Different Residues in the GABA<sub>A</sub> Receptor α<sub>1</sub>T60-α<sub>1</sub>K70 Region Mediate GABA and SR-95531 Actions

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Although γ-aminobutyric acid type A receptor agonists and antagonists bind to a common site, they produce different conformational changes within the site because agonists cause channel opening and antagonists do not. We used the substituted cysteine accessibility method and two-electrode voltage clamping to identify residues within the binding pocket that are important for mediating these different actions. Each residue from α<sub>1</sub>T60 to α<sub>1</sub>K70 was mutated to cysteine and expressed with wild-type β<sub>2</sub> subunits in Xenopus oocytes. Methanethiosulfonate reagents reacted with α<sub>1</sub>T60C, α<sub>1</sub>D62C, α<sub>1</sub>F64C, α<sub>1</sub>S68C, and α<sub>1</sub>K70C. γ-Aminobutyric acid (GABA) slowed methanethiosulfonate modification of α<sub>1</sub>F64C, α<sub>1</sub>R66C, and α<sub>1</sub>S68C, whereas SR-95531 slowed modification of α<sub>1</sub>D62C, α<sub>1</sub>F64C, and α<sub>1</sub>R66C, demonstrating that different residues are important for mediating GABA and SR-95531 actions. In addition, methanethiosulfonate reaction rates were fastest for α<sub>1</sub>F64C and α<sub>1</sub>R66C, indicating that these residues are located in an open, aqueous environment lining the core of the binding pocket. Positively charged methanethiosulfonate reagents derivatized α<sub>1</sub>F64C and α<sub>1</sub>R66C significantly faster than a negatively charged reagent, suggesting that a negatively charged subsite important for interacting with the ammonium group of GABA exists within the binding pocket. Pentobarbital activation of the receptor increased the rate of methanethiosulfonate modification of α<sub>1</sub>D62C and α<sub>1</sub>S68C, demonstrating that parts of the binding site undergo structural rearrangements during channel gating.

Few studies of ligand-gated ion channels (LGICs) have addressed the question of how the binding of compounds with divergent structure leads to dramatic functional differences.

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1 The abbreviations used are: LGIC, ligand-gated ion channel; GABA, γ-aminobutyric acid; GABA<sub>A</sub>, γ-aminobutyric acid type A; SCAM, substituted cysteine accessibility method; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate; MTSET, 2-(trimethylammonium)ethyl methanethiosulfonate; MTS, methanethiosulfonate; MTS, methanethiosulfonate; MTSET, 2-aminoethyl methanethiosulfonate; MS-2, mercaptoethanol; AChBP, acetylcholine-binding protein.

For example, agonist binding induces conformational changes that result in channel opening, whereas binding of competitive antagonists does not. Distinguishing the specific amino acid residues involved in the binding of agonists and antagonists will help to elucidate the structural rearrangements that govern the pharmacological effects of these compounds. In this paper, we examined the molecular determinants important for the binding of the agonist GABA and the competitive antagonist SR-95531 to the γ-aminobutyric acid type A (GABA<sub>A</sub>) receptor and explored the conformational changes that occur within the GABA-binding site during channel activation by a barbiturate.

GABA<sub>A</sub> receptors are heteropentameric chloride channels that mediate fast synaptic inhibition in the brain and are members of an evolutionarily related superfamily of LGICs that also includes nicotinic acetylcholine, glycine, and serotonintype 3 receptors (1). To date, 16 different GABA<sub>A</sub> receptor subunit isoforms (α<sub>1</sub>–6, β<sub>1</sub>–3, γ<sub>1</sub>–3, δ, ε, π, and θ) have been cloned (2–7). Most native receptors are thought to contain α, β, and γ subunits (8) in a 2:2:1 stoichiometry (9), although functional channels that lack benzodiazepine modulation can be formed without the γ subunit (10, 11).

The neurotransmitter recognition site, where agonists such as GABA and muscimol and antagonists such as SR-95531 and bicuculline bind, is located at the interface between the α and β subunits because residues have been identified on both subunits that are important for ligand recognition. On the α<sub>1</sub> subunit, residues identified include Phe<sup>64</sup> (12, 13), Arg<sup>66</sup>, Ser<sup>68</sup> (14), Arg<sup>119</sup>, and Ile<sup>120</sup> (15, 16). On the β<sub>2</sub> subunit, residues Tyr<sup>157</sup>, Thr<sup>160</sup> (17), Thr<sup>202</sup>, Ser<sup>204</sup>, Tyr<sup>205</sup>, Arg<sup>207</sup>, and Ser<sup>209</sup> (17, 18) have been identified. Based on work on the related nicotinic acetylcholine receptor, residues that contribute to forming the binding site are located in at least six different non-contiguous extracellular N-terminal regions of the α and β subunits. These regions have been designated loops A–F (19). Residues within these loops likely have different functional roles. Some residues may directly contact ligand, some may be important for maintaining the structural integrity of the binding site, and others may mediate local conformational movements within the site.

