Mapping the $\rho_1$ GABA$_C$ Receptor Agonist Binding Pocket

CONSTRUCTING A COMPLETE MODEL*  

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$\gamma$-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian brain. The GABA receptor type C (GABA$_C$) is a ligand-gated ion channel with pharmacological properties distinct from the GABA$_A$ receptor. To date, only three binding domains in the recombinant $\rho_1$ GABA$_C$ receptor have been recognized among six potential regions. In this report, using the substituted cysteine accessibility method, we scanned three potential regions previously unexplored in the $\rho_1$ GABA$_C$ receptor, corresponding to the binding loops A, E, and F in the structural model for ligand-gated ion channels. The cysteine accessibility scanning and agonist/antagonist protection tests have resulted in the identification of residues in loops A and E, but not F, involved in forming the GABA$_C$ receptor agonist binding pocket. Three of these newly identified residues are in a novel region corresponding to the extended stretch of loop E. In addition, the cysteine accessibility pattern suggests that part of loop A and part of loop E have a $\beta$-strand structure, whereas loop F is a random coil. Finally, when all of the identified ligand binding residues are mapped onto a three-dimensional homology model of the amino-terminal domain of the $\rho_1$ GABA$_C$ receptor, they are facing toward the putative binding pocket. Combined with previous findings, a complete model of the GABA$_C$ receptor binding pocket was proposed and discussed in comparison with the GABA$_A$ receptor binding pocket.

GABA$_A$ and GABA$_C$ receptors are both GABA-gated chloride channels but with very different pharmacological properties. In fact, that is the basis for their classification. Although both types of receptors can be activated by GABA, the GABA$_C$ receptor can be specifically antagonized by bicusculine. In contrast, the GABA$_A$ receptor is insensitive to bicusculine but can be selectively antagonized by 1,2,5,6-tetrahydrodipyridine-4-yl)methylphosphinic acid (1). The functional properties of these two types of GABA receptors are also different. For example, the GABA$_C$ receptor has higher GABA sensitivity but slower activation and deactivation kinetics than the GABA$_A$ receptor (2). The GABA$_C$ receptor also shows almost no desensitization (2), whereas the GABA$_A$ receptor exhibits strong desensitization (3). Although the functional differences could arise from differences in the structural architecture of the GABA binding pockets, distinct antagonist profiles of GABA$_A$ and GABA$_C$ receptors indicate that their agonist/antagonist binding pockets are not the same. Studies over the last 15 years have shaped a relatively complete model for the GABA$_A$ receptor (4–9). In contrast, structural information on the GABA$_C$ receptor agonist binding pocket is far from complete. Some candidate binding residues in several potential “binding loops” are still undefined.

GABA-gated ion channels belong to the ligand-gated ion channel family, which also includes nicotinic acetylcholine receptors, serotonin-3 receptors, and glycine receptors. The study of nicotinic receptors in the past two decades has shaped a structural model for the ligand-gated ion channel family. In this model, six loops designated A–F have been identified to participate in the formation of the agonist binding pocket (10). This structural model was further confirmed and extended by the crystal structure of the homologous acetylcholine-binding protein, AChBP (11). Cumulating evidence suggests a similar general structural architecture for all members of this ligand-gated ion channel family. For example, in the GABA receptor, two ligand binding domains identified in the $\beta_2$ subunit (4) and the $\rho_1$ subunit (2) can be mapped to the homologous residues in loops B and C. One binding domain identified in the $\alpha_5$ subunit (5, 12) and also in the $\rho_1$ subunit (13) can be mapped to loop D in the complementary face. More recently, two residues contributed from loop A of the $\beta_2$ subunit (7), three residues from loop F (8), and two residues from loop E of the $\alpha_5$ subunit of the GABA$_A$ receptor have been identified as binding residues (9). Furthermore, the secondary structure of the GABA receptor is in agreement with the AChBP-based structural model (6–8). Due to agonist specificity and sequence diversity, key residues involved in agonist binding in different ligand-gated ion channels are not the same. Nevertheless, the similarity of the general architecture in this receptor family suggests that the AChBP-based structural model can be used as a guideline in search of the complete model of the GABA receptor agonist binding pocket.

