Structure and Dynamics of the GABA Binding Pocket: A Narrowing Cleft that Constricts during Activation

David A. Wagner and Cynthia Czajkowski
Department of Physiology, University of Wisconsin, Madison, Wisconsin 53706

Photo-affinity labeling and mutagenesis studies have identified several amino acids that may contribute to the ligand binding domains of ligand-gated ion channels. These types of studies, however, only generate a one-dimensional, static description of binding site structure. In this study, we used the substituted cysteine accessibility method not only to identify binding pocket residues but also to elicit information about binding site dynamics and structure. Residues surrounding the putative loop C ligand binding domain of the GABA\(_{\alpha}\) receptor (\(\beta_2V199\) to \(\beta_2S209\)) were individually mutated to cysteine, and the mutant subunits were coexpressed with wild-type \(\alpha_1\) subunits in Xenopus oocytes. N-biotinylaminoethyl methanethiosulfonate (MTSEA-biotin) reacts with cysteines introduced at positions G203, S204, Y205, P206, R207, and S209. This accessibility pattern is not consistent with either an \(\alpha\)-helix or \(\beta\)-strand. Instead, G203–S209 seems to form a water-accessible extended coil, whereas V199–T202 appears to be buried in the protein or membrane. Coapplication of either GABA or the competitive antagonist SR-95531 significantly slows MTSEA-biotin modification of cysteines introduced at positions S204, Y205, R207, and S209, demonstrating that these residues line and face into the GABA binding pocket. MTSEA-biotin reaction rates reveal a steep accessibility gradient from G203–S209 and suggests that the binding pocket is a deep narrowing cleft. Pentobarbital activation of the receptor significantly slows MTSEA-biotin modification of cysteines at S204, R207, and S209, suggesting that the binding site may constrict during gating.

Key words: GABA; GABA\(_{\alpha}\) receptor; binding site; substituted cysteine accessibility method; cysteine mutagenesis; agonist efficacy; protein structure

GABA\(_{\alpha}\) receptors share their fundamental structure and functional properties with an evolutionarily related superfamily of ligand-gated ion channels (LGICs) that also includes nicotinic acetylcholine (nACh), 5-HT\(_3\), glycine, and GABA\(_{\alpha}\) receptors (Ortells and Lunt, 1995). For these receptors, neurotransmitter binding induces allosteric changes in the protein that result in channel opening and fast synaptic response. A complete understanding of the function of these receptors will not only require detailed structural information regarding the protein domains involved in agonist binding, transduction, and gating but will also necessitate knowledge of the relative movements of these domains, and their constituent amino acid residues, when the receptor undergoes transitions from the unliganded, closed state to the fully liganded, open state.

Although high-resolution crystal structures of liganded and unliganded receptors may ultimately help us to answer these questions, this data has proven to be notoriously elusive, and precise information regarding the structure and dynamics of the GABA binding domain remains scarce. Methods such as photo-affinity labeling and site-directed mutagenesis have led to the identification of several individual amino acid residues on the \(\alpha\) and \(\beta\) subunits that may contribute to the ligand binding pocket (Sigel et al., 1992; Amin and Weiss, 1993; Smith and Olsen, 1994; Westh-Hansen et al., 1997). This work, and parallel work on the closely related nicotinic acetylcholine receptor, indicates that agonist binding takes place at inter-subunit interfaces (Czajkowski et al., 1993) and may be coordinated by residues from at least six polypeptide loops designated loop A–loop F (Corringer et al., 2000).

One means of extracting more detailed information about the secondary structure, solvent accessibility, and dynamics of a protein domain is the substituted cysteine accessibility method (SCAM) (Javitch et al., 1995; Xu and Akabas, 1996; Wilson and Karlin, 1998; Basiry et al., 1999). SCAM entails the mutation of a residue to cysteine and the subsequent observation of the functional effect (if any) caused by reaction of the introduced cysteine with a sulfhydryl reactive reagent (Karlin and Akabas, 1998). Previously, we used SCAM on the F64 region (loop D) of the GABA\(_{\alpha}\) \(\alpha_1\) subunit to define the secondary structure of this region as a \(\beta\)-strand and identified \(\alpha_1\)F64, \(\alpha_1\)R66, and \(\alpha_1\)S68 as residues likely to line the GABA binding pocket (Boileau et al., 1999).

