The GABA<sub>A</sub> Receptor α<sub>1</sub> Subunit Pro<sup>174</sup>–Asp<sup>191</sup> Segment Is Involved in GABA Binding and Channel Gating*<sup>a</sup>

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J. Glen Newell and Cynthia Czajkowski†

From the Department of Physiology, University of Wisconsin-Madison, Madison, Wisconsin 53706

The GABA-binding site undergoes structural rearrangements during the transition from agonist binding to channel opening. To define possible roles of the GABA<sub>A</sub> receptor α<sub>1</sub> subunit Pro<sup>174</sup>–Asp<sup>191</sup> segment in these processes, we used the substituted cysteine accessibility method to characterize this region. Each residue was individually mutated to cysteine, expressed with wild-type β<sub>2</sub> subunits in Xenopus laevis oocytes, and examined using two-electrode voltage clamp. Most mutations did not alter GABA EC<sub>50</sub> values. The D183C mutation produced a 7-fold reduction in GABA sensitivity. There were no significant changes in this fact.

The GABAA<sub>R</sub> α<sub>1</sub> subunit segment between Pro<sup>174</sup> and Asp<sup>191</sup> is homologous in position to the putative “loop F” of the nAChR (see Fig. 1) (11). Studies of this segment of the nAChR γ/δ and δ subunits have identified negatively charged amino acid residues that influence acetylcholine binding, channel gating, and perhaps potassium ion interactions (see Fig. 1) (12–16). Based on the crystal structure of a soluble acetylcholine-binding protein (AChBP), a protein homologous to the extracellular domain of the nAChR, the secondary structure of the loop F region is predicted to be a random coil (17). Strikingly, the loop F protein sequence is poorly conserved among all GABA<sub>A</sub> subunit isoforms and other related ligand-gated ion channel subunits and may represent a unique structural element that could account for differences in agonist affinity, dimensions of binding pockets, and access pathways important for receptor-ligand interactions. Therefore, an analysis of the structure and the role(s) of the α<sub>1</sub> subunit Pro<sup>174</sup>–Asp<sup>191</sup> segment in ligand binding and ion channel activation is fundamental for understanding GABA<sub>A</sub>R function.

The development of SCAM has proved to be very powerful tool for identifying residues important for the pharmacology of both agonists and antagonists. Originally developed to identify the channel-lining residues of ligand-gated ion channels (18), SCAM has gained widespread use in the study of the ligand binding domains of these channels (2, 3, 9, 10, 19–25). The method entails introduction of successive cysteine residues, one at a time, within a protein domain and expression of recombinant receptors in heterologous systems. Solvent accessibility of a given cysteine is determined by monitoring changes in function following application of a sulphydryl-specific modifying agent (GABA<sub>A</sub>R)<sup>−</sup> reveals that the GABA-binding sites are located at β-α subunit interfaces. Consistent with the agonist-binding site of nicotinic acetylcholine receptors (nAChR), the GABA-binding site is formed by amino acid residues clustered in non-continuous protein segments of the extracellular amino-terminal domains of adjacent subunits. Multiple residues have been implicated in the formation of this binding site using a variety of approaches, including site-directed mutagenesis, photoaffinity labeling, and the substituted-cysteine accessibility method (SCAM). These include Phe<sup>64</sup>, Arg<sup>66</sup>, Arg<sup>119</sup>, and Ile<sup>120</sup> of the α<sub>1</sub> subunit (2–7), in addition to Tyr<sup>97</sup>, Leu<sup>99</sup>, Tyr<sup>157</sup>, Thr<sup>160</sup>, Thr<sup>202</sup>, Ser<sup>204</sup>, Tyr<sup>205</sup>, Arg<sup>207</sup>, and Ser<sup>209</sup> of the β<sub>2</sub> subunit (8–10). Of these residues, it is likely that some contact agonist/antagonist molecules directly, some maintain the overall structure of the binding site, while others mediate conformational dynamics within the site during allosteric transitions among the resting, active, and desensitized states.