The substituted cysteine accessibility method was first implemented in ion channels to identify pore-lining residues in nicotinic and GABA receptors (14–16). This approach is based on a functional effect of modification of the substituted cysteine by a water-soluble sulfhydryl-reactive compound. If such a modification occurs in a functionally important domain of the receptor, it is likely to produce a change in receptor function.
By testing the functional effect of a water-soluble cysteine modification reagent and blockability of this effect by agonist and antagonist, the substituted cysteine accessibility method has also been successfully used to probe the binding sites of the GABA_{A} (6, 17) and nicotinic receptors (18, 19). With this approach, novel residues involved in agonist binding were identified, and the secondary structures of the newly identified domain were predicted (6, 7).

In this study, using cysteine accessibility scanning and agonist/antagonist protection tests, we have identified amino acid residues involved in agonist binding in novel regions of the \( \rho_{1} \) GABA_{A} receptor subunit corresponding to loops A and E in nicotinic receptor subunits. The newly identified binding residues in loop A and part of loop E are in comparable positions as in the GABA_{A} receptor. Moreover, in the extended stretch of loop E, a region previously unexplored in GABA-gated ion channels, we have identified three novel binding residues in the \( \rho_{1} \) GABA_{A} receptor. In contrast to the GABA_{A} receptor, loop F in the \( \rho_{1} \) homomeric GABA_{A} receptor does not appear to be involved in agonist binding. Using the AChBP crystal structure as a template, we have built a homology model for the amino-terminal domain of the \( \rho_{1} \) GABA receptor. These newly discovered binding residues, along with previously identified binding residues, are mapped to the homology model to construct a complete agonist binding pocket for the GABA_{A} receptor.

**Experimental Procedures**

**Mutagenesis and cRNA Preparation**—The cDNA encoding the human \( \rho_{1} \) GABA receptor subunit was cloned into the pGEMHE vector in the T7 orientation (20). The residues in the amino-terminal segments corresponding to loops A, E, and F in nicotinic receptor subunits were mutated to cysteine, one at a time, using the PCR-based QuickChange method of site-directed mutagenesis (Stratagene, Hercules, CA). The mutations were confirmed by automated DNA sequencing. The fragments containing unique mutations were subcloned and resequenced to avoid additional mutations. The wild type and mutant cDNAs were then linearized by NheI digestion. The cRNAs were then transcribed by T7 RNA polymerase. After degradation of the DNA template by RNase-free DNase I, the cRNAs were purified and resuspended in diethyl pyrocarbonate-treated water. cRNA yield and integrity were examined on a 1% agarose gel.

**Oocyte Preparation and Receptor Expression**—Female Xenopus laevis (Xenopus I, Ann Arbor, MI) were anesthetized by 0.2% MS-222. The ovaries were surgically removed from the frog and placed in calcium-free frog Ringer (OR2) in ice-cold solution containing (in mM): 82.5 NaCl, 2.5 KCl, 1 MgCl_{2}, 1 Na_{2}HPO_{4}, 5 HEPES, 50 units/ml penicillin, and 50 µg/ml streptomycin, pH 7.5. The frog was then allowed to recover from surgery before being returned to the incubation tank. The oocytes were cut into small pieces and digested with 0.3% collagenase A (Roche Applied Science) with constant stirring at room temperature for 1.5–2 h. The dispersed oocytes were thoroughly washed with the above solution plus 1 mM CaCl_{2}. The stage VI oocytes were selected, and the follicular layer (if still present) was manually removed to prevent the influence of the cysteine modification reagent on the reference electrode. The oocytes were voltage-clamped at −70 mV to measure GABA-induced currents using a GeneClamp 500 (Axon Instruments, Foster City, CA). For the cysteine accessibility test, 1 mM MTSET was applied for 1 min. The current responses to GABA at a concentration inducing ~20% of the maximum GABA response (−EC_{20}) were then examined before and after MTSET modification. For agonist/antagonist protection, a super-saturation concentration (>100-fold of EC_{50}) of agonist/antagonist was co-applied with 1 mM MTSET for 1 min to ensure adequate protection in the binding pocket.

**Drug Preparation**—GABA (Calbiochem) stock solution (100 mM) was prepared daily from the solid. 3-Aminopropylphosphonic acid (3-APA, Sigma) stock solution (100 mM) was prepared and frozen in aliquots before use. MTSET (Toronto Research Chemicals, Toronto, Canada) stock solution (100 mM) was prepared in pure water and frozen in aliquots before use. Oocytes were incubated at 16 °C before use. MTSET (1 mM) was freshly prepared from the stock solution for each oocyte immediately before use.