In the present study, we performed SCAM analysis on residues \(\beta_2V199–S209\), which comprise the putative loop C domain of the GABA binding pocket. These experiments identified four residues that face into the GABA binding pocket: S204, Y205, R207, and S209. Residues that influence GABA affinity but are not part of the pocket were also identified: F200, S201, T202, and G203. Accessibility and rate of reaction studies indicate that loop C has an extended conformation that may traverse the GABA binding pocket from its rim to its depths. Finally, we demonstrate that this region of the binding pocket experiences structural rearrangements consistent with a constriction of the binding pocket during pentobarbital-mediated gating of the receptor.
RESULTS
Cysteine mutation of the β2 loop C region

Mutations at β2-Y205 and β2-T202 cause large shifts in EC50: GABA values of αβ2mut and αβ2mutY2 GABA receptors but have no effect on direct activation of receptors by pentobarbital (Amin and Weiss, 1993), indicating that these residues may contribute to the ligand binding pocket. These residues align with putative ligand binding domains of the nACH α subunit (Dennis et al., 1988) and the glycine receptor α subunit (Vandenberg et al., 1992), and this region has been termed loop C (Corringer et al., 2000). To fully evaluate the contribution of the loop C region to ligand binding and gating in the GABAα receptor, 11 cysteine mutants were made at positions V199, F200, S201, T202, G203, S204, Y205, P206, R207, L208, and S209 (Fig. 1). The β2 cysteine mutants were subcloned into pGH19 (Lim et al., 1992; Robertson et al., 1996) for expression in Xenopus laevis oocytes. All β2 cysteine mutants were verified by double-stranded DNA sequencing. The β2 cysteine mutants have been named, using the single letter code, as wild-type residue, residue name, and mutated residue.

Expression in oocytes and voltage-clamp analysis. Oocytes from Xenopus laevis were prepared and injected with cRNA as described previously (Boileau et al., 1998). GABAα receptor rat α1, β2, or β2 cysteine mutants in pH91 were expressed by injection of cRNA into oocytes at 20 ng of current, EC50 is the concentration of agonist that evokes a current half-maximal, [A] is the concentration of GABA, and EC50 is the GABA-EC50 for the mutant in question. Measurement of MTSEA-biotin effects. All oocytes were tested for stability of I GABA before addition of MTSEA-biotin (Toronto Research Chemicals Inc., North York, Canada) by applying a 5 sec pulse of GABA every 10 min until the peak currents varied by <3% from one trial to the next. The stability was usually observed after only three to six trials (30–60 min). GABA concentrations ranged between EC50 and EC90. After the GABA response stabilized, we bath applied freshly diluted MTSEA-biotin (2 mM) for 2 min, washed for 5 min, and then recorded I GABA at the same concentration used before MTSEA-biotin treatment. The covariant effect of MTSEA-biotin was calculated as (I GABApost/I GABApre) − 1.

Rate of reaction assays. The rate at which MTSEA-biotin covalently modified introduced cysteines was determined by observing the effects of sequential applications of MTSEA-biotin on I GABA. The protocol was as follows: apply GABA (EC50–EC90) for 5 sec, wash for 30 sec, apply MTSEA-biotin for 5–20 sec, wash for 2.5 min, and repeat sequence (see Fig. 4). This protocol was repeated until the reaction was complete (I GABA no longer changed). To accommodate for the disparate rates at which MTSEA-biotin reacts with the various mutants, the concentration and time period for sequential applications of MTSEA-biotin were varied as follows: G203C, 1 μM, 5 sec; S204C, 1 μM, 10 sec; Y205C, 200 μM, 10 sec; P206C, 200 μM, 20 sec; R207C, 200 μM, 20 sec; and S209C, 1 mM, 10 sec. The effects of agonists and antagonists on reaction rates were assayed by coapplying GABA (EC50–EC90), SR-95531 (IC50–IC90), or pentobarbital (50 μM or 1 mM) with the MTSEA-biotin.

Statistical analysis. All data gathered with the rate of reaction protocol was plotted as I GABA versus cumulative time of MTSEA-biotin exposure The pseudo-first-order rate constant (k) was determined by fitting the plotted data to a single exponential decay equation: y = (span − span x e−kt) + plateau, where span = max – plateau. The second-order rate constant (k2) was determined by dividing the pseudo-first-order rate constant by the concentration of MTSEA-biotin used (Pascual and Karlin, 1998). To verify the accuracy of our protocol, k2 was determined at two different concentrations of MTSEA-biotin for several of the mutants.