In the present study, we examined the binding site region surrounding α<sub>1</sub>F64 (loop D) of the GABA<sub>A</sub> receptor. In the homologous region of the serotonin-type 3 receptor, White and colleagues (20) used alanine-scanning mutagenesis and determined that different amino acid residues contribute to the binding of agonists and antagonists. We hypothesized that the region surrounding α<sub>1</sub>F64 of the GABA-binding site also contains unique residues important for agonist and antagonist binding, and we tested this hypothesis by using the substituted cysteine accessibility method (SCAM).

SCAM has been used on a variety of ion channels to elucidate channel lining and binding site residues, to determine the
location of channel gates and selectivity filters, and to identify regions of the protein that are involved in conformational rearrangements during state changes (21). In this method, individual amino acid residues are mutated to cysteine and the ability of sulfhydryl-specific reagents to modify covalently each introduced cysteine is assessed by observing the effect of the reagent on receptor function. We measured the rates of sulfhydryl modification of accessible introduced cysteine residues in the presence and absence of GABA and SR-95531. We identified a subsite important for agonist binding that includes Tyr59, Thr60, Ile61, Asp62, Val63, Phe64, Phe65, Arg66, Gln67, Ser68, Trp69, and Lys70, where the number reflects the position in the mature α1 subunit protein. The cysteine mutants were subcloned into pGH19 (23, 24) for expression in Xenopus oocytes (0.3 ng of cRNA/subunit/oocyte, except for α1,F64C where currents were increased after application of MTS reagents. *, cysteine substitution was not tolerated at these positions.

**Fig. 1.** Effects of MTS reagents on wild-type and mutant GABA<sub>A</sub> receptors. A, structures and lengths of MTS reagents. Shown are the portions of the MTS reagents that covalently modify an introduced cysteine. Lengths were measured after energy minimization (<0.5 kcal/Å; Chemsketch, ADC, Toronto, Ontario, Canada). B, amino acid residues α1,T60C–α1,K70 were individually mutated to cysteine and expressed with β<sub>2</sub> subunits in Xenopus oocytes. By using two-electrode voltage clamping, the accessibility of the introduced cysteines to MTSEA, MTSET, and MTSES was examined. The absolute change in GABA-mediated current after MTS treatment is plotted for wild-type (WT) and mutant receptors (percent effect = [(I<sub>MTSEA, MTSET, or MTSES</sub> − I<sub>GABA, before</sub>) × 100]/I<sub>GABA, before</sub>). †, percent effect reflects inhibition of current for all mutants except T60C where currents were increased after application of MTS reagents.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—The α<sub>1</sub> cysteine mutants were engineered using the Altered Sites I®<sup>®</sup> in vitro Mutagenesis Systems (Promega Corp., Madison, WI) or by recombinant PCR as described previously (14, 22). Cysteine substitutions were made in the rat α<sub>1</sub> subunit at positions Tyr<sup>59</sup>, Thr<sup>60</sup>, Ile<sup>61</sup>, Asp<sup>62</sup>, Val<sup>63</sup>, Phe<sup>64</sup>, Phe<sup>65</sup>, Arg<sup>66</sup>, Gln<sup>67</sup>, Ser<sup>68</sup>, Trp<sup>69</sup>, and Lys<sup>70</sup>, where the number reflects the position in the mature α<sub>1</sub> subunit protein. The cysteine mutants were subcloned into pH19 (23, 24) for expression in Xenopus laevis oocytes. The presence of the mutations was verified by restriction endonuclease digestion and double-strand cDNA sequencing. The mutants have been named, using the single letter amino acid code, as wild-type residue, residue number, and mutated residue.