**Data Analysis**—The dose-response relationship of the GABA-induced current in recombinant GABA_{A} receptors was least squares fit to the following Hill equation

\[
I = \frac{I_{\text{max}}}{1 + \left(\frac{[\text{GABA}]}{EC_{50}}\right)^n}
\]

where the GABA-induced current (I) is a function of the GABA concentration, EC_{50} is the GABA concentration required for inducing a half-maximal current, n is the Hill coefficient, and I_{max} is the maximum current. This equation was then used to normalize the dose-response curve for each individual oocyte. The average of the normalized currents for each GABA concentration was used to plot the data. All the data were presented as mean ± S.E. (standard error).

**Statistics**—The statistical significance of the effect of MTSET on the GABA-induced current in wild type and mutant receptors was evaluated by analysis of variance with the Dunnett post test. The significance of agonist/antagonist protection of MTSET modulation was evaluated by grouped student t test using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

**Homology Modeling**—Three-dimensional modeling of the \( \rho_{1} \) GABA receptor amino-terminal domain was made based on the crystal structures of AChBP deposited in the Protein Data Bank (Protein Data Bank code 1BB (11)). Low sequence homology between GABA receptor and AChBP did not allow straightforward or confident modeling by commonly used automatic methods such as Swiss-Model (21). Thus, a manual method was used. An electron density map of AChBP was calculated to 1.8 Å resolution, based on the coordinates of one of the monomers in the 1H9B Protein Data Bank entry. The AChBP model was then embedded in the electron density. With the sequence alignment as a guide, residues of the AChBP model were graphically mutated using the molecular graphics program QUANTA (Accelrys, San Diego, CA). Residues were mutated to residue types as indicated by sequence alignment along randomly chosen stretches of the AChBP sequence. The new side chain atoms were automatically best fit into the electron density by the QUANTA program. The best rotamers were chosen to fit density around the side chain. Other parts of the model were deleted and insertions and deletions were made, structural regularization and the best rotamers were used to model surrounding and inserted residues. Manual movements of residues and side chains were made when the QUANTA automatic features were not able to resolve steric clashes. The parts of the sequence that were homologous provided many points between which regularization could be anchored. After the entire sequence was mutated and manually modeled, the structure was energy-minimized using the Crystallography and NMR System (CNS) program suite (22). The structural alignment program TOP (23) was used to align the modeled monomer with each of the five AChBP monomers in the 1H9B structure. Finally, the entire pentameric structure, with sequences mutated to that of the GABA receptor, was energy-minimized using CNS.

**Visualization of the Binding Pocket**—For three-dimensional presentation of the binding pocket, the newly identified binding residues along with the previously identified ones were mapped onto the homology model using the molecular graphics program Deep View. The resulting image was saved as a POVRay 3.5 scene file. The final image of the model was rendered by POVRay 3.6 software.

**Results**

**Location of the Scanning Regions**—To determine the scanning regions, we used the AChBP-based structural model of the amino-terminal domain of ligand-gated ion channels as a reference. Fig. 1 shows the alignment of the \( \rho_{1} \) GABA receptor subunit and AChBP partial sequences. We defined six potential binding loops in the GABA receptor \( \rho_{1} \) subunit (Fig. 1, underlined residues). Since the critical residues for agonist binding in...
loops B, C, and D of the $\rho_1$ GABA receptor subunit have been previously identified (highlighted in bold) (2, 13), in this study, we selected loops A, E, and F of the $\rho_1$ GABA receptor subunit for cysteine scanning. The bold letters in the AChBP highlight the amino acid residues previously identified to participate in the formation of the agonist binding pocket of nicotinic receptors.