Statistical analysis. When determining IC50, EC50, or k2, complete data sets were obtained from individual oocytes. Curve fitting was subsequently performed on the data from each oocyte, and the resultant parameters were used in statistical analysis. Statistical analysis for significant differences was performed by one-way ANOVA with Dunnett’s post hoc test for multiple independent samples. In the case of EC50 and IC50 results, analysis for significance was performed using log values. All curve fits and statistical analysis were performed using Prism software (GraphPad Software Inc., San Diego, CA).
R207C mutations caused 70- to 300-fold shifts in EC_{50-GABA} values relative to wild type, whereas the T202C, G203C, and Y205C mutations resulted in 4800- to 18,000-fold increases in EC_{50-GABA} values (Fig. 2A, Table 1). All of these mutations (with the exception of R207C) also caused significant shifts in the IC_{50} values relative to wild type, whereas the T202C, G203C, and R207C mutations caused 70- to 300-fold shifts in EC_{50-GABA} values. In oocytes expressing wild-type α_1β_2 receptors, the ratio of current elicited by a saturating concentration of P4S to current elicited by a saturating GABA concentration (I_{max-P4S}/I_{max-GABA}) was 0.50 (Fig. 2B, C). In oocytes expressing α_1β_2-F200C receptors, the I_{max-P4S}/I_{max-GABA} ratio (0.45) was not significantly different from wild type, indicating that this mutation has no effect on agonist efficacy. However, oocytes expressing α_1β_2-S201C and α_1β_2-R207C receptors had significantly reduced I_{max-P4S}/I_{max-GABA} ratios of 0.12 and 0.20, respectively (Fig. 2B, C). These results demonstrate that mutation of either β_2-S201 or β_2-R207 to cysteine reduces agonist efficacy at the GABA binding site. A reduction in efficacy can also result in a reduction of the Hill coefficient (Colquhoun, 1998) and may explain why α_1β_2-S201C receptors have a significantly reduced Hill coefficient (n_H) for GABA (Fig. 2A, Table 1). It was not possible to test the remaining mutants that caused EC_{50-GABA} shifts (T202C, G203C, and Y205C) for changes in efficacy because their severely reduced affinities require concentrations of GABA near or above 1 M to elicit maximal responses.

**Reaction of introduced cysteines with MTSEA-biotin**

One of the caveats of SCAM analysis is that the data gathered describes the structure of a mutant receptor that may not be the same as the structure of a wild-type receptor. Because of this, the results of SCAM studies are most reliable if the introduced mutations do not cause large changes in the functional properties of the receptor. Unfortunately, in domains that are functionally significant, even small changes in structure can translate into noticeable changes in receptor behavior. This seems to be the case for the region in question (β2V199–S209) in which 6 of the 11 mutations caused significant changes in EC_{50-GABA} values. However, the fact that none of the mutations significantly affected direct activation by pentobarbital and no significant difference in I_{max-GABA} was detectable suggests that the global structure of the receptor was not altered by any of the cysteine mutations, and it this could be attributable to the fact that SR-95531, a much larger molecule than GABA, may enjoy extra binding interactions that make it more tolerant to a single point mutation within the binding pocket.

**Determining agonist efficacy in cysteine mutants**

Receptor occupancy and gating of LGICs can be most simply described by the model represented in Scheme 1 (del Castillo and Katz, 1957).

In this model, the microscopic affinity for agonist is represented by the dissociation constant (K_D) and agonist efficacy is represented by E, where E is the ratio of the number of fully liganded receptors that are open to the number of fully liganded receptors that are closed (Colquhoun, 1998). When using highly efficacious agonists (E > 10), changes in efficacy have little effect on maximum current and can be difficult or impossible to detect. For instance, if using an agonist with E = 20, a mutation that causes a twofold reduction of efficacy will only produce a 5% change in I_{max}. To determine whether the cysteine mutations that shift EC_{50-GABA} also cause shifts in efficacy, experiments were performed using piperidine-sulfonic acid (P4S), which acts as a partial agonist (E ≈ 1) at α_1 containing GABA_A receptors (Krogsgaard-Larsen et al., 1980; O’Shea et al., 2000).

In oocytes expressing wild-type α_1β_2 receptors, the ratio of current elicited by a saturating concentration of P4S to current elicited by a saturating GABA concentration (I_{max-P4S}/I_{max-GABA}) was 0.50 (Fig. 2B, C). In oocytes expressing α_1β_2-F200C receptors, the I_{max-P4S}/I_{max-GABA} ratio (0.45) was not significantly different from wild type, indicating that this mutation has no effect on agonist efficacy. However, oocytes expressing α_1β_2-S201C and α_1β_2-R207C receptors had significantly reduced I_{max-P4S}/I_{max-GABA} ratios of 0.12 and 0.20, respectively (Fig. 2B, C). These results demonstrate that mutation of either β_2-S201 or β_2-R207 to cysteine reduces agonist efficacy at the GABA binding site. A reduction in efficacy can also result in a reduction of the Hill coefficient (Colquhoun, 1998) and may explain why α_1β_2-S201C receptors have a significantly reduced Hill coefficient (n_H) for GABA (Fig. 2A, Table 1). It was not possible to test the remaining mutants that caused EC_{50-GABA} shifts (T202C, G203C, and Y205C) for changes in efficacy because their severely reduced affinities require concentrations of GABA near or above 1 M to elicit maximal responses.