The GABA<sub>A</sub>R α<sub>1</sub> subunit segment between Pro<sup>174</sup> and Asp<sup>191</sup> is homologous to the competitive antagonist, SR-95531. N-Biotinylaminomethyl methanethiosulfonate modified P174C-, R176C-, S177C-, V178C-, V180C-, A181C-, D183C-, R186C- and N188C-containing receptors. The pattern of accessibility suggests that this protein segment is aqueous-exposed and adopts a random coil conformation. Both GABA and SR-95531 slowed covalent modification of V178C, V180C, and D183C, indicating that these residues may line the GABA-binding site. Further, pentobarbital-induced channel activation accelerated modification of V180C and A181C and slowed the modification of R186C, suggesting that this region of the α<sub>1</sub> subunit may act as a dynamic element during channel-gating transitions.

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† To whom correspondence should be addressed: Dept. of Physiology, Rm. 197 MSC, University of Wisconsin-Madison, 1300 University Ave., Madison, WI 53706. Tel.: 608-265-5683; Fax: 608-265-5512; E-mail: czajkowski@physiology.wisc.edu.

‡ To whom correspondence should be addressed: Dept. of Physiology, University of Wisconsin-Madison, Madison, Wisconsin 53706.
and SR-95531 (Sigma) were prepared in water, while Noethyl methanethiosulfonate (100 mM) (MTSEA-biotin, Biotium, Hayward, CA) was prepared in buffer with a pH of 7.4, supplemented with 100 μg/ml gentamicin and 100 μg/ml bovine serum albumin. Oocytes were used 2-7 days after injection for electrophysiological recordings.

Modification of Introduced Cysteine Residues by MTSEA-biotin—MTSEA-biotin was the sulfhydryl-specific reagent used in this study. It is a relatively impermeant compound with dimensions (14.5 Å) and a relatively high diffusion coefficient (31) allowing entrance into the oocyte cytoplasm after injection of cRNA. The reagent reacts with extracellular cysteine residues of the GABA A receptor (30).

To measure the sensitivity to SR-95531, GABA (EC50) was applied following exposure to high concentrations of MTSEA-biotin. The effect of MTSEA-biotin was calculated as the difference in the amplitude of the GABA-evoked current before and after application of MTSEA-biotin.

Rate of Modification of Introduced Cysteine Residues—Rates were measured only for those cysteine mutants that had a 40% change in Icalm following MTSEA-biotin treatment (2 min, 2 mM). The rate at which MTSEA-biotin modified introduced cysteine residues was measured using low MTSEA-biotin concentrations as described previously in Section 2. In general, the concentration of MTSEA-biotin used was 50 μM, with the exception of A181C (500 μM) and R186C (5 μM). The experimental protocol was as follows: GABA (EC50) application (5 s); ND96 washout (25 s); MTSEA-biotin application (10–20 s); ND96 washout (2–2.5 min). The amplitude was recorded until Icalm no longer changed following the MTSEA-biotin treatment (i.e., the control reaction had proceeded to apparent completion). The individual abilities of GABA, SR-95531, and pentobarbital to alter the rate of cysteine modification by MTSEA-biotin were determined by co-application of GABA and MTSEA-biotin using low concentrations of SR-95531 and pentobarbital (500 μM) during the MTSEA-biotin pulse. In all cases, the wash times were adjusted to ensure that currents obtained from test pulses of GABA and pentobarbital were stabilized. This ensured complete wash-out of drugs and that any reductions in the current amplitude were the result of MTSEA-biotin application.