**Functional Properties of the Cysteine Mutants**—Wild type and single cysteine mutants were individually expressed in *Xenopus* oocytes. The functional expression and dose-response relationship were examined for all the constructs. Fig. 2A shows dose-response relationships of representative mutants with significant changes in GABA sensitivity. By least squares fitting to individual GABA dose-response relationships with Equation 1, we have derived EC$_{50}$ values for the wild type and mutant receptors (Table I). The fold changes in EC$_{50}$ for all mutants (normalized to the wild type receptor) are plotted in Fig. 2B. The alternating pattern in EC$_{50}$ changes between L166C and V171C in loop E and between S223C and F227C in loop F may suggest a $\beta$-strand structure in these two regions.
Novel GABAC Receptor Binding Sites

EC₅₀ values determined from the GABA dose-response relationship in the individual cysteine mutants of the p₁ homomeric GABA receptor
NF, nonfunctional; LE, low expression; SO, spontaneously opening.

<table>
<thead>
<tr>
<th>Loop A</th>
<th>Mutant</th>
<th>EC₅₀</th>
<th>μV</th>
<th>Loop E</th>
<th>Mutant</th>
<th>EC₅₀</th>
<th>μV</th>
<th>Loop F</th>
<th>Mutant</th>
<th>EC₅₀</th>
<th>μV</th>
</tr>
</thead>
<tbody>
<tr>
<td>W133C</td>
<td>NF</td>
<td>V155C</td>
<td>0.88±0.06</td>
<td>L216C</td>
<td>0.60±0.03</td>
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<tr>
<td>V134C</td>
<td>0.66±0.02</td>
<td>M156C</td>
<td>7.21±0.94</td>
<td>K217C</td>
<td>0.75±0.06</td>
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<tr>
<td>P135C</td>
<td>NF</td>
<td>L157C</td>
<td>1.53±0.20</td>
<td>T218C</td>
<td>1.03±0.04</td>
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<tr>
<td>D136C</td>
<td>387.6±62.3</td>
<td>R158C</td>
<td>LE</td>
<td>D219C</td>
<td>3.02±0.25</td>
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<tr>
<td>M137C</td>
<td>0.72±0.07</td>
<td>V159C</td>
<td>0.97±0.05</td>
<td>E220C</td>
<td>1.48±0.11</td>
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<tr>
<td>F138C</td>
<td>5.94±0.43</td>
<td>Q160C</td>
<td>SO</td>
<td>R221C</td>
<td>0.51±0.02</td>
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<tr>
<td>F139C</td>
<td>NF</td>
<td>P161C</td>
<td>0.85±0.05</td>
<td>I222C</td>
<td>0.50±0.03</td>
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<tr>
<td>V140C</td>
<td>0.39±0.07</td>
<td>D162C</td>
<td>0.71±0.02</td>
<td>S223C</td>
<td>0.66±0.01</td>
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<tr>
<td>R141C</td>
<td>3.65±0.44</td>
<td>G163C</td>
<td>NF</td>
<td>L224C</td>
<td>3.38±0.36</td>
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<tr>
<td>S142C</td>
<td>2.41±0.17</td>
<td>K164C</td>
<td>0.69±0.10</td>
<td>S225C</td>
<td>0.53±0.02</td>
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<tr>
<td>K143C</td>
<td>LE</td>
<td>V165C</td>
<td>2.17±0.12</td>
<td>Q226C</td>
<td>17.6±3.58</td>
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<tr>
<td>R144C</td>
<td>1.66±0.06</td>
<td>L166C</td>
<td>13.04±1.56</td>
<td>F227C</td>
<td>0.38±0.32</td>
<td></td>
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<tr>
<td>S145C</td>
<td>8.50±0.51</td>
<td>Y167C</td>
<td>0.52±0.05</td>
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<tr>
<td>F146C</td>
<td>SO</td>
<td>S168C</td>
<td>399.9±70.7</td>
<td></td>
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<tr>
<td>I147C</td>
<td>3.92±0.38</td>
<td>R170C</td>
<td>10.84±2.23</td>
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<tr>
<td></td>
<td></td>
<td>V171C</td>
<td>0.65±0.06</td>
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Note that in loop A, mutants W133C, P135C, and F139C are not functional. Interestingly, the F146C mutant exhibited a high leak current in the absence of GABA, which was blocked by picrotoxin (data not shown). This suggests that the mutation created a spontaneously opening channel. This residue is aligned to an interface residue, Lys-94, in AChBP. In loop E, G163C was nonfunctional. This residue is conserved across the subunits in the ligand-gated ion channel family and is located in the subunit interface in AChBP. In the same loop, the expression of the mutant R158C was extremely low, and the mutants L166C and S168C showed a significant increase in EC₅₀, suggesting that they may be involved in ligand binding. In fact, these three residues are aligned to the ligand binding residues Arg-104, Leu-112, and Met-114 in the AChBP (11) (Fig. 1). The mutant Q160C also showed spontaneous opening in the absence of agonist. This residue is presumably aligned to another ligand binding residue, Val-106, in AChBP. In loop F, only minor changes in channel function were observed (Table I), with the most significant change being a 20-fold increase in the EC₅₀ (Q226C).