**Figure 2. GABA dose–response curves and P4S currents.** A, GABA dose–response relationships for wild-type α_1β_2 receptors (○) and three representative mutants: α_1β_2-R207C (▲), α_1β_2-S201C (●), and α_1β_2-Y205C (▼). Data were fit by nonlinear regression as described in Materials and Methods. All data points are normalized to I_{max-GABA} and are shown as mean responses ± SEM from four or more cells. B, Current traces recorded from oocytes expressing wild type or α_1β_2-S201C. Arrows indicate a 5 sec application of saturating P4S (wild type, 1 mM; S201C, 10 mM) or GABA (wild type, 1 mM; S201C, 100 mM). Line break in current trace represents 5 min wash with ND96. C, Bar graph denoting P4S efficacy of wild-type and mutant receptors as I_{max-P4S}/I_{max-GABA} where values given as mean ± SEM follow: α_1β_1, 0.50 ± 0.03, n = 4; α_1β_1-F200C, 0.45 ± 0.06, n = 4; α_1β_1-S201C, 0.12 ± 0.01, n = 3; and α_1β_1-R207C, 0.21 ± 0.02, n = 4. *p < 0.01 indicates values that are significantly different from wild type calculated using a one-way ANOVA with a Dunnett’s post hoc test.
MTSEA-biotin treatment had no significant effect on therefore, the region is likely to be a turn or random coil. possible that these residues react with MTSEA-biotin without any residue from V199C to T202C, it seems likely that this region V199C-, F200C-, S201C-, T202C-, and L208C-containing recep-
tion about the dimensions of the binding site crevice. MTSEA-methanethiolsulfonate (MTS) reagent can also provide informa-
tion. The reactive disulfide is near the end of the tail,

MTSEA-biotin treatment alters change in GABA-mediated current (Fig. 3). Therefore, if assume that MTSEA-biotin has modified the introduced cysteine.

is likely that the changes in EC_{50,GABA} represent small local effects. In addition, cysteine substitution of five of the residues mutated had no discernable effect on any of the receptor properties that we assayed, making them ideal candidates for this study.

Reaction of wild-type αβ2 GABA_{A} receptors with the sulfhydryl-specific reagent MTSEA-biotin caused no significant change in GABA-mediated current (Fig. 3). Therefore, if MTSEA-biotin treatment alters I_{GABA} in a mutant receptor, we assume that MTSEA-biotin has modified the introduced cysteine. MTSEA-biotin treatment significantly decreased I_{GABA} in 6 of the 11 mutant receptors tested (Fig. 3). For each affected mutant, I_{GABA} was inhibited as follows: G203C, −36 ± 9%; S204C, −26 ± 5%; Y205C, −98 ± 1%; P206C, −47 ± 13%; R207C, −55 ± 8%; S209C, −85 ± 1% (mean ± SEM; % inhibition = 100 × [(I_{GABA,post MTSEA-biotin}/I_{GABA,pre MTSEA-biotin}) − 1]). Because six of the seven consecutive residues from G203–S209 reacted with MTSEA-biotin, the accessibility pattern of this region is not predictive of an α-helix or β-strand and, therefore, the region is likely to be a turn or random coil. MTSEA-biotin treatment had no significant effect on I_{GABA} from V199C-, F200C-, S201C-, T202C-, and L208C-containing receptors. Because we cannot detect reaction of MTSEA-biotin with any residue from V199C to T202C, it seems likely that this region is buried in the hydrophobic core of the subunit, but it is also possible that these residues react with MTSEA-biotin without affecting I_{GABA}.

Observation of reaction of an introduced sulfhydryl with a methanethiol-sulfonate (MTS) reagent can also provide information about the dimensions of the binding site crevice. MTSEA-biotin is composed of two distinct structural domains: a flexible tail ~14 Å long and 2.5 Å in diameter, and a 4 × 5 Å planar head group. The reactive disulfide is near the end of the tail, ~12 Å from the head group. Therefore, any residue that reacts with MTSEA-biotin must be accessible via an aqueous pathway >2.5 Å in diameter and <12 Å deep. GABA is a linear molecule ~6 Å long and 3 Å in diameter, and the dimensions of SR-95531 are ~16 Å long and 6 Å in diameter.

Measurement of MTSEA-biotin reaction rates

The rate at which MTSEA-biotin reacts with a cysteine side chain is determined by the physical environment of the sulfhydryl group (e.g., steric hindrance to reaction) and the ionization of the sulf-