Two-electrode Voltage Clamp Analysis—Oocytes under two-electrode voltage clamp were perfused continuously with ND96 at a rate of ~5 ml/min. The holding potential was ~80 mV. The volume of the recording chamber was 200 μl. Standard two-electrode voltage clamp procedures were carried out using a GenClamp500 amplifier (Axon Instruments, Inc.). Borosilicate electrodes were filled with 3 M KCl and had resistances of 0.5–3.0 MΩ in ND96. Stock solutions of GABA (Sigma) and SR-95531 (Sigma) were prepared in water, while N-biotinylaminomethyl methanethiosulfonate (100 mM) (MTSEA-biotin, Biotium, Hayward, CA) was prepared in dimethyl sulfoxide (MeSO4). All compounds were prepared fresh daily and MTSEA-biotin was diluted appropriately in ND96 such that the final concentration of MeSO4 was ≤2%. This solvent concentration did not affect recombinant GABA A receptors.

To measure the sensitivity to GABA, the agonist (0.0001–1 mM) was applied via gravity perfusion or by pipetor application (~5–8 s) with a 3–15-min washout period between each application to ensure complete recovery from desensitization. Peak GABA-activated current (Icalm) was recorded. To correct for slow drift in the maximum amplitude of the receptor, the coefficient of variation of time and concentration-response data were normalized to a low concentration of GABA (EC50). Concentration-response curves were generated for each recombinant receptor, and the data were fit by non-linear regression analysis using GraphPad Prism software (San Diego, CA; graphpad.com). Data were fit to the following equation:

\[ I = I_{max}(1 + (EC_{50}/[A]))^{n}, \]

where \( I \) is the peak amplitude of the current for a given concentration of GABA ([A]), \( I_{max} \) is the maximum amplitude of the current, \( EC_{50} \) is the concentration required for half-maximal receptor activation, and \( n \) is the Hill coefficient.

To measure the sensitivity to SR-95531, GABA (EC50) was applied via gravity perfusion followed by a brief (20 s) washout period before co-application of GABA (EC50) and increasing concentrations of SR-95531. The response to the application of SR-95531 and GABA was normalized to the response elicited by the agonist alone. Concentration-inhibition curves were generated for each recombinant receptor, and the data were fit by non-linear regression analysis using GraphPad Prism software. Data were fit to the following equation:

\[ I = I_{max}(1 + (IC_{50}/[A]))^{n}, \]

where \( IC_{50} \) is the concentration of the agonist ([A]) that reduces the amplitude of the GABA-evoked current by 50% and \( n \) is the Hill coefficient. \( K_{i} \) values were calculated using the Cheng-Prussof correction:

\[ K_{i} = IC_{50}/(1 + (IC_{50}/[A]))^{n}, \]

where [A] is the concentration of GABA used in each experiment and \( EC_{50} \) is the concentration of GABA that elicits a half-maximal response for each receptor.

Modification of Introduced Cysteine Residues by MTSEA-biotin—MTSEA-biotin was the sulfhydryl-specific reagent used in this study. It is a relatively impermeant compound (31) with dimensions (14.5 Å) and a relatively high diffusion coefficient (31) allowing entrance into the oocyte cytoplasm after injection of cRNA. The reagent reacts with extracellular cysteine residues of the GABA A receptor (30).
fig. 2. A, concentration-response curves of GABA-activated current for wild-type (■) and recombinant α1β2 receptors carrying the D183C mutation (□) expressed in Xenopus oocytes. Data were normalized to peak I_GABA for each experiment. Data represent the mean ± S.E. of at least three independent experiments. B, concentration-dependence of SR-95531-mediated reduction of I_GABA (EC50) for wild-type (■) and recombinant receptors carrying the D183C mutation (□). Data represent the mean ± S.E. of at least three independent experiments. The EC50 values, Kd values, and calculated Hill coefficients are summarized in Table I.

For all rate experiments, the decrease in I_GABA was plotted as a function of the cumulative time of MTSEA-biotin exposure and fit to a single-exponential decay function using GraphPad Prism software. A pseudo-first order rate constant (k1) was determined and the second order rate constant (k2) was calculated by dividing k1 by the concentration of MTSEA-biotin used in the assay (33). Second order rate constants were determined using at least two different concentrations of MTSEA-biotin.