Accessibility of the Engineered Cysteine by MTSET—To further delineate the binding pocket of the p₁ GABAC receptor, we examined the accessibility of the individual engineered cysteines in putative loops A, E, and F to a water-soluble thio-reactive compound, MTSET. The oocytes were treated with 1 mM MTSET for 1 min. The currents induced by a GABA concentration (~EC₅₀) before and after the MTSET treatment were recorded for each cell. The normalized current change upon treatment was calculated and plotted in Fig. 3. MTSET treatment of the wild type receptor did not significantly alter the GABA-induced current (Fig. 3). MTSET treatment of the mutant that was statistically different from the wild type control.

FIG. 3. The accessibility to MTSET of the cysteine mutants in loops A, E, and F. The wild type and individual cysteine mutants were examined for accessibility of a water soluble thio-reactive compound, MTSET (1 mM for 1 min) using a GABA concentration ~EC₅₀ for each mutant as a test pulse. Examples of current traces of mutants D136C (loop A), M156C (loop E), and L224C (loop F) before and after MTSET treatment are shown above each plot. The normalized changes of the GABA-induced current after MTSET treatment ((Iafter/Ibefore)) are presented as bar graphs. A filled bar represents an MTSET effect on the mutant that was statistically different from the wild type control. NF, nonfunctional mutant; LE, low expression; SO, spontaneously opening; SOM, spontaneously opening after modification.
induced by GABA, accessibility of residues in other regions of the receptor distant from the agonist binding pocket would potentially be altered in the presence of GABA. Therefore, a competitive antagonist protection experiment would further test whether these residues line the binding pocket.

Protection of Accessible Residues by 3-APA—3-APA is a GABA<sub>C</sub> receptor-competitive antagonist, presumably competing with the agonist for the same binding site but without inducing a similar conformational change (24). A saturating concentration of 3-APA was co-applied with 1 mM MTSET to the oocytes expressing individual cysteine mutants of the GABA<sub>C</sub> receptor. Fig. 5 shows that 3-APA has a protection pattern similar to that for GABA. That is, the mutants D136C, F138C, and V140C in loop A and the mutants V155C, M156C, V159C, S168C, and R170C in loop E were efficiently protected by this competitive antagonist, whereas no residue in loop F was protected. These data suggest that D136C, F138C, and S142C in loop A and V155C, M156C, V159C, S168C, and R170C in loop E are most likely lining the agonist/antagonist binding pocket of the GABA<sub>C</sub> receptor.

DISCUSSION

Identification of Novel Binding Residues in the Binding Pocket—If a residue directly interacts with the agonist, then mutation of the residue would be expected to decrease the binding affinity. With the cysteine mutations, we have identified several residues that, when mutated, reduced agonist sensitivity of the GABA receptor. The sensitivity decrease by mutation could be due to the reduction of binding affinity or a decrease in the maximum open probability (25). A pure gating effect on EC<sub>50</sub>, however, is limited in the G<sub>15</sub> homomeric GABA receptor. For example, the previously established three-bind-to-open model (26) predicts that a decrease in gating efficiency of 100-fold in the G<sub>15</sub> homomeric GABA receptor would only result in an increase in the EC<sub>50</sub> of 4-fold (27). Thus, the mutations with an increase in EC<sub>50</sub> for more than 4-fold, such as D136C (loop A), L166C, S168C, R170C (loop E), and Q226C (loop F), likely have a decreased binding affinity in addition to any changes in gating efficacy. The binding affinity decrease could be a direct effect of the mutation in the binding site, or the mutation could also allosterically influence binding by changing the conformation of the binding site from a distant location. These two possibilities can be further differentiated by a combination of cysteine accessibility and protection tests.