<p>| Table 1. Apparent affinities of wild-type and mutant receptors for GABA, SR-95531, and pentobarbital |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Receptor</th>
<th>GABA</th>
<th>SR-95531</th>
<th>PB</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC_{50} (μM)</td>
<td>n</td>
<td>wt/mut</td>
<td>EC_{50} (μM)</td>
</tr>
<tr>
<td>α₁β₂ Wild type</td>
<td>4.3 ± 1.2</td>
<td>1.1</td>
<td>8</td>
</tr>
<tr>
<td>α₁β₂-V199C</td>
<td>3.1 ± 0.4</td>
<td>1.4</td>
<td>4</td>
</tr>
<tr>
<td>α₁β₂-F200C</td>
<td>1292 ± 170*</td>
<td>1.1</td>
<td>4</td>
</tr>
<tr>
<td>α₁β₂-S201C</td>
<td>725 ± 160*</td>
<td>0.5*</td>
<td>4</td>
</tr>
<tr>
<td>α₁β₂-T202C</td>
<td>61100 ± 10900*</td>
<td>1.0</td>
<td>5</td>
</tr>
<tr>
<td>α₁β₂-G203C</td>
<td>20480 ± 6900*</td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td>α₁β₂-S204C</td>
<td>1.5 ± 0.3</td>
<td>1.1</td>
<td>6</td>
</tr>
<tr>
<td>α₁β₂-Y205C</td>
<td>78000 ± 7070*</td>
<td>1.4</td>
<td>5</td>
</tr>
<tr>
<td>α₁β₂-P206C</td>
<td>2.6 ± 1.0</td>
<td>1.2</td>
<td>4</td>
</tr>
<tr>
<td>α₁β₂-R207C</td>
<td>310 ± 30*</td>
<td>1.3</td>
<td>4</td>
</tr>
<tr>
<td>α₁β₂-L208C</td>
<td>2.7 ± 0.5</td>
<td>1.5</td>
<td>5</td>
</tr>
<tr>
<td>α₁β₂-S209C</td>
<td>5.4 ± 1.1</td>
<td>1.1</td>
<td>3</td>
</tr>
</tbody>
</table>

EC_{50} and K values are presented as mean ± SEM. An asterisk indicates that the value is significantly different from wild type (p < 0.01). PB, Pentobarbital; wt, wild type; mut, mutant.

Figure 3. Effects of MTSEA-biotin on wild-type and mutant GABA_{A} receptors. A, Representative current traces demonstrating the effect of MTSEA-biotin treatment (2 mM, 2 min) on currents from wild-type and Y205C-containing receptors. For wild-type traces, [GABA] is 3 μM, and for Y205C traces, [GABA] is 30 μM. B, Effect of MTSEA-biotin treatment on all mutants shown as % change = (I_{GABA,post MTSEA-biotin}/I_{GABA,pre MTSEA-biotin}) − 1) × 100. Results represent the mean ± SEM of at least three experiments. Black bars indicate that the percent change is significantly different from wild type (p < 0.01). Gray bars indicate no significant difference from wild type (p > 0.05).
of MTSEA-biotin. This protocol was repeated until I
in a region in which the sulfhydryl group is in an aqueous
steep rate gradient implies that this stretch of amino acids starts
the most N-terminal residue tested (G203C, 258,000 M
itude (Table 2). The fastest reaction rate was recorded for the
pocket. Pentobarbital at EC50–EC70 significantly slowed the re-
we infer a gating-related structural rearrangement of the binding
ations. Therefore, if the rate at which MTSEA-biotin reacts with
activated by either of these compounds have similar conforma-
may also allosterically modulate the GABAA receptor (Uchida et
95531, a classical competitive agonist for GABA, binds within the
GABA binding pocket. GABA (at EC60–EC80 concentrations) and
the presence of GABA and in the presence of SR-95531. SR-
of various compounds on the reaction rate of MTSEA-biotin with
Figure 4. Measurement of MTSEA-biotin reaction rates. A, B, Examples
of traces recorded during experiments measuring the reaction rate of
MTSEA-biotin with α1β2-R207C receptors. Downward deflections repre-
sent inward current elicited by a 5 sec application of 300 μM GABA
(=EC50). Arrows indicate either 10 sec application of 200 μM MTSEA-
biotin (A) or a 20 sec coapplication of MTSEA-biotin plus 1 μM SR-95531
(B). C, Normalized IGABA plotted as a function of cumulative time of
MTSEA-biotin exposure. Single exponential curve fits illustrate the effect
of various compounds on the reaction rate of MTSEA-biotin with α1β2-
R207C receptors. Data points are normalized to the current measured at
t = 0 and are presented as mean ± SEM. PB, Pentobarbital.

State-dependent changes of binding site conformation
According to Scheme 1, receptor activation has two distinct steps,
binding of agonist and isomerization of the receptor from the
closed to open state. The closed-to-open transition involves a
global allosteric rearrangement of the receptor that not only
opens a gate but also changes the structure of the binding pocket.
We examined gating-related structural changes of the GABA
binding pocket by measuring the effect of the barbiturate pento-
barbital on k2 values for the MTSEA-biotin reaction.