Statistical Analysis—log (EC50) and log (Kd) values were analyzed using a one-way analysis of variance, followed by a post-hoc Dunnett’s test to determine levels of significance between wild-type and mutant receptors. Differences among the second order (k2) rates of covalent modification of the various mutants were assessed using the false positive discovery rate method (34). This method limited the expected percent of false positives to 5%. The false positive discovery rate is a more meaningful measure of error in large screening experiments than the more traditional approach of limiting the probability of one or more false positives (also known as experiment-wise error control). Before analysis, the rates were transformed to a log scale to obtain more normally distributed residuals. Results are reported in the original scale. Even using this approach, clear trends in the data did not always achieve significance as has been noted in other large assays using SCAM (35).

Structural Modeling—The mature protein sequences of the rat α1 and β2 subunits were homology-modeled with a subunit of the AChBP (17). The crystal structure of the AChBP was downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (code 1H9B) and loaded into Swiss Protein Bank Viewer (SPDBV, ca.expasy.ord/spdbv). The α1 protein sequence from Thr14–Ile277 and the β2 protein sequence from Ser10–Leu218 were aligned with the AChBP primary amino acid sequence as depicted in Cromer et al. (36) and threaded onto the AChBP tertiary structure 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 (<0.5 kcal/Å). The first 100 iterations were carried out using Simplex minimization (37) followed by 1000 iterations using the Powell conjugate gradient method (38). A β2/α1, GABA-binding site interface was assembled by overlaying the monomeric subunits on the AChBP scaffold, the resulting structure was imported into SYBYL, and energy was minimized. Our model is quite similar to models recently published for the nAChR and GABAAR ligand binding domains (36, 39).

It is worth noting that positioning of the α1 subunit forms a random turn/coil. Cysteine substitutions are positions 187 and 189 were not tolerated.
Table I
Concentration-response data for GABA activation and SR-95531 inhibition of wild-type and mutant receptors expressed in Xenopus oocytes

| Receptor | GABA | EC50  | nH   | n  | mut/wt | SR-95531 | Kf  | nH   | n  | mut/wt |
|----------|------|-------|------|----|--------|----------|-----|------|----|--------|
| α1β2     |      | μM    |      |    |        |          | μM |      |    |        |
| α1(P174C)β2 | 1.6 ± 0.5 | 1.50 ± 0.2 | 3 | 1 | 0.33 ± 0.06 | 0.94 ± 0.06 | 5 | 1 |
| α1(A175C)β2 | 4.3 ± 1.5 | 0.98 ± 1.5 | 3 | 2.7 | 0.10 ± 0.02 | 1.48 ± 0.04 | 3 | 0.3 |
| α1(R176C)β2 | 1.7 ± 0.4 | 1.10 ± 0.2 | 3 | 1.1 | 0.20 ± 0.09 | 1.00 ± 0.08 | 3 | 0.6 |
| α1(S177C)β2 | 3.0 ± 0.3 | 1.44 ± 0.1 | 4 | 2.9 | 0.10 ± 0.02 | 1.41 ± 0.31 | 3 | 0.5 |
| α1(V178C)β2 | 4.4 ± 1.0 | 1.21 ± 0.3 | 3 | 2.8 | 0.13 ± 0.02 | 1.39 ± 0.23 | 3 | 0.4 |
| α1(V180C)β2 | 2.3 ± 1.1 | 1.40 ± 0.3 | 3 | 1.4 | 0.05 ± 0.01 | 1.19 ± 0.04 | 3 | 0.2 |
| α1(A181C)β2 | 1.9 ± 0.3 | 1.01 ± 0.4 | 3 | 1.2 | 0.14 ± 0.05 | 1.80 ± 0.50 | 3 | 0.5 |
| α1(E182C)β2 | 2.6 ± 0.7 | 1.05 ± 0.3 | 3 | 1.6 | 0.08 ± 0.01 | 1.56 ± 0.15 | 3 | 0.3 |
| α1(D183C)β2 | 11.0 ± 2.0 | 1.90 ± 0.3 | 3 | 6.9* | 0.42 ± 0.10 | 1.18 ± 0.31 | 3 | 1.3 |
| α1(G184C)β2 | 1.9 ± 0.3 | 1.30 ± 0.1 | 3 | 1.2 | 0.12 ± 0.04 | 1.51 ± 0.31 | 3 | 0.4 |
| α1(S185C)β2 | 5.5 ± 1.0 | 1.10 ± 0.2 | 3 | 3.4 | 0.90 ± 0.03 | 1.04 ± 0.02 | 3 | 2.7 |
| α1(R186C)β2 | 7.1 ± 1.7 | 1.40 ± 0.1 | 3 | 4.4 | 1.30 ± 0.50 | 0.90 ± 0.06 | 3 | 3.9 |
| α1(L187C)β2 |      |        |      |    |        |          |     |      |    |        |
| α1(N188C)β2 | 1.4 ± 0.4 | 1.10 ± 0.1 | 3 | 0.9 | 0.15 ± 0.03 | 1.25 ± 0.09 | 3 | 0.5 |
| α1(Q189C)β2 |      |        |      |    |        |          |     |      |    |        |
| α1(Y190C)β2 | 1.6 ± 0.6 | 1.10 ± 0.2 | 3 | 1 | 0.34 ± 0.07 | 1.18 ± 0.08 | 3 | 1 |
| α1(D191C)β2 | 2.3 ± 1.1 | 1.50 ± 0.2 | 3 | 1.4 | 0.77 ± 0.20 | 1.27 ± 0.30 | 3 | 2.3 |