The mutants W133C, F139C (loop A), and G163C (loop F) were not functional. These mutations probably influence receptor trafficking or assembly, although we cannot rule out the possibility that the mutations directly disrupt the agonist binding site. For those mutants with minor changes in the GABA sensitivity, the role of these residues in agonist binding is probably less significant, although they might still line the binding pocket.

To further determine whether a residue lines the binding pocket, we tested the accessibility and agonist/antagonist protection of the engineered cysteines. A residue that lines the binding pocket would become less accessible when the agonist is bound. However, due to the ability of agonist to induce a global conformational change related to channel opening, it is possible that the agonist could change the conformation outside the binding pocket and make that site less accessible to the cysteine modification reagent. As a further test, we examined protection by a competitive antagonist. Although recent studies suggest that a competitive antagonist can also induce a conformational change beyond the binding site to stabilize the GABA receptor in the closed state (17, 28, 29), this antagonist-induced conformational change is distinct from that induced by the agonist (24). Thus, only when the agonist and antagonist both protect a residue from modification do we consider that residue
Fig. 6. 3-APA, at a saturation concentration, did not change the reaction rate of MTSET at L166C. A, current traces induced by 3 µM GABA and modification by MTSET (100 µM) in the presence or absence of 3-APA (1 mM). B, normalized and averaged data of MTSET modification with single exponential fits (lines). Note that in the presence of 1 mM 3-APA, the rate of the current decay induced by MTSET modification did not change. C, the average MTSET reaction rate determined by fitting data from individual oocytes with a single exponential function. The rates were second-order rates (normalized to the MTSET concentration). Note that there was no significant difference in the rates in the presence or absence of 3-APA. D, dose-dependent inhibition of the 15 µM GABA-induced current by 3-APA, showing saturation of 3-APA inhibition at 1 mM. The continuous line is the least squares fit of the data to a Hill equation. The error bars are smaller than the symbol.

In this way, we have identified three novel binding residues in loop A (Asp-136, Phe-138, and Val-140) and five novel binding residues in loop E (Val-155, Met-156, Val-159, Ser-168, and Arg-170). By these criteria, no binding residues were identified in loop F, suggesting that this loop in the $\rho_1$ GABA receptor is not directly involved in agonist binding. Note that V140C, V155C, and V159C showed no significant change in GABA EC50, suggesting that they may not directly contact the agonist but may still line the binding pocket. In contrast, the L166C and Q226C mutants were 15–20-fold less sensitive to GABA, suggesting a reduced binding affinity. The two engineered cysteine residues, however, were not protected by agonist or antagonist, indicating that they are likely outside the binding pocket. The reduction of binding affinity by these mutations could be due to an indirect/distant effect on the binding pocket.

Since our goal was to detect dramatic changes in accessibility caused by direct physical occlusion of binding residue by agonist/antagonist, we examined MTSET modification at a single time. At least in terms of detecting minor changes in accessibility, this approach is less sensitive than an examination of the kinetic network. Perturbation of this allosteric network, either by agonist binding or by mutation at one of the key positions, could alter the energy landscape to stabilize the receptor in the open state. In the amino-terminal domain of the $\rho_1$ GABA receptor, these key positions, including residues Tyr-102 (loop D), Phe-146 (in loop A), Gln-160 (in loop E), and Ser-255 (in loop F), are distributed in multiple binding (or coupling) loops. The coordinated movement of these loops induced by agonist could be an important mechanism for channel activation. It is interesting that a mutation at a different position (L99C) in loop A of the $\beta_2$ subunit also created a spontaneously opening channel (7). In contrast, the mutant V140C in the homologous position of the $\rho_1$ subunit was not spontaneously active. This difference in key sites of the gating determinants may contribute to the distinct gating kinetics of $\text{GABA}_A$ and $\text{GABA}_C$ receptors.

Secondary Structure Prediction—As indicated in Fig. 2, the alternating change in EC50 upon cysteine mutation in the segments L166C–V171C of loop E and S223C–F227C of loop F suggests a $\beta$-strand structure. The $\beta$-strand structure of segment L166C–V171C of loop E is further supported by the alternating accessibility pattern in this region (Fig. 5). In loop F, however, the pattern of accessibility to MTSET suggests a random coil. In addition, an alternating pattern of accessibility to MTSET in the segment Asp-136–Val-140 in loop A also suggests a $\beta$-strand structure. The secondary structure predictions in these loops of the $\rho_1$ GABA receptor are in agreement with the crystal structure of the homologous protein, AChBP, and similar to the predictions in the homologous regions of the $\alpha_2$ (8, 9) and $\beta_2$ (7) subunits of the $\text{GABA}_A$ receptor.

