$  and  $V_{max}$  of the modified transporters as well as caused alteration in their inhibitor specificity. Taking this observation together with the influence on  $\beta$ -alanine binding by loop V substitutions, we suggest that these three external loops form a pocket on the

transporter into which the substrate binds. Recent studies with monoamine transporters using chimeric transporters also concluded that this region is involved in substrate binding (23, 24). It is likely that the location identified in this work is only the initial binding site of the substrate, and upon binding to this structure the substrate may move to a secondary site that is more involved in the transport process. The conclusion reached in the present investigation should be rigorously substantiated by other approaches. In the absence of x-ray crystallographic data on any membrane transporter, the immediate techniques that can supplement this work are the use of biophysical as well as genetic means of second-site suppressors. We presently are expressing the neurotransmitter transporters in bacteria and yeast to provide a ready system for obtaining second-site suppressors for inactive mutations.

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