RESULTS

Expression and Functional Characterization of GABAαR α1 Subunit Cysteine Mutants—Cysteine substitutions were engineered at eighteen individual positions in the GABAαR α1 subunit (Pro174, Ala175, Arg176, Ser177, Val178, Val179, Val180, Ala181, Glu182, Asp183, Gly184, Ser185, Arg186, Leu187, Asn188, Gln189, Tyr190, and Asp191) and co-expressed with wild-type β2 subunits in X. laevis oocytes for functional analysis using the two-electrode voltage clamp method. Expression of most mutant subunits produced GABA-activated channels with the exception of L187C and Q189C (Fig. 2 and Table I). The lack of functional expression of receptors carrying the L187C and Q189C mutations may indicate a role for these residues in receptor synthesis/assembly as they are conserved in all GABAαR and glycine receptor subunits. Expression of D183C produced a significant 7-fold rightward shift in EC50 relative to wild-type values (EC50 = 1.6 μM). However, the Kf values for the competitive antagonist, SR-95531, for mutant receptors were not significantly different from wild-type values (Kf = 330 nM). Hill coefficients were not significantly different from wild type (Table I). In general, the maximum current amplitude was 1–10 μA for wild-type and mutant receptors, with the exception of R186C (<300 nA).

These data suggest that cysteine substitution within this domain of the GABAαR α1 subunit protein is well tolerated. A major assumption of SCAM is that the side chain of the introduced cysteine is in a similar position as the side chain of the native residue. Since GABA and SR-95531 bind equally well to both mutant and wild-type receptors, it is likely that the structures of the receptors are similar.

Modification of Introduced Cysteine Residues by MTSEA-biotin—To define the surface accessibility of the α1 subunit P174C-D191C segment, wild type and mutant receptors were exposed to MTSEA-biotin (2 μM) for 2 min (Fig. 3). MTSEA-biotin had no effect on wild-type receptors. MTSEA-biotin significantly reduced I(GABA) at P174C (60.5 ± 1.1%, n = 3), R176C (39.3 ± 6.3%, n = 3), S177C (72.4 ± 1.7%, n = 3), V178C (88.1 ± 3.2%, n = 4), V180C (65.2 ± 2.0%, n = 4), A181C (76.3 ± 2.0%, n = 4), D183C (46.0 ± 5.7%, n = 6) and R186C (44.8 ± 1.7%, n = 4). MTSEA-biotin potentiated I(GABA) at N188C (31.3 ± 10%, n = 3). An apparent lack of reaction (as in the case of A175C, V179C, E182C, and D191C) may indicate that no reaction has occurred or that the outcome of modification is functionally silent. It should be noted that most residues in this region were modified, although the magnitude of the effect of modification did not always achieve statistical significance (e.g. G184C, S185C, and Y190C).