Comparison with the Residues Lining the $\text{GABA}_C$ Receptor Agonist Binding Pocket—$\text{GABA}_A$ and $\text{GABA}_C$ receptors share many agonists, such as GABA and muscimol, but have distinct competitive antagonists, such as bicuculline for $\text{GABA}_A$ and 1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid for $\text{GABA}_C$. Agonist sharing suggests that they have similar overall architecture in the agonist/antagonist binding pocket, whereas the distinct antagonist profile suggests that their agonist/antagonist binding pockets are not identical. Thus, when we use agonist and competitive antagonist protection of the modification of the engineered residues as criteria for binding site residues, it would result in identification of some common and distinct residues lining the binding pocket. Fig. 7 shows all...
the binding residues identified to date in the $\alpha_1$ (GABA$_C$) and $\alpha_2/\beta_2$ (GABA$_\lambda$) subunits. In loops B, C (2), and D (5, 13), the binding residues identified by site-directed mutagenesis are almost identical. In loop D, two additional binding residues, Arg-66 and Ser-68, in the $\alpha_1$ subunit are recognized by the substituted cysteine accessibility method (6, 17). The corresponding residue Arg-104 in the $\alpha_2$ subunit seems to line the binding pocket.\footnote{V. Torres and D. S. Weiss, personal communication.}

In loop E, our results indicate that Ser-168 and Arg-170 line the binding pocket of the $\alpha_1$ GABA receptor, similar to the homologous residues (Thr-129 and Arg-131) in the $\alpha_1$ subunit (9). In addition, we have identified three more binding residues (V155C, M156C, and V159C) in the extended region of loop E, but it belongs to a different $\beta$-strand, of the $\alpha_1$ subunit. This region in the $\alpha_1$ subunit has not yet been explored. In loop F, we could not find any residue meeting our criteria for a binding site lining residues in the $\alpha_1$ GABA receptor. In contrast, three residues (Val-178, Val-180, and Asp-183) in loop F of the $\alpha_2$ subunit have been identified as binding site residues (8). This major difference between GABA$_C$ and GABA$_\lambda$ could, in part, underlie the functional and pharmacological diversities between these two types of GABA receptors.

**Model for the $\rho_1$ GABA$_C$ Receptor Agonist/Antagonist Binding Pocket**—Using cysteine scanning accessibility, we have successfully identified novel residues in the $\rho_1$ GABA receptor involved in GABA binding. With these data and previously identified binding residues in other loops, we were able to construct a model for the $\rho_1$ GABA receptor binding pocket. For a three-dimensional presentation of the agonist/antagonist binding pocket, we first built a structural model for the amino-terminal domain of the $\rho_1$ GABA receptor by homology modeling using AChBP as a template. The binding residues experimentally identified to date, as highlighted in Fig. 7, were then mapped onto this structural model and highlighted with the side chain (Fig. 8). The residues from loops A (red), B (cyan), and C (green) form the principal face of the binding pocket, whereas residues Tyr-102 and Arg-104 in loop D (yellow) and Val-155, Met-156, Val-159, Ser-168, and Arg-170 in loop E (purple) form the complimentary face of the binding pocket.
binding pocket is formed by residues from loops D (yellow) and E (purple). Loop F is in the vicinity of the binding pocket but does not directly contribute to agonist binding. Note that all these presumed binding residues are pointing toward the putative GABA binding pocket with the exception of Val-155 and Val-159. Mutation of these residues to cysteine did not significantly alter the GABA EC<sub>50</sub> of the receptor. Thus, it is possible that these residues are not in the binding pocket in the absence of agonist/antagonist, but a local conformational change induced by binding of the agonist or antagonist can alter their orientation and thus change their accessibility. Although the precise location of each residue still awaits the high resolution structure of the GABAC receptor, the excellent agreement of most experimentally identified binding residues and the homology model further strengthens our conclusion that the identified residues are actually lining the agonist binding pocket of the receptor.

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Mapping the $\rho_1$ GABA$\gamma$ Receptor Agonist Binding Pocket: CONSTRUCTING A COMPLETE MODEL

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