**Expression in Oocytes—**X. laevis oocytes were prepared as described previously (25). cRNA transcripts were generated using the mMessage M<sub>T</sub> kit (Ambion, Austin, TX). GABA<sub>A</sub> receptor rat α<sub>1</sub> or α<sub>2</sub> mutants were expressed with wild-type rat β<sub>2</sub> subunits by injection of cRNA into oocytes (0.3 ng of cRNA/subunit/oocyte, except for α1,F64C<sub>β</sub> and α1,R66C<sub>β</sub> that were injected at 7 ng of cRNA/subunit to ensure high levels of receptor expression). Mean maximal responses to GABA ranged from 1 to 10 μA. The oocytes were stored in ND96 medium (in mM: 96 NaCl, 2 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, and 5 HEPES, pH 7.4) supplemented with 100 μg/ml gentamicin and 100 μg/ml bovine serum albumin for 2–14 days and used for electrophysiological recordings.
Rate of MTS Modification—The rate of MTS reagent covalent modification of introduced cysteines was determined by measuring the outcome of sequential applications of MTS reagents on $I_{\text{GABA}}$. The protocol was as follows: $EC_{20}$–$EC_{95}$, GABA was applied for 5 s; the cell was washed for 30 s; MTS reagent was applied for 5–20 s; the cell was washed for 2.5 min; and the response was repeated until $I_{\text{GABA}}$ no longer changed indicating that the reaction was complete. Before the rate of MTS modification was measured, GABA was applied every 3 min until $I_{\text{GABA}}$ stabilized to within 3% demonstrating that the observed changes in $I_{\text{GABA}}$ after application of MTS reagent were due to the effects of the MTS reagent. Concentration of MTS reagent and time of application varied as follows: $a$D62C: MTS, 1 mM, 20 s; MTSES, 10 nM, 5 s; MTSET, 100 nM, 5 s; $a$R66C: MTS, 500 nM, 20 s; MTSET, 1 mM, 5 s; MTSEA, 10 mM, 10 s; $a$S68C: MTS, 150 mM, 10 s; MTSET: 100 mM, 5 s; MTSEA: 100 mM, 5 s. The effects of agonists and antagonists on the rate of MTS modification were tested by co-applying GABA (EC$_{20}$–EC$_{95}$), SR-95531 (IC$_{90}$–IC$_{95}$), or pentobarbital (500 μM) with MTS for all mutants except $a$D62C, in which case they were co-applied with MTSEA. For these studies, $I_{\text{GABA}}$ was stabilized before the rate of MTS reaction was measured as follows: apply GABA (EC$_{20–90}$) for 5 s, wash for 30 s, apply GABA, SR-95531, or pentobarbital at high concentration for 5–20 s, wash for 2.5 min, and repeat the procedure. This procedure was repeated until the peak of the GABA (EC$_{20–60}$) current was within 3% of the previous GABA (EC$_{20–60}$) current peak.

For all rate experiments, the decrease in current was plotted versus cumulative time of MTS exposure. We assume that the concentration of MTS reagent does not change significantly during the reaction, and thus, we can determine a pseudo first–order rate constant from the rate of decrease in $I_{\text{GABA}}$. Peak current at each time point was normalized to the initial peak current, and a pseudo first-order rate constant ($k_1$) was determined by fitting the data with a single exponential decay equation: $y = y_0 + a + b e^{-kt}$. Because the data are normalized to $I_{\text{GABA}}$ at time 0, span = $t = t_0$. The second-order rate constant ($k_2$) for MTS reaction was determined by dividing the calculated pseudo first-order rate constant by the concentration of MTS reagent used (26). To verify the accuracy of this protocol, second-order rate constants were determined using at least two different concentrations of MTS reagents for several mutants.

$EC_{50}$ Analysis—Concentration-response experiments were performed as described previously (14). In brief, these trials used a low concentration of GABA (EC$_{20}$–EC$_{90}$) immediately before the test concentration of agonist to correct for any slow drift in GABA responses that may occur during the experiment. Currents elicited by each test concentration were normalized to the corresponding low concentration current before curve fitting. Concentration–response data were fit to the following equation: $I = I_{\text{max}}/1 + (EC_{50}/[A])^n$, where $I$ is the peak response to a given concentration of GABA; $I_{\text{max}}$ is the maximum amplitude of current; $EC_{50}$ is the concentration of GABA that produces a half-maximal response; $[A]$ is the concentration of GABA; and $n$ is the Hill coefficient.