The pattern of solvent accessibility is not indicative of either a β-strand or an α-helix, suggesting that this domain of the GABAαR α1 subunit adopts either a loop or a random coil conformation (Fig. 6).

MTSEA-biotin Rates of Reaction—The rate at which MTSEA-biotin reacts with a cysteine side chain depends mainly on the ionization of the thiol group and the access route to the engineered cysteine (18). A residue in a relatively open, aqueous environment will react faster than a residue in a relatively restrictive, non-polar environment. To gain insight into the physico-chemical environment of the loop F region of the GABA-binding site, we determined the reaction rate of MTSEA-biotin with several accessible cysteine mutants (Fig. 4). The rate MTSEA-biotin modified A181C was ~400-fold faster than the slowest reacting cysteine mutant, V180C. The rank order k2 values were A181C > R186C = R176C = S177C > D183C ≈ V180C = V178C (Table II).

Effects of GABA and SR-95531 on MTSEA-biotin Rate Constants—To determine whether a given cysteine residue lines the neurotransmitter binding pocket, the rate of MTSEA-biotin modification of an introduced cysteine is measured in the presence of GABA and the competitive antagonist, SR-95531. We identify a residue as being within or near the binding site if the rate of covalent modification of the introduced cysteine is slowed in the presence of both agonists and antagonists, which presumably promote different conformational changes within the site. SR-95531 slowed the rate of modification at V178C, V180C, and D183C by factors of 3.6, 1.9, and 3.5, respectively (Fig. 4, Table II). GABA slowed the rate of reaction at R176C, V178C, V180C, and D183C by factors of 2.4-, 1.9-, 1.8-, and 3.5-fold, respectively. Protection of V178C, V180C, and D183C from covalent modification by MTSEA-biotin by GABA and SR-95531 suggests that the slowing of the MTSEA-biotin reaction rates from steric block rather than allosteric changes induced in the protein. It is
interesting to note that R176C was protected only by GABA but not SR-95531. S177C was protected significantly only by SR-95531. While the effects of GABA failed to reach statistical significance for this mutant, there was a clear trend in the data to suggest that GABA also slowed the MTSEA-biotin reaction rate (Table II).

**Effects of Pentobarbital on MTSEA-biotin Rate Constants**—At wild-type α1β2 or α1β2γ2 GABA<sub>2</sub>R, the apparent affinity for direct activation by pentobarbital ranges from 500–700 μM (8, 10). Further, the mean single channel conductances elicited by GABA and pentobarbital are not different, suggesting that the open states produced by both ligands is similar (40). Moreover, mutations that compromise the affinity of GABA have thus far not affected the affinity or efficacy of barbiturates (8, 10), suggesting that the actions of pentobarbital are mediated from a site distinct from the GABA-binding site. Therefore, pento-

**Table II**

Second order rate constants for MTSEA-mediated modification of accessible cysteine residues in the absence and presence of SR-95531, GABA, and pentobarbital