Pentobarbital directly activates the GABAα receptor but does
not bind at the same location as GABA (Ito et al., 1996). Because
the single channel conductances of GABAα receptors activated
by GABA and pentobarbital are similar (Jackson et al., 1982; Akk
and Steinbach, 2000), it is likely that the open states of receptors
activated by either of these compounds have similar conforma-
tions. Therefore, if the rate at which MTSEA-biotin reacts with
an introduced cysteine is altered in the presence of pentobarbital,
we infer a gating-related structural rearrangement of the binding
pocket. Pentobarbital at EC50–EC70 significantly slowed the re-
action rate of MTSEA-biotin with cysteines at positions S204,
G203 (Fig. 5, Table 2).

Identification of binding site residues
To identify residues in loop C that line the GABA binding pocket,
we measured the second-order rate constant (k2) for reaction of
MTSEA-biotin with each accessible introduced cysteine both in
the presence of GABA and in the presence of SR-95531. SR-
95531, a classical competitive agonist for GABA, binds within the
GABA binding pocket. Although evidence suggests that SR-95531
may also allosterically modulate the GABAα receptor (Uchida et
al., 1996; Ueno et al., 1997), it does not activate the receptor and
clearly does not induce the same change in receptor structure as
GABA. Therefore, if the rate at which MTSEA-biotin reacts with
an introduced cysteine is altered by SR-95531 and GABA,
then it is likely that both compounds are sterically interfering with
the reaction and that the sulfhydryl side chain is facing into the
GABA binding pocket. GABA (at EC60–EC80 concentrations) and
SR-95531 (at IC50–IC95 concentrations) significantly slowed the
reaction rate of MTSEA-biotin with cysteines introduced at positions
S204, Y205, R207, and S209 (Fig. 5, Table 2). Therefore,
these residues face into the GABA binding pocket.
concentration of pentobarbital was for G203C, in which 50 μM pentobarbital, undergo a change in environment during gating. These residues, which we have demonstrated to be facing into the binding pocket, is attributable to activation of the receptor. Therefore, the only reaction rate that was affected by a modulatory concentration of pentobarbital was for G203C, in which 50 μM pentobarbital caused an increase in $k_{2}$ (Fig. 5, Table 2). This is direct evidence that allosteric modulation of $I_{\text{GABA}}$ by pentobarbital involves a structural rearrangement near the GABA binding pocket that makes G203C more accessible.

The rate of reaction of MTSEA-biotin with a cysteine introduced at position P206 was significantly increased in the presence of 50 μM pentobarbital, a concentration that robustly potentiates GABA by pentobarbital to decrease the MTSEA-biotin $k_{2}$ values at these positions is attributable to activation of the receptor. Therefore, these residues, which we have demonstrated to be facing into the binding pocket, undergo a change in environment during gating.

The only reaction rate that was affected by a modulatory concentration of pentobarbital was for G203C, in which 50 μM pentobarbital caused an increase in $k_{2}$ (Fig. 5, Table 2). This result confirms the hypothesis that the ability of 1 mM pentobarbital to decrease the MTSEA-biotin $k_{2}$ values at these positions is attributable to activation of the receptor. Therefore, these residues, which we have demonstrated to be facing into the binding pocket, undergo a change in environment during gating.

Figure 5. Summary of effect of GABA, SR-95531, and pentobarbital on the rate at which MTSEA-biotin modifies introduced cysteines. Second-order rate constants were calculated for each reaction, and for each mutant, the rates were normalized to the control rate (rate measured when no other compound is present). *p < 0.01 indicates that rate is significantly different from control rate. All data represent the mean ± SEM of at least three experiments.

Table 2. Summary of second-order rate constants for reaction of MTSEA-biotin with introduced sulphydryls

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Control $k_{2}$ (M$^{-1}$s$^{-1}$)</th>
<th>GABA $k_{2}$ (M$^{-1}$s$^{-1}$)</th>
<th>SR-95531 $k_{2}$ (M$^{-1}$s$^{-1}$)</th>
<th>1 mM PB $k_{2}$ (M$^{-1}$s$^{-1}$)</th>
<th>50 μM PB $k_{2}$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_{1}\beta_{2}$-G203C</td>
<td>257,600 ± 32,700</td>
<td>184,400 ± 14,300</td>
<td>317,700 ± 19,500</td>
<td>579,200 ± 990</td>
<td>400,100 ± 7300</td>
</tr>
<tr>
<td>$\alpha_{1}\beta_{2}$-S204C</td>
<td>12,060 ± 980</td>
<td>6050 ± 980</td>
<td>3735 ± 310</td>
<td>5656 ± 1180</td>
<td>14,990 ± 3300</td>
</tr>
<tr>
<td>$\alpha_{1}\beta_{2}$-Y205C</td>
<td>955 ± 150</td>
<td>410 ± 15</td>
<td>285 ± 5</td>
<td>870 ± 40</td>
<td>ND</td>
</tr>
<tr>
<td>$\alpha_{1}\beta_{2}$-P206C</td>
<td>276 ± 41</td>
<td>715 ± 70</td>
<td>398 ± 16</td>
<td>385 ± 25</td>
<td>352 ± 17</td>
</tr>
<tr>
<td>$\alpha_{1}\beta_{2}$-R207C</td>
<td>585 ± 40</td>
<td>100 ± 9</td>
<td>135 ± 10</td>
<td>370 ± 32</td>
<td>690 ± 65</td>
</tr>
<tr>
<td>$\alpha_{1}\beta_{2}$-S209C</td>
<td>120 ± 2</td>
<td>67 ± 11</td>
<td>40 ± 7</td>
<td>77 ± 8</td>
<td>125 ± 3</td>
</tr>
</tbody>
</table>