$EC_{50}$ Analysis—$IC_{50}$ values were measured as described previously (18). SR-95531 IC$_{50}$ values were measured by applying a fixed concentration of GABA (EC$_{20}$–EC$_{90}$) immediately followed by co-application of the same concentration of GABA and a test concentration of SR-95531. Inhibition was calculated as $I_{\text{GABA}} - i_{\text{SR-95531/GABA}}$. Data were fit to the following equation: inhibition = $1 - 1/(1 + (IC_{50}/[A])^{n})$, where IC$_{50}$ is the concentration of the antagonist that blocks half of $I_{\text{GABA}}$; $[A]$ is the concentration of antagonist, and $n$ is the Hill coefficient. $K_v$ values were calculated using the Cheng-Prusoff/Chou equation (27, 28): $K_v = IC_{50}/[A]/1 + [A]/EC_{90}$, where $[A]$ is the concentration of GABA used, and EC$_{90}$ is the concentration of GABA that elicits a half-maximal response.

Statistical Analysis—Data analysis was carried out using nonlinear regression analysis included in the GraphPad Prism software package (San Diego, CA; www.graphpad.com). Statistical analysis was conducted using a one-way analysis of variance, followed by a post hoc Dunnett's test.

Measurement of Length of MTS Reagents, GABA, and SR-95531—All compounds were measured after energy minimization (<0.5 kcal/A; Chemsketch, ADC, Toronto, Ontario, Canada). All MTS reagents were measured from the sulfur to the center of the base of the tetrahedron formed by the terminal tertiary group. GABA was measured from the nitrogen to the base of the tetrahedron formed by the terminal tertiary group. SR-95531 was measured from the carbon of the methyl group to the center of the base of tetrahedron formed by the carboxyl group.

Structural Modeling—The mature protein sequences of the rat $\alpha_1$ and $\beta_3$ subunits were homology modeled with a subunit of the acetylcholine-binding protein (AChBP) (29). The crystal structure of the AChBP was downloaded from the RCSB Protein Data Bank (code 1B9B).
and loaded into Swiss Protein Data bank Viewer (SPDBV, ca.expasy. org/spdbv). The α1 protein sequence from Thr12–Ile227 and the β2 protein sequence from Ser16–Leu198 were aligned with the AChBP sequence using the alignment function of SPDBV. The aligned sequences of the α1 and β2 subunits were threaded onto an AChBP subunit using the “Interactive Magic Fit” function of SPDBV. The threaded subunits were imported into SYBYL (Tripos, Inc., St. Louis, MO) where energy minimization was carried out with the first 100 iterations carried out using Simplex minimization followed by 10,000 iterations using the Powell method.

### RESULTS

**Modification of Cysteine Mutants by MTS Reagents**—We reported previously that when mutated to cysteine, alternating residues from α1T60 to α1S68 are accessible to covalent modification by MTSEA-biotin, suggesting that this region of the GABA-binding site forms a β-strand (14). We also determined that the presence of GABA inhibits the reaction of MTS compounds at α1F64C, α1R66C, and α1S68C, indicating that these residues may face into the agonist-binding pocket. In the present study, we used MTS reagents of different size and charge to explore the physicochemical environment of this region of the GABA-binding site. The MTS reagents used were MTSEA (3.7 Å long), which covalently adds a positively charged ethyl-ammonium group, MTSET (4.5 Å), which adds a positively charged ethyl-trimethylammonium group, and MTSES (4.8 Å), which adds a negatively charged ethyl-sulfonate group (Fig. 1).

In order to determine the ability of the MTS reagents to react with each introduced cysteine, mutant α1 and wild-type β2 subunits were co-expressed in Xenopus oocytes, and \( I_{\text{GABA}} \) (EC40–60) was measured before and after a 2-min MTS application. Because the MTS reagents did not affect the amplitude of \( I_{\text{GABA}} \) at wild-type α1β2 receptors, we assumed that current changes observed in mutant receptors were due to covalent modification of the introduced cysteine residues (Fig. 1). In general, the residues that were reported previously (14) to be modified by MTSEA-biotin (α1T60C, α1D62C, α1F64C, α1R66C, and α1S68C) were also accessible to modification by MTSEA, MTSET, and MTSES (Fig. 1). Reaction with MTS reagents reduced GABA current by 14 (α1K70C, MTSET) to 96% (α1F64C, MTSEA). In contrast to all other mutants, covalent modification of α1T60C caused an increase in \( I_{\text{GABA}} \) suggesting that the GABA EC50 value for this mutant receptor decreases following covalent modification.