| Receptor       | Control | SR-95531 | GABA | Pentobarbital |
|----------------|---------|----------|------|---------------|
|                | k<sub>2</sub> | n | k<sub>2</sub> | n | k<sub>2</sub> | n | k<sub>2</sub> | n |
| α1(R176C)β2    | 4074 ± 683 | 5 | 4647 ± 1368 | 4 | 1690 ± 329* | 3 | 5037 ± 1183 | 3 |
| α1(S177C)β2    | 3306 ± 869 | 6 | 816 ± 85* | 3 | 1442 ± 70 | 3 | 1740 ± 901 | 3 |
| α1(V179C)β2    | 879 ± 90 | 4 | 245 ± 67** | 3 | 455 ± 117* | 5 | 866 ± 63 | 3 |
| α1(V180C)β2    | 780 ± 50 | 4 | 413 ± 23* | 3 | 436 ± 52* | 4 | 1071 ± 122** | 3 |
| α1(A181C)β2    | 291,900 ± 63,000 | 4 | 160,945 ± 23,000 | 4 | 271,390 ± 72,000 | 4 | 676,074 ± 109,000*** | 3 |
| α1(D183C)β2    | 1263 ± 259 | 3 | 357 ± 57** | 3 | 366 ± 85** | 3 | 1177 ± 218 | 3 |
| α1(R186C)β2    | 4674 ± 703 | 3 | 4665 ± 911 | 3 | 5178 ± 1176 | 3 | 1467 ± 559* | 3 |
barbital can be used as a pharmacological tool to assess gating-induced changes in the GABA-binding site. The rate of modification at R186C was slowed 3.2-fold in the presence of pentobarbital, while the rates of covalent modification at V180C and A181C were accelerated 1.4- and 2.3-fold, respectively (Table II, Fig. 5). Thus, these residues act as reporters of barbiturate-mediated channel gating.

FIG. 5. Summary of the effects of GABA, SR-95531, and pentobarbital on MTSEA-biotin second order rate constants. Data were normalized to control second order rate constants (rate measured when no other compound was present). Co-application of GABA (5 × 10−3 M) or SR-95531 (40 × 10−6 M) slowed reaction of MTSEA-biotin at receptors containing the following mutations: R176C, V178C and D183C, suggesting that they line the GABA-binding site. Data represent the mean ± S.E. for at least three experiments. R176C was protected only by GABA, and while not significant, there is a clear trend in the data to suggest that S177C was protected by both agonist and antagonist. The rate of covalent modification at V180C, A181C, and R186C is significantly altered by pentobarbital (500 μM), *p < 0.05.

**Discussion**

**Structure of the GABA Binding Pocket**—Previous work has shown that the GABA binding pocket is composed of aromatic (α1Phe64, β2Tyr157, β2Tyr205), hydroxylated (β1Tyr160, β1Thr202, β2Ser204, β2Ser209), and charged amino acid residues (α3Arg66, β2Arg207). Here, our data demonstrate that GABA and SR-95531 protect V178C, V180C, and D183C also indicate that residues in loop F are near the agonist-binding site. An additional residue, Arg176, may be important for interactions with the agonist alone as modification of R176C was protected by GABA and not SR-95531. Barbiturate-mediated receptor activation did not alter MTSEA modification of R176C, suggesting that the observed slowing of the derivatization of R176C by GABA was a function of steric block, as opposed to channel-gating phenomena. Ligands of divergent chemical structure such as GABA and SR-95531 likely have different contact points within the GABA-binding site (3). However, the amino acid residues identified here need not be contact points for agonist/antagonist molecules, but they may be important for stabilizing the structure of the GABA-binding site or mediating local movements important for activation and/or desensitization.

When mapped onto a homology model of the GABA binding site, these residues appear to be located at the putative entrance of the binding site (Fig. 6). Using this model, we measured distances between loop F GABA-binding site residues and core GABA-binding regions. For example, approximate distances (α–β, in Å) include the following: Asp183-Phe200 (9.0), Asp183-Thr202 (16.0), Asp183-Tyr205 (15.0), and Asp183-Arg207 (12.0); (α–α, in Å) Asp183-Phe54 (12.0) Asp183-Arg66 (9.0). Whether these distances reflect the binding site in a resting, open, or desensitized state is unknown. The AChBP was crystallized in an ill-defined state, lacks an ion channel, and shows little cooperativity in ligand binding (39, 41). In addition, the loop F region was not well defined in the AChBP structure (17).