Second-order rate constants ($k_{2}$) were calculated by dividing pseudo-first-order rate constants ($k_{1}$) by the concentration of MTSEA-biotin used during rate experiments, which were as follows: G203C, 1 μM; S204C, 10 μM; Y205C, P206C, and R207C, 200 μM; and S209C, 1 mM. Concentrations of GABA and SR-95531 present during the MTSEA-biotin reaction, which varied according to the affinity of the mutant receptor for each compound, were always between EC$^{50}$–IC$^{90}$ for GABA and EC$^{95}$–IC$^{95}$ for SR-95531. An asterisk indicates that the rate is significantly different from control (p < 0.01). PB, Pentobarbital; ND, not determined. Values are mean ± SEM.

DISCUSSION

SCAM analysis of the $\beta_{2}$V199–S209 (loop C) region of GABA$\text{A}_{\alpha}$ receptor identified several amino acid residues that face into the GABA binding pocket and mediate agonist affinity ($K_{D}$) and efficacy. In addition, we provide evidence that the ligand binding pocket is a deep narrowing structure that constrains during gating.

Mutations that affect $K_{D\text{-GABA}}$

Mutation to cysteine causes significant shifts in EC$^{50}$-GABA for six residues: F200C, S201C, T202C, G203C, Y205C, and R207C. According to the model shown in Scheme 1, shifts in EC$^{50}$-GABA can be caused by changes in affinity of the closed receptor for GABA ($K_{D \text{(C)}}$) and/or changes in the ability of GABA to induce opening of the receptor (efficacy or $E$). Determining which of these parameters is responsible for mutation-induced EC$^{50}$ shifts is difficult (Colquhoun, 1998). However, for receptors that require the binding of two ligands for efficient opening and have relatively low $E$ values, a large increase in EC$^{50}$ that is not accompanied by a significant reduction in $I_{\text{max}}$ can be attributed to a reduction in $K_{D \text{(C)}}$ (Amin and Weiss, 1993; Anson et al., 1998). In fact, for the GABA$\text{A}_{\alpha}$ receptor, a 50-fold increase in EC$^{50}$-GABA would have to be accompanied by a >99% reduction in $I_{\text{max}}$ for the shift in the dose–response curve to be caused solely by changes in efficacy. Because none of the mutations in this study cause significant reductions in $I_{\text{max}}$-GABA and the shifts in EC$^{50}$ values range from 70-fold (R207C) to 18,000-fold (Y205C), it is clear that the in-
creases in EC\textsubscript{50-GABA} values reflect, at least in part, a reduction in ligand affinity (K\textsubscript{D}) at the GABA binding site. Although I\textsubscript{max} comparisons between different mutant receptors are problematic because of poor control of expression levels, we feel confident that we would detect a >99% reduction in I\textsubscript{max-GABA}. Moreover, five of the six mutations that shift EC\textsubscript{50-GABA} (all except R207C) also significantly reduce affinity for SR-95531, further suggesting that mutation of F200, S201, T202, G203, Y205, and R207 to cysteine alters the microscopic binding affinity of ligands at the GABA binding site.

When mutation of an amino acid disrupts agonist affinity, it has been used as evidence that the residue in question is located in the binding pocket. This, however, is not proof. Our result, that there is no detectable reaction of MTSEA-biotin with cysteines introduced at the positions F200–T202, suggests that these side chains are not facing into the water-accessible GABA binding pocket. Rather, these residues are likely to be buried in the protein or membrane lipid. Caution, however, must be taken with the interpretation of the accessibility results. The possibility that MTSEA-biotin modifies an introduced cysteine without affecting I\textsubscript{GABA} must also be considered. However, it is unlikely that addition of the large biotin moiety would have no discernible affect on I\textsubscript{GABA} if F200C, S201C, and T202C actually face into the binding pocket. Thus, although mutation of F200, S201, and T202 to cysteine results in large shifts in EC\textsubscript{50-GABA}, we believe these residues are not lining the GABA binding pocket.