At any given position, the magnitude of the MTS effect on \( I_{\text{GABA}} \) was dependent on the specific MTS reagent used (Fig. 1). The observed differences in MTS effects may be due to the charge and/or size of the functional group tethered within the binding site. However, it is also possible that the MTS reactions did not go to completion due to their varied intrinsic reactivities (21). To test this possibility, we measured the rate at which each MTS reagent modified α1D62C, α1F64C, α1R66C, and α1S68C, making sure that each reaction was followed to completion.

### MTS Reaction Rate Constants

The rates of reaction of MTS modification of a binding site were measured as described under “Experimental Procedures.” \( k_2 \) values represent mean second-order rate constants ± S.D. of at least three experiments. The free solution (free sol.) rates were reported by Karlin and Akabas (21) and reflect the rate at which each MTS compound reacts with 2-mercaptoethanol, in solution.

### Table I

<table>
<thead>
<tr>
<th>Receptor</th>
<th>MTSES ( k_2 ) s⁻¹</th>
<th>NR ( ^* )</th>
<th>MTSET ( k_2 ) s⁻¹</th>
<th>NR ( ^* )</th>
<th>MTSEA ( k_2 ) s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1D62Cβ2</td>
<td>8.2 ± 0.4</td>
<td>&gt;4</td>
<td>1</td>
<td>&gt;4</td>
<td>1</td>
</tr>
<tr>
<td>α1D62Cβ2</td>
<td>28 ± 2</td>
<td>&gt;4</td>
<td>3.46^*</td>
<td>&gt;4</td>
<td>3.46^*</td>
</tr>
<tr>
<td>α1F64Cβ2</td>
<td>600 ± 10</td>
<td>&gt;4</td>
<td>72.8^*</td>
<td>&gt;4</td>
<td>72.8^*</td>
</tr>
<tr>
<td>α1R66Cβ2</td>
<td>2610 ± 160</td>
<td>&gt;4</td>
<td>320^p</td>
<td>&gt;4</td>
<td>320^p</td>
</tr>
<tr>
<td>α1S68Cβ2</td>
<td>8.0 ± 0.7</td>
<td>&gt;4</td>
<td>0.97</td>
<td>&gt;4</td>
<td>0.97</td>
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</table>

^* NR is no reaction.

### Table II

<table>
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<tr>
<th>Receptor</th>
<th>EC50 (μM)</th>
<th>Mutant/wild-type</th>
<th>KI (nM)</th>
<th>Mutant/wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1β2</td>
<td>8.2 ± 0.4</td>
<td>&gt;4</td>
<td>1</td>
<td>&gt;4</td>
</tr>
<tr>
<td>α1D62Cβ2</td>
<td>28 ± 2</td>
<td>&gt;4</td>
<td>3.46^*</td>
<td>&gt;4</td>
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<tr>
<td>α1F64Cβ2</td>
<td>600 ± 10</td>
<td>&gt;4</td>
<td>72.8^*</td>
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<td>2610 ± 160</td>
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<td>320^p</td>
<td>&gt;4</td>
</tr>
<tr>
<td>α1S68Cβ2</td>
<td>8.0 ± 0.7</td>
<td>&gt;4</td>
<td>0.97</td>
<td>&gt;4</td>
</tr>
</tbody>
</table>

\( ^a \) Mutant EC50 and KI values were statistically significant from wild type (\( p < 0.001 \)).

\( ^b \) Mutant EC50 values and SR-95531 KI values were measured using two-electrode voltage clamping in oocytes as described under “Experimental Procedures.” All EC50 and KI values are expressed as the average of at least three independent experiments ± S.D. GABA EC50 values were reported previously by Boileau et al. (14).
pocket and along the pathway; 3) the ionization (acid dissociation) of the substituted cysteine’s sulfhydryl group; and 4) steric restrictions in forming an activated complex between the thiolate of the substituted cysteine and the MTS reagent. MTS reagents react preferentially with the ionized thiolate (RS−) form of cysteine (30, 31). Of the residues tested, covalent modification of α1F64C was the fastest, indicating that this is the most accessible residue in loop D. For example, MTSET modified α1F64C with a $k_2$ of $-5,500,000$ M$^{-1}$ s$^{-1}$, which was about 340-fold faster than reaction at α1R66C. Reaction at α1R66C, in turn, was about 40-fold faster than modification at α1S68C (Table I). At α1D62C, MTSEA was the only reagent tested that significantly altered $I_{GABA}$. There are two possible explanations for this result. Either MTSET and MTSES do not react with α1D62C or covalent modification by these reagents does not change $I_{GABA}$, implying that any apparent modification is functionally silent. To test these possibilities, we measured the ability of MTSEA to modify covalently α1D62C after application of MTSET or MTSES. If MTSET or MTSES modified α1D62C, then reaction with MTSEA should not occur, and no change in $I_{GABA}$ should be observed. Application of MTSET or MTSES prior to MTSEA had no effect on the ability of MTSEA to inhibit $I_{GABA}$ (data not shown), indicating MTSET and MTSES do not react with α1D62C. It should be noted that the reaction rate of MTSA with α1D62C was very slow ($k_2 = 16$ M$^{-1}$ s$^{-1}$) indicating that α1D62C has limited accessibility.