Previous work has demonstrated that the nAChR loop F is involved in agonist binding. Using a chemical cross-linker, Czajkowski and Karlin identified several negatively charged residues in loop F (δAsp180, δGlu182, and δGlu189) within 9 Å of the Cys193/Cys194 loop of the α subunit (13). These data suggest that, at least in some cases, the loop F domain of the δ subunit is in close proximity to residues on the α subunit that are within the core of the ACh-binding site. In addition, recent studies have shown that naturally occurring mutations in the loop F protein chain of the δ subunit (D175N, N182Y) alter ACh microscopic binding affinity and channel gating (16).
**Structural Rearrangements during Gating Transitions**—

Allosteric proteins such as ligand-gated ion channels cycle through a number of affinity states, including a low affinity resting state, an active open channel state of moderate affinity and two desensitized states of high and very high affinity, respectively (42). During these state transitions, a molecule of GABA likely contacts a number of different residues. Residues important in the initial docking of the ligand may be different than residues involved in stabilizing ligand binding in open and desensitized states. It is likely that the GABA-binding site undergoes a series of transitions in which alternate domains of the protein are brought into closer contact with the ligand during active and desensitized states. It is equally possible that the protein are brought into closer contact with the ligand and desensitized states. It is likely that the GABA-binding site than residues involved in stabilizing ligand binding in open and desensitized states (1), further complicating analysis of agonist binding segments.

Methanethiosulfonate reagents can be used as reporter molecules to detect agonist- or drug-induced changes in protein regions that are distant from the agonist or modulator binding site. GABA-induced structural rearrangements have been reported in the benzodiazepine-binding site (19) and in the $\alpha_1$ subunit M2-M3 loop (43). The allosteric modulators, diazepam and propofol, induce changes in the $\alpha_1$ subunit M3-spanning segment (35, 44). In addition, we have previously demonstrated movements within the GABA-binding site in response to pentobarbital gating of the channel (3, 10).

To test the hypothesis that movement of loop F is a plausible ion channel activation mechanism (14), we measured the rate of covalent modification of accessible amino acid residues in the presence of pentobarbital (50 $\mu$M). The ability of pentobarbital to alter the rates of modification of the loop F segment provides an indirect measure of changes that occur within this region of the binding cleft in the transition from the resting to the active/desensitized states. Co-application of pentobarbital and MTSEA-biotin should capture a receptor state that differs from that captured by application of MTSEA-biotin alone. Pentobarbital-mediated acceleration of the rate of modification at V180C (a GABA-binding site residue) and A181C and the concomitant slowing of the rate of modification of R166C indicate that Val$^{180}$ and Ala$^{181}$ move to a more accessible environment, while Arg$^{186}$ becomes less accessible. These data demonstrate that the loop F region of the GABA binding site undergoes conformational rearrangements during receptor activation and/or desensitization. Other movements within the binding site may also be needed to trigger channel gating. For example, rotations and/or tilting movements of the $\beta_3$ subunit may move the loop C region of the GABA-binding site closer to $\alpha_1$ subunit binding segments (45).

**CONCLUSIONS**

SCAM analysis has enabled us to identify novel residues of the $\alpha_1$ subunit (Val$^{175}$, Val$^{186}$, and Asp$^{186}$) that contribute to forming the GABA-binding site. Further, we provide evidence that the domain defined by Pro$^{174}$—Asp$^{191}$ adopts a random coil/turn conformation. Barbiturate-mediated channel activation suggests that this segment of the protein undergoes conformational movements during channel gating. We speculate that this loop of the protein is a dynamic element that may move closer to the core of the binding site during allosteric transitions to higher affinity states. While this is a plausible channel-gating mechanism, corroboration of these SCAM observations will require studies using chemical cross-linkers to understand the relative positions of amino acids in this domain during the transduction of agonist binding to channel opening and desensitization.

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