In contrast, the large shifts in EC\textsubscript{50-GABA} values caused by mutation of Y205 and R207 likely reflect disruptions of residues that line the binding site. Because MTSEA-biotin reacts with cysteines at positions S204, Y205, R207, and S209 and both GABA and SR-95531 significantly slow their modification, we believe that cysteines at positions S204, Y205, R207, and S209 and both GABA pocket from its rim to its depths. This type of structure correlates with the water-filled tunnels in the nACh receptor identified by electron microscopy (Miyazawa et al., 1999). We hypothesize that, in the GABA\textsubscript{A} receptor, at least a portion of these tunnels mediate local (i.e., in or near the binding pocket) allosteric transitions that translate agonist binding into channel opening.

**Structure of the GABA binding pocket**

Assessment of the accessibility of introduced cysteines to reaction with MTSEA-biotin reveals direct structural information about loop C of the GABA binding pocket. MTS reagents react 10\textsuperscript{5}–10\textsuperscript{9} times faster with ionized sulfhydryl groups than they do with protonated sulfhydryls (Roberts et al., 1986). Therefore, an introduced cysteine that reacts with an MTS reagent is likely to be oriented with its side chain in an aqueous environment in which ionization of the sulfhydryl is more probable (Pascual and Karlin, 1998).

Patterns of accessibility can be used to discern the secondary structure of a region. For example, after mutation to cysteine, alternating residues of the \(\alpha\) loop D domain are accessible to MTSEA-biotin, indicating that the region is a \(\beta\)-strand (Boileau et al., 1999). Here we show that six of seven sequential cysteine mutants in the \(\beta_2\) loop C domain (G203C–R207C and S209C) are available for reaction with MTSEA-biotin. This accessibility pattern does not suggest a regular secondary structure, indicating that the region in question may be an extended coil or loop. This result agrees with secondary structure predictions for the N-terminal domain of the nACh receptor in which the loop C region is predicted to be a coil (Le Novère et al., 1999).

Additional structural information emerges from the rates at which the introduced cysteines react with MTSEA-biotin. Two main factors influence these rates: (1) ionization of the sulfhydryl side chain, which is more likely in an aqueous environment, and (2) steric hindrance (i.e., how difficult is it for the MTSEA-biotin molecule to physically approach and interact with the sulfhydryl group). The fast reaction rate measured for G203C (\(k_2 \approx 250,000\) M\textsuperscript{-1}s\textsuperscript{-1}) indicates that the side chain of this residue is in an aqueous and sterically unrestricted environment such as would exist at the mouth of the binding pocket. The >2000-fold slower reaction rate measured for S209C (\(k_2 \approx 120\) M\textsuperscript{-1}s\textsuperscript{-1}) indicates that the side chain of this residue is poorly ionized (in a relatively hydrophobic environment), located in a sterically confined region, or both. These are the conditions one might expect to find near the deepest point of the binding pocket. Significantly, the reaction rates for the introduced cysteines between G203 and S209 sequentially decline, almost continually, with progression along the peptide chain (Fig. 6, Table 2). This rate gradient is highly suggestive of a protein domain that traverses an aqueous pocket from its rim to its depths. This type of structure correlates with the water-filled tunnels in the nACh receptor identified by electron microscopy (Miyazawa et al., 1999). We hypothesize that, in the GABA\textsubscript{A} receptor, at least a portion of these tunnels lie at an \(\alpha/\beta\) interface. The fact that none of the introduced cysteines before G203 appear to react with MTSEA-biotin suggests that the polypeptide chain may turn at this glycine (a residue that allows for maximum flexibility) and dive into the hydrophobic core of the protein or the lipid membrane.

**Structural rearrangements involved in receptor gating**

It has been speculated that the allosteric transition underlying gating of LGICs is primarily from quaternary rearrangements of the N-terminal domains of subunits with little change in tertiary or secondary structure (Corringer et al., 2000). The results presented here suggest that activation of the receptor involves movement of the \(\alpha_1\) and \(\beta_2\) domains of the GABA binding site toward each other. Three residues that face into the binding pocket (S204, R207, and S209) experience reductions in accessibility to MTSEA-biotin during gating. This is exactly the result we would expect if convergence of two subunits were to decrease the vol-
Some of the results presented in this study are graphically summarized in Figure 6. This region appears to consist of two structurally distinct domains. The C-terminal residues of this domain (S204–S209) predominantly line the GABA binding pocket and are in an aqueous environment. The N-terminal residues (V199–T202) do not appear to be in an aqueous environment and thus are not part of the binding pocket. G203 seems to be a transition residue between these two domains in that it is easily modified by MTSEA-biotin (i.e., in an aqueous environment). The structure of these domains with the cysteine (P206C) suggests that the entire region implies both domains are critically involved in receptor gating and that side chains at positions S204, R207, and S209 experience a change in environment concomitant with gating of the receptor. Thus, the loop C region of the GABA binding site contains dynamic elements that respond to both modulators and channel activation. The agonist-mediated binding site movements may be the initial trigger that drives channel opening.

REFERENCES


Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (50%) of an enzymatic reaction. Biochem Pharmacol 22:309–318.