In free solution, the rates of MTSET, MTSES, and MTSEA with 2-mercaptopethanol (2-ME) are 76,000, 212,000, and 17,000 M$^{-1}$ s$^{-1}$, respectively (21) (Table I). The rate constants depend on the charges of the reactants. Because the net charge of 2-ME is −1, positively charged MTS reagents react faster than negatively charged MTS reagents with this compound (31). Interestingly, the reaction rate constants of MTSET and MTSEA with α1F64C were −30-fold faster than the their rates of reaction with 2-ME in free solution (Table I). The rate of reaction of the MTS reagents with α1F64C is influenced by the intrinsic electrostatic potential of the GABA-binding site, which arises from fixed charges and dipoles in the protein. The faster rates of MTSET and MTSEA modification of α1F64C compared with the rates of modification of a simple thiol in solution are likely due to these intrinsic properties of the protein and suggest that the short range interactions of MTSET and MTSEA with the GABA-binding site are stronger than those with a simple thiol. Similar fast rates were measured for MTSET and MTSEA reaction with the acetylcholine-binding site cysteines, α1C192/193, in reduced, wild-type Torpedo nicotinic acetylcholine receptors, $k_2 \sim 3 \times 10^6$ M$^{-1}$ s$^{-1}$ (31).

Intrinsic Negative Electrostatic Potential in the GABA-binding Site—The intrinsic electrostatic potential at a substituted cysteine can be examined by determining the rate of reactions of MTS reagents that differ in charge (26, 31, 32). We examined the electrostatic potential at α1F64C, α1R66C, and α1S68C by comparing the rates of reaction of the positively charged MT-SET and the negatively charged MTSES (Fig. 2B). Because MTSET and MTSES are approximately equivalent in size and have a common reaction mechanism, differences in their respective rates of reaction at a given residue are likely due to their opposite charges. The second-order rate constants for MTSET modification of α1F64C, α1R66C, and α1S68C were 235-, 2320-, and 10.4-fold faster than that for MTSES, respectively (Table I and Fig. 2B). In comparison, the second-order rate constant for MTSET modification of 2-ME is 12.5-fold faster than that for MTSES. To factor out the intrinsic differences in the reactivities of the two MTS reagents and the extent of ionization of the respective thiolis, we divided the ratio of the rates of the two reagents at an introduced cysteine by the ratio of the rates for the two reagents with 2-ME (31–33). For α1F64C, the ratio of ratios is $\rho = 235/12.5 = 18.8$. For α1R66C and α1S68C, $\rho = 185.6$ and 0.84, respectively. A ratio of ratios that is significantly larger than one indicates that there is a negative potential experienced by that thiol. A ratio of ratios of $\sim 1$ indicates that there is no charge selectivity for the reaction with this residue. We can estimate the effective electrostatic potential at an introduced cysteine as shown in Equation 1,

$$\phi = -\frac{1}{2}(\epsilon_{MTSET} - \epsilon_{MTSES})(RT/F)\ln(\rho)$$

(Eq. 1)

where $\epsilon$ is the charge of the MTS reagent; $R$ is the gas constant; $T$ is absolute temperature, and $F$ is Faraday’s constant (31, 32).

The calculated electrostatic potential at α1F64C is −37 mV and the potential at α1R66C is −66 mV. The negative potential may be smaller at α1F64C because of the nearby positively charged arginine at position 66. The data indicate that there is a substantial negative potential experienced by α1F64C and α1R66C and that a negative subsite exists within the GABA-binding pocket that interacts with the positive charge on MTSET and MTSEA during their reaction with α1F64C or α1R66C.

Expression and Functional Analysis of α1R66 Mutations—Because GABA is zwitterionic, it is plausible that both a positively and a negatively charged subsite are involved in its binding. One residue within loop D that could be part of a positive subsite is α1R66. Previously, we determined that cysteine substitution at α1R66 increased the GABA EC$50$ value 320-fold (14). We mutated α1R66 to other residues including alanine, histidine, leucine, serine, glutamine, and the positively charged lysine. In each case, the GABA EC$50$ values were increased by more than 2 orders of magnitude compared with wild-type receptors (R66A, 1000 ± 510 μM; R66H, 6200 ± 1500 μM; R66L, 1400 ± 400 μM; R66S, 2700 ± 375 μM; R66Q, 5300 ± 1700 μM; R66K, 5600 ± 350 μM; WT, 8.2 ± 0.4 μM). We calculate that the change in free energy due to α1R66 mutation is $\sim 3$–4 kcal/mol. These data suggest that the positively charged arginine at position 66 may play a critical role in agonist binding.

We also measured the $K_v$ values for the antagonist SR-95531 in oocytes expressing α1D62C, α1F64C, α1R66C, or α1S68C.
Whereas mutations at α1F64 and α1R66 altered GABA EC₅₀ values, SR-95531 Kᵣ values were only altered by cysteine substitution at α1F64 (180-fold, Table II and Fig. 3). Because SR-95531 is a larger molecule than GABA, it is likely to be stabilized by different amino acid residues within the binding pocket. These data indicate that within the GABA-binding site there are distinct residues important for agonist and antagonist binding. In this paper, the term “GABA subsite” refers to residues within the overall binding pocket that are important for GABA binding. Similarly, the term “SR-95531 subsite” refers to residues within the pocket that contribute to SR-95531 binding. There may be residues within the binding pocket that are involved in both GABA and SR-95531 binding, and thus the subsites may overlap.

Effects of GABA, SR-95531, and Pentobarbital on MTS Reaction Rate Constants—To identify potential agonist and antagonist subsites, we measured the rates of MTS covalent modification of α1D62C, α1F64C, α1R66C, and α1S68C in the presence and absence of GABA or SR-95531 (Table III and Fig. 4). We reasoned that if these residues line the binding pocket, then the presence of SR-95531 or GABA should slow the rate of MTS reaction due to steric hindrance. Both GABA and SR-95531 significantly slowed the rate of covalent modification at α1F64C and α1R66C, suggesting that these residues line a common agonist/antagonist-binding region. However, the rate of covalent modification of α1D62C was only slowed by SR-95531, whereas the rate of modification of α1S68C was only slowed by GABA. These results suggest that α1D62C may form part of an antagonist subsite, whereas α1S68C appears to form part of an agonist subsite.

GABA not only binds to the receptor but also gates the channel. Therefore, the slowing of the rate of reaction at α1S68C in the presence of GABA could be due to conformational changes that occur when the channel opens and desensitizes rather than to a direct physical block of position α1S68C by GABA. To distinguish between these possibilities, we measured the rate of covalent modification of α1S68C in the presence of pentobarbital, which directly activates the channel (34, 35) by binding to a site distinct from GABA (17).

The rates of covalent modification of α1F64C and α1R66C were not altered in the presence of a directly activating concentration of pentobarbital (500 μM), suggesting that the opening of the channel does not change the ability of the MTS reagents to modify these residues. However, the rates of covalent modification of α1D62C and α1S68C were significantly accelerated in the presence of a high concentration of pentobarbital (500 μM) (Fig. 5 and Table III). Because opening of the channel with pentobarbital accelerated covalent modification and GABA slowed covalent modification of α1S68C, we predict that GABA sterically blocks access to this residue.

Covalent modification of α1D62C by MTSEA was slowed in the presence of SR-95531 and accelerated in the presence of pentobarbital. Although the data are consistent with SR-95531 causing a steric block and α1D62C lining part of an antagonist subsite, it is feasible that SR-95531 could induce a conformational change in the receptor that leads to a slowing of MTSEA reaction at α1D62C.

DISCUSSION

Recently, the crystal structure of the AChBP was solved (29). The AChBP is a homologue of the extracellular N-terminal domain of the nicotinic acetylcholine receptor and binds several ligands of this receptor. The nicotinic acetylcholine receptor and the GABA<sub>A</sub> receptor are related proteins and are members of a LGIC superfamily of receptors. Thus, by using the AChBP structure as a template, we can begin to model the GABA-binding site (Fig. 6). Our secondary structure prediction of loop

![Fig. 4](image-url)
environment of cysteine mutants in loop D. A residue in a relatively open, aqueous environment will have a faster rate of reaction than a residue in a relatively restrictive, nonpolar environment (26). In loop D, the fastest MTS reaction rate occurs at α1F64C, followed by α1R66C, α1S68C, and α1D62C (Table I). In addition, MTS reagents produce the largest inhibition of K_{GABA} at α1F64C, followed by α1R66C, α1S68C, and α1D62C (Fig. 1). Our data indicate that α1F64C and α1R66C are located in an aqueous and sterically unrestricted environment. Such an environment is thought to exist within the core of the binding pocket, and we predict that these residues lie within that core. Our prediction agrees with the AChBP structure where aligned residues are located in the center of the acetylcholine-binding pocket (Fig. 6). In contrast, we predict that α1D62C is located in a sterically confined region and/or its sulfhydryl chain is in a relatively hydrophobic environment that is poorly ionized, because its rate of covalent modification by MTSEA (k2 = 16 M^{-1} s^{-1}) is −150,000-fold slower than that of α1F64C. Again, this is consistent with the structure of the AChBP where the aligned position is at the periphery of the binding site on a region of the β2 strand that is twisting away and below the binding site.

We measured rates of reaction of differently charged MTS reagents to identify charge-specific regions of the binding pocket (Table I). Positively charged MTSET reacts significantly faster than negatively charged MTSES at α1F64C and α1R66C (Fig. 2B). We conclude that the difference in rates is due to a negative electrostatic potential located within the binding pocket. Based on the AChBP structure, residues in the binding pocket that could potentially form this negative subsite include α1E182, α1D183 in the loop F region of the GABA-binding site. Negatively charged residues in the homologous region of the muscle nicotinic acetylcholine receptor (γ1D174/δD180) have been identified that are important for acetylcholine binding (36, 37). Alternatively, the negative subsite could be formed by π electrons of aromatic amino acid side chains (38). Several aromatic residues have been identified that are important for GABA binding (β2Y97 (39), β2Y157, β2Y205, (17)). Experiments are in progress to test these hypotheses.

We speculate that the amino group of GABA is oriented away from α1F64 and α1R66 and faces toward this negative subsite, whereas the carboxylate group of GABA may be stabilized, at least in part, by α1R66. Consistent with this hypothesis, removal of the bidentate positive charge at α1R66 increases GABA EC_{50} values several hundred-fold. In addition, muscimol, a high affinity agonist of the GABA_A receptor, has been shown to photofluorify label the receptor at α1F64 (12), and the photochemistry of this reaction indicates that the carboxylate-like part of the muscimol molecule reacts with α1F64 (40).

In order to elucidate differences between agonist and antagonist binding, we measured rates of covalent modification in the presence and absence of GABA and SR-95531. Covalent
modification of $\alpha_{D62C}$ is slowed by SR-95531 but not GABA, whereas modification of $\alpha_{S68C}$ is slowed by GABA but not SR-95531. In addition, cysteine mutagenesis of $\alpha_{R66}$ causes a change in GABA EC$_{50}$ values but not SR-95531 $K_v$ values, whereas mutagenesis of $\alpha_{F64}$ causes a change in both GABA EC$_{50}$ and SR-95531 $K_v$ values. Based on these data, we conclude that different amino acid residues within the loop D region of the binding pocket are important for mediating the effects of GABA and SR-95531. This is most likely due to differences in ligand structure and/or ligand positioning within the site.

Most GABA$_A$ receptor agonists and antagonists contain a positively and a negatively charged functional group ~5 Å apart (41), similar to the GABA molecule. It is possible that these different classes of compounds bind with their interchange portion in the same orientation. We have provided evidence that the carboxylate group of GABA likely binds near $\alpha_{R66}$ and $\alpha_{F64}$. Thus, like GABA and muscimol, we predict that the negatively charged region of SR-95531 is oriented near $\alpha_{R66}$ and $\alpha_{F64}$.

One problem with this prediction is that mutation of $\alpha_{R66}$ dramatically alters GABA binding but does not affect SR-95531 binding. The larger size of SR-95531 indicates that this molecule likely utilizes more attachment points than GABA within the binding pocket. It is likely that the ring structures of SR-95531, which is larger than GABA, prevents complete closure of the site.

Further studies identifying specific residues involved in agonist and antagonist recognition within other parts of the binding pocket will lead to a better understanding of the mechanisms behind ligand agonism and antagonism and the process of channel